Helicobacter pylori in the 21st Century

Edited by

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Through the application of molecular and cellular microbiology we now recognize the diversity and dominance of microbial life forms that exist in all environments on our planet. These microbes have many important planetary roles, but for humans a major problem is their ability to colonize our tissues and cause disease. The same techniques of molecular and cellular microbiology have been applied to the problems of human and animal infection since the 1990s and have proved to be immensely powerful tools in elucidating how microorganisms cause human pathology. This series has the aim of providing information on the advances that have been made in the application of molecular and cellular microbiology to specific organisms and the diseases they cause. The series is edited by researchers active in the application of molecular and cellular microbiology to human disease states. Each volume focuses on a particular aspect of infectious disease and will enable graduate students and researchers to keep up with the rapidly diversifying literature in current microbiological research.

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Foreword

Unlike books that have been written before about *Helicobacter pylori*, this volume, 30 years after Warren’s original description of a case at Royal Perth Hospital, presents a mature understanding of how the bacterium colonizes its host and causes disease. As such, it will stay on my laboratory shelf for years to come, perhaps with a few minor details added as new signalling pathways are discovered.

Thirty years after Robin Warren first saw spiral bacteria on human gastric biopsies, Sutton and Mitchell’s new book is a timely reminder that many clinical and basic aspects of *H. pylori* science are still controversial and worthy of further research. While much of our knowledge of the bacterium is a logical extrapolation of findings in other infectious diseases, the unique ability of *Helicobacter* to colonize the human gastric mucosa for many decades provides important insights into host–pathogen interactions, mucosal immunity and the whole immune system.

The editors themselves have been entrenched in *Helicobacter* microbiology for many years and are very well respected in their fields. Associate Professor Phil Sutton, an immunologist, has spent the past several years developing new animal models of *Helicobacter* infection whereby the fine details of the mucosal immune response can be isolated and teased out with the goal of designing vaccines against the organism. Similarly, Professor Hazel Mitchell is now Professor of Microbiology at the University of New South Wales, with many publications on all aspects of *Helicobacter* since she was one of the first in the world to complete a PhD on the biology and immunology of this intriguing organism.

With such eminent editors, it is no surprise that their co-authors include the leading lights in specific aspects of *Helicobacter* science. These specialists have each spent many years supervising teams of scientists and no doubt any one of them could easily produce an authoritative book on their special area of interest if they had the time. However, thanks to years of experience, every one of them has produced a concise chapter that not only emphasizes the proven facts but also mentions aspects of the disease that are unusual, controversial or defy explanation.

The chapter on epidemiology is written by Hoda Malaty, who trained in David Graham’s faculty in Houston and who managed many of the seminal epidemiological studies using endoscopy, ELISA serology tests and $^{13}$C-urea breath tests. There is no doubt that *Helicobacter* is less common now as the standard of living increases around the world, but questions of transmission, acquisition and spontaneous loss of the infection are not all that well understood. Clinicians treating refractory patients need to consider these puzzling aspects of the disease. Twin studies, too, have been described here, to give a taste of the ongoing controversy, which is: ‘Are host factors or bacterial factors the most important in determining disease outcome?’
Many of the unsolved dilemmas are then addressed further by Professor Kwong Fock from Singapore, a well-known academic and clinician with first-hand experience of the great disparities between Helicobacter incidence and Helicobacter-related diseases in the Asia Pacific region. Of particular interest is the question: 'If Helicobacter causes gastric cancer, why is it that some ethnic and geographic groups have one without the other?' Once again the host/cultural factors versus bacterial factors, especially various types of toxigenic Helicobacter strains, are carefully considered. Recent molecular/epidemiological studies of the mosaicism and phosphorylation sites of the gene encoding the cytotoxin-associated gene A product (CagA) could explain some of this variation. Presently, however, one must admit that a unifying hypothesis does not exist to explain the great disparities in disease phenotypes in Asia. Fock's chapter points out where modest efforts into carefully selected new studies are likely to pay off.

In a related chapter, the whole subject of gastric carcinogenesis, its histology, aetiology and relationship with Helicobacter, is placed into perspective by Pelayo Correa, a pathologist and pioneer of gastric cancer histological studies, and M. Blanca Piazuelo. With direct experience in the USA and South America, particularly the high cancer areas in Colombia, Correa and Piazuelo establish the background knowledge relating cancer risks to diets and lifestyle and then bring us up to date with new advances in the basic science of gastric carcinogenesis, particularly the exciting areas of Helicobacter toxin types, nitric oxide generation and oxidative stress. This well-referenced chapter is essential reading for anyone starting out in the field of gastric carcinogenesis.

Rounding off the clinical section is the chapter on antimicrobial resistance by Francis Mégraud of Bordeaux, who expertly addresses this emerging problem that is leading to global reductions in cure rate. Mégraud has authored many clinical studies of Helicobacter treatment and therefore has studied the resistance patterns of persisting isolates. His chapter is up to date with authoritative explanations of the pharmacokinetics of antimicrobials as they apply to the human stomach. In addition, he explains new technologies being introduced to rapidly evaluate antimicrobial resistance using molecular methods which thus avoid the several days' delay of 'old-fashioned' bacterial culture.

Bridging the clinical and basic sections, the chapter on taxonomy and extragastric Helicobacter infections by Hazel Mitchell, Nadeem Kaakoush and Phil Sutton will still be of great interest to clinicians. Mitchell has orchestrated a careful and impartial review of the non-gastric diseases that might be associated with H. pylori infection. Actually, it is very likely that some of these so-called associations are coincidental, related to socio-economic status for example. But in response to this, Mitchell has put the most robust linkages at the front of the chapter ('idiopathic thrombocytic purpura' and iron deficiency), then those with highly variable associations and likely reporting bias, most notably atherosclerosis, are discussed later. The review of the epidemiologic and basic research for each of these associations is an excellent resource for hypothesis-driven research plans. Similarly, attempts to unravel the highly variable association between H. pylori and atherosclerotic diseases could justify a career in clinical epidemiology. The chapter then flows easily into an expertly crafted discussion of the 'hygiene' link to allergic and 'autoimmune' disorders diseases, e.g. asthma and colitis, again using a clinical review followed by an in-depth description of hypothesis-driven basic immunological studies. Surprisingly, these have already revealed potential causative pathways that could lead to novel therapies for atopic diseases. With half the world still infected with H. pylori, modest increases in ‘non-gastric’ disease risk could actually drive numerically large amounts of morbidity, so it is very easy to justify further investigation into this emerging area of Helicobacter discovery.

In their chapter on H. pylori-induced acquired immunity and immunoregulation, Karen Robinson and John Atherton start off by listing the many paradoxes associated with Helicobacter immunology – for example, that B-cell-deficient animals can still be immunized and that some immunizations can actually increase Helicobacter colonization. To whet our appetite further,
they discuss tantalizing data whereby some human diseases characterized by hyperactive immune systems might be ameliorated or prevented by chronic gut infections such as \textit{Helicobacter}.

Phil Sutton, Alison Every and Stacey Harbour’s chapter on host genetic factors and susceptibility to \textit{H. pylori} pathogenesis systematically describes the known workings of the mucosal immune system and how its components are influenced by various \textit{Helicobacter} infections in several animal models. Since \textit{Helicobacter} cannot usually be eradicated by even a robust serological immune response, it is important to understand the workings of the cell-mediated immune response too, as this is the component that correlates with protection from \textit{Helicobacter} in animal models. Interaction between \textit{Helicobacter} immunity and other pathogens, such as helminths, is discussed as part of the ‘clean’ versus ‘dirty’ human bacterial ecology hypothesis as it could affect the human propensity to develop hyperactive immune responses in autoimmune diseases, particularly atopic conditions such as asthma.

Maria Kaparakis, Cody Allison and Melanie Hutton from Richard Ferrero’s group at Monash University in Melbourne start their review of innate immune initiators and effectors in \textit{H. pylori} infection by pointing out the apparent paradox: namely that gastric epithelial cells are not usually considered to be a component of the immune system, yet colonization of this mucosa generates mucosal inflammation and a strong antibody response. However, these gastric epithelial cells are all very competent innate immune responders and this immediate part of the immune response might be used by \textit{H. pylori} to improve its nutrition in the gastric mucus layer. The chapter then catalogues evidence for and against a major role for each of the relevant cytokines, assigning importance to the Toll-like receptors (TLR) 4 and TLR2 (from epithelia) and the neutrophil-activating protein HP-NAP (from \textit{H. pylori}), but not lipopolysaccharide or flagellin (to which TLR5 is unreactive). Ferrero’s specialty is the nucleotide-binding oligomerization (NOD) receptors, an interesting group that carries out an inflammatory role but might also trigger release of the cellular antibiotics such as ‘defensins’. The authors make the point that much work is needed to further clarify this area.

Thomas Blanchard and John Nedrud have prepared a careful review of \textit{H. pylori} vaccines, reflecting their long experience in this area. So that immunologists new to the field do not try to ‘reinvent the wheel’, the authors systematically explain the evolution of vaccines for \textit{H. pylori} with tales of success and failure in various animal models, finishing with comprehensive lists of antigens, administration routes and results. They critically review the outcome of many studies, explaining results according to whether the vaccine causes protection, sterilizing immunity, or some lesser degree of effect. In each case they characterize the underlying immune response, T-helper 1 or T-helper 2 type, and consider why the results might not have been appropriate or sufficient for human use.

Going into some detail, Blanchard and Nedrud explain, similarly to the other immunological chapters, how the \textit{H. pylori} immunity is unrelated to the serological immune response and that, in some models, antibodies actually enhance the colonization with \textit{H. pylori} compared with a knockout mouse unable to produce antibody (µMT antibody-deficient mice). These data are reminiscent of the human situation, where \textit{H. pylori} has never been a major pathogen in AIDS or immune-deficient patients, supporting the hypothesis that robust immunity might be necessary for conversion from the asymptomatic to the symptomatic (ulcer) phenotype. In their chapter, these authors try to make sense of a plethora of experimental data but it seems that there is much work to be done. With so many adjuvants, mouse strains, vaccination routes and even knockout animals to test these days, it is difficult to say which will mimic the human situation and be an appropriate model. Of interest is a description of some animal models whereby gastritis worsens or activates when an \textit{H. pylori} challenge is given subsequent to a vaccine. Thoughtful consideration of this effect, which seems to resolve after \textit{H. pylori} eradication, is worthwhile in case there are autoimmune implications to empirical attempts to eradicate \textit{H. pylori} in humans. However, as far as we know, \textit{H. pylori} disease is not the cause of idiopathic autoimmune gastritis.
In great detail Blanchard and Nedrud catalogue even more obscure factors that interact with gastric immunity, most notably the adipokine leptin. The fact that leptin influences immunization against *H. pylori* is an example to us that there may be other as yet undiscovered factors that are playing a key role in the mucosal defence, or lack thereof, against *H. pylori*. After discussing poor results from studies in monkeys and humans using live *Salmonella* vectors and various other strategies, the authors summarize by saying that there are many strategies that should work, but have yet to be properly trialled, so *H. pylori* vaccinology will remain an exciting and busy area of research for years to come.

In a personal chapter, based on a lifelong interest in the lipopolysaccharides of *H. pylori* and their importance in gastric adaptation and pathogenesis, Professor Tony Moran provides a very detailed review of the *H. pylori* lipopolysaccharide, cell wall structures, interactions with the immune systems and the intricacies of the Lewis antigen mimicry. Clearly, fundamental understanding of the bacterial cell wall structures, particularly its lipid, is essential if we are to develop new means of eradicating or immunizing against *Helicobacter*. Not just a list of references and summaries of each, Moran's well-known personal expertise in this area allows him to synthesize the data and critically review the field so that new investigators can see what has been done, what strengths, weaknesses and inconsistencies there are in the existing body of work, and what is needed to make progress in this exciting but tough scientific niche.

In a very comprehensive chapter about virulence factors of *H. pylori*, accompanied by beautiful and detailed illustrations, Steffen Backert, Hitomi Mimuro, Dawn Israel and Richard Peek from Nashville summarize current knowledge in this area. Their long experience in the field is reflected first in a relevant critique of the latest methodology, especially cell biology and new transgenic animal models. They successfully tease out the interactions between the many adhesins, cytokines, toxins and signalling proteins, with carefully linked text and illustrations. Their synthesis incorporates new information from the post-genomic/proteomic era to explain the fundamental workings of the vacuolating cytotoxin VacA and CagA. By carefully reading the text and following the numbered steps in the illustrations readers will see why, for 60,000 years, *H. pylori* has been able to successfully colonize – but not kill – its unlucky human host. This is a very detailed, heavy-duty chapter about the workings of VacA and CagA, a really great reference especially valued because of its thoughtful and expertly done illustrations.

In a related chapter on *H. pylori* adhesion to the gastric surface, authors Sara Lindén, Anna Arnqvist, Susanne Teneberg and Tony Moran represent three of the world's premier groups studying the interaction between *H. pylori* and the various carbohydrate moieties present on the gastric epithelial cells. Put simply, these structures help make *H. pylori* stick. In a complicated area, these experts have created a structured chapter starting with consideration of the oral cavity, salivary, then gastric and epithelial adhesive structures. Some of these are well studied, such as the Bab adhesins, but others remain a mystery. Clearly, however, *H. pylori* has an efficient means of reaching and then tethering itself to the gastric mucosa, and a comprehensive explanation of the current state of the art is presented here.

Hilde de Reuse, Markus Heimesaat and Stefan Bereswill provide a fast-moving and readable overview of the *H. pylori* genome, ‘Helicobacteromics’, emphasizing the variability and plasticity as vast bacterial numbers balance their existence against the human immune system on the quite variable environments of the human gastric mucosa. *H. pylori* does this by genome shuffling and randomly disabling or enabling its outer membrane proteins to vary their interactions with the host, aided by its rather poor DNA repair repertoire, so that small mutations continually occur whereby new variations of all its genes are constantly being tested. Understanding how the *H. pylori* genome works also explains how the bacterium imports blocks of genes such as the *cagA* pathogenicity island, a secretion system that has a distinctly different G+C content from the rest of the organism's DNA, showing that it was imported 'en bloc' from a different organism, though which one we have no idea! After finding a home in the human stomach, *H. pylori* then discarded many genes because it now lived exclusively in a rather hostile but constant environmental niche, with few competitors, and its excursions into
the outside world were probably rare and rather short-lived, as its new host was almost always in the same human family. The authors explain how tracing human migrations using polymorphisms in essential housekeeping genes (via multilocus sequence typing, MLST) has become an area of research with wide interest and general implications for the human family. These authors enhance their chapter with their own deep insights into the genomic strategies whereby *H. pylori* survives in its acidic niche.

To sum it up then, this is an industrial-strength book containing up-to-date reviews on Helicobacter science, each presented by a world expert in that particular area. The first third of the book contains reviews of the issues of interest to clinicians, i.e. epidemiology, microbiology, pathophysiology and therapeutics. The rest of the book delves minutely into a diverse range of fundamental issues arising in the bacterium itself (carbohydrates, lipids and toxins) and their effects on the host's immune systems, innate and adaptive. This book is action-packed, fact-filled and thoughtfully assembled. I look forward to having it on my laboratory shelf for years to come.

Barry Marshall
December 2009
1 Epidemiology of *Helicobacter pylori* Infection

**H.M. Malaty**

1.1 Introduction

*Helicobacter pylori* infection is now recognized as a worldwide problem. *H. pylori* infection is the most common cause of chronic gastritis, and has been strongly linked to peptic ulcer disease and gastric cancer. *H. pylori* is estimated to infect one-half of the world’s population. The epidemiology of infection reveals that given the right circumstances it is readily transmissible. Infection is generally acquired in childhood, but disease manifestations typically do not appear until adulthood and often only after long periods of latency. The infection has a high morbidity rate, but a low mortality rate, and is curable with antibiotic therapy.

1.2 Relationship between *H. pylori* Infection and Associated Diseases

*H. pylori* infection is causally related to chronic gastritis and peptic ulcer disease, and indirectly related to gastric adenocarcinoma and primary gastric B-cell lymphoma (Forman et al., 1990, 1991; Tytgat et al., 1993; Peura and Graham, 1994). Infection with *H. pylori* leads to the development of gastric damage, and of those infected approximately 25% may ultimately develop low gastric acid production (achlorhydria), which is associated with an increased susceptibility to the development of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume; Graham et al., 1988). Approximately 17% of infected patients develop peptic (gastric and duodenal) ulcers, and one-quarter of such patients experience an ulcer complication (Tytgat et al., 1993). Numerous trials have shown that ulcer relapse is prevented after cure of the infection (Graham et al., 1992). Histological and serological studies have also shown that infection precedes the ulcer and thus *H. pylori* infection is now accepted as one of the two major causes of peptic ulcers, the other being use of non-steroidal anti-inflammatory drugs (NSAIDs). In an evaluation of 100 consecutive duodenal ulcer and 154 gastric ulcer patients in a Veteran Affairs population in Houston, Texas, 99% of those with duodenal ulcers were found to be positive for *H. pylori*, 30% of whom were also found to be using NSAIDs (Al-Assi et al., 1996), and 92% of patients with gastric ulcers were infected with the bacterium, 58% of whom were also taking NSAIDs. These findings were confirmed in a recent study by Gisbert and Calvet (2009) that reviewed the real prevalence of *H. pylori*-negative duodenal ulcer and its possible causes. This study reported that, in truly *H. pylori*-negative patients, the single most common cause of duodenal ulcer is, by far, the use of NSAIDs.

More sophisticated approaches looking for evidence of cyclooxygenase-1 inhibition
have shown that NSAID use is much more common than expected in peptic ulcer patients (Al-Assi et al., 1996) and probably accounts for a sizeable proportion of cases. Additional possibilities include other infections such as herpes simplex virus and other drugs such as alendronate (Gisbert and Calvet, 2009), as well as misdiagnosis by the endoscopist. Peptic ulcer disease is a chronic relapsing disorder, with typical morphological and endoscopic appearances. One important reason for the observed differences in the ulcer prevalence and ulcer recurrence rates in clinical trials is the interpretation of the findings by endoscopists, who are possibly the most likely source of variability in such studies.

The exact prevalence of H. pylori infection in gastric cancer patients remains difficult to estimate, because infection can be lost from individuals with cancer or its precursor conditions (see Correa and Piazuelo, Chapter 3, this volume). In a study by Yamaji et al. (2002), sera from 10,234 consecutive Japanese individuals who participated in a health examination programme were tested for the presence of antibodies against H. pylori by immunoglobulin G (IgG) ELISA. While this study confirmed the relationship between serum positivity and gastric cancer, these authors pointed out that, particularly in the elderly, a weak H. pylori antibody response carried a high risk for gastric cancer. A Swedish population-based case–control study, using an immunoblot for the cytotoxin-associated gene A protein (CagA) (rather than an IgG ELISA) to detect infection, showed the CagA immunoblot to be a more sensitive assay for detecting past infection. The study reported that the estimated proportion of non-cardia adenocarcinoma attributable to H. pylori was 71% for both histological subtypes (odds ratio (OR) = 21, 95% confidence interval 8.3, 53.4), ranking with the association reported between smoking and lung cancer (Ekstrom et al., 2001).

Atrophic gastritis and intestinal metaplasia are well-established precursor lesions of the intestinal type of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). A study from Japan followed up 1526 patients with duodenal ulcers, gastric ulcers, gastric hyperplasia or non-ulcer dyspepsia, of whom 1246 had H. pylori infection and 280 did not, for a mean period of 7.8 years (Uemura et al., 2001). Gastric cancer was shown to develop in 36 (2.9%) of the infected subjects compared with none of the uninfected patients. Among the patients with H. pylori infection, those with severe gastric atrophy, corpus-predominant gastritis and intestinal metaplasia were at significantly higher risk for gastric cancer. The authors detected gastric cancers in 21 (4.7%) of the 445 patients with non-ulcer dyspepsia, ten (3.4%) of the 297 with gastric ulcers, five (2.2%) of the 229 with gastric hyperplastic polyps, and none of the 275 with duodenal ulcers. The study concluded that gastric cancer develops in persons infected with H. pylori but not in uninfected persons. Another study from Japan reported the results of a multicentre study to evaluate the relationship between H. pylori infection, atrophic gastritis and intestinal metaplasia (Asaka et al., 2001). The study reported that the prevalence of atrophic gastritis increased from 9.4% in individuals less than 20 years of age to >70% in those aged 60 years or older, and was strongly associated with H. pylori infection. The overall prevalence of atrophic gastritis in H. pylori infection was 82.9%, compared with 9.8% among those uninfected (OR = 44.8). Intestinal metaplasia was present in 43.1% of H. pylori-positive persons compared with 6.2% among the uninfected (OR = 11.5). The authors concluded that atrophic gastritis and intestinal metaplasia were strongly associated with H. pylori and not with ageing. These data suggest that the risk of development of early gastric cancer will continue to remain high in Japan.

### 1.3 Geographical Distribution of the Prevalence of H. pylori Infection

The prevalence of H. pylori infection varies from country to country, with the largest differences being observed between developed and developing countries (Megraud et al., 1989; Graham et al., 1991a; Taylor and Blaser, 1991; Bardhan et al., 1998; Redlinger et al., 1999) (Fig. 1.1). The epidemiology of H. pylori infection in developing countries such
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3

as India, Saudi Arabia and Vietnam is characterized by a rapid rate of acquisition of infection such that approximately 80% of the population is infected with the bacterium by the age of 20 (Megraud et al., 1989; Al-Moagel et al., 1990; Graham et al., 1991a). Because the infection is most often acquired in childhood (Mitchell et al., 1992; Malaty et al., 1996a, 1999, 2001, 2002), unless the infection is treated, it persists during adulthood. The prevalence of infection varies between and within countries and within subpopulations within the same country (Pateraki et al., 1990; Perez-Perez et al., 1990; Graham et al., 1991b; EUROGAST Study Group, 1993; Breuer et al., 1996). For example, a study conducted in Houston, Texas, of 485 asymptomatic individuals (50% black and 50% white) aged 15–80 years of age showed that the prevalence of H. pylori infection increased with age (Graham et al., 1991b). These results are similar to patterns observed in other developed countries such as the UK, Australia and France where an increasing prevalence of infection with age has been observed. The increase in prevalence of infection with age can be attributable either to new acquisition of infection among the adult population or to the presence of different birth cohorts, each with a different rate of acquisition in childhood, within the population. Current data suggest that the increase with age is actually related to different birth cohorts, and reflects that each successively younger cohort has had a lower rate of acquisition of infection than those born earlier (Parsonnet et al., 1992; Banatvala et al., 1993).

H. pylori infection has been shown to follow the routes of human migration by their geographical origin, and several studies have examined the effect of immigration on the prevalence of the infection. One recent study examined H. pylori strains among three major ethnic groups in Malaysia (namely the Malay, Chinese and Indian populations), reporting that while the majority of the Malay and Indian H. pylori isolates share the same origin, the origin of the Malaysian Chinese H. pylori is distinctive (Tay et al., 2009). The study concluded that the Malay population was likely to have been initially H. pylori-free and gained the pathogen recently from cross-infection from other populations.

It has been also established that the prevalence of H. pylori is inversely related to socio-economic status (Graham et al., 1991b; Malaty et al., 1996a,b, 2001) with the major variable being the status during childhood, the period of highest risk of acquisition. Attempts to understand the different rates of infection in defined groups have focused on differences in socio-economic status as defined by occupation, family income level and living conditions. Each of these variables measures a different component of the socio-economic complex. Within the USA, studies in a cohort of blacks and white Hispanics examined the relationship between current and childhood socio-economic status and the prevalence of H. pylori infection (Malaty et al., 1992). The study showed that there was an inverse correlation between low socio-economic status during childhood and the prevalence of H. pylori infection, irrespective
of the present social class. These results were later confirmed in a study conducted in Korea (Malaty et al., 1996a). For populations in which the social class is more or less homogeneous, such as China and Russia, density of living has been shown to be the most significant risk factor (Sitas et al., 1991; Mitchell et al., 1992; Malaty et al., 1996b). Similar findings have been reported in a number of other countries (Monno et al., 2008; Cheng et al., 2009). A study of the effect of childhood conditions carried out in monozygotic and dizygotic twins, reared together or apart and differing in socio-economic status, revealed that the strongest effects on the acquisition of H. pylori infection were density of living and low household income during childhood (Malaty et al., 1998). Variation in acquisition of infection among ethnic and racial groups appears to be primarily related to differential exposure (e.g. cultural background; social, dietary and environmental factors) (Graham et al., 1991b; Mitchell et al., 1992; Malaty et al., 1998) and not to possible differences in genetic predisposition (Malaty et al., 1992).

The finding regarding socio-economic status during childhood holds true for all subgroups in the USA. For instance, in a paediatric study conducted in Arkansas, children (aged 11–20 years) from families with an income of <US$5000/year had prevalence rates of up to 60%, whereas those with a family income of >US$25,000/year had only a 15% rate of infection (Fiedorek et al., 1991). This phenomenon has also been observed in other countries. In recently developed countries such as Korea, although approximately 80% of individuals more than 20 years of age are infected (reflecting the fact that Korea has become a developed country only within the past 20 years) the prevalence of infection in young children is inversely related to the socio-economic class of their family. Those aged 10–19 years who were from a high socio-economic class had a 20% frequency of H. pylori infection, while those of the same age who were from a low socio-economic class had a 60% frequency (Malaty et al., 1996a). A cross-sectional study conducted in children residing in industrial and rural areas of Italy reported that the seroprevalence of H. pylori infection was significantly higher in children residing in rural areas compared with those residing in industrial areas (Dore et al., 2002).

The study also found that, in rural areas, children having dogs (most commonly shepherd dogs) were at greatest risk for H. pylori acquisition, but this effect was not observed among children from the industrial areas.

1.4 Changing Prevalence of H. pylori Infection

While the rate of H. pylori infection in the USA is low among white and economically advantaged Americans, minority populations have a high frequency of infection. Data from the USA indicate that black children aged 5–9 years have an overall infection frequency of 30%, which is three times higher than that in white children (Malaty et al., 2002). Around the world, the frequency of infection in children aged 3–19 years ranges from approximately 35% in Russia, to 20% in China and Poland, 12% in Korea and the USA, and <10% in France, Belgium and Finland (Ashorn et al., 1995; Malaty et al., 1996a,b; Matysiak-Budnik et al., 1996; Mitchell, 1997). Despite high rates of infection in certain pockets of the globe, the frequency of H. pylori infection is declining worldwide. For instance, in one study conducted in Matsumoto, Japan, the rate of infection declined by up to 20% between 1986 and 1994 among individuals aged 9–70 years (Kumagai et al., 1998).

In two cross-sectional studies conducted in Russia, the effect of recent improvements in the standard of living on the prevalence of H. pylori infection was examined in Russian children resident in St Petersburg. In both the first study conducted in 1995 and the second conducted a decade later, H. pylori status was evaluated using the same ELISA method to measure anti-H. pylori IgG (Malaty et al., 1996b; Tkachenko et al., 2007). These studies showed that the overall prevalence of H. pylori infection in 1995 was 44%, which decreased to 13% 10 years later. In both studies, the prevalence of infection increased with age. Interestingly, while in 1995 the prevalence of infection among children younger than 5 years of age was 30%, a decade later the prevalence in the
same age group was only 2%. The study concluded that improvements in the standard of living in Russia had resulted in a marked reduction in H. pylori transmission. Different rates of acquisition of H. pylori form the basis for the differences in the prevalence of infection between and among populations. The changes in Russia are a dramatic example of how sensitive H. pylori acquisition appears to be to improvement in standards of living and/or the availability of H. pylori treatment (Malaty et al., 1996b; Tkachenko et al., 2007).

1.5 Routes of Transmission

To date, there is still an ongoing debate about the exact mode of transmission of H. pylori infection. Person-to-person transmission through faecal–oral or oral–oral transmission has been supported by numerous research studies. The majority of data support the notion that transmission is within families (Mitchell et al., 1993; Weyermann et al., 2009). In one study, H. pylori status was determined in 41 families, with ‘family’ being defined as a husband and wife living together with biological children (Malaty et al., 1991). Prior to disclosure of the results of H. pylori testing, one of the parents was randomly selected as the index case. The results revealed that, if the index case (either the mother or father) was positive, the children and spouse in that family were also likely to test positive for H. pylori (Fig. 1.2). If the index case was negative, then the children and spouse were likely to be negative as well. Hence in that study performed in the USA, there was no difference whether the index case was the father or the mother (Malaty et al., 1991). H. pylori strains isolated from two Hispanic families living in Houston were characterized on the basis of their cagA, vacA (vacuolating cytotoxin) and iceA (induced by contact with the epithelium) genotypes. While H. pylori isolates from children and their mothers had the same genotype, they were different from those associated with the children’s father (Yamaoka et al., 2000). This finding emphasizes the importance of contact between mother and child for the transmission of the bacterium. In countries where the father has little contact with the children (e.g. in Japan), infection appears to be almost always linked to the mother (Malaty et al., 2000a). Thus, close contact and level of household sanitation appear to be the important variables (Nouraei et al., 2009; Weyermann et

![Fig. 1.2. The dynamics of transmission of Helicobacter pylori (Hp) infection in family members and Hp status of the index parent. (Adapted from Malaty et al., 1991.)](image-url)
Another study examined the entire genome of *H. pylori* by PCR-based, random amplified polymorphic DNA (RAPD) fingerprinting, among 32 members of 11 families, for the presence of genetic homogeneity (Roma-Giannikou *et al.*, 2003). In this study RAPD fingerprinting confirmed that closely related *H. pylori* strains were involved in intra-familial dispersion and strongly supported the hypothesis of transmission of *H. pylori* from person to person or from a common source. Transmission between siblings by their birth order has also been reported. A study conducted on 684 rural Colombian children aged 2–9 years found that the odds of infection increased with the number of 2–9-year-old siblings in the household (Goodman and Correa, 2000). That study concluded that *H. pylori* infection seems to be transmitted most readily among siblings who are close in age, and most frequently from older siblings to younger ones. These findings support the concept that the likely sources of transmission are person-to-person transmission and/or exposure to a common source of infection.

The rationale for the oral–oral route of transmission is based on the premise that *H. pylori* is present in the gastric juice and thus could reach the oral cavity through reflux or vomitus. An early study reported that *H. pylori* could be cultivated uniformly from vomitus and concluded that *H. pylori* is potentially transmissible during episodes of gastrointestinal tract illness, particularly with vomiting (Parsonnet *et al.*, 1999). The mechanism of spread of the organism, whether by the faecal–oral or oral–oral route, raises the possibility of transmission of this organism from infected patients to hospital staff, particularly those involved in endoscopy. Evidence for an increased risk of *H. pylori* infection in endoscopists is contradictory, varying from none to a fivefold increase (Nelson and Muscarella, 2006).

The transmission of *H. pylori* through exposure to contaminated food or water is still very controversial. There is mounting evidence which suggests that the prevalence of *H. pylori* infection has a strong correlation with access to clean water, suggesting a transmission route to the host. Studies from Peru and Kazakhstan found contaminated water to be a risk factor for *H. pylori* transmission (Klein *et al.*, 1991; Nurgalieva *et al.*, 2003). A study among unrelated asymptomatic individuals between 10 and 60 years of age examined various aspects of the local household environment and access to water (Nurgalieva *et al.*, 2003). The authors reported that the transmission of *H. pylori* could be waterborne or water-washed or both (related to poor sanitary practices) and concluded that reducing the rate of *H. pylori* transmission will require an improvement in overall sanitation, including waste disposal, clean water and safe food, as well as in household hygiene practices. In a study from India that examined 500 adults of varying ages ranging from 30 to 79 years, three biopsy samples were collected from each subject to assess *H. pylori* infection (Ahmed *et al.*, 2007). Based on detection by PCR amplification of the gene encoding 16S rRNA from *H. pylori*, the prevalence of infection among people who drank water from wells was 92%, compared with 75% in those who drank tap water (*P* < 0.001). *H. pylori* infection prevalence was found to be higher in people with a low clean water index (88%) than in those with a higher clean water index (33%) (*P* < 0.001). The results of the study suggested that the risk of acquisition and transmission of *H. pylori* can be prevented to a large extent by regular boiling of water used for drinking purposes. However, the lack to date of established culture methods for the detection of viable *H. pylori* in the environment, in particular drinking water supplies, has prevented the development of true epidemiological and risk assessments.

A study from Japan investigated the presence of *H. pylori* in cow’s milk as this is one of the foods that most Japanese children consume (Fujimura *et al.*, 2002). Detection of *H. pylori* was demonstrated by semi-nested PCR, a culture method and by electron microscopy. *H. pylori* was cultured from one raw milk sample, whereas it was not cultured in pasteurized milk samples. The study concluded that there is a possibility that cow’s milk is a transmission vehicle in childhood *H. pylori* infection, although the study failed to confirm the survival of *H. pylori* in pasteurized milk.
1.6 Changes in the Environment and Human Behaviour that Influence the Prevalence of *H. pylori* Infection

It is now apparent that the decrease in the age-adjusted prevalence of *H. pylori* infection in industrialized countries is related to both improved living conditions during childhood and loss of infection. Although *H. pylori* infection is chronic, and possibly lifelong, spontaneous elimination of the infection was reported as early as 1992 using serological tests and was confirmed using breath tests (Klein et al., 1994) and histology (Guelrud et al., 1994) in both developed and underdeveloped countries. These observations have subsequently been confirmed in a number of populations (Matysiak-Budnik et al., 1996; Cranstrom et al., 1997; Roosendaal et al., 1997). A longitudinal study, conducted over a 12-year period in 212 black and white children living in the same community and attending the same schools, has provided additional insights into the changing pattern of infection (Malaty et al., 1999). The study showed differences to exist between the two races. At ages 7–9 years, 19% of children had *H. pylori* infection (40% of blacks versus 11% of whites; *P* = 0.001). After a 12-year period of observation, more black children remained infected (or were more likely to become re-infected) compared with white children, in whom the infection was lost in 50% during the observation period. This suggested that the higher rate of acquisition and the lower rate of loss of infection among black children might be due to differences in access to healthcare facilities or more intense exposure, for whatever reason. The high rate of loss of *H. pylori* infection among white children was not related to *H. pylori* ‘eradication therapy’ as the infection had not yet been diagnosed.

A cohort study conducted in Japanese children and adults from a typical mountain village evaluated the seroepidemiology of *H. pylori* infection over a 9-year period, and also compared the results with the age-specific seroprevalence from two independent cross-sectional surveys conducted in the same population, over the same period (Kumagai et al., 1998). The study also found the rate of disappearance of *H. pylori* infection to be greater than the rate of acquisition, and these results were confirmed in the two cross-
sectional studies. Of particular interest was the observation that there was a decrease in prevalence in every age group (Fig. 1.3). The fall in prevalence that occurred during the observation period did not reflect changes in the rate of acquisition of *H. pylori* in childhood but rather the higher rate of loss of infection. The continuing change in the epidemiology of *H. pylori* infection complicates our understanding of the actual prevalence of *H. pylori*. The higher rate of loss of infection as compared with acquisition may be related to changes in standards of living in successive generations, or to changes in medical practices leading to increased use of antimicrobials for other common infections.

It is impossible to entirely separate environmental factors from genetic influences. An early twin study in the Swedish population suggested a genetic component for acquiring *H. pylori* infection (Malaty et al., 1994) and a follow-up study in the same twin population has questioned if there are genetic influences for peptic ulcer disease in common with genetic influences for *H. pylori* infection (Malaty et al., 2000b). A comparison of monozygotic and dizygotic cross-twin and cross-trait correlations in this Swedish population demonstrated that, despite the similarity in heritability for the two traits (peptic ulcer disease and *H. pylori* infection), the genetic influences for susceptibility to peptic ulcer disease were independent of the genetic effects for acquiring *H. pylori* infection. It is feasible that the relationship between *H. pylori* and peptic ulcer disease could be mediated by familial environmental factors, such as environmental experiences or situations that are shared by family members. Examples of familial environmental factors that may mediate the association between *H. pylori* and peptic ulcer disease are diet, smoking and drug consumption (e.g. alcohol, caffeine and/or NSAID consumption).

### 1.7 The Prevalence of Infection-induced Ulcers is Falling in Western Countries

Since the early 1980s, the occurrence of peptic ulcer disease has declined remarkably in the USA, Europe, Australia and Japan (Vogt and Johnson, 1980; Wylie, 1981; Smith, 1997). The risk of peptic ulcers was highest among those born at the beginning of the 20th century and has decreased in all subsequent generations (Susser, 1982; Sonnenberg et al., 1985). However, this is not exactly the case in Asia (see Fock, Chapter 2, this volume). Due to the rapidity of this change in the pattern of peptic ulcer disease in successive generations (birth cohort phenomenon), it is far more likely to be due to changes in environmental factors rather than to changes in the genes of the affected patients (Sonnenberg et al., 1985). Another possibility is that a change has occurred in the prevalence of particularly virulent *H. pylori* strains (e.g. *cag* pathogenicity island-positive; see Backert et al., Chapter 11, this volume). However, serological studies have shown that the prevalence of identified *H. pylori* putative virulence factors has not changed in any population over time, which can be interpreted in two ways. Either we have not examined the critical factors, or this is not the correct explanation. The latter appears most likely.

In the past there has been interest in identifying genetic factors that might play an important role in the aetiology of peptic ulcer disease (Jensen, 1972; McConnell, 1980). However, the pattern of inheritance is not simple Mendelian and it has been suggested that the genetic basis of peptic ulcer disease is multifactorial (see Sutton et al., Chapter 7, this volume). Initially, the strongest evidence to support a genetic influence in peptic ulcer disease came from studies showing an increased risk of duodenal ulcer in individuals with hyperpepsinogenemia (Neiderman et al., 1964; Rotter et al., 1979). This evidence was discounted, however, when it became evident that elevated serum pepsinogen I was also a feature of *H. pylori* infection (Asaka et al., 1992; Fraser et al., 1992). The fact that family members of patients with peptic ulcer disease are at increased risk can be explained by the fact that they are at increased risk of infection (clustering effect of infection in families), they are more likely to have a similar strain, or they share(d) a common environment. The marked geographical variation in the incidence rates of peptic ulcer disease in different parts of the world along with the rapid rate of change in
incidence provides strong evidence that environmental factors are the most important features that modulate the development of an ulcer.

1.8 Conclusions

Epidemiological studies of *H. pylori* show acquisition in early childhood. However, infection often remains asymptomatic in children and, except for peptic ulcer disease (which is rare in childhood), a relationship between abdominal pain and *H. pylori* infection is not demonstrated. It is inappropriate to prescribe anti-*H. pylori* therapy without a firm diagnosis. The use of multiple antibiotic-containing regimens in a paediatric patient with an ulcer but without *H. pylori* cannot provide any benefit to the patient or the community. Most studies are in favour of intra-familial transmission, and *H. pylori* prevalence in mothers is a crucial determinant for the child’s risk of being infected. The prevalence of *H. pylori* infection in a community depends on the rate of acquisition of the infection (i.e. incidence of the infection). The rate of acquisition of *H. pylori* infection varies remarkably between populations and is higher in non-industrialized countries than in industrialized countries. Several gastrointestinal and non-gastrointestinal diseases (see Mitchell et al., Chapter 5, this volume) have been reported to have a significant association with *H. pylori* infection. We therefore need to discover why ulcers or cancer occur in so few of those infected with *H. pylori* and how this subgroup can be identified and treated.

References


as a marker of past infection. *Gastroenterology* 121, 784–791.


2 Helicobacter pylori Infection in Asia

K.M. Fock

2.1 Introduction

Helicobacter pylori is a micro-aerophilic, spiral-shaped, Gram-negative bacterium that colonizes the human stomach. Globally, H. pylori infection affects 50% of the population (Correa and Piazuelo, 2008). Although H. pylori was first described in 1892 by the Italian pathologist Giulio Bizzozero, its role in the pathogenesis of gastritis and peptic ulcer disease was discovered in Perth, Australia, by Barry Marshall and Robin Warren. This discovery has led to a major change in the treatment of peptic ulcer disease. In 1994, the International Agency for Research on Cancer (IARC) listed H. pylori as an aetiological factor in the pathogenesis of gastric carcinoma (Anwar et al., 1994). As two-thirds of cases of stomach cancer occur in Asia, H. pylori and gastric cancer has been the subject of intensive research in the Asia Pacific region. This chapter focuses on the epidemiology of H. pylori infection and gastric cancer in Asia, strategies for preventing gastric cancer targeted at H. pylori eradication and treatment regimens used in Asia.

2.2 Epidemiology of H. pylori Infection

A study of the epidemiology of H. pylori in the Asia Pacific region reveals that there is a wide variation in the prevalence of H. pylori infection, both between countries and within countries, as well as between ethnic communities within countries. In general, the seroprevalence rates in developing or less-developed countries are higher than in developed countries (Table 2.1). In Iran, the reported H. pylori seroprevalence rate (from children to adults) was 71% (Alizadeh et al., 2009). In India, the reported overall seroprevalence rate was 79% (Graham et al., 1991). In Vietnam, the H. pylori seroprevalence rate was 75% (Hoang et al., 2005). On the other hand, the seroprevalence rates in more developed countries in the Asia Pacific region are generally lower. For example in Australia, the overall seroprevalence rate has been reported to be 15% (Moujaber et al., 2008). In Asian countries that became developed or industrialized in recent years, the seroprevalence rates are higher than in Australia, but still considerably lower than in less-developed countries. Among North-east Asian countries, the overall seroprevalence rate was 58% in China (Wang and Wang, 2003), 39% in Japan (Fujisawa et al., 1999), 55% in Taiwan (Teh et al., 1994) and 60% in South Korea (Yim et al., 2007). Among South-east Asian countries, the reported seroprevalence rate was 36% in Malaysia (Goh and Parasakthi, 2001), 31% in Singapore (Fock, 1997) and 57% in Thailand (Deankanob et al., 2006).

Table 2.1 shows that, in general, countries with a high seroprevalence of H. pylori...
infection have a high or intermediate risk of gastric cancer, with the exceptions of Thailand, India and possibly Vietnam. Much research has been undertaken to understand this phenomenon, variously referred to as the ‘Indian dilemma’, the ‘Thai dilemma’, etc. This phenomenon could be due to differences in host genetic factors or bacterial virulence factors in H. pylori strains prevalent in these regions – a topic addressed in Section 2.4 on molecular epidemiology.

In each country, differences in seroprevalence rates between different geographic regions and also between different ethnic groups have been reported. In Australia, for example, the Anglo-Celtic population has been shown to have a lower seroprevalence rate compared with the Aboriginal population, the rates being 38% (Lin et al., 1998) and 68% (Windsor et al., 2005), respectively. In China, seroprevalence rates have been noted to be higher in regions with higher gastric

### Table 2.1. Seroprevalence of Helicobacter pylori and gastric cancer incidence in Asia.

<table>
<thead>
<tr>
<th>Country/region</th>
<th>H. pylori seroprevalence (%)</th>
<th>Age-standardized incidence rate of gastric cancer (per 100,000)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Low gastric cancer risk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>15.1</td>
<td>9.8</td>
<td>4.1</td>
</tr>
<tr>
<td>India</td>
<td>79</td>
<td>5.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Thailand</td>
<td>57</td>
<td>4.3</td>
<td>2.9</td>
</tr>
<tr>
<td>Intermediate gastric cancer risk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong Kong</td>
<td>58.4</td>
<td>19.3</td>
<td>9.6</td>
</tr>
<tr>
<td>Malaysia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>35.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>26.7–57.8</td>
<td>11.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Malay</td>
<td>11.9–29.3</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Indian</td>
<td>49.4–52.3</td>
<td>12.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Singapore</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>48.3</td>
<td>21.4</td>
<td>10.8</td>
</tr>
<tr>
<td>Malay</td>
<td>27.9</td>
<td>6.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Indian</td>
<td>48.1</td>
<td>7.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Taiwan</td>
<td>54.5</td>
<td>18.6</td>
<td>10.5</td>
</tr>
<tr>
<td>Vietnam</td>
<td>74.6</td>
<td>21.8</td>
<td>10.0</td>
</tr>
<tr>
<td>High gastric cancer risk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>58.07</td>
<td>41.4</td>
<td>19.2</td>
</tr>
<tr>
<td>Japan</td>
<td>39.3</td>
<td>62.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Korea</td>
<td>59.6</td>
<td>69.7</td>
<td>26.8</td>
</tr>
<tr>
<td>Iran</td>
<td>71</td>
<td>51.2</td>
<td>15.4</td>
</tr>
</tbody>
</table>
cancer incidence rates. For example, the seroprevalence of \textit{H. pylori} in Changle, northern China, was reported to be 80% (Wong et al., 1999) compared with 62.5% in Guangzhou, southern China (Chen et al., 2007). In Malaysia, the seroprevalence rate has been reported to be lower in west Malaysia (26–31%) than in east Malaysia (43–55%) (Goh and Parasakthi, 2001). In Taiwan, the highest seroprevalence rate (63%) has been reported in rural areas where the aborigines live and in which gastric cancer rates are highest. This compares with a prevalence rate of 40.5% in urban areas, where gastric cancer rates were lowest (Teh et al., 1994). In Vietnam, differences in \textit{H. pylori} seroprevalence rates have been reported between an urban area (Hanoi; 79%) and a rural area (Hatay; 69%) (Hoang et al., 2005).

While it is generally agreed that \textit{H. pylori} infection has been declining in Asia, the data supporting this impression are limited. In a study from Guangzhou province in China, the overall \textit{H. pylori} seroprevalence rate was reported to have decreased from 62.5 to 47% in the period between 1993 and 2003 (Chen et al., 2007). Similarly, in Japan, the overall seroprevalence rate declined from 73% in 1974 to 55% in 1984 and declined even further to 39% in 1994 (Fujisawa et al., 1999). In South Korea, the seroprevalence rate decreased from 67% in 1998 to 60% in 2005 (Yim et al., 2007). In particular, lower seroprevalence rates have been observed in the younger population.

In Australia, the prevalence rate in those aged 1–4 years has been reported to be 4% but increased to 23% among those aged 50–59 years (Moujaber et al., 2008). In Malaysia, among those aged less than 45 years, reported seroprevalence rates ranged from 25 to 41%, whereas among those aged more than 45 years, the rates ranged from 31 to 57% (Goh and Parasakthi, 2001). In Singapore, the seroprevalence rate in those aged less than 3 years has been reported to be 3%, but rose to 71% among those aged more than 65 years (Fock, 1997). In Taiwan, the seroprevalence rate among subjects less than 10 years of age was 27% compared with 72% in adults older than 40 years (Teh et al., 1994). In a study of asymptomatic subjects from New Delhi, India, a stepwise increase in seroprevalence rate was evident with increasing age. Among subjects less than 10 years old, the rate was 39%, which increased to 52% among those aged 10–19 years and 60% among those aged 20–29 years; by 30–39 years, the seroprevalence rate was 68% (Chen et al., 2007). In Thailand, among those aged 5–9 years, the seroprevalence rate has been reported to be 17.5%, which was found to increase to 75% among those aged 30–49 years (Moodley et al., 2009).

Current evidence would therefore indicate that in most countries in Asia the rate of \textit{H. pylori} infection has been decreasing over the last 40–50 years, with an overall decline in \textit{H. pylori} seroprevalence in Asia similar to that observed in Western developed countries. While the awareness and diagnosis of \textit{H. pylori} infection have led to increased use of eradication therapies, the major decline in \textit{H. pylori} seroprevalence is probably associated with socio-economic development in Asia. With development, there is an improvement in public health measures, personal hygiene and living conditions. Consequently, childhood infections have decreased, leading to a lower seroprevalence rate of \textit{H. pylori} in the younger generations, thus lowering the overall seroprevalence rate in the population.

The decline in \textit{H. pylori} infection in Asia has been matched by a decline in gastric cancer incidence and gastric cancer mortality, although there are uncertainties regarding the quantification of this association. In Japan, for example, with the fall in \textit{H. pylori} prevalence between 1986 and 1996 the fall in gastric cancer mortality has been greater in younger age groups (20–39 years) in comparison with the overall gastric cancer mortality rate, which has long been declining (Kobayashi et al., 2004).

A study in Singapore, where the population comprises immigrants from China and India and the Malays who were early settlers in the area, has demonstrated that Singaporean Chinese and Singaporean Indians have similar \textit{H. pylori} seroprevalence rates and yet have significantly different gastric cancer incidence rates (Ang et al., 2005). The fact that these ethnic groups live on a small island with an area of 704 km\(^2\) makes it ideal for studying host genetic factors, bacterial virulence factors or dietary factors that could influence carcinogenesis.
2.3 Gastric Cancer Epidemiology in Asia

Gastric cancer arises as a consequence of a complex interaction between host factors, *H. pylori* infection and environmental factors. Correa’s hypothesis outlines the steps towards the development of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). Gastric cancer is the fourth most common cancer in the world, with 934,000 new cases diagnosed annually. It is the second most common cancer in terms of mortality, with 700,000 deaths. Two-thirds of the cases are found in Asia and about 42% of patients are from China. The Asia Pacific can be subdivided into regions of high, intermediate and low gastric cancer risk. High-risk regions are mainly localized in East Asian countries such as China, Japan and Korea, where the age-standardized incidence rate (ASR) is greater than 20 per 100,000. The intermediate-risk areas, such as Singapore, Malaysia and Taiwan, have an ASR of 11–20/100,000. Low-risk areas (ASR < 10/100,000) include countries such as India, Australia and New Zealand. As stated above, gastric cancer risk correlates in general with *H. pylori* prevalence, with notable exceptions being the Indian subcontinent and the Indian immigrant populations of the South-east Asian countries of Singapore and Malaysia. The other exception is Thailand, where ethnic Chinese have a higher gastric cancer incidence than indigenous Thais, despite similar *H. pylori* infection rates. Parkin et al. (2002) estimated that about 63% of the gastric cancer cases worldwide are attributable to *H. pylori* infection. In a landmark study, Uemura et al. (2001) prospectively studied 1526 Japanese patients, of whom 1246 had *H. pylori* infection and 280 were uninfected. Subjects underwent endoscopy with biopsy at baseline and between 1 and 3 years after enrolment. Over a mean follow-up period of 7.8 years, gastric cancer developed in 2.9% of patients with *H. pylori* infection, but none of the uninfected patients developed gastric cancer, resulting in a relative risk of 34.5 (95% confidence interval (CI) 7.1, 166.7) for gastric cancer. This Asian study showed conclusively that *H. pylori* is an important aetiological agent in gastric carcinogenesis in Asia.

2.4 Molecular Epidemiology of *H. pylori*

The first complete genome sequence of *H. pylori*, reported in 1997 (Tomb et al., 1997), showed that *H. pylori* strain 26695 has a circular genome of 1,667,867 base pairs and 1590 predicted coding sequences, and that it has well-developed systems for motility, for scavenging iron, and for DNA restriction and modification (see de Reuse et al., Chapter 13, this volume). In addition, multiple adhesins, lipoproteins and other outer membrane proteins (OMPs) have been identified (see Moran, Chapter 10, this volume; Lindén et al., Chapter 12, this volume). There is an immense diversity in *H. pylori* strains in terms of these characteristics. The clinical outcome of *H. pylori* infection is also diverse and includes chronic gastritis, with or without intestinal metaplasia, atrophic gastritis, peptic ulcer disease and gastric cancer. The reason for the differences observed in clinical outcome following *H. pylori* infection have been related to variability in bacterial virulence factors, host genetics (see Sutton et al., Chapter 7, this volume), diet or a combination of these factors.

Multilocus sequence typing of the seven core housekeeping genes (*atpA, efp, mutY, ppa, trpC, ure1, yphC*) of *H. pylori* isolates from different geographic regions has been conducted. On the basis of these analyses, six main geographic strains were identified, termed hpAfrica1, hpAfrica2, hpNEAfrica, hpEastAsia, hpAsia2 and hpEurope (Linz et al., 2007). The hpEurope strain is common in Europe and countries colonized by Europeans, while hpEastAsia characterizes strains from East Asia. The hpEastAsia strain has been further classified into hspMaori (Polynesians), hspAmerind (native Americans) and hspEAsia (East Asia) subpopulations. The hpAsia2 strain was isolated from South and South-east Asia. It has been observed that populations with high gastric cancer rates correspond almost exactly to populations with hpEastAsia strains (Yamaoka et al., 2008). In South Asian countries where *H. pylori* sero-prevalence rates are high but gastric cancer prevalence rates are low, *H. pylori* strains have
been reported to be predominantly hpAsia2. Similarly, in Africa, most strains have been shown to be hpNEAfrica, hpAfrica1 or hpAfrica2, and the gastric cancer rates are also correspondingly lower than in East Asia (Linz et al., 2007).

A number of H. pylori putative virulence genes have been proposed to play key roles in gastric carcinogenesis. These include the genes encoding cytotoxin-associated gene A protein (CagA), vacuolating cytotoxin (VacA) (see Backert et al., Chapter 11, this volume) and OMPs (McNamara and El-Omar, 2008).

The presence of the cagE gene, which is located on the cag pathogenicity island (cagPAI) and has attracted considerable attention, has been associated with gastric cancer in some studies (Erzin et al., 2006; Fock et al., 2006a), but studies that failed to show this association have also been reported (Chomvarin et al., 2008; Ghasemi et al., 2008).

The cagA gene, also situated on the cagPAI, has also been associated with gastric cancer. A meta-analysis of 16 studies (comprising a total of 2284 cases and 2770 controls) showed that infection with CagA-positive strains of H. pylori increased the risk for gastric cancer (Huang et al., 2003). Individually, H. pylori and CagA seropositivity significantly increased the risk for gastric cancer by 2.28- and 2.87-fold, respectively. However, among H. pylori-infected populations, infection with CagA-positive strains further increased the risk for gastric cancer by 1.64-fold (95% CI 1.21, 2.24) overall and by 2.01-fold (95% CI 1.21, 3.32) for non-cardia gastric cancer. In Asia, however, where the prevalence of CagA seropositivity is high, many reports have failed to demonstrate an association between cagA genotypes and gastric cancer.

More recent studies have shown that CagA is characterized by the presence of repeated five-amino-acid sequences (Glu-Pro-Ile-Tyr-Ala), designated EPIYA motifs, that are located at the C terminus of the protein. Four different EPIYA motifs (EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D) have been defined and, based on these, the CagA protein has been classified into Western and East Asian types (see Correa and Piazuelo, Chapter 3, and Backert et al., Chapter 11, this volume). The Western type, prevalent in Europe, America, Australia and Africa, contains EPIYA-A and EPIYA-B, followed by up to five repeated sequences of EPIYA-C, whereas the East Asian strain, which is dominant in Japan, Korea and China, possesses EPIYA-A, EPIYA-B and EPIYA-D (Nguyen et al., 2008). The East Asian strain has been shown to be more virulent than the Western CagA strains with respect to clinical outcomes. Azuma et al. (2004) demonstrated that, in the gastric antrum and body, grades of inflammation, activity and mucosal atrophy are significantly higher in patients infected with East Asian CagA-positive strains than in those infected with Western CagA-positive strains. In addition, Satomi et al. (2006) have shown that in Okinawa, Japan, where both Western and East Asian CagA are present, the prevalence of East Asian CagA-positive strains is significantly higher in patients with gastric cancer (85%) compared with patients with duodenal ulcer (27%), with Western strains predominating in patients with duodenal ulcers. Similar results have been reported by Vilaichone et al. (2004), where both East Asian and South Asian types are found in infected groups in Thailand. The authors showed that 85% of H. pylori isolates from ethnic Chinese people living in Thailand harboured East Asian CagA, whereas only 18% of isolates from indigenous Thais carried East Asian CagA (Vilaichone et al., 2004). Interestingly, gastric cancer is common among Thais of Chinese stock but rare among indigenous Thais. This study supports the theory that the differences in the EPIYA motifs of CagA may be associated with the risk of developing gastric cancer. In Malaysia and Singapore, which have multi-ethnic populations comprising Chinese, Malays and Indians, gastric cancer rates are significantly higher among the Chinese than the Malays and Indians. Interestingly, although the seroprevalence of H. pylori in Chinese and Indians is similar, the gastric cancer incidence in Chinese is three times that in Indians (Ang et al., 2005). A recent collaborative study between Singapore, Malaysia and Australia showed that the East Asian strain was present in 90% of H. pylori strains isolated from Chinese subjects, compared with 38% from Malay subjects and only 7% from Indian subjects (Schmidt et al., 2009). This observation could
explain the lower incidence of gastric cancer in Indian Singaporeans compared with Chinese Singaporeans.

The protein VacA, encoded by the \textit{vacA} gene, is generally thought to be a virulence factor (see Backert \textit{et al.}, Chapter 11, this volume). Genotypic variations in the \textit{vacA} gene have been reported in \textit{H. pylori} strains from different geographic regions and postulated to be associated with different disease outcomes (McNamara and El-Omar, 2008). Variations in the \textit{vacA} gene structure can occur at both the signal region (\textit{s1} and \textit{s2}) and the middle region (\textit{m1} and \textit{m2}). The \textit{s1/m1} genotype is more toxic for a wider range of epithelial cells than is \textit{s1/m2} and gastric cancer patients usually are infected with the \textit{s1/m1} type. The \textit{vacA s2/m2} strains are virtually non-toxic to epithelial cells. All East Asian \textit{H. pylori} strains are \textit{vacA s1}. Within East Asian countries, the \textit{m1} type is predominant in Japan and Korea, which have a higher incidence of gastric cancer. In contrast, the \textit{m2} type is predominant in southern parts of East Asia (Vietnam, Hong Kong), where the incidence of gastric cancer is relatively lower. This suggests that the \textit{s1/m1} type could be more virulent than the \textit{s1/m2} type. The \textit{vacA s1/m2} genotype is also predominant in South Asia, where the incidence of gastric cancer is lower (Yamaoka \textit{et al.}, 2008). However, a comparative study from Singapore that compared the prevalence rate of \textit{vacA s1/m1} genotypes in Chinese subjects with gastric cancer, and in patients with functional dyspepsia, failed to show any difference between the two groups (Fock \textit{et al.}, 2006a). This was a cross-sectional study and it is possible that infected patients with functional dyspepsia could develop gastric cancer with increasing age.

Genomic differences in the structure of the \textit{OipA} gene, encoding outer inflammatory protein A, have been noted between East Asian strains and Western strains of \textit{H. pylori} and may account for the geographic differences in gastric cancer rates (Fock \textit{et al.}, 2006b; Yamaoka \textit{et al.}, 2008). Functional and intracellular signalling differences associated with AlpAB adhesin have also been observed between Western and East Asian strains (Lu \textit{et al.}, 2007). Currently it is not established with certainty which of these genotypes of OMPs is crucial for gastric carcinogenesis.

## 2.5 Host Factors

Scientific evidence clearly supports the importance of host factors in gastric cancer pathogenesis (for details see Sutton \textit{et al.}, Chapter 7, this volume). The risk of developing gastric cancer is increased up to threefold in individuals with an immediate relative suffering from gastric cancer, and 10% of cases of gastric cancer show familial clustering (Fox and Wang, 2007). Strong associations have been shown between host genetic polymorphisms in the interleukin (IL)-1β gene cluster (El-Omar \textit{et al.}, 2000; Machado \textit{et al.}, 2001; Furuta \textit{et al.}, 2004; Palli \textit{et al.}, 2005), tumour necrosis factor-α, IL-10 (El-Omar \textit{et al.}, 2003) and in the IL-8 promoter (Lee \textit{et al.}, 2005; Taguchi \textit{et al.}, 2005), and the risk of gastric cancer in Caucasian populations. In contrast, results from studies conducted in Asia are conflicting, with a meta-analysis examining the role of polymorphisms in the genes encoding IL-1β and IL-1 receptor antagonists in gastric cancer risk showing an association in Caucasians but not in Asians (Camargo \textit{et al.}, 2006).

## 2.6 \textit{H. pylori} Eradication as a Strategy for Primary Prevention of Gastric Cancer in Asia

Because \textit{H. pylori} infection accounts for two-thirds of gastric cancer cases globally, it would seem logical to prevent gastric cancer by eradicating this bacterium in the community. Public health measures such as improvements in sanitation and water supply and better housing to reduce overcrowding, while not specifically targeting \textit{H. pylori}, have resulted in the reduction of this infection in many countries over the period following World War II. While the development of a vaccine for mass vaccination would appear a logical step, this approach has proved to be elusive owing to difficulties encountered in vaccine development (see Blanchard and Nedrud, Chapter 9, this volume).

Antimicrobial eradication of \textit{H. pylori} infection in patients with gastric cancer has been examined as a possible approach to reduce the recurrence rate of early gastric
cancer in patients (see Mégraud, Chapter 4, this volume). The first study to provide evidence that \textit{H. pylori} eradication had a direct impact on gastric recurrence was reported by Uemura \textit{et al.} (1997). In this non-randomized trial, patients with early gastric cancer treated by endoscopic resection were offered \textit{H. pylori} eradication therapy. During a 3-year follow-up, metachronous gastric cancer developed in 9% of the untreated group, as compared with 0% in patients treated with \textit{H. pylori} eradication therapy (Uemura \textit{et al.}, 1997). Since then, several randomized trials have been conducted in China and Japan.

At a consensus meeting convened to discuss strategies to prevent gastric cancer in the Asia Pacific region, a group of experts recommended a test-and-treat approach for \textit{H. pylori} infection in asymptomatic adults residing in high gastric cancer risk areas and communities, to prevent gastric cancer (Fock \textit{et al.}, 2008). This recommendation was based on a meta-analysis of randomized controlled studies of \textit{H. pylori} eradication conducted in Asia that showed a reduced gastric cancer incidence (odds ratio = 0.56; 95 % CI 0.4, 0.8) following \textit{H. pylori} eradication (Fock \textit{et al.}, 2008; Moayyedi \textit{et al.}, 2008). The consensus report emphasized that this practice should not replace the current practice of gastric cancer surveillance in two East Asian countries (namely Japan and Korea) as well as in Matsu, Taiwan. Based on a number of studies that have suggested that eradication of \textit{H. pylori} may be either ineffective or less effective in people with precancerous lesions such as intestinal metaplasia and atrophic gastritis, the group further recommended that the age to commence this screen-and-treat strategy was approximately 10 years, prior to the rise in the ASR of gastric cancer. In addition to preventing gastric cancer, eradication of \textit{H. pylori} in the adult population should also reduce the rate of peptic ulcer disease and its associated complications. Furthermore, data would suggest that a reduction in dyspeptic symptoms and functional dyspepsia would be likely to occur (Ford \textit{et al.}, 2005).

There are some concerns, however, that such an approach may increase the risk of other diseases, which might outweigh any potential benefits of a population screen-and-treat strategy (see Mitchell \textit{et al.}, Chapter 5, this volume). While there have been a number of studies suggesting that \textit{H. pylori} eradication increases the risk of gastro-oesophageal reflux, the preponderance of evidence based on meta-analyses of \textit{H. pylori} eradication and post hoc analyses of peptic ulcer trials indicates that \textit{H. pylori} does not lead to the development of erosive oesophagitis or new symptomatic reflux (Malfertheiner \textit{et al.}, 2002; Vakil \textit{et al.}, 2006). Furthermore, studies within the general population support the view that \textit{H. pylori} is unlikely to increase significantly the risk of reflux symptoms or Barrett's oesophagus and oesophageal adenocarcinoma (Moayyedi \textit{et al.}, 2001; Moayyedi and Talley, 2006).

A further concern is that antibiotic resistance to \textit{H. pylori} in the community could further increase; however, given that most treatments are for 1 week, the impact is likely to be limited.

### 2.7 Treatment Regimens for \textit{H. pylori} Infection in Asia

In Asia, the first-line therapy for \textit{H. pylori} infection is a proton pump inhibitor (PPI)-based triple therapy using a combination of amoxicillin and clarithromycin for 7 days. While metronidazole has been an acceptable alternative to amoxicillin with similar eradication rates, it has not been as widely used in Asia where the amoxicillin-containing combinations are preferred due to high metronidazole resistance rates in the region, and regulatory restrictions exist in some areas such as Japan. In patients who have penicillin allergy, metronidazole is issued in place of amoxicillin, or a bismuth-based quadruple therapy is used. In parts of Asia there are increasing rates of primary resistance to clarithromycin. For example, in Japan it has been reported that resistance to clarithromycin increased from 19 to 28% between 2002 and 2005 (Kobayashi \textit{et al.}, 2007). In Korea, primary resistance increased to 14% in 2003 and in China to 18% in 2002 (Kim \textit{et al.}, 2004; Cheng and Hu, 2005). Some data indicate that primary resistance to clarithromycin reduces treatment efficacy by 66% in the PPI, amoxicillin and clarithromycin
combination. Metronidazole resistance reduces the efficacy of the PPI, amoxicillin and metronidazole combination by 30%, and the PPI, clarithromycin and metronidazole combination by 18% (Fischbach and Evans, 2007). Bismuth-based quadruple therapy is an effective alternative to PPI-based triple therapy, but this regimen is not available in some Asian countries. At the second Asia Pacific consensus on the management of *H. pylori* infection, the group recommended the following salvage therapy following failed PPI-based triple therapy: (i) a standard triple therapy that has not been used previously; (ii) bismuth-based quadruple therapy; (iii) levofloxacin-based triple therapy. The choice of salvage therapy depends on the local pattern of antibiotic resistance, drug availability and the local prevalence of tuberculosis in the context of rifabutin use. The group considered that, at that time, there were insufficient data to recommend sequential therapy as an alternative first-line therapy in Asia. Sequential therapy consists of a 5-day treatment of a PPI and one antibiotic (usually amoxicillin) followed by a 5-day treatment with the same PPI and two antibiotics (usually clarithromycin and metronidazole). This strategy has generated much interest, as data suggest that eradication rates were as good, if not better, than those achieved with triple therapy.

### 2.8 Conclusions

In conclusion, the role of *H. pylori* in the pathogenesis of peptic ulcer disease was first discovered 25 years ago in Australasia, and its role in gastric carcinogenesis was recognized by the IARC in 1994. Since then, and with the development of eradication therapy, *H. pylori*-positive peptic ulcer disease has seen a decline in Asia. Gastric cancer too has declined in incidence, although it is still a major health issue in Asia. It remains to be seen if *H. pylori* eradication through a population search-and-treat strategy, combined with public health measures to improve sanitation and living standards, could eliminate this dreaded disease with a high mortality from Asia.

### References


Satomi, S., Yamakawa, A., Matsunaga, S., Masaki, R., Suto, H., Ito, Y., Yamazaki, Y., Kuriyama, M.,


3 Gastric Adenocarcinoma

P. Correa* and M.B. Piazuelo

3.1 Introduction

Gastric adenocarcinoma has been considered an infectious disease since 1994, when the International Agency for Research on Cancer categorized *Helicobacter pylori* infection as a class I human carcinogen (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). The pathogenesis of gastric adenocarcinoma represents a prototype for bacteria-induced and inflammation-driven malignancies. While *H. pylori* infection plays a primary role, other environmental and host factors modulate its outcome. Gastric cancer is a major cause of global morbidity and mortality. It is the fourth most common cancer worldwide, after cancers of the lung, breast and colorectum. In 2002, there were approximately 934,000 new cases of gastric cancer, accounting for 9% of all new cancers, and 700,000 deaths, making it second only to lung cancer as a cause of cancer death (Table 3.1) (Parkin et al., 2005).

Although the incidence of gastric cancer is declining in many countries (Jemal et al., 2008), the global burden is rising. Approximately two-thirds of the cases occur in developing countries, with a wide variation in incidence rates worldwide. The highest incidence rates are reported in Eastern Asia, Central and Eastern Europe, and Central and South America (Parkin et al., 2005). Most cases of gastric adenocarcinoma are diagnosed when the tumour has invaded the muscularis propria. Overall, the 5-year survival rate for gastric cancer is under 20%. In Japan, where gastric cancer is the most common cause of cancer-related deaths, mass screening programmes for gastric cancer are well developed. As a result, more than 70% of gastric cancers are diagnosed at an early stage, providing a 5-year survival rate of over 90% (Miyahara et al., 2007). During recent decades, the relative proportion of carcinomas originating in the gastric cardia has increased (Blot et al., 1991; Devesa et al., 1998; Newnham et al., 2003). However, this matter is controversial due to the difficulty of distinguishing tumours originating in the distal oesophagus, gastro-oesophageal junction and gastric cardia. El-Serag et al. (2002) reported that in the USA the incidence of adenocarcinoma of the cardia peaked in 1991 and has subsequently declined steadily. Approximately 90% of stomach cancers are adenocarcinomas (Coleman et al., 1993). The model for development of the most frequent type of gastric adenocarcinoma (intestinal type) consists of the sequential changes in the gastric mucosa shown in Fig. 3.1 (Correa et al., 1975). This model is discussed below.

Gastric Adenocarcinoma

3.2 Aetiology

The aetiology of gastric adenocarcinoma eluded scientists for centuries. During most of the 20th century, the prevalent hypotheses were focused on chemical carcinogens, especially those possibly ingested in the diet. After numerous attempts at identifying chemical carcinogens in foods, especially fish and meat, no consistent findings were reported in populations at high risk throughout the world. A search for contaminants in food provided limited information which was not generally applicable to most high-risk populations. One dietary item associated with increased cancer risk across populations is the excessive intake of salt (Joossens et al., 1996; Tsugane, 2005; Tsugane and Sasazuki, 2007). The role of salt intake in cancer aetiology is in general not considered to be that of a primary (genotoxic) carcinogen, but more probably that of an adjuvant that may increase cancer risk by indirect mechanisms such as enhancing cell replication (Charnley and Tannenbaum, 1985). In addition, co-culture of gastric epithelial cells with *H. pylori* grown under high salt conditions has shown an increase in tyrosine-phosphorylated cytotoxin-associated gene A protein (CagA) and in secretion of the pro-inflammatory cytokine interleukin (IL)-8 by the epithelial cells compared with co-culture of the cells and *H. pylori* grown under low salt conditions (Loh et al., 2007). Another recognized role of diet in gastric carcinoma is the protective effect of adequate intake of fresh

<table>
<thead>
<tr>
<th></th>
<th>New cases</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1,352,132</td>
<td>1,178,918</td>
</tr>
<tr>
<td>Stomach</td>
<td>933,937</td>
<td>700,349</td>
</tr>
<tr>
<td>Liver</td>
<td>626,162</td>
<td>598,321</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>1,023,152</td>
<td>528,978</td>
</tr>
<tr>
<td>Breast</td>
<td>1,151,298</td>
<td>410,712</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>462,117</td>
<td>385,892</td>
</tr>
<tr>
<td>Cervix and uteri</td>
<td>493,243</td>
<td>273,505</td>
</tr>
<tr>
<td>Pancreas</td>
<td>232,306</td>
<td>227,023</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>300,522</td>
<td>222,506</td>
</tr>
<tr>
<td>Prostate</td>
<td>679,023</td>
<td>221,002</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>300,571</td>
<td>171,820</td>
</tr>
<tr>
<td>Bladder</td>
<td>356,557</td>
<td>145,009</td>
</tr>
<tr>
<td>All sites but skin</td>
<td>10,862,496</td>
<td>6,723,887</td>
</tr>
</tbody>
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Fig. 3.1. Gastric precancerous multistep model. (Adapted from Correa et al., 1975.)
fruits and vegetables (González et al., 2006; Larsson et al., 2006). Again it is not considered to have a primary role, but rather one of ameliorating the effects of another (as yet unknown) carcinogen.

That rather hopeless panorama changed drastically when Warren and Marshall published their seminal letter in The Lancet in 1983 (Warren and Marshall, 1983). Although the main focus of these letters addressed the effects of H. pylori infection on non-neoplastic outcomes such as peptic ulcers, a mention was made, in passing, that the infection could play a role in gastric cancer causation. The debate that followed was mostly settled by the International Agency for Research on Cancer report (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Although several viral infections and one parasitic infection play a role in cancer causation, up to now H. pylori is the only bacterium directly implicated in cancer causation in man. Furthermore, H. pylori infection is the single most important cause of infection-associated cancer globally, contributing to 5.5% of the global cancer burden, approximately 25% of all infection-associated cancers, and 63% of all stomach cancer (Parkin, 2006). The long-term decline in gastric cancer mortality in developed countries has resulted, in part, from interrupting H. pylori transmission through improved basic sanitation, housing and socio-economic status. H. pylori infection plays a primary role, but its effects are modulated by other factors, mainly related to environmental forces and host genetic susceptibility (see Sutton et al., Chapter 7, this volume). Approximately one-half of the world’s population is infected with this bacterium, but only a very small fraction of infected people will develop cancer. Gastric cancer incidence rates differ considerably worldwide, from about 70 per 100,000 per annum in high-risk countries, to less than five per 100,000 per annum in low-risk populations (Ferlay et al., 2004).

The model of gastric cancer causation is outlined in Fig. 3.2 (Correa et al., 2004). In this model, the primary neoplastic stimulus is provided by the H. pylori infection, which can be intense with more virulent bacteria and mild with the less virulent strains. The outcome of the infection is modulated by the genetic susceptibility of the host, mostly documented for the inflammatory cytokine polymorphisms; in particular, polymorphisms of the gene encoding IL-1β (IL1B) have been associated with gastric cancer in some populations but not in others (El-Omar et al., 2000; Lee et al., 2003; Machado et al. 2003; Alpizar-Alpizar et al., 2005; Sicinschi et al., 2006). Two meta-analyses concluded that the IL1B-511T genotype was associated with gastric cancer susceptibility in Caucasian populations, but not in Asians (Camargo et al., 2006; Loh et al., 2009). IL-1β is a proinflammatory cytokine induced by H. pylori infection and is a powerful inhibitor of gastric acid secretion. It promotes hypochlorhydria, favouring further colonization of H. pylori and a more severe gastritis. Over decades, gastric atrophy and adenocarcinoma may develop (El-Omar, 2001). In addition, there is evidence showing that, for subjects with the IL1B-511T genotype and infected with more virulent H. pylori strains, the gastric cancer risk is 87 times greater than for subjects with genotype IL1B-511CC and infected with less virulent H. pylori strains (Figueiredo et al., 2002).

Environmental influences are of several kinds. Socio-economic conditions may determine the timing and severity of the first infection, usually during childhood in developing countries (Goodman and Correa, 1995, 2000; Camargo et al., 2004). Co-infections with parasites, more prevalent in certain lower socio-economic strata, may modulate the immune reaction to the bacterial infection towards an anti-inflammatory T-helper (Th) type 2 response (Whary and Fox, 2004; Whary et al., 2005), which possibly lowers the risk of a neoplastic outcome.

A well-established additional risk factor for gastric cancer is cigarette smoking. A significant dose-dependent association between tobacco smoke and gastric cancer risk has been demonstrated (González et al., 2003; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Nishino et al., 2006). In the European Prospective Investigation into Cancer and Nutrition cohort, it was estimated that 17.6% of gastric cancer in this European population was attributable to cigarette smoking (González et al., 2003).
Another infectious agent associated with gastric cancer is Epstein–Barr virus (EBV). EBV has been also classified as a class I oncogenic pathogen by the World Health Organization (WHO) and has been strongly linked to the development of nasopharyngeal carcinoma, Burkitt’s lymphoma and Hodgkin’s disease. For gastric carcinogenesis, the pathogenic role of EBV remains to be established. Multiple studies around the world have identified the presence of EBV in the malignant cells of approximately 9% of cases (Lee et al., 2009). EBV-associated adenocarcinomas are predominant in men and in younger individuals (Truong et al., 2009), develop more frequently in the proximal stomach (cardia or corpus) and generally show the diffuse histological type. EBV is closely associated with remnant (post-gastrectomy) cancer and highly prevalent in the uncommon lymphoepithelial carcinoma. There is no association between EBV-positive adenocarcinomas and H. pylori infection (Lee et al., 2009).

The interaction of the factors mentioned above, as outlined in Fig. 3.2, may determine if the gastric inflammation follows the path of non-atrophic gastritis, which is not associated with elevated risk of gastric cancer, or that of multifocal atrophic gastritis, the initial step in the precancerous process.

![Fig. 3.2. Schematic representation of bacterial, human and environmental factors that may interact and influence the clinical outcome of Helicobacter pylori infection. Abbreviations/gene names: cagA, cytoxin-associated gene A; CCND1, cyclin D1; CDH1, E-cadherin; GSTM, glutathione S-transferase μ; GSTT1, glutathione S-transferase θ1; IL1B, IL10 and IL8, interleukins-1β, -10 and -8; MTHFR, 5,10-methylenetetrahydrofolate reductase; NAT1 and NAT2, N-acetyl transferases 1 and 2; GSTT1, glutathione S-transferase θ1; vacA, vacuolating cytotoxin; XRCC1, X-ray repair cross-complementing protein 1. (Adapted from Correa et al., 2004, with permission.)](image-url)
3.3 Pathology

3.3.1 Macroscopy

Anatomically, gastric adenocarcinomas are classified as cardia or non-cardia (distal). Non-cardia adenocarcinomas most commonly arise in the antrum–corpus junction of the lesser curvature, the incisura angularis, and may disseminate to the antrum or the corpus, following the topographic distribution observed for precancerous lesions (Correa et al., 1970). The macroscopic appearance of early gastric cancer (described below) follows the classification established by the Japanese Endoscopic Society (Japanese Gastric Cancer Association, 1998). Advanced gastric carcinomas are grossly classified according to the Borrmann classification: type I for well-circumscribed polypoid or fungating lesions, type II for polypoid tumours with marked central ulceration, type III for the ulcerated tumours with infiltrative margins and type IV for diffusely infiltrating (linitis plastica) tumours (Lewin and Appelman, 1996).

3.3.2 Early gastric cancer

Early gastric carcinomas are defined as invasive adenocarcinomas in which the invasion is limited to the mucosa or submucosa, with or without lymph node metastasis (Lewin and Appelman, 1996). Early carcinomas may be multiple, synchronous or metachronous. Penetration of the muscularis mucosa is the criterion for sub-classifying early gastric cancer into intramucosal and submucosal types. Improvement in diagnostic techniques worldwide and implementation of screening programmes have resulted in more frequent diagnoses of gastric cancer at this stage. Gastric cancers can be classified endoscopically according to the growth pattern. The Japanese classification defines the following types: I, protruded or polypoid; II, superficial; IIa, elevated; IIb, flat; IIc, depressed; III, excavated (Japanese Gastric Cancer Association, 1998). In eastern Asia, up to 70% of all gastric cancers are diagnosed as early gastric cancers (due to mass population screening), whereas in the western hemisphere, the proportion of early gastric cancers is only about 15% (Siewert, 2005; Oda et al., 2006). In Japan, the 5-year survival rate following resection of early gastric cancer reaches 90–100% (Yamamoto and Kita, 2005).

3.3.3 Adenocarcinoma of the gastric cardia

Because of its borderline location between the oesophagus and stomach, many discrepancies exist in the literature regarding the cause and classification of these tumours. According to internationally accepted criteria, three entities are included under the term adenocarcinoma of the oesophagogastric junction (AEG): adenocarcinoma of the distal oesophagus (AEG I), true carcinoma of the cardia (AEG II) and sub-cardial gastric carcinoma (AEG III) (Siewert and Stein, 1998). The association between H. pylori and gastric cancer is stronger for neoplasias involving the distal stomach or non-cardia than for those involving the proximal stomach or cardia (Lochhead and El-Omar, 2008). In addition, a stronger association exists for cardia adenocarcinomas and EBV than for distal tumours (Lee et al., 2009).

3.3.4 Histopathology

Gastric adenocarcinomas display high histological variability. They may be composed of tubular, acinar or papillary structures, or consist of a mixture of discohesive, isolated cells with variable morphologies, sometimes in combination with glandular, trabecular or alveolar solid structures. Mucin production is variable and pools of extracellular mucin may be present. Several classification systems have been proposed, but the most frequently used are the WHO and Laurén classifications. The WHO classification (Hamilton and Aaltonen, 2000) includes the following categories: papillary, tubular, signet-ring cell, mucinous and undifferentiated. The prevailing histological classification, which originated in the studies published by Laurén and Jarvi, characterizes
two main types, intestinal and diffuse, based on their display of features resembling intestinal mucosa (Jarvi and Laurén, 1951; Laurén, 1965). The two types follow distinct precancerous processes and have different clinical and epidemiological implications.

**Intestinal-type adenocarcinoma**

Intestinal-type gastric cancer is believed to develop through a sequence of steps involving recognized precancerous lesions. Chronic infection with *H. pylori* is the main recognized aetiological factor. The intestinal type is most common in men and in the elderly, and has a better prognosis than the diffuse type.

Non-cardia gastric adenocarcinoma is most commonly of the intestinal type.

**Precancerous process.** The widely accepted model for development of gastric adenocarcinoma of the intestinal type consists of the following sequential changes in the gastric mucosa: chronic active gastritis, atrophy, intestinal metaplasia and dysplasia (Fig. 3.1) (Correa et al., 1975; Correa, 1992). This process usually takes decades after persistent infection with *H. pylori*. Upon colonization of the gastric mucosa, *H. pylori* induces an immune response that attracts abundant polymorphonuclear neutrophils and mononuclear inflammatory cells to the lamina propria. The well-orchestrated inflammatory response is mediated by cytokines, chemokines and other mediators, which facilitate chemotraction of haematopoietic cells, recruit effector cells and determine the natural history of the infection (Peek and Crabtree, 2006). Unless treated and eradicated, the infection becomes chronic, leading initially to non-atrophic chronic gastritis. The localization of *H. pylori* colonization within the stomach is mostly determined by local acid production. Initially, *H. pylori* colonizes the antral mucosa, probably because the acid secretion in the corpus inhibits colonization. Acid suppression, either by use of pharmacological agents or surgical intervention (vagotomy), leads to the extension of *H. pylori* colonization to the corpus and fundus. The same effect in colonization may also be seen in genetic or other medical conditions with reduced acid output (Lee et al., 1995).

In some patients, the infection may result in ulcer formation. Subjects who develop duodenal ulcers are not at increased risk of gastric cancer (Uemura et al., 2001). In contrast, subjects with gastric ulcers typically have multifocal atrophic gastritis and high risk of gastric cancer (Hansson et al., 1996; Uemura et al., 2001). In *H. pylori*-associated gastritis, alterations in the cell cycle have been documented (Peek et al., 1997, 2000; Moss et al., 2001; Crabtree et al., 2004). Imbalance between proliferation and apoptosis may lead to progressive loss (atrophy) of gastric glands. The development of atrophy starts in small foci in the mucosa, so-called multifocal atrophic gastritis. As atrophy increases, foci coalesce covering more extensive areas of the mucosa, and the lamina propria may show proliferation of fibrocollagenous tissue.

Some of the glands may then be replaced by epithelium with intestinal morphology, a stage called intestinal metaplasia. The classification of intestinal metaplasia most widely used consists of complete and incomplete types, based on the preservation or lack, respectively, of digestive enzymes such as sucrase, trehalase and alkaline phosphatase (Matsukura et al., 1980). Jass and Filipe (1981) proposed the following classification, according to the phenotypic characteristics. Type I (complete) is characterized by the presence of absorptive enterocytes, Paneth cells and goblet cells secreting sialomucins, and corresponds to the small intestine phenotype (Fig. 3.3A). Types II and III (incomplete type) are characterized by more irregular glands, with cells containing multiple mucus vacuoles of different sizes, without a brush border and lacking absorptive enterocytes. Metaplastic cells secrete sialomucins and/or sulfomucins: neutral and acid sialomucins in type II and sulfomucins in type III. Studies have shown that type I does not increase the risk of malignant transformation, whereas type III is associated with an increased risk of developing adenocarcinoma (Rokkas et al., 1991; Filipe et al., 1994).

A small proportion of subjects with intestinal metaplasia will develop dysplasia (also called intraepithelial neoplasia), which is subclassified as low grade and high grade, according to the degree of cytological and
architectural alterations. In low-grade dysplasia, epithelial nuclei are elongated (sometimes with a cigar-like appearance), present slight pseudostratification and maintain polarity with respect to the basement membrane. The glands may show some irregularity in shape and size (Fig. 3.3B). In high-grade dysplasia (a term equivalent to carcinoma in situ), there is severe cellular and architectural atypia. Cigar-like, pseudostratified nuclei alternate with rounded nuclei that show loss of polarity. Glandular structures may be markedly irregular and often show branching and folding (Rugge et al., 2000). Differences between Western and Japanese pathologists exist in this category. For Western pathologists, invasion to the lamina propria is the hallmark of carcinoma and is the criterion considered by the authors in this chapter. For Japanese pathologists, the presence of neoplastic epithelium, even in the absence of proven invasion, is grounds for a diagnosis of carcinoma. The Padova classification provides equivalent terms to accommodate both Western and Japanese classifications (Rugge et al., 2000). Histologically, intestinal-type adenocarcinomas are composed of cohesive tumoural epithelial cells forming acinar or tubular structures often resembling intestinal epithelium, infiltrating the gastric wall (Fig. 3.4A). Sometimes, the tumoural cells form papillary structures or clusters. The tumours exhibit a variable degree of differentiation.

Fig. 3.3. Microphotographs of gastric precancerous lesions. (A) Intestinal metaplasia, complete type: glands with alternating goblet cells and absorptive enterocytes. (B) Low-grade dysplasia originating in intestinal metaplasia: nuclei are enlarged, hyperchromatic and crowded, but maintain the polarity.

Fig. 3.4. Microphotographs of gastric adenocarcinoma. (A) Intestinal type: cohesive tumour cells forming irregular tubular structures that resemble the intestinal epithelium. (B) Diffuse type: non-cohesive tumour cells with large, irregular and hyperchromatic nuclei infiltrate diffusely into the lamina propria. There are scattered signet-ring cells containing intracytoplasmic mucin that push the nuclei to the periphery (arrows).
Well-differentiated adenocarcinomas display well-formed glands, closely resembling metaplastic intestinal epithelium. Moderately differentiated adenocarcinomas exhibit intermediate characteristics between well differentiated and poorly differentiated. Poorly differentiated adenocarcinomas are composed of highly irregular glands that are recognized with difficulty, or cells arranged in small or large clusters with mucin secretion. In general, intestinal-type adenocarcinomas are associated with intestinal metaplasia of the surrounding mucosa.

**Diffuse-type adenocarcinoma**

Diffuse-type adenocarcinoma is relatively more frequent in women and younger patients and in populations at low risk of gastric cancer, and occurs more commonly in the body of the stomach. Its development does not follow the sequential steps outlined above for the intestinal type. Genetic predisposition seems to play an important role. Although environmental agents seem to play a less important role than genetic factors, *H. pylori* infection has been also associated with the development of diffuse-type adenocarcinoma (Parsonnet et al., 1997; Rugge et al., 1999). Microscopically, diffuse-type gastric adenocarcinomas are composed of poorly differentiated tumour cells lacking cohesion that diffusely infiltrate the gastric wall. In some cases, signet-ring cells containing intracytoplasmic mucin and nuclei flattened against the side of the cell are observed (Fig. 3.4B). In general, diffuse-type adenocarcinomas show a great propensity to spread locally and have poorer prognosis than the intestinal-type tumours. Within this group, a well-characterized syndrome, hereditary diffuse gastric cancer, is an autosomal dominant disorder that accounts for less than 1% of all cases of gastric cancer. It was initially described in three New Zealand Maori families with early-onset diffuse gastric cancer (Guilford et al., 1998). Since then, at least 151 families have been reported in different countries (Kaurah et al., 2007; Norton et al., 2007; Carneiro et al., 2008). This syndrome is associated with germline mutations in the gene encoding E-cadherin (*CDH1*) in 30–40% of the families. Although uncommon, this disease constitutes an important health issue due to its severity, high penetrance, early age at presentation and the unavailability of effective screening tools (Lynch et al., 2008).

### 3.4 The *H. pylori*/Gastric Cancer Enigmas

Although *H. pylori* infection has been recognized as a class I carcinogen, incongruence between infection prevalence and gastric cancer incidence rates has been reported. Holcombe (1992) called attention to the high prevalence of infection in the face of low cancer rates, which he called the ‘African enigma’. Similar observations have been made in other geographic areas. Miwa et al. (2002) compared the infection prevalence rates with the cancer incidence rates in Asia and called attention to the fact that in some countries (mostly northern, like Japan and Korea) both rates are high, while in others (mostly southern, like Thailand and Bangladesh) infection prevalence rates are high but cancer rates are low. In larger countries, like India and China, intra-country contrasts in subpopulations may reflect the two situations. In addition, the immune response to *H. pylori* infection, in terms of immunoglobulin (Ig) G subclass, has been compared between the population of Soweto, South Africa, and populations of Germany and Australia (Mitchell et al., 2002). The IgG1/IgG2 ratio was significantly lower in Germans and Australians than in Sowetans, reflecting a Th1 immune response bias in the former and a Th2 bias in the latter. Obviously, the Germans and Australians represent wealthy (‘developed’) societies, while the Sowetans represent lower socio-economic (‘developing’) societies. The north–south geographical gradient reported in Asia is also applicable to the comparison between Germans and Sowetans. Climatic conditions are known to influence helminthic infections, which have an extracorporeal component in their life cycle. Warm climates with abundant rainfall favour helminthic life cycles (Birrie et al., 1994). Dietary patterns are usually different in
populations with contrasting gastric cancer risks. In Colombia, South America, high-altitude inhabitants of the Andes Mountains have a very high prevalence of infection and very high gastric cancer incidence rates. By contrast, inhabitants of the Pacific coast, who also have a very high prevalence of infection, have very low gastric cancer rates (Correa et al., 1976). The diet in the mountains is rich in starchy foods like potatoes, while coastal dwellers consume abundantly fresh fish. A survey of intestinal parasites reported a very much higher prevalence, especially of helminths, in the coastal compared with the mountain inhabitants. Blood levels of IgE, a marker of Th2 response, were much higher in the coastal than in the mountain inhabitants (Whary et al. 2005). Gastric mucosa biopsies from both populations revealed that eosinophilic infiltration (a marker of allergic or Th2 response) was much more prominent in inhabitants of the coast (Piazuelo et al., 2008). A Th2 bias in immunological response has been reported in Chinese patients infected with Schistosoma japonicum. In those subjects, IgG1/IgG2 ratios were 5.88, higher than the 4.0 ratio observed in patients without the parasite (Du et al., 2006). Experimental evidence has been provided by Fox et al. (2000) in mice co-infected with Helicobacter felis and the nematode Heligmosomoides polygyrus Their study showed that the immune reaction to the bacterial infection was biased towards a Th2 response by the nematode co-infection, which was associated with a considerable reduction in Helicobacter-induced gastric atrophy, a known premalignant lesion. Taken together, the available data indicate that the predominant anti-inflammatory Th2 response to H. pylori, probably brought about by helminthic infections, may prevent the progression of the gastric precancerous process to more advanced lesions such as intestinal metaplasia and dysplasia.

Variations in the virulence potential of different H. pylori strains have also been studied as a possible explanation for the discrepancy between cancer rates and infection prevalence (Yamaoka et al., 2008). Several virulence-associated genes have been identified. A major focus of enquiry has been the cag pathogenicity island (PAI), an approximately 40 kb locus containing 31 genes, inserted in the glutamate racemase gene. The marker of the island is the cytotoxin-associated gene cagA (Covacci et al., 1993; Tummuru et al., 1993; Censini et al., 1996). Other genes in the island code for a type IV secretion system, a syringe-like structure that injects CagA and probably other proteins into the cytoplasm of the gastric epithelial cells (see Backert et al., Chapter 11, this volume). The geographic distribution of different H. pylori genotypes led Covacci et al. (1999) to suggest co-evolution of man and H. pylori species. According to their hypothesis, H. pylori travelled along with man during major migrations. On the basis of nucleotide sequences, Covacci et al. (1999) were able to differentiate Asian from European and African strains. The genetic geography of H. pylori coincides with human populations and supports the hypothesis that H. pylori was already established in the human stomach at least 100,000 years ago, before intercontinental migrations. The antiquity of human infection was corroborated using carbon dating of South American mummies, 800–3000 years old, found colonized with Helicobacter species (Allison et al., 1999).

Several studies have shown that cagA-positive bacteria lead to more inflammation and more severe outcomes, including peptic ulcer and gastric carcinoma (Blaser et al., 1995; Peek et al., 1995; Parsonnet et al., 1997). It has been suggested that cagA-positive genotypes may partially explain the African and related enigmas (Yamaoka et al., 2008). In Latin America, cagA positivity may partially explain the enigmas. The prevalence of cagA-positive strains in Colombian communities with a high risk for gastric cancer was shown to be higher than in low-risk communities: 90.4% versus 81.1% (Bravo et al., 2002). Patients from a high-risk area in Costa Rica were reported to have a higher proportion of cagA-positive strains than patients from a low-risk area: 78.8% versus 53.6% (Con et al., 2006). This difference is not found in most Asian countries, where the prevalence of cagA positivity is almost 100% (Hatakeyama, 2009).

A similar paradox is the dichotomy outlined in Fig. 3.2, namely non-atrophic gastritis, which does not increase gastric
cancer risk, versus multifocal atrophic gastritis–intestinal metaplasia, which is clearly a marker of a precancerous process. The paradox is clearly demonstrated in patients with duodenal ulcer, causally associated with virulent \textit{H. pylori} genotypes: most duodenal ulcer patients carry \textit{cagA}-positive, \textit{vacA} \textit{s1/m1} bacteria. Although patients with duodenal ulcer may have antral gastritis for decades, the lesion remains non-atrophic in nature. Such patients are not at increased gastric cancer risk when compared with the general population (Hansson et al., 1996).

The \textit{CagA} protein of \textit{H. pylori} isolates from different populations exhibit diverse sequence polymorphisms in their C-terminal regions containing EPIYA (glutamic acid–proline–isoleucine–tyrosine–alanine) motifs (see Backert et al., Chapter 11, this volume). According to the amino acid sequences surrounding those motifs, the segments are designated EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D. Most isolates contain EPIYA-A and EPIYA-B motifs. The EPIYA-C motifs, most typically present in one to three copies, characterize the denominated Western \textit{CagA}, present in isolates from European, American and Australian patients. The EPIYA-D motifs are characteristic of strains from East Asian patients (Hatakeyama, 2009). EPIYA-C and EPIYA-D motifs contain major tyrosine phosphorylation sites, and are associated with cell damage. Increasing numbers of phosphorylation sites lead to more severe gastric lesions (Naito et al., 2006; Sicinschi et al., 2010), but East Asian \textit{CagA} has shown more oncogenic potential than Western \textit{CagA} (Hatakeyama, 2009). The possible role of EPIYA motifs in gastric carcinogenesis has been investigated in Malaysia and Singapore, where three distinct ethnic groups (Chinese, Indian and Malay) share the same geographic environment (Schmidt et al., 2009). In this population, Chinese have higher gastric cancer rates than Indians. While EPIYA-C was found to predominate in the Indian population with a higher proportion of strains having four or more motifs, EPIYA-D predominated in the Chinese. Interestingly, in the Malays who have a low cancer rate and a lower prevalence of \textit{H. pylori} infection, EPIYA-C and EPIYA-D motifs were found to have a similar prevalence (Schmidt et al., 2009).

Another major virulence factor is the vacuolating cytotoxin VacA, a secreted protein toxin that is responsible for gastric epithelial vacuolar degeneration and interference with cell membrane function (Cover and Blaser, 1992; Cover, 1996). Several alleles have been identified that are associated with bacterial virulence in the signal (s), medial (m) or intermediate (i) domains. The combination of s- and m-region alleles determines the production of the cytotoxin and is associated with the pathogenicity of the bacterium (see Backert et al., Chapter 11, this volume). In general, \textit{s1} and \textit{m1} variants are associated with more severe gastric lesions, including gastric cancer, than \textit{s2} and \textit{m2} strains (Atherton et al., 1995; Atherton et al., 1997; Cover and Blanke, 2005). In Colombia, the prevalence of \textit{vacA s1} and \textit{vacA m1} genotypes in isolates from communities with high gastric cancer risk was significantly higher than in communities with low risk: 93.2% versus 83.7% for \textit{s1} genotypes and 83.3% versus 70.2% for \textit{m1} genotypes (Bravo et al., 2002). In addition to \textit{cagA} and \textit{vacA}, \textit{H. pylori} expresses many other putative virulence factors, such as outer inflammatory protein A (\textit{oipA}), blood group antigen binding adhesin A (\textit{babA}) and adherence-associated proteins A and B (\textit{alpA/B}). Structures of these genes also vary among populations (see Lindén et al., Chapter 12, this volume). Polymorphisms of \textit{oipA} and \textit{babA} have also been linked to cancer risk (Yamaoka et al., 2008). It has been proposed that the combination of high-virulence polymorphisms of \textit{cagA}, \textit{vacA}, \textit{oipA}, \textit{babA} and \textit{alpA/B} may largely explain the African and related enigmas (Yamaoka et al., 2008). Populations of Asian extraction tend to carry more virulent \textit{H. pylori} strains than those of African extraction. Other bacterial factors may influence infection outcome and potential carcinogenesis. Tumour necrosis factor-\(\alpha\) (TNF\(\alpha\))-inducing protein (Tip\(\alpha\)), secreted by \textit{H. pylori}, is a potent inducer of TNF\(\alpha\) and various other chemokine genes. \textit{H. pylori} isolates from gastric cancer patients have been shown to secrete significantly larger amounts of Tip\(\alpha\) compared with those from chronic gastritis patients (Suganuma et al., 2008).

The genetic identity of \textit{H. pylori} strains has been analysed by multilocus sequence
typing (MLST) of housekeeping genes. The geographic sources of H. pylori reflect major events in human settlement history. MLST of 370 strains from 27 geographical, ethnic and/or linguistic human groupings assigned the strains to four main clusters: hpEurope, hpEastAsia, hpAfrica1 and hpAfrica2 (Falush et al., 2003). Further analyses split hpEastAsia into hspMaori and hspAmerind subpopulations. An expanded data set including 769 H. pylori isolates from 51 ethnic sources identified two new populations designated hpAsia2 and hpNEAfrica (Linz et al., 2007). Before Columbus discovered the New World, strains in the Americas were assumed to be all hspAmerind, and in Oceania hspMaori. After Columbus, human migrations from Europe and Africa changed the original predominant genotypes. In the Americas, the original hspAmerind strains became rare in Native American populations. hpEurope strains became more common, probably replacing or modifying the original strains. Similarly, in Oceania, hpEurope strains became more frequent. Before the MLST technology, a study of isolates from native Peruvians described the relatedness of Latin American and Spanish strains, suggesting that H. pylori had been brought to the New World by European conquerors and colonists about 500 years ago (Kersulyte et al., 2000). Re-analysis of the native Peruvian strains with MLST reported that 20% of the isolates had hspAmerind markers (Devi et al., 2006). A correlation between high gastric cancer rates and hpEastAsian genotypes has been noted, contrasting with low cancer rates in Africa, where most strains are hpAfrica (Yamaoka et al., 2008). MLST technology has been also applied to H. pylori isolates from the three ethnic groups residing in Malaysia, which have different rates of gastric cancer: Chinese (with the highest rate), Indian and Malay (Tay et al., 2009). Phylogenetic analysis assigned the isolates to previously identified H. pylori ancestral populations, hpEastAsia, hpAsia2 and hpEurope. Chinese isolates predominantly fell into hpEastAsia populations. Malays carried isolates related to those of Indian origin, apparently acquired relatively recently. Malaysian Indian/Malay isolates were found to differ from the Ladakh isolates from India (Linz et al., 2007) and revealed a new subpopulation, hspIndia, within hpAsia2 (Tay et al., 2009). These studies contribute substantially to our understanding of the ancestral origins of Asian populations, and their innate H. pylori flora.

3.5 Mechanisms of Carcinogenesis

The mechanisms by which H. pylori induces gastric cancer are not well understood. The intense inflammatory infiltrate induced by more virulent strains has been proposed as an explanation for the higher cancer rates. It has been suggested that higher cell replication may immortalize mutations or other DNA alterations that may arise during the repeated cycles of cell division. The gastric precancerous process in its initial stage is characterized by non-atrophic gastritis. However, the stage that apparently marks the entrance into the precancerous process is gastric atrophy (gland loss). Such loss is generally multifocal, initiated around the incisura angularis and the corpus–antrum junction. As the precancerous process advances, intestinal metaplasia sets in and the inflammatory infiltrate tends to gradually become less prominent. It would then appear that a separate set of events takes place after the initial stage of inflammation subsides. Some evidence suggests that inflammation, per se, is not a sufficient cause of neoplastic transformation. In subjects with duodenal ulcer, severe antral gastritis persists for decades if the H. pylori infection is not eradicated. However, duodenal ulcer patients are not at higher cancer risk than the general population. Comparisons of the histopathological parameters of the gastric mucosa in populations with contrasting cancer risks do not reveal more severe inflammation in subjects from the high-risk area (Piazuelo et al., 2008). However, a significantly higher prevalence of intestinal metaplasia is observed in subjects from high-risk areas (Correa et al., 1970; Bravo et al., 2002).

The link between genomic characteristics and functional mechanisms which can be associated with carcinogenesis has not been established. Oxidative and nitrosative stress
has been proposed as the relevant pathway to
H. pylori-related gastric carcinogenesis
(Mannick et al., 1996; Pignatelli et al., 1998, 2001; Xu et al., 2004). Several enzymes induced by H. pylori infection may contribute: (i) the inducible nitric oxide synthase (iNOS) enzyme results in expression of nitric oxide, capable of inducing genetic damage to epithelial cell DNA; (ii) arginase is an enzyme that plays a role in the polyamine metabolism by inducing \( L^{-}\)-ornithine, a step in the putrescine→sperrmidine→spermine cycle; (iii) ornithine decarboxylase stimulates the polyamine cycle progression; and (iv) spermine oxidase activates spermidine→spermine transformation, a reversible step that may induce \( \text{H}_2\text{O}_2 \) expression, capable of inducing DNA damage (Xu et al., 2004).

A recent study addressed the issue of oxidative and nitrosative damage in H. pylori isolates from Colombian populations with contrasting gastric cancer risks (Asim et al., 2008). Bacterial strains from subjects from a high cancer risk population induced significantly higher levels of iNOS in macrophages, a clear indicator of excessive nitrosative stress. The same isolates from the high-risk population induced significantly higher expression of spermine oxidase and \( \text{H}_2\text{O}_2 \), clearly indicating excessive oxidative stress. The same isolates from the high-risk population induced significantly higher expression of spermine oxidase and \( \text{H}_2\text{O}_2 \), clearly indicating excessive oxidative stress. All the strains tested in that study were \( \text{cagA} \)-positive, \( \text{vacA} \) s1/m1, indicating that H. pylori may partially be responsible for gastric carcinogenesis, and contribute to the cancer/infection enigmas by mechanisms related to oxidative and nitrosative stress not linked to these known virulence genes.

The causal role of oxidative damage is supported by chemoprevention trials using antioxidant agents. Supplementing the diet with ascorbic acid and \( \beta \)-carotene for 6 years resulted in a significant regression of precancerous lesions in Colombia (Correa et al., 2000; Mera et al., 2005). That trial also showed the same effects after curing the H. pylori infection. However, combining these two interventions did not have an additive effect in the regression of precancerous lesions. Such results can be interpreted as indicating that the role of H. pylori infection in carcinogenesis is driven by oxidative damage which could be avoided by curing the infection or by providing an adequate supply of antioxidants.

Evidence suggests that H. pylori influences stomach carcinogenesis through the development of chronic multifocal atrophic gastritis (Ohata et al., 2004), which may disseminate proximally to the fundic mucosa and is usually accompanied by intestinal metaplasia in advanced stages. Although multifocal atrophic gastritis and intestinal metaplasia are histopathological diagnoses, reduction of the functional fundic gland mucosa may be assessed by serum pepsinogen (PG) levels. Serum PG levels are considered a reliable marker for the extent of gastric atrophy/metaplasia (Miki, 2006). Ohata et al. (2004) followed a cohort of 4655 asymptomatic male subjects in Japan for 7.7 years. At baseline, H. pylori infection was evaluated by serum specific antibodies, and the presence of gastric mucosal atrophy was documented by serum PG levels. Chronic atrophic gastritis (CAG) was diagnosed if serum PGI levels were below 70 \( \mu \)g/l and the PGI/PGII ratio was less than 3.0. Forty-five new cases of gastric cancer were detected during the period of observation. No cancer developed in subjects without H. pylori infection or CAG. A stepwise increase in gastric cancer risk was observed from H. pylori+/CAG− subjects to H. pylori+/CAG+ subjects and finally to H. pylori−/CAG+ subjects. It appears that the early stages of the process are driven by H. pylori infection but the advanced stages are driven by gastric atrophy (Table 3.2).

The highest hazard ratio and incidence of gastric cancer are observed in the last group, representing subjects who cleared their infection and had advanced gastric atrophy. Apparently, the H. pylori-induced damage persists after the bacterium is no longer present in the gastric mucosa, as observed in intestinal metaplasia, which no longer provides a favourable niche for H. pylori colonization.

### 3.6 Cancer Control

The prognosis of gastric cancer is largely determined by the stage of tissue invasion at the time of diagnosis. If detected while the
tumour cells are confined to the mucosa and submucosa (early carcinoma), surgical resection, either endoscopically or by laparotomy, results in a 5-year survival rate above 90% (Yamamoto and Kita, 2005). Detection at such an early (mostly asymptomatic) stage requires extensive screening programmes in the population, so far available almost exclusively in Japan. In most Western countries, most cases are detected after the tumour cells have invaded the muscularis propria. In the USA, two-thirds are diagnosed at this stage (Jemal et al., 2008). Five-year survival rates at this stage are below 20% in most populations. The inescapable conclusion from the Western experience is that prevention is the only hope of controlling the disease.

The key to effective prevention is the endoscopic identification of advanced precancerous lesions, namely dysplasia. In Japan and some other countries, the word ‘adenoma’ is equivalent to dysplasia (Rugge et al., 2000). The Japanese model of prevention and early diagnosis is very expensive. It requires an extensive infrastructure of advanced endoscopy services and outstanding expertise in the diagnosis of minimal lesions, not available in most other countries. It is also combined with special equipment and expertise in double-contrast barium X-rays and photofluorography. Some attempts to set up such programmes in Latin America have been conducted in specific subpopulations in Venezuela and Costa Rica. Those efforts have resulted in the identification of a limited number of early-stage carcinomas that have been successfully resected surgically. The impact on the general population, however, has been very limited (Oliver, 1994; Pisani et al., 1994; Rosero-Bixby and Sierra, 2007).

The strategies for prevention vary according to the cancer risk. In many Western countries the incidence is so low that screening and early detection programmes are not cost-effective. No organized screening and early detection programmes exist in most high-risk countries, with the exception of Japan and to some degree South Korea (Choi et al., 2009). In high-risk countries with limited economic resources, there is a need to develop, pilot (test) and implement prevention strategies that are cost-effective and viable. Some investigators advocate elimination of *H. pylori* infection in the general population. In the absence of a vaccine, bacterial eradication can be achieved with antibiotics in approximately 90% of cases. However, treating millions of persons with antibiotics is problematic not only in terms of the cost, but also in making other pathogenic bacteria resistant to antibiotics, a potentially serious health threat that is becoming increasingly recognized in several countries. Some investigators have reported that unwanted effects may become apparent when *H. pylori* infection is eradicated. The complex of reflux oesophagitis, Barrett’s oesophagus and adenocarcinoma of the lower oesophagus is a prime example of possible unexpected complications of eradication of *H. pylori* infection (Blaser, 1999, 2005). Other possible consequences might be bronchial asthma and other allergic and autoimmune diseases, which may result from the absence of some minor infections and their effect in modulating the immune response to other antigens (the so-called hygiene hypothesis) (Strachan, 1989; Zaccone et al., 2006; see Mitchell et al., Chapter 5, this volume).

The classic epidemiological model of prevention consists of identifying the subjects

<table>
<thead>
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<th>H. pylori</th>
<th>Chronic atrophic gastritis</th>
<th>Hazard ratio</th>
<th>Gastric cancer incidence per 100,000 person-years</th>
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<tr>
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<td>Negative</td>
<td>1.00 (referent)</td>
<td>0</td>
</tr>
<tr>
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<td>Negative</td>
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<td>107</td>
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<tr>
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<tr>
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<td>Positive</td>
<td>61.85</td>
<td>871</td>
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</tbody>
</table>

Table 3.2. Incidence rate and hazard ratio of gastric cancer according to serological diagnosis of *Helicobacter pylori* infection and chronic atrophic gastritis. (From Ohata et al., 2004.)
at the highest risk of developing the disease. In the case of *H. pylori*, even in the very high-risk communities, only approximately 50 of every 100,000 persons are expected to develop gastric cancer in a year. The challenge is to identify them before they develop invasive disease. As discussed above, the cancer risk is determined by host genetic susceptibility factors, bacterial genotypes and immune modulation of the response to *H. pylori* infection. What is lacking at the present time is a strategy to identify such individuals in the community. Although the techniques needed to assess genetic susceptibility, bacterial genotypes and immune response to the infection are known, no attempts have been made to develop cost-effective high-throughput methods to apply to the general population in high-risk countries.

As discussed above, serum PGs are specific indicators of gastric atrophy, a well-known marker of elevated cancer risk. Some studies have reported the use of serum PG levels in cancer patients and controls. While very low PGI levels and low PGI/PGII ratios are very specific indicators of invasive gastric cancer, these markers may not be sensitive indicators of cancer risk. It has been estimated that approximately one-half of the invasive cancers are not associated with low serum PG markers. The reason for this is that PGI is exclusively expressed in oxyntic mucosa and is drastically reduced when the oxyntic mucosa becomes atrophic and metaplastic. However, antrum-restricted atrophy and metaplasia, not reflected in serum PG levels, may be associated with high cancer risk. The real promise of serum PG levels is the identification of subjects with cancer precursors, before they develop invasive disease (Miki, 2006). *H. pylori*-induced atrophy classically starts in the incisura angularis and the antrum–corpus junction. The area covered by multifocal atrophy advances with time and extends to the neighbouring antral and corpus mucosa. The latter situation leads to lower PGI levels and PGI/PGII ratio. A sound strategy for prevention in high-risk countries would be to detect subjects with low serum PG markers. Once identified, a battery of host, bacterial and immune markers as well as endoscopic monitoring could be effective in detecting advanced precancerous lesions. Their progression to invasive carcinomas could be avoided by endoscopic monitoring and appropriate interventions. Such a strategy could be reinforced by curing *H. pylori* infection and providing an adequate supply of antioxidants.

### 3.7 Epilogue

The natural history of *H. pylori* infection and gastric cancer is changing drastically as the 21st century advances. The steady decline in the frequency of both conditions will probably continue and become more accentuated. However, the health burden of gastric adenocarcinoma will continue to be considerable for decades to come. Special attention should be given to addressing the issue of whether the gradual decrease in *H. pylori* infection and gastric cancer incidence will be followed by increases in other conditions such as oesophageal adenocarcinoma, bronchial asthma and autoimmune diseases. As of now, the health burden of gastric adenocarcinoma is much greater than that of the conditions just mentioned. However, it is not clear whether this imbalance will persist in future decades.

The role of infection and chronic inflammation in the causation and progression of major health burdens is being increasingly recognized. Such is the case for neoplasias with aetiologies much less clearly understood than those of gastric carcinoma, in which an infectious agent has been identified. Carcinomas of the colon, pancreas, breast, prostate and probably other organs may be influenced by infections and chronic inflammation. As an example, clinical trials utilizing anti-inflammatory drugs such as aspirin and cyclooxygenase-2 inhibitors have reported promising results in the prevention and progression of cancer. The present scientific interest in solving the enigmas of the aetiology and pathogenesis of gastric cancer may throw light on the general subject of infection/inflammation-induced carcinogenesis.
References


4 Antimicrobial Resistance and Approaches to Treatment

F. MÉGRAUD

4.1 Introduction

The discovery of Helicobacter pylori in 1982 was a tremendous breakthrough in relation to the treatment of gastroduodenal diseases (Marshall and Warren, 1984). For the first time it was possible to treat a cause and, this cause being infectious, to easily cure the disease. However, as for any bacterial agent, resistance has developed and in many places we are now faced with multi-resistant bacteria for which antimicrobial susceptibility testing is mandatory. This chapter presents the basis of H. pylori treatment, the currently recommended treatment regimes, and the problems linked to antimicrobial resistance.

4.2 Basis for H. pylori Treatment

4.2.1 Rationale to use antisecretory drugs

The antibiotics to be used to treat H. pylori infection have to be chosen from among the numerous drugs naturally active against the bacterium. Indeed, H. pylori is intrinsically resistant to only a few compounds.

However, the particularity of H. pylori is to live in the specialized niche of the gastric mucus, outside the interior milieu, and which, unlike most of our body, is not at a neutral pH but rather presents a pH gradient. This gradient ranges from pH 1.82 to 5.5 in the corpus and from pH 3.5 to 5.4 in the antrum (Quigley and Turnberg, 1987). However, given the important production of urease by H. pylori and consequently of ammonia in the immediate environment of the bacterium, the pH is higher when H. pylori is present (Marshall et al., 1990).

These conditions impose important restrictions for antimicrobial use. The first concern is chemical stability: clarithromycin, for example, has been shown to degrade rapidly at gastric pH (Erah et al., 1997). Second, most antibiotics have decreased activity when the pH is decreased (Table 4.1). For this reason, the first attempts using an antibiotic alone were unsuccessful and it became mandatory to add an antisecretory drug, i.e. a drug that diminishes gastric acid secretion, to the antibiotics. The first to be used was ranitidine, a histamine H2-receptor antagonist (Hentschel et al., 1993), but when the more potent antisecretory drugs – the proton pump inhibitors (PPIs) – emerged, they quickly became the standard (Gisbert et al., 2003). The PPIs omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole can all be used for this purpose (Vergara et al., 2003). These drugs also have an anti-H. pylori activity per se (Megraud et al., 1991; Suerbaum et al., 1991), but this occurs at concentrations which are most likely unachievable in the gastric mucosa and therefore this activity is only theoretical. However, these drugs have
an added value for the treatment: they lead to pain relief, which is a major benefit for the patient.

Metronidazole has the property of being a pro-drug, which is reduced by nitroreductases to an active compound inside the bacterium. This reduction depends on the redox potential of the bacterial cell environment. Along these lines, it has been shown in vitro that an anaerobic pre-incubation of the agar plates decreases the minimal inhibitory concentration (MIC) of metronidazole as compared with agar plates that have been left in contact with air (Cederbrant et al., 1992). Therefore, redox potential rather than pH appears to be an important factor of activity when metronidazole is used; however, this cannot be controlled in vivo.

4.2.2 Rationale for the use of absorbable antibiotics

The possibility of a direct topical action of a drug on bacteria in the stomach following oral administration was first considered to be important for the drug’s activity. Indeed, this is true for drugs like bismuth salts which have an ‘antiseptic-like’ activity, i.e. a short contact leads to the destruction of the bacterium (Armstrong et al., 1987). However, this does not appear to be true for antibiotics whose activity requires prolonged contact with the bacterium in order to be active, and for these a systemic diffusion is required.

Orally delivered antibiotics are absorbed in the intestine, circulate in the bloodstream and then finally diffuse into the gastric mucus through the epithelial layer. Antibiotics that are not absorbed have never been shown to be effective for H. pylori eradication. However, all antibiotics do not have the same characteristics in this respect. To date, diffusion has mainly been studied in vivo for amoxicillin, clarithromycin and metronidazole. By increasing the pH, the intragastric concentration of amoxicillin increases, while that of metronidazole decreases and clarithromycin is not affected (Goddard et al., 1996).

The difference between amoxicillin and clarithromycin has also been studied in an in vitro model using epithelial cells, with contrasting results (Matysiak-Budnik et al., 2002). Unlike amoxicillin, clarithromycin is able to accumulate intracellularly where a high concentration can be reached, conferring several advantages. The first advantage is that clarithromycin can act on the few bacteria which have penetrated intracellularly. Even if H. pylori is not an intracellular pathogen, recent data have pointed out the occurrence of intracellular bacteria and their importance in the natural history of infection (Dubois and Boren, 2007). Second, the slow release of clarithromycin into the mucus allows a sufficient concentration of the

<table>
<thead>
<tr>
<th>Table 4.1. Distribution of the minimal inhibitory concentrations (MIC &lt;sub&gt;90&lt;/sub&gt;) of various antibiotics against wild-type Helicobacter pylori at various pH.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agent</strong></td>
</tr>
<tr>
<td>Penicillin</td>
</tr>
<tr>
<td>Ampicillin</td>
</tr>
<tr>
<td>Cephalexin</td>
</tr>
<tr>
<td>Erythromycin</td>
</tr>
<tr>
<td>Clarithromycin</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>Tetracycline</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
</tr>
<tr>
<td>Metronidazole</td>
</tr>
<tr>
<td>Bismuth subcitrate</td>
</tr>
</tbody>
</table>
antibiotic over time to kill the bacteria. In contrast, amoxicillin is not able to penetrate intracellularly but instead diffuses between the epithelial cells, which is effective only when blood concentrations of this antibiotic are high. Given this, a good approach would be to administer amoxicillin several times daily, or even better intravenously, in order to increase its activity, but due to practical problems this approach is not used.

The antibiotics more recently recommended for *H. pylori* eradication have not been studied from a pharmacological point of view. However, it is well known that fluoroquinolones (levofloxacin) and rifampicins (rifabutin) are able to diffuse intracellularly. Furazolidone is like metronidazole, a small molecule that easily crosses the epithelial barriers.

### 4.2.3 Rationale for the use of a combination of antibiotics

The limitations of antibiotic efficacy mentioned previously led to the use of a combination of two antibiotics. The rationale for this approach is: (i) to increase efficacy by an additive (or eventually synergistic) effect; and (ii) to avoid the emergence of resistance. Resistance to antibiotics in *H. pylori*, similar to the case of *Mycobacterium tuberculosis*, arises essentially via mutations (Table 4.2).

The probability of observing mutations for two antibiotics in a given bacterial cell is the product of the probability of having it occur for each antibiotic separately. For example, if the probability of mutations is $10^{-7}$ for clarithromycin and $10^{-5}$ for metronidazole, it will be $10^{-12}$ for both, i.e. a frequency so rare that it should not occur.

### 4.3 Currently Recommended Treatment

#### 4.3.1 First-line therapy

The combinational use of a PPI with two antibiotics was proposed in 1993 in Italy by Bazzoli et al. (1993; clarithromycin and metronidazole) and in France by Lamouliaie et al. (1993; clarithromycin and amoxicillin). These treatments, known as PPI-based triple therapies, were recommended as first-line treatments at the first Maastricht Conference held in 1995 (Anonymous, 1997) and then reconfirmed at other consensus conferences held around the world, including the Maastricht III Conference (Malfertheiner et al., 2007). The only controversy arising from these consensus meetings relates to the length of treatment regimens, which ranges from 7 to 14 days. A recent meta-analysis by Fuccio et al. (2007) was in favour of 7 days.

Given that amoxicillin resistance seldom occurs, the combination of clarithromycin and amoxicillin was originally preferred, despite the subsequent observation that the combination of clarithromycin and metronidazole is slightly more effective on susceptible strains. An alternative was also to use amoxicillin and metronidazole. All of these treatments reached an eradication rate of more than 80%, the threshold that was defined as being acceptable at consensus conferences (Anonymous, 1997).

There is a large body of data published on these various combinations. In particular, the causes of failure have been documented (Table 4.3). Pre-treatment with a PPI is not a risk factor for failure (Janssen et al., 2005). Instead, antimicrobial resistance, which concerns essentially clarithromycin, while

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Gene(s) concerned</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrolides</td>
<td><em>rrn</em> 23S</td>
<td>Versalovic et al. (1996)</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td><em>gyrA</em></td>
<td>Moore et al. (1995)</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td><em>rrn</em> 16S</td>
<td>Gerrits et al. (2002a); Trieber and Taylor (2002)</td>
</tr>
<tr>
<td>Rifampicins</td>
<td><em>rpoB</em></td>
<td>Heep et al. (1999)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td><em>pbp1</em></td>
<td>Gerrits et al. (2002b)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td><em>rdxA</em>, <em>frxA</em></td>
<td>Goodwin et al. (1998)</td>
</tr>
</tbody>
</table>
not the explanation for all cases, appears to be the main reason for failure. Details on resistance prevalence, impact on treatment success and modes of detection are presented below. At the last Maastricht Conference in 2007, the recommendation was made to perform susceptibility testing for clarithromycin, or not to use this antibiotic if the prevalence of resistance in a specific country/region was in the range of 15–20% or higher (Malfertheiner et al., 2007).

Alternative first-line treatments are now emerging, especially the use of PPI, amoxicillin and a fluoroquinolone such as levofloxacin. This combination, first used by Cammarota et al. (2000) in Italy, resulted in eradication rates between 92 and 95% and has been recommended as the first choice of salvage therapy. However, similar to clarithromycin, resistance to levofloxacin easily occurs and jeopardizes the success of treatment. Another alternative treatment that has been claimed not to be influenced by antimicrobial resistance is the so-called ‘sequential therapy’. The concept is to first treat with a PPI and amoxicillin for 5 days and then replace amoxicillin with clarithromycin plus metronidazole for the following 5 days. The rationale is to decrease the bacterial load during the first 5 days using amoxicillin, then to eradicate the remaining bacteria by the combination of clarithromycin and metronidazole (Zullo et al., 2003). Decreasing the bacterial load reduces the chance of mutations to almost nil, given that mutations occur at a low frequency. Another postulated mechanism to explain the efficacy of the sequential therapy is the impact of amoxicillin on the bacterial cell wall and then on efflux pumps, which would prevent the binding of clarithromycin to the ribosome (Zullo et al., 2007a). However, the role of efflux pumps in macrolide resistance is usually not considered a major one.

This sequential therapy has proved to be successful (Gisbert et al., 2010). However, antimicrobial susceptibility was not tested in all studies and the number of cases where efficacy is shown on resistant strains is small (Table 4.4) and deserves further study.

Performing antimicrobial susceptibility testing is the best way to ensure successful treatment of a patient and should be carried out whenever possible for clarithromycin and also for levofloxacin, since new tools to

Table 4.3. Factors implicated in the failure of proton pump inhibitor-based triple therapies to eradicate Helicobacter pylori.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance to antibiotics</td>
<td>Megraud (2004); Fischbach and Evans (2007)</td>
</tr>
<tr>
<td>Lack of compliance</td>
<td>Wermeille et al. (2002)</td>
</tr>
<tr>
<td>High gastric acidity</td>
<td>Villoria et al. (2008); Zhao et al. (2008)</td>
</tr>
<tr>
<td>High bacterial load</td>
<td>Moshkowitz et al. (1995)</td>
</tr>
<tr>
<td>cagA-negative status</td>
<td>Suzuki et al. (2006)</td>
</tr>
<tr>
<td>Presence of intracellular bacteria</td>
<td>Lai et al. (2006)</td>
</tr>
<tr>
<td>Altered immunity</td>
<td>Borody et al. (2002)</td>
</tr>
</tbody>
</table>

cagA, cytotoxin-associated gene A.

Table 4.4. Impact of Helicobacter pylori clarithromycin resistance on the success of sequential treatment (number of patients in whom H. pylori was eradicated/number treated).

<table>
<thead>
<tr>
<th></th>
<th>Standard therapy</th>
<th>Sequential therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible/R resistant</td>
<td>Susceptible/R resistant</td>
</tr>
<tr>
<td></td>
<td>3/11</td>
<td>15/19</td>
</tr>
<tr>
<td></td>
<td>51/54</td>
<td>58/59</td>
</tr>
<tr>
<td></td>
<td>86/91</td>
<td>108/114</td>
</tr>
<tr>
<td></td>
<td>8/8</td>
<td>10/12</td>
</tr>
<tr>
<td>Total</td>
<td>145/153</td>
<td>176/185</td>
</tr>
<tr>
<td>%</td>
<td>94.7</td>
<td>95.1</td>
</tr>
</tbody>
</table>
determine antibiotic susceptibility are now available (see Section 4.4.3).

Lastly, the sole treatment that appears to be only modestly influenced by resistance is bismuth-based quadruple therapy. This treatment can be advocated as a first-line treatment in areas of high antibiotic resistance, as was the recommendation at the Maastricht III Conference (Malfertheiner et al., 2007).

### 4.3.2 Second-line therapy

In individuals in whom initial eradication therapy has failed, unless there is the possibility of showing their efficacy by testing clarithromycin and levofloxacin susceptibility, subsequent treatments using these drugs should be avoided. The PPI-based triple therapies to be considered are indicated in Table 4.5. The amoxicillin–metronidazole combination has been used in a large study in France (Lamouliatte et al., 2003) among others, but led to less than 80% eradication as a second-line treatment. The tetracycline–metronidazole combination appears more effective, but few records exist (Realdi et al., 1999). The amoxicillin–rifabutin combination should be used as little as possible because of the fear of rifampicin resistance in *M. tuberculosis*.

Bismuth-based triple and, even better, quadruple therapies are of great interest in this context. Indeed, the Maastricht Conferences always recommended bismuth-based quadruple therapy as a second-line treatment. Given the potential adverse events due to bismuth salts a systematic review was performed, including 35 randomized controlled trials with 4763 patients (Ford et al., 2008). No serious adverse event was reported in these trials. The total number of adverse events observed with bismuth-based therapies was indeed not significantly different, with the exception of dark stools, from those observed with control therapies comprising antisecretory-based triple therapies or association of antibiotics: odds ratio (OR) = 1.01 (95% confidence interval (CI) 0.87, 1.16). In addition, there was no increase in the withdrawal of therapy when bismuth salts were administered (Ford et al., 2008).

Non-bismuth quadruple therapies (including three antibiotics and a PPI) have also been used. A meta-analysis reporting on five randomized trials found that pooled estimates of these trials showed a superiority of quadruple therapy over triple therapy: OR = 2.86 (95% CI 1.73, 4.73) in intention-to-treat analysis (Essa et al., 2009).

### 4.3.3 Specific problems

Specific problems may arise in some countries because certain drugs are not available. Such is the case in France, for example, where bismuth salts and tetracycline are currently not on the market. If, in addition, the doctor is faced with a patient supposedly allergic to β-lactams who is infected with a strain resistant to clarithromycin and levofloxacin, it may become impossible to find an adequate treatment. This point highlights the need to consider the treatment of *H. pylori* infection seriously and therefore to apply susceptibility testing as often as possible.

**Table 4.5.** Therapies used as second-line treatment for *Helicobacter pylori* eradication in cases of clarithromycin and levofloxacin resistance.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Regimen</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI-based</td>
<td>PPI–amoxicillin–metronidazole</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>PPI–tetracycline–metronidazole</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>PPI–amoxicillin–rifabutin</td>
<td>10 days</td>
</tr>
<tr>
<td>Bismuth-based</td>
<td>Bismuth–tetracycline–metronidazole</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>Bismuth–tetracycline–furazolidone</td>
<td>14 days</td>
</tr>
<tr>
<td></td>
<td>Bismuth–tetracycline–metronidazole–PPI</td>
<td>7–14 days</td>
</tr>
<tr>
<td>Dual therapies</td>
<td>PPI–amoxicillin</td>
<td>14 days (high dosage)</td>
</tr>
</tbody>
</table>

PPI, proton pump inhibitor.
4.3.4 Adjuvant therapy

Antisecretory drugs are already an adjuvant therapy, i.e. an additional drug that has limited effects by itself but improves the efficacy of the other drugs given at the same time. In addition, attempts have been made to improve eradication rates by other means.

The most interesting is the use of probiotics. A meta-analysis of trials (14 trials, 1671 patients) where probiotics were added to triple therapy regimens found a beneficial effect of this supplementation. The pooled eradication rate in this meta-analysis was 83.6% (95% CI 80.5, 86.7) with probiotics versus 74.8% (95% CI 71.1, 78.5) without, by intention-to-treat analysis (Tong et al., 2007). While some probiotics are able to decrease the load of bacteria, it appears that their beneficial effect is essentially the result of decreasing adverse events (from 38.5 to 24.7%) and thereby probably facilitating better compliance.

Another adjuvant therapy comprises the addition of lactoferrin. A meta-analysis conducted on nine randomized trials representing 1343 patients found a beneficial effect, with the pooled eradication rate with lactoferrin being 86.5% (95% CI 84.0, 89.1) versus 74.4% (95% CI 71.1, 77.7) without lactoferrin, by intention-to-treat analysis (Zou et al., 2009). The positive effect of lactoferrin on adverse events was particularly notable regarding nausea. The same beneficial effects have been noted in another meta-analysis (Sachdeva and Nagpal, 2009).

Vegetable products may also be interesting for this purpose. Cranberry juice, for example, has been reported to improve eradication in a subgroup of patients, but further studies are needed (Shmuely et al., 2007).

4.4 Antimicrobial Resistance

4.4.1 Prevalence

Antibiotics with high resistance rates: clarithromycin, levofloxacin, metronidazole

The prevalence of antimicrobial resistance is by essence an evolving phenomenon in time and space. Therefore only recent data are of interest and they vary from place to place.

We are essentially interested in the so-called primary resistance, i.e. the H. pylori resistance found in patients not having been previously treated for H. pylori infection. This primary resistance is most likely the consequence of treatments administered to the patient for other types of infection. Examples are respiratory infections for clarithromycin, urinary tract and respiratory infections for levofloxacin, and gynaecological and dental infections for metronidazole. In such cases the antibiotic used as a monotherapy may have selected resistant H. pylori mutants, but was not able to lead to eradication. Another possibility, which probably occurs only in a minority of cases, is the transmission of resistant organisms to a new host. Since H. pylori is essentially transmitted from parents to children, and clarithromycin has been mainly administered to children for less than two decades, the transmission of clarithromycin-resistant strains is considered to be rare. The situation is different for resistance to fluoroquinolones, as these antibiotics are not used to treat children. In Portugal, a country in which the new fluoroquinolones have been widely used (in adults) for almost 20 years, fluoroquinolone resistance is now being observed in children (4.5%) (Lopes et al., 2005). Such transmission of clarithromycin-resistant organisms will also occur in the future, when yesterday’s children become adults and have children themselves.

Another point to highlight when considering the prevalence of H. pylori resistance is how representative were the cases studied. An interesting design was applied in a Swedish study which selected a random sample of 3000 subjects in the population and contacted them by mail. Seventy-four percent responded and a random sample of one-third of these respondents was offered an endoscopy. The H. pylori strains obtained (333), which were definitely representative of the population as a whole, were then tested for antimicrobial susceptibility (Storskrubb et al., 2006). Interestingly, the authors found only 1.5% of the strains resistant to clarithromycin and 16.2% resistant to metronidazole. It may be argued that it is more pertinent to test the strains of patients seeking medical attention and this has been done many times.
In order to get meaningful prevalence values with small confidence intervals, it is important to test large numbers of strains and therefore to perform multicentre studies. An important Japanese study carried out between 2002 and 2005 gathered 3707 strains (from 36 centres) and showed a clarithromycin resistance rate of 22.7% (Kobayashi et al., 2007).

In Europe, multicentre studies have also been conducted. The first in 1991 (involving 12 centres from 11 countries) surveyed only metronidazole resistance (Anonymous, 1992), while subsequent studies tested more antibiotics: in 1998 (22 centres from 17 countries) (Glupczynski et al., 2001) and in 2008–2009 (31 centres from 17 countries). Despite the fact that the centres involved were not exactly the same and that the protocols used evolved between the studies, information was collected on the development of H. pylori resistance over time. For example, clarithromycin resistance that was 9.9% in 1998 has doubled over the last decade.

There is a marked difference between northern and southern European countries. In northern countries where there is a strict policy for antibiotics resulting in more restrictive use, the resistance rates are much lower (4.2% in the north and 18.4% in the south for clarithromycin in 1998).

A European multicentre study conducted recently over a 4-year period in 17 paediatric centres from 14 countries showed a resistance rate to clarithromycin among 1233 patients of 24% (Koletzko et al., 2006).

A further source of information on resistance corresponds to the data generated by clinical trials where strains were cultured and tested, and those from monocentre studies. The problem with monocentre studies, however, is that these are usually referral centres, which gather difficult-to-treat patients, hence giving higher resistance rates.

The results of recent studies are presented in Table 4.6.

**Antibiotics with low resistance rates:**

- amoxicillin
- tetracycline
- rifabutin

In most of the studies performed, resistance to these three antibiotics was nil or less than 1% with a few exceptions for tetracycline (Korea: 5.3%, Bulgaria: 4.4%) (Kim et al., 2001; Boyanova et al., 2008). Indeed, concerning amoxicillin, the prevalence rates observed in some studies must be treated with caution if the strains were not studied in detail.

Rifabutin resistance can be found in patients having received treatment for tuberculosis (Suzuki, S. et al., 2009).

**Multiple resistance**

H. pylori strains resistant to two or more antibiotics are still not very common in patients receiving a first treatment (<10%). After failure of a clarithromycin-based triple therapy, the risk of finding a clarithromycin-resistant strain is approximately 65%. It is in the same range after failure of a levofloxacin-based triple therapy (Perna et al., 2007; Nishizawa et al., 2008). After several treatment attempts, it was possible to find strains resistant to three, four and even five antibiotics in 15% of the cases, for example in Germany (Wueppenhorst et al., 2009).

Results concerning resistance to metronidazole have shown low reproducibility (Glupczynski et al., 2002) and limited clinical relevance on an individual basis (see next section).

**4.4.2 Clinical impact of antibiotic resistance**

Data on the clinical impact of antibiotic resistance exist essentially for clarithromycin and metronidazole, with contrasting figures. Indeed, for clarithromycin, they can explain the majority of treatment failures. The most recent study published in this respect is the meta-analysis of Fischbach and Evans (2007) (Table 4.7). For the triple therapy PPI–clarithromycin–amoxicillin, there was a reduction in H. pylori eradication of 66% (95% CI 58.2, 74.2) when the strain was resistant to clarithromycin, versus susceptible strains. This result is in the range of those previously published by Megraud (2004), which indicated a 70% decrease in eradication from 87.8% to 18.3%.

For the triple therapy PPI–clarithromycin–metronidazole, the decrease in efficacy was
Table 4.6. Prevalence of *Helicobacter pylori* resistance in adults, worldwide. Data are from studies including more than 100 strains isolated totally or partially since the year 2000.

<table>
<thead>
<tr>
<th>Country</th>
<th>Year(s)</th>
<th>Study</th>
<th>Method</th>
<th>No. of strains</th>
<th>Clari&lt;sup&gt;R&lt;/sup&gt; (%)</th>
<th>Metro&lt;sup&gt;R&lt;/sup&gt; (%)</th>
<th>Tetra&lt;sup&gt;R&lt;/sup&gt; (%)</th>
<th>Amox&lt;sup&gt;R&lt;/sup&gt; (%)</th>
<th>FQR (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>2002</td>
<td>MonoC</td>
<td>DD</td>
<td>164</td>
<td>3</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>NP</td>
<td>Aguemon <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>2005–2007</td>
<td>MultiC</td>
<td>AD</td>
<td>613</td>
<td>17.8</td>
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### Far East

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<th>RT (%)</th>
<th>PCR (%)</th>
<th>FISH (%)</th>
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<th>Metronidazole</th>
<th>Tetracycline</th>
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<td></td>
<td></td>
<td></td>
<td>Lwai-Lume et al. (2005)</td>
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</table>

Abbreviations: AD, agar dilution; Amox, amoxicillin; Clari, clarithromycin; DD, disc diffusion; FISH, fluorescence in situ hybridization; FQ, fluoroquinolones; Metro, metronidazole; MonoC, monocentre; MultiC, multicentre; NP, not performed; PCR, polymerase chain reaction; R, resistant; RT, real time; Tetra, tetracycline.
35% (95% CI 25.4, 45.4) when the strain was resistant to clarithromycin, versus susceptible strains. In contrast, for the same triple therapy (PPI–clarithromycin–metronidazole), when the strain was resistant to metronidazole instead of clarithromycin, the reduction in efficacy was 18% (95% CI 13.4, 24.0). The combination of PPI–amoxicillin–metronidazole was also studied and showed a 30% (95% CI 21.8, 38.2) reduction in efficacy. The impact of metronidazole resistance appears to be even lower when the regime contains a bismuth salt. For the bismuth-based triple therapy bismuth–tetracycline–metronidazole, a decrease in efficacy of 26% (95% CI 14.2, 37.8) was observed. However, for the bismuth-based quadruple therapy where a PPI was added, the decrease in efficacy observed was only 14% (95% CI 5.4, 22.6) (see Table 4.7) (Fischbach and Evans, 2007).

Concerning the resistance to fluoroquinolones, only two studies were found in the literature. In one study the triple therapy PPI–amoxicillin–levofloxacin, administered to 33 patients for 10 days, led to eradication in 75% of those harbouring susceptible strains, but in only 33.3% of those with resistant strains (Perna et al., 2007). In another trial using gatifloxacin instead of levofloxacin over 7 days, 100% of susceptible strains were eradicated, but only one-third of resistant strains (Nishizawa et al., 2008). Resistance to the other antibiotics used may also have an impact but the clinical trials performed either did not test for resistance or did not include resistant strains to these antibiotics.

### 4.4.3 Detection of antibiotic resistance

**Phenotypic methods**

As for any bacterium, the standard method of agar diffusion can be applied to determine the antibiotic sensitivity of *H. pylori* strains. However, *H. pylori* being a fastidious organism, the E test (an agar diffusion method using a strip with an antimicrobial gradient which gives the exact MIC of the strain) appears to be more adapted and provides MIC values. The agar dilution method is, nevertheless, considered to be the reference method being the most accurate, and can be simplified by including in the agar the concentration of antibiotic considered to be the cut-off for resistance. This is commonly performed for metronidazole and has also been proposed with a four-sector agar plate in which one sector does not include any antibiotics and the three others include, for example, clarithromycin, amoxicillin and another antibiotic (Caristo et al., 2008).

Cut-off values for resistance have been officially validated only for clarithromycin, by the National Committee for Clinical Laboratory Standards (NCCLS), now the Clinical and Laboratory Standards Institute (CLSI), as follows: susceptible, <0.25 mg/l; intermediary, 0.25–0.75 mg/l; and resistant, ≥1 mg/l.

The thresholds commonly used for the other antibiotics are: fluoroquinolones, 1 mg/l; rifabutin, 1 mg/l; tetracycline, 2 mg/l; amoxicillin, 1 mg/l; and metronidazole, >8 mg/l. For amoxicillin, MICs of 0.25 and 0.5 mg/l are considered to correspond to a decreased
susceptibility, but the clinical impact has not been studied.

Currently, the minimal antibiogram to be performed, i.e. the least number of antibiotics with high clinical relevance to test, includes only two antibiotics: clarithromycin and a fluoroquinolone (ciprofloxacin can be tested if levofloxacin is not available, but must never be used clinically due to its low efficacy in vivo). It can be performed either by agar diffusion or by E test. If agar diffusion is used, an inhibition diameter of 20 mm is applied as the threshold. An E test can be used as a control when the diameter is less than 20 mm.

**Genotypic methods**

Given that *H. pylori* resistance to antibiotics essentially occurs by point mutations, it is possible to detect these mutations by molecular methods. Numerous methods have been applied to the detection of clarithromycin resistance and some to the detection of fluoroquinolone and tetracycline resistance. Such methods have proved more difficult to develop for amoxicillin and metronidazole.

**CLARITHROMYCIN. Mutations.** Macrolides target the peptidyl transferase loop on domain V of 23S ribosomal RNA (rrn) and produce an arrest in peptide elongation. Resistance occurs in *H. pylori* by mutations, especially at positions 2142 (A2142G, A2142C) and 2143 (A2143G), which probably lead to a conformational modification of the target site and a decrease in fixation (Versalovic et al., 1996; Occhialini et al., 1997). These mutations lead to a cross-resistance between all macrolides. Despite the fact that *H. pylori* contains two rRNA operons, heterozygosity is rarely found.

Such mutations occur spontaneously and are thus selected when the bacteria are exposed to the antibiotic. In vitro, the frequency of these mutations ranges from $3.2 \times 10^{-7}$ to $6.0 \times 10^{-8}$ but could be higher in vivo due to oxidative stress (Kuwahara et al., 2009). The most commonly found mutations do not appear to have a biological cost and appear to be stable, while other possible mutations are either deleterious to the bacteria or too costly to be maintained.

**Detection.** Numerous methods have been applied for clarithromycin resistance, either on strains or directly on biopsy specimens (Table 4.8). Those that are currently commercially available are discussed further below.

**Mutation detection by real-time PCR.** One of the most effective methods is real-time polymerase chain reaction (PCR). This method can be performed using the fluorescence resonance energy transfer (FRET) principle in a LightCycler™ apparatus. First, primers have to be designed for the 23S rRNA genes in order to specifically detect *H. pylori*. Second, a specific biprobe must be designed inside the amplicon. It comprises: (i) a detection probe 5’-labelled with LC-Red 640 whose hybridization site includes the nucleotides where the mutation may occur; and (ii) a fixation probe 3’-labelled with fluorescein, which hybridizes three to five nucleotides upstream of the others. When the fixation probe is excited, there is an energy transfer to the detection probe because of its proximity, which results in emission of a signal. This allows the possibility of following amplicon synthesis in real time in order to detect *H. pylori* in a gastric biopsy. When the amplification is finished, a melting curve analysis (MCA) of the amplicons is conducted. Because of the mismatch, the melting temperature of the mutant is lower than that of the wild-type strain.

Different designs of this reaction have been published (Matsumura et al., 2001; Oleastro et al., 2003; Schabereiter-Gurtner et al., 2004) and one is now commercially available (ClariRes Assay; Ingenetix, Vienna, Austria).

**Advantages of real-time PCR are:**

- rapid results (2 h);
- limited manipulations;
- no risk of contamination with amplicons; and
- application to specimens other than gastric biopsies.

**Limitations of real-time PCR are:**

- possible amplification of bacteria having a similar *rrn*23S, including *Helicobacter helimannii* and possibly others not currently in databases;
• detection of mutations only inside the chosen amplicon;
• difficulty in identifying the exact mutation (position 2642 or 2643) in some formats; and
• need for an expensive thermocycler.

Mutation detection by dual-priming oligonucleotide-based multiplex PCR. Another method is dual-priming oligonucleotide (DPO)-based multiplex PCR (Seeflex®ClaR-H. pylori ACE detection; Seegene, Seoul, Korea). The DPO primer system differs from a conventional system by introducing a poly(I) linker between two unequal segments of primer sequences. This addition increases the specificity sufficiently to discriminate between single-base changes by using a one-step PCR, thus allowing an accurate multiplex PCR. To determine H. pylori resistance to clarithromycin, two forward and two reverse DPO primers were designed against the 23S rRNA gene: HP-F, HP-R, A2142G-F and A2143G-R, respectively. This four-primer combination amplifies three fragments: a common H. pylori sequence, an A2142G mutant and an A2143G mutant in a multiplex PCR. The mutant-specific DPO primers A2142G-F and A2143G-F were designed to contain either a single- or a two-base variation in the middle of the 3′ segment to maximize the ability of DPO to discriminate between single-base changes resulting from disruptions in the annealing of the 3′ segment.

When an H. pylori strain is a wild-type, neither A2142G-F nor A2143G-R anneals to the template, and PCR amplification occurs between HP-F and HP-R, resulting in the production of a 621 bp amplicon. In the case of the A2143G mutation, PCR amplification occurs simultaneously between HP-F and HP-R and between HP-F and A2143G-R, resulting in the production of a 621 bp and a 475 bp amplicon, respectively. Similarly, in the case of the A2142G mutation, PCR amplification occurs simultaneously between HP-F and HP-R and between HP-F and A2143G-R, resulting in the production of a 621 bp and a 193 bp amplicon, respectively. The detection of the A2142C mutation is not performed, as this mutation has rarely been identified (Woo et al. 2009). A specific apparatus has been designed to minimize post-PCR

Table 4.8. Genotypic methods used to detect macrolide resistance in Helicobacter pylori.

<table>
<thead>
<tr>
<th>Technique</th>
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</tr>
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<td>Using 23S rDNA amplification</td>
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<tr>
<td>Sequencing</td>
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<td>Restriction fragment length polymorphism (RFLP)</td>
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<td>Real-time polymerase chain reaction (PCR)</td>
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<td>Line probe assay (InnoLipa)</td>
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<td>Dual-priming oligonucleotide (DPO)-PCR</td>
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<td>Without using 23S rDNA amplification</td>
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<td>Fluorescence in situ hybridization (FISH)</td>
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<td>Electrocatalytic detection</td>
<td>Lapierre et al. (2003)</td>
</tr>
<tr>
<td>Microelectronic chip array</td>
<td>Xing et al. (2005)</td>
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</table>
manipulations. A procedure to minimize contamination with amplicons is available.

Advantages of DPO-based multiplex PCR are:

- rapid results (less than 5 h);
- no need for expensive apparatus; and
- limited risk of contamination.

Limitations of DPO-based multiplex PCR are:

- manipulation required;
- possible amplifications of bacteria having a similar \( rrn23S \); and
- detection of only the mutations for which primers are designed (e.g. A2142C is not included).

*Mutation detection by DNA-strip test*. One of the first methods developed to detect \( H. pylori \) and the mutations associated with its resistance to macrolides was a DNA-strip test (Van Doorn *et al.*, 1999). A new test based on this technology is now commercially available which also includes the detection of the mutations associated with fluoroquinolone resistance (GenoType HelicoDR; Hain LifeSciences, Nehren, Germany). It consists of a multiplex PCR followed by a hybridization with biotin-labelled specific oligonucleotides immobilized on a strip and visualized by a streptavidin/alkaline phosphatase-mediated staining reaction (Cambau *et al.*, 2009).

Advantages of the DNA-strip test are:

- rapid results (less than 6 h); and
- no need for expensive apparatus.

Limitations of the DNA-strip test are:

- manipulation required;
- risk of contamination;
- possible amplification of bacteria having a similar \( rrn23S \); and
- detection of only the mutations for which hybridization is carried out.

*Mutation detection by fluorescence in situ hybridization*. It is also possible to detect \( H. pylori \) and its resistance to clarithromycin without PCR by using a fluorescence *in situ* hybridization (FISH) method. The principle includes the use of specific probes, one targeting the 16S rRNA gene labelled with a Cy3 fluorochrome (red) allowing the specific detection of \( H. pylori \), and three others targeting the mutated 23S rRNA labelled with fluorescein (green). These four probes are used simultaneously. When a susceptible organism is present, it hybridizes only with the first probe and \( H. pylori \) appears red. When a resistant organism is present, the 23S rDNA probe as well as one of the other probes hybridize and \( H. pylori \) appears yellow due to the overlapping of the red and green colours (Trebesius *et al.*, 2000).

In contrast to the other methods, this method must be used on slides and is adapted to formalin-fixed paraffin-embedded histological preparations. It is commercially available as the SeaFAST \( H. pylori \) Combi Kit (SeaPro Theranostics, International b.v., Lelystad, Netherlands).

Advantages of FISH are:

- rapid results;
- no need for special apparatus;
- no impact of PCR inhibition;
- bacteria are visualized; and
- test can be performed in a pathology laboratory.

Limitations of FISH are:

- manipulation required;
- possible difficulty in interpretation;
- results are observer-dependent; and
- detection of only the mutations for which probes are available.

When a mutation occurs, it concerns only a single organism within a whole population. However, the descendants of this organism will also have the mutation, so along with successive replications, the population will be enriched in resistant organisms. However, it is only when the antibiotic is administered that the resistant organisms will be selected and eventually constitute the whole population. Molecular methods appear to be more sensitive in the detection of resistant organisms than phenotypic methods, as they are able to detect resistant organisms at a lower proportion in a mixed population of susceptible and resistant organisms.

The correlation between the presence of resistant organisms and \( H. pylori \) eradication with clarithromycin-based triple therapy has
been performed essentially using phenotypic methods and shows good results. However, among the strains classified as susceptible, failure to eradicate can still occur on occasion. While this can be explained by causes other than antimicrobial resistance, it may also be due to resistant mutants present at a low level and not detected phenotypically. Such resistant mutants are prone to emerge once the clarithromycin-based treatment is administered.

Molecular methods also allow detection of *H. pylori* in faecal samples, as anything present in the stomach is eliminated in faeces. However, except in cases where there is a very short transit time, these bacteria do not remain viable in faeces (Parsonnet *et al.*, 1999).

The detection of specific antigens of *H. pylori* has been used for diagnosis, and the detection of specific DNA fragments is now possible, with the advantage of also providing information on clarithromycin susceptibility. A nested PCR targeting the 23S rDNA can be performed on faeces followed by restriction of the amplicons or sequencing (Rimbara *et al.*, 2005; Kawai *et al.*, 2008). The ClariRes real-time PCR assay can also be used for this purpose. Two published studies (Schabereiter-Gurtner *et al.*, 2004; Lottspeich *et al.*, 2007) showed a marked difference with regard to the sensitivity in detecting *H. pylori* in stools: 98% and 63%, respectively. This difference may relate to the maintenance of the samples prior to testing, or the need for duplicate testing of samples. However, the difficulty in detecting targets that are not present in high quantities in stool samples, e.g., *H. pylori*, has often been raised, due to the presence of inhibitors of the Taq polymerase in faeces. These inhibitors have been identified as complex polysaccharides of vegetable origin, which follow the DNA extraction procedure and are difficult to eliminate (Monteiro *et al.*, 1997). Therefore, special care must be taken in DNA preparation.

**FLUOROQUINOLONES. Mutations.** Fluoroquinolones target the A subunit of bacterial DNA gyrase, an enzyme that supercoils DNA. This enzyme is comprised of four subunits: two A subunits encoded by the *gyrA* gene and two B subunits encoded by the *gyrB* gene. Resistance to fluoroquinolones has been associated with mutations in the so-called ‘quinolone resistance-determining region’ (QRDR) of the *gyrA* gene in *H. pylori* as in other bacteria (Moore *et al.*, 1995).

The amino acids in positions 87 and 91 of *gyrA* are very important, and a mutation in either may lead to a high level of resistance. However, it is possible to find resistant strains without a mutation in these positions (Tankovic *et al.*, 2003; Bogaerts *et al.*, 2006; Cattoir *et al.*, 2007).

While there is a cross-resistance between the different fluoroquinolones, the impact of such mutations on MICs appears to be less important for some of the newer fluoroquinolones, including sitafloxacin, garenoxacin and finafloxacin (Suzuki, H. *et al.*, 2009). In contrast to most fluoroquinolones, the activity of finafloxacin increases when the pH decreases.

**Detection.** The above mutation can be detected by designing a real-time PCR based on the QRDR of *gyrA*. The FRET-MCA principle has also been applied. However, the situation is complicated by the fact that there is a polymorphism, i.e. all of the mutations (eventually present) are not linked to resistance but generate peaks when the MCA is performed. Nevertheless, using two biprobes, Glocker and Kist (2004) were able to detect the relevant mutations in the triplets for aa87 and aa91 leading to resistance. The allele-specific PCR methodology has also been applied to resistance detection (Nishizawa *et al.*, 2007). However, only one kit (GenoType HelicoDR) is currently commercially available that allows the detection of both fluoroquinolone resistance and clarithromycin resistance. This is a DNA-strip test, the principle of which was presented above. Nine bands can be detected for *gyrA*, five corresponding to susceptible phenotypes which had to be included because of the polymorphism observed (N87 (AAC or AAT), T87 (AAC or ACT) and D91 (GAT)), and four corresponding to resistant phenotypes (N87K (AAA), D91W (AAT), D91G (GGT) and D91Y (TAT)). The sensitivity and specificity of this approach, based on 105 gastric biopsy specimens, were 87% and 98%, respectively (Cambau *et al.*, 2009).
Tetracyclines. Mutations. Tetracyclines target the 30S ribosomal subunit where they prevent the fixation of the aminoacyl-tRNA, resulting in the termination of protein synthesis and leading to a truncated peptide. Resistance is due to a mutation(s) in the nucleotide triplet in position 926–928 of the h1 loop of the 16S rRNA (AGA926–928TTC). There is some cross-resistance with minocycline and doxycycline (Gerrits et al., 2002a; Trieber and Taylor, 2002).

Mutations in only one or two of the nucleotides at the same site can also be encountered. In this case the resistance level is lower (4 mg/l). The need for a triple mutation may explain the rarity of such strains (Dailidiene et al., 2002; Gerrits et al., 2002a; Nonaka et al., 2005). However, some resistant strains where the above mutation(s) was not present have been described and the mechanism for this has been linked to efflux pumps (Wu et al., 2005).

Detection. A PCR–restriction fragment length polymorphism (RFLP) method was the first approach used for detecting tetracycline resistance. The PCR amplifies part of the 16S rDNA and the amplicons are then submitted to the Hinfl restriction enzyme. Strains having triple mutations associated with a high resistance level exhibit three bands, while those susceptible or with reduced susceptibility have only two bands (Ribeiro et al., 2004).

Two real-time PCRs have also been developed which can distinguish single, double or triple base-pair mutations directly on biopsy specimens (Glocker et al., 2005; Lawson et al., 2005). However, in one of the studies where 1000 isolates were screened, the test was able to detect only ten out of 18 strains that possessed reduced susceptibility (Lawson et al., 2005).

Rifamycins. Mutations. Rifamycins, including rifabutin, which is used in H. pylori treatment, target the β subunit of the DNA-dependent RNA polymerase subunit β encoded by the rpoB gene. Resistance is associated with mutations at codons 524, 525 and 585 of the rpoB gene (Heep et al., 1999). Another mutation at position 149 has also been described (Heep et al., 2000). However, some resistant strains may harbour a mutation other than those described (Glocker et al., 2007b; Chisholm and Owen, 2009). There is also cross-resistance between all compounds of this group.

Detection. The detection of rifamycin resistance has been performed only by PCR and sequencing. The rarity of this resistance and the presence of numerous silent mutations may be the reasons for the lack of development of a molecular test.

Amoxicillin. Mutations. Amoxicillin, along with other β-lactams, inhibits peptidoglycan synthesis. The first amoxicillin-resistant H. pylori (MIC 8 mg/l) was described in 1996 in the Netherlands. This resistance was linked to a mutation in the gene coding for penicillin binding protein (PBP)-1A (Ser414Arg), which leads to a decreased affinity for β-lactams (Gerrits et al., 2002b). Further studies have shown that other mutations could also be involved.

While β-lactamase-producing H. pylori strains had never been isolated in the past, a recent publication reports on such a strain (MIC 256 mg/l) which had a type bla (TEM1) β-lactamase (Tseng et al., 2009). The possible spread of this strain is alarming, in view of the limited armamentarium we have to eradicate H. pylori.

Detection. No molecular test has been developed to detect the mutation in pBP1A.

Metronidazole. Mutations. Metronidazole must be reduced inside the bacterial cell to be active and induce DNA alterations. This reduction is due to the nitroreductase encoded by rdxA (Goodwin et al., 1998) and possibly the flavin oxidoreductase encoded by frxA and other enzymes (Mendz and Megraud, 2002; Marais et al., 2003). As stated in Section 4.2.1, this reduction depends on the redox potential in the intracellular environment (Kaakoush et al., 2009). The presence of mutations either in or upstream of the rdxA and frxA genes has been associated with
metronidazole resistance. Efflux mechanisms could also be involved (Van Amsterdam et al., 2005).

**Detection.** The link between mutations and metronidazole resistance is not strong enough to develop a molecular test. An original approach proposed was detection of the protein RdxA by immunoblotting. Using a rabbit anti-RdxA antiserum, a 24 kDa band can be observed in susceptible strains but not in resistant strains (Latham et al., 2002). However, this technique has never been used further.

### 4.5 Conclusions

The difficulty of treating patients infected with *H. pylori* is now common in many parts of the world, essentially due to an increase in antimicrobial resistance. This chapter is a plea to consider *H. pylori* infection as any infectious disease and to carry out susceptibility testing before prescribing a treatment. The aim of this approach is to achieve a greater than 90% success rate and minimize the selection of resistance, as advocated by Graham and Shiotani (2008). The comparison of regimens that include antibiotics for which *H. pylori* resistance is known to be high, without breaking down the results according to susceptibility testing, has limited value.

There is no excuse for failing to perform susceptibility testing, because *H. pylori* treatment is not an emergency and commercial kits are readily available for quickly testing susceptibility to the most relevant antibiotics (clarithromycin, levofloxacin). Metronidazole remains the only antibiotic for which testing is not currently recommended and this drug can be used in many different combinations, including with bismuth salts, which appears to be quite successful as a quadruple therapy.

New drugs are desperately needed, but few appear to be in the pipeline. New combinations must be tested. In this respect the sequential treatment is promising even if its impact on resistant strains is still not well documented.

### References


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5 Extragastric Manifestations of *Helicobacter pylori* Infection

**H.M. Mitchell,** *N.O. Kaakoush and P. Sutton*

### 5.1 Introduction

The initial recognition by Marshall and Warren that *Helicobacter pylori* was the principal aetiological agent of gastritis proved to be a seminal finding that led to the later recognition that *H. pylori* also played a major role in peptic ulcer disease, gastric cancer and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Marshall and Warren, 1984; Marshall et al., 1985; International Agency for Research on Cancer, 1994; McColl et al., 1997; Wotherspoon, 1998). This knowledge has resulted in the introduction of therapies targeted against *H. pylori* that not have only been shown to prevent ulcer recurrence and lead to regression of low-grade MALT lymphoma, but also have the potential, if subjects are treated early, to prevent progression to gastric cancer (Graham et al., 1992; Isaacson et al., 1999; Du and Isaacson, 2002; see Mégraud, Chapter 4, this volume).

In addition to studies investigating the role of *H. pylori* in gastroduodenal disease, since the late 1980s an increasing number of studies have focused on the role of *H. pylori* infection in a range of extragastric diseases. The ability of *H. pylori* to induce chronic gastric inflammation that in many cases is lifelong makes the possibility that this bacterium could lead to extragastric disease, through induction of systemic effects, a feasible hypothesis.

To date, associations between *H. pylori* infection and a range of cardiovascular, respiratory, hepatobiliary, dermatological, neurological, autoimmune, allergic and haematological diseases, as well as growth and weight disorders and retardation, have been investigated. However, there is by no means consensus with regard to the findings of such studies. As discussed in an excellent review by Goodman et al. (2006), many studies examining the association between *H. pylori* infection and extragastric manifestations have major limitations in regard to experimental design and statistical power. In particular, many studies include a limited number of subjects, involve biases in the selection of controls and often fail to recognize confounders such as age, ethnic background and socioeconomic status. Given that acquisition of *H. pylori* infection is strongly linked to these factors, failure to match patients and controls for such factors is highly likely to confound these studies (Mitchell, 2001; Goodman et al., 2006). In addition, the biological plausibility of a number of the investigated association studies is questionable.

Given the plethora of extragastric diseases investigated in relation to their possible association with *H. pylori* infection, it is not feasible
to cover all of these in this chapter. Thus, extragastric diseases for which there is currently strong or mounting evidence for a role of *H. pylori* infection are discussed in depth.

5.2 Idiopathic Thrombocytopenic Purpura

Perhaps the most compelling and mysterious association of *H. pylori* infection and extragastric disease is that with ITP. While the term ‘ITP’ initially arose from ‘idiopathic thrombocytopenic purpura’, reflecting the unknown aetiology of the condition at the time, with increased understanding of the pathogenic mechanisms behind this condition, ITP is now increasingly referred to as ‘immune or immune-mediated thrombocytopenia’ (Rodeghiero et al., 2009). This often mild pathological condition is characterized by the presence of low platelet counts. Individuals with ITP are frequently asymptomatic or may develop bruising (purpura). However, in a subset of patients in whom platelet counts are particularly low, severe bleeding can result, with significant morbidity and mortality.

This condition can be subdivided into primary and secondary ITP, with secondary ITP including those resulting from an identified underlying disease, drug exposure or infection. For example, ITP is believed to develop as a secondary outcome of a wide range of conditions, including autoimmune diseases such as systemic lupus erythematosus and lymphoproliferative diseases such as chronic lymphocytic leukaemia, as well as chronic infections including HIV (Cines et al., 2009).

The precise mechanism behind ITP is still unclear, but appears related to the production of platelet-reactive autoantibodies. In individuals with ITP, platelets coated with these autoantibodies are removed more quickly than they can be generated, resulting in a deficiency. These autoantibodies can react with a number of platelet surface glycoproteins, including GP Ib-IXa, GP IIb-IX and GP IIb-IIIa (McMillan, 2000).

In 1998, Gasbarrini and colleagues investigated whether this condition could also be caused by chronic *H. pylori* infection. Of 18 ITP patients examined, 11 were found to be positive for *H. pylori*. These infected patients were treated with an *H. pylori* triple therapy eradication regime, then blood platelet and anti-platelet autoantibody levels were assessed 2 and 4 months later (Gasbarrini et al., 1998). While there was no change in these parameters in ITP patients who were *H. pylori*-negative, in infected patients in whom *H. pylori* was eradicated a significant increase in platelet numbers was observed. Further, anti-platelet autoantibodies disappeared in six out of eight successfully treated patients. In contrast, however, in three patients in whom therapy failed to eradicate *H. pylori*, there was no increase in platelets, nor a change in autoantibody levels. This last point is very important, as overall these data indicate that the resolution of ITP in treated patients was strongly associated with successful eradication of *H. pylori* infection and not due to the incidental treatment of another, unidentified bacterial infection – although this possibility cannot be completely discounted.

This initial observation has been supported by the majority of similar, subsequent trials (Veneri et al., 2002; Suvajdzic et al., 2006; Campuzano-Mayà, 2007), although some studies did not find the same effect (Ahn et al., 2006; Estrada-Gomez et al., 2007). Further, in a prospective study in which *H. pylori*-negative ITP patients were treated with an *H. pylori* eradication regime, no effects on their platelet counts were observed, providing important support for the role of *H. pylori* eradication per se, rather than other effects of the medication on ITP being responsible for resolution of this condition (Asahi et al., 2006). For a recent systematic review on studies that have evaluated ITP following *H. pylori* eradication, see Stasi et al. (2009).

While evidence based on observational and treatment studies would support the role of *H. pylori* in ITP, the big question that remains is how *H. pylori* infection could induce autoantibodies against platelets. While this remains unknown, it has been proposed that it could possibly be related to the infection inducing cross-reactive antibodies. For example, one study has reported that antibodies eluted from the surface of platelets...
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obtained from some ITP patients react with the H. pylori virulence factor cytotoxin-associated gene A protein (CagA), although as this also occurred for samples from three H. pylori-uninfected individuals, the significance is unclear (Takahashi et al., 2004). The best explanation for the pathogenic mechanism behind ITP and H. pylori infection has thus far been provided by Asahi et al. (2008), who found that H. pylori infection of ITP patients was associated with a change in the balance of Fcγ receptors on their macrophages. This receptor imbalance is believed to result in increased macrophage activation, which was redressed following H. pylori eradication and shown to precede an improvement in platelet parameters in these ITP patients. The authors proposed that activation of macrophages in H. pylori-infected ITP patients played two roles in this autoimmune condition: in platelet destruction and by supporting the generation of autoimmune antibodies (Asahi et al., 2008).

Therefore, in contrast to many other diseases with which H. pylori has been associated, there is strong evidence indicating that infection with H. pylori is a key factor in the development of ITP. While the precise mechanism behind this association requires further investigation, the 2006 Maastricht III Consensus Report recommended that H. pylori infection should be sought for and treated in patients with ITP (Malfertheiner et al., 2007).

5.3 Iron-deficiency Anaemia

Iron is crucial for the production of haemoglobin (Hb), an essential transporter of oxygen. Most of the body’s iron is stored in Hb inside red blood cells, excluding approximately 30% that is stored as ferritin and haemosiderin in the bone marrow, spleen and liver. Iron deficiency can result from a number of mechanisms. For example, if the body loses iron in relatively high quantities, this can, over time, result in depletion of its iron stores. Iron deficiency can also develop when iron intake is low or metabolism of iron is inefficient. The metabolic uptake and use of iron is limited by several factors that include its low solubility and the ability of chelators to impair iron absorption. Gastric acidity has been shown to assist in the conversion of iron to absorbable forms and thus high gastric pH can impede absorption of iron by the stomach. Additionally, some disorders disrupt the integrity of the enteric mucosa, which can result in the inhibition of iron uptake (Gasche et al., 2004).

Iron-deficiency anaemia (IDA) develops due to a lack of metabolic iron, a common causal factor in human subjects being chronic blood loss. It is estimated that this condition affects roughly 15% of the population worldwide (Clark, 2008).

5.3.1 Mechanisms by which H. pylori infection may produce iron deficiency

In the 1990s, researchers began investigating the involvement of H. pylori infection in IDA. As opposed to some conditions with which H. pylori infection has been correlated, associations with IDA are biologically plausible. One proposed mechanism may be blood loss as a result of infection-driven gastric erosion, development of atrophy or peptic ulcer disease (Milman et al., 1998; Muhsen and Cohen, 2008), although not all research supports this possibility (Carnicer et al., 1997; Marignani et al., 1997; Barabino et al., 1999; Konno et al., 2000; Ashorn et al., 2001; Sugiyama et al., 2002; Kostaki et al., 2003; Yoshimura et al., 2003). For example, Dufour et al. (1993) observed that, following eradication of H. pylori, resolution of IDA occurred in patients diagnosed with gastritis who at the time of endoscopy had no bleeding lesions and no blood in their faecal stream. Another example is a case series by Annibale et al. (1999) on 30 patients with a long history of IDA, in whom H. pylori-associated asymptomatic gastritis was the only pathological finding detected in their gastrointestinal tract. Following H. pylori eradication therapy and discontinuation of iron replacement therapy, 75% of patients recovered from IDA (P < 0.001) and ferritin values (mean ± standard error) increased from 5.7 ± 0.7 μg/l to 24.5 ± 5.2 μg/l after 6 months. Significantly, after 12 months the percentage of patients who had
recovered from IDA increased to 91.7% (Annibale et al., 1999).

Another putative mechanism is a reduction in the host’s ability to absorb iron (Lee, 2007). A low gastric pH and sufficient quantities of ascorbic acid have been shown to be required for efficient absorption of iron (Muhsen and Cohen, 2008). *H. pylori* increases stomach pH by buffering the secreted acid and damaging the epithelial layer due to atrophy. In addition, several studies have observed a reversible negative impact by *H. pylori* on gastric ascorbic acid (Ruiz et al., 1994; Zhang et al., 1998). The effect of *H. pylori* on iron absorption is supported by the findings of Ciacci et al. (2004) who analysed iron absorption and ferritin levels in 55 individuals without occult blood in their stools. This showed that the levels (mean ± standard deviation) of ferritin (ng/ml) and iron absorption (delta iron, μg/dl) were significantly lower in *H. pylori*-positive versus *H. pylori*-negative subjects: 59.3 ± 22.2 versus 76.5 ± 23.7 (*P* = 0.008) and 86.8 ± 42.7 versus 132.5 ± 55.7 (*P* = 0.001), respectively. When the results were divided by gender, only the *H. pylori*-positive women retained significantly lower iron absorption rates and ferritin levels (Ciacci et al., 2004). Iron absorption improved significantly, although only in females, after clearance of *H. pylori* infection, with iron absorption in individual patients after *H. pylori* eradication being on average significantly higher (95% confidence interval (CI) 17.022, 32.746; *P* < 0.001) than before treatment (Ciacci et al., 2004).

A third possible mechanism is the enhanced capacity of the bacterium to scavenge iron in the gastric mucosa (Choe et al., 2003; Lee, 2007) (Fig. 5.1). Park et al. (2006) performed proteomic analyses on *H. pylori*

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![Fig. 5.1](image-url)  
**Fig. 5.1.** Enhanced capacity of *Helicobacter pylori* to scavenge iron in the gastric mucosa. Iron is present in its free form and bound to transferrin within the mucous layer (A). Infection with *H. pylori* results in the inflammation of the epithelial layer (B, C) and in neutrophil migration to the site of infection (D). Neutrophils secrete lactoferrin, which has the capacity to capture iron from transferrin (D). Through its ability to target lactoferrin via receptors, *H. pylori* infection may decrease the amount of iron available for uptake (E).
strains isolated from patients with and without IDA, demonstrating that these strains could be distinguished by their protein expression patterns and that a number of proteins had higher levels of expression in IDA strains. Furthermore, it was shown that strains recovered from patients with IDA possessed enhanced iron uptake abilities and iron-dependent rapid growth compared with those from patients without IDA (Yokota et al., 2008). Recently, iron-repressible outer membrane proteins (IROMP) were identified under iron-restricted conditions but not under iron-rich conditions in H. pylori strains associated with IDA (Lee et al., 2009). In the iron-depleted state, specific H. pylori strains associated with IDA demonstrated an advantage in iron acquisition due to a higher expression of IROMP. These results suggest that some strains possess an advantage in iron acquisition, thus providing a possible explanation as to why some patients infected with H. pylori are more likely to develop IDA.

5.3.2 Evidence for and against a role for H. pylori infection in iron deficiency

Since the late 1980s many studies have examined the association between H. pylori infection and development of IDA. Having reviewed the literature, Goodman et al. (2006) concluded that ‘overall the evidence shows a positive association, and plausible mechanisms by which the bacterium could cause, perpetuate, or worsen IDA have been proposed, but support for a causal relationship is not strong’. While at the time Goodman et al. were not convinced that H. pylori was a cause of IDA, they clearly did not rule out this possibility. In 2008, Muhsen and Cohen performed comprehensive literature searches and subsequent analyses on the relationship between H. pylori infection and iron stores. Their analyses indicated that evidence supporting a role for H. pylori in IDA was not strong, being based on case reports, observational epidemiological studies and a very limited number of intervention trials (Muhsen and Cohen, 2008). Their review identified 15 observational epidemiological studies that reported supportive findings linking H. pylori and decreased iron stores (Yip et al., 1997; Milman et al., 1998; Peach et al., 1998; Collett et al., 1999; Choe et al., 2000, 2001; Parkinson et al., 2000; Berg et al., 2001; Seo et al., 2002; Kaffes et al., 2003; Nahon et al., 2003; Weyermann et al., 2005; Yang et al., 2005; Baggett et al., 2006; Cardenas et al., 2006a). However, due to shortcomings in the design of many of these studies they could not conclude whether the infection per se caused changes in iron levels (Muhsen and Cohen, 2008). These authors found a limited number of trials that attempted to resolve IDA using anti-H. pylori therapy; of these, most had shortcomings in either design or number of subjects, and of those that were well-designed conflicting results were reported. Nevertheless, based on the results of three well-designed studies (Choe et al., 2001; Baggett et al., 2006; Cardenas et al., 2006a), Muhsen and Cohen (2008) calculated that there was a 2.8-fold (95% CI 1.9, 4.2) increased risk of developing IDA in H. pylori-infected patients. Additionally, based on the results of four studies (Milman et al., 1998; Parkinson et al., 2000; Baggett et al., 2006; Cardenas et al., 2006a), they determined that the risk of developing iron deficiency alone was approximately 40% higher in H. pylori-infected patients.

However, the number of studies supporting a role for H. pylori in IDA would have been higher if Muhsen and Cohen (2008) had not missed two studies performed in 2007. In one study, 29 male IDA patients were given a triple therapy regimen for eradication of H. pylori. Following treatment, all patients had normal Hb levels within 4–69 months, which was also accompanied by a significant decrease in H. pylori immunoglobulin (Ig) G antibodies (Hershko et al., 2007). Interestingly, four of the patients achieved normal Hb levels without receiving any oral iron treatment (Hershko et al., 2007). In a second study consisting of 70 patients aged 4–16 years who had undergone upper endoscopy for recurrent gastrointestinal complaints during a 2-year period, significantly lower levels of serum iron and ferritin were observed in H. pylori-positive patients than in those without infection (P < 0.001) (Suoglu et al., 2007). IDA was observed in 20 (57.1%) and six (17.1%) patients in the H. pylori-positive and -negative
groups, respectively ($P < 0.01$). These authors found $H.\text{ pylori}$ infection to be the only variable that had a significant effect on IDA (odds ratio (OR) = 6.444; 95% CI 2.134, 19.453; $P = 0.001$) (Suoglu et al., 2007).

Further studies published since 2008 have continued to provide controversial results, with some supporting and some opposing a role for $H.\text{ pylori}$ in the development of IDA. For example, a case–control study on 325 adult patients found that gastric atrophy was strongly associated with IDA, and that this was likely to be causative in some patients and contributory in others (Kaye et al., 2008). In that study, 40 of 156 (25.6%) of patients with IDA were shown to have significant body atrophy compared with only seven of 169 (4.1%) controls ($P < 0.001$). Additionally, a statistically significant association was found between $H.\text{ pylori}$ infection and IDA in 117 women with uncomplicated pregnancy, all 18 women with IDA being $H.\text{ pylori}$-positive as compared with only 50% of women without IDA (Mulyaim et al., 2008).

In a paediatric study, Cherian et al. (2008) found no association between $H.\text{ pylori}$ infection or helminthic infection and the presence of IDA in 181 African refugee children less than 16 years of age. In contrast, iron deficiency was corrected following antibiotic eradication of $H.\text{ pylori}$ infection in three children who had symptomatic IDA with no obvious clinical cause (Cardamone et al., 2008). However, in a study involving 200 $H.\text{ pylori}$-infected Bangladeshi children, Sarker et al. (2008) found that iron supplementation cure rates of IDA were comparable between patients who received $H.\text{ pylori}$ treatment and those who did not. In a further study, Muhsen et al. (2009) examined the association between $H.\text{ pylori}$ seropositivity and low ferritin levels in sera from 509 Israeli Arab children. Low ferritin levels were found in a significantly higher proportion of $H.\text{ pylori}$-seropositive (14.5%) than -seronegative (8.6%) children. Interestingly, a higher frequency of low ferritin levels was detected among children with $H.\text{ pylori}$-CagA seropositivity (Muhsen et al., 2009). Additionally, while Fayed et al. (2008) reported a high prevalence of $H.\text{ pylori}$ infection in 25 patients <18 years of age with refractory IDA, no significant influence of $H.\text{ pylori}$ infection was observed with regard to the severity of IDA.

In a study conducted in six Latin American countries (Argentina, Brazil, Bolivia, Cuba, Mexico and Venezuela) and following adjustment of the statistical analyses to include confounding factors, no association was found between $H.\text{ pylori}$ infection and IDA (Santos et al., 2009). Recently, Gessner and colleagues revisited their previous results, which had suggested that treatment of $H.\text{ pylori}$ infection did not reduce the prevalence of iron deficiency or anaemia among Alaskan native children and, based on a study by Cardenas et al. (2006b), they hypothesized that 14 months was not sufficient time for the resolution of $H.\text{ pylori}$-associated gastric problems (Gessner et al., 2006). Their new study showed that 40 months after resolution of $H.\text{ pylori}$ infection the prevalence of iron deficiency and anaemia was reduced, suggesting that for some children $H.\text{ pylori}$ likely plays a role in haematological outcomes (Fagan et al., 2009). Gessner (2009) also investigated reasons for the high incidence of IDA in Alaskan native individuals (50,964 children and 30,154 pregnant or postpartum women) and concluded that the increased risk observed supported the role of a region-specific environmental factor, but that nutritional iron deficiency or $H.\text{ pylori}$ infection was unlikely to be the sole or major aetiology of this high anaemia prevalence.

### 5.3.3 Conclusion

Although overall there is evidence to support a positive association between $H.\text{ pylori}$ infection and IDA, and plausible mechanisms have been proposed for this association, data indicating otherwise are also available; moreover, support for a direct causal link is minimal due to inadequate study design and the presence of a multitude of confounding factors in many studies. Although the association between $H.\text{ pylori}$ and IDA requires further investigation, the 2006 Maastricht III Consensus Report recommended that $H.\text{ pylori}$ infection should be sought for and treated in patients with unexplained IDA (Malferttheiner et al., 2007).
5.4 Cardiovascular Disease

Cardiovascular disease remains the principal cause of death in the USA, Europe and much of Asia despite interventions such as lifestyle changes and the use of a range of pharmacological approaches to lower plasma cholesterol concentrations (Ross, 1999).

Coronary heart disease (CHD) commonly results from the accumulation of atheromatous plaques within the walls of the coronary arteries that supply the myocardium of the heart with oxygen and nutrients. As a result of this accumulation, a reduced blood supply to the heart muscle occurs, which in turn can lead to ischaemic heart disease (IHD). Although for many years the process of atherogenesis was considered to be due to the accumulation of lipids within the artery wall, current evidence suggests this is a multifactorial process in which chronic inflammation plays a pathophysiological role in the evolution, progression and destabilization of atherosclerosis (Ross, 1999; Shah, 2002). The possibility that chronic inflammation resulting from infection could play a role in atherogenesis was first raised in the late 1800s (Shah, 2002). Indeed in 1911, Frothingham stated ‘The sclerosis of old age may simply be a summation of lesions arising from infections or metabolic toxins’ (Frothingham, 1911). Given the postulated role of chronic infections in the genesis and development of vessel wall injury and atheromatous plaques, the associations between a range of chronic infective agents (including viruses and bacteria) and cardiovascular disease have been investigated since the late 1970s.

5.4.1 H. pylori and cardiovascular disease

In 1994 Mendall and colleagues first investigated the specific association between H. pylori infection and CHD. In this case–control pilot study, the H. pylori serostatus of 111 consecutive patients with documented CHD was compared with that in 74 controls, all of whom were white men aged 45–65 years. After adjustment for age, cardiovascular risk factors, current social class and features of their childhood environment known to be risk factors for H. pylori infection, a weak positive association was shown to exist between H. pylori seropositivity and CHD (OR = 1.9) (Mendall et al., 1994). Following this report, at least 20 epidemiological studies were conducted between 1994 and 1997 in which the association between H. pylori antibody levels and either CHD or stroke was investigated. A review of these studies by Danesh et al. (1997) showed that many comprised small numbers of subjects and failed to adjust for potential confounding factors, including selection bias in relation to controls and socio-economic status. While a positive association between H. pylori and CHD was observed in many studies, Danesh et al. (1997) noted that, in studies where attempts were made to adjust for potential confounding factors and where controls were sampled from approximately the same population as the cases, the association tended to be weaker.

In 1998, Pasceri et al. investigated for the first time the association between both H. pylori and CagA status and IHD in 88 patients with IHD and 88 age- and sex-matched controls from a similar social background. Following adjustment for age, sex, major cardiovascular risk factors and social class, this showed a significant association between H. pylori seropositivity and IHD (OR = 2.8; 95% CI 1.3, 7.4; P < 0.001). Further investigation showed that the prevalence of CagA-positive strains in IHD patients (43%) was significantly higher than in the controls (17%; adjusted OR = 3.8; 95% CI 1.6, 9.1; P < 0.001). Conversely, the prevalence of CagA-negative strains was not significantly different in patients and controls, a finding that led these authors to suggest that the association between H. pylori infection and IHD may be related to more virulent H. pylori strains (Pasceri et al., 1998).

While several subsequent case–control and cohort studies further assessed the possible association between CagA status and vascular disease, these studies resulted in conflicting results (Koenig et al., 1999; Gunn et al., 2000; Figura et al., 2002; Mayr et al., 2003). In an attempt to clarify this issue, Pasceri et al. (2006) conducted a meta-analysis on ten retrospective case–control studies (1527 cases/1661 controls) and three prospective cohort studies (701 cases/1439 controls) that examined the association between CagA status and IHD,
plus four retrospective case–control studies (513 cases/590 controls subjects) that examined the association between CagA status and cerebral ischaemia. While in the case–control studies a positive association was observed between IHD and CagA-positive strains (OR=1.87; 95% CI 1.46, 2.40), no significant association was found with CagA-negative strains (OR = 1.15; 95% CI 0.83, 1.60) (Pasceri et al., 2006). Interestingly, in the three prospective cohort studies, the association of CagA-positive strains with IHD was weaker, but still significant (OR = 1.26; 95% CI 1.05, 1.51).

In the four retrospective case–control studies, CagA-positive strains were shown to be significantly associated with cerebral ischaemia (OR = 2.43; 95% CI 1.89, 3.13); however, again no association was observed for CagA-negative strains. Based on the results of the three prospective cohort nested case–control studies in which a weak association was found between CagA-positive H. pylori and the occurrence of fatal and non-fatal myocardial infarction in follow-up, they concluded that while these findings 'virtually ruled out any major relationship between CagA status and IHD they did not rule out a minor role in IHD' (Pasceri et al., 2006).

A further meta-analysis of nine studies (4241 subjects), comprising six retrospective case–control studies and three nested prospective case–control studies that had assessed the association between CagA seropositivity and acute coronary events, was published by Franceschi et al. (2009). This found that CagA seropositivity was significantly, although weakly, associated with the occurrence of acute coronary events (OR = 1.34; 95% CI 1.15, 1.58; P = 0.0003) (Franceschi et al., 2009), a finding consistent with that of Pasceri et al. (2006).

5.4.2 Effect of H. pylori on intimal-medial thickness

Given the known positive association between cardiovascular risk factors and carotid artery intimal-medial thickness (O’Leary et al., 1991, 1992), a number of studies have examined the relationship between H. pylori and intimal-medial thickness. While the majority of studies found no association between intimal-medial thickness and H. pylori positivity per se (Espinola-Klein et al., 2000), a number have reported a link with CagA-positive strains. For example, in a large prospective study which investigated the relationship between CagA status and progression of carotid atherosclerosis, Mayr et al. (2003) showed that infection with CagA-positive strains was associated with a greater increase of carotid intimal-medial thickness during 5-year follow-up, compared with CagA-negative and H. pylori-negative subjects. While Diomedi et al. (2004) also reported an association between CagA-positive H. pylori strains and increased intimal-medial thickness in patients with atherosclerotic stroke, Markus et al. (2002) found neither H. pylori nor CagA-
positive strains to be a major risk factor for early arteriosclerosis as assessed by carotid artery intimal-medial thickness.

5.4.3 Potential effect of *H. pylori* on risk factors for ischaemic heart disease

To date, a large number of studies have focused upon the effect of *H. pylori* on a range of risk factors for atherosclerosis including plasma levels of lipids, fibrinogen, C-reactive protein (CRP), total cholesterol and cytokines. In 1998, a meta-analysis of 18 epidemiological studies examined the possible link between *H. pylori* and vascular risk factors including systolic blood pressure, diastolic blood pressure, body mass index, plasma viscosity, white cell count, and concentrations of total cholesterol, high-density lipoprotein cholesterol, fibrinogen, blood glucose and CRP. However, no strong correlations were found between *H. pylori* seropositivity and any of these vascular risk factors (Danesh and Peto, 1998). Since then, numerous studies have further investigated the possible association between *H. pylori* and a range of risk factors for IHD with, as previously, conflicting results. A recent review by Manolakis et al. (2007) provides a good coverage of this topic. In a recent study, Siddiqui (2009) examined the effect of *H. pylori* eradication on blood levels of CRP, homocysteine, folate and vitamin B12 in 46 healthy African Americans over 40 years of age with *H. pylori* CagA positivity. In subjects in whom eradication was successful (*n* = 43), eradication induced no significant change in homocysteine, folate or B12 levels; however, CRP levels decreased significantly 2 months after eradication (*P* = 0.02) (Siddiqui, 2009). These findings, however, are by no means consistent (Manolakis et al., 2007).

5.4.4 *H. pylori* and the possibility of a direct role in atherosclerosis

A substantial number of studies have investigated the possibility of a direct role for *H. pylori* in atherosclerosis. For example, Kaplan *et al*. (2006) investigated the presence of *H. pylori* DNA in atherosclerotic plaque specimens from the carotid arteries of 52 patients who had undergone carotid endarterectomy and in macroscopically healthy regions of the ascending aorta of 52 control patients who had undergone coronary artery bypass grafting. This showed a significantly higher detection rate of *H. pylori* DNA in atherosclerotic plaques (17.3%) as compared with none in controls (*P* = 0.003) (Kaplan *et al*., 2006). Consistent with these findings, Arias *et al*. (2006) detected *H. pylori* DNA in 83% of atherosclerotic carotid tissue samples, 64% of which were CagA-positive, while Farsak *et al*. (2000) detected *H. pylori* DNA in 37% of endarterectomy specimens as compared with none in controls (*P* < 0.001). Interestingly Ameriso *et al*. (2001) not only detected *H. pylori* DNA in 20/38 atherosclerotic plaques but also provided immunohistochemical evidence of *H. pylori* infection in 50% of positive plaques. However, many other studies have failed to detect *H. pylori* in atherosclerotic plaques. For example, Blasi *et al*. (1996) failed to detect *H. pylori* DNA in plaque specimens collected from 47 *H. pylori*-seropositive patients, while Danesh *et al*. (1999) found *H. pylori* DNA in only 1/39 atheromatous specimens collected from patients at carotid surgery and Weiss *et al*. (2006) were unable to detect *H. pylori*-specific DNA in atherosclerotic plaques from 36 patients undergoing carotid endarterectomy for symptomatic carotid artery stenosis. More recently Reszka *et al*. (2008) reported a similar frequency of *H. pylori* DNA to be present in aortic wall samples from patients with stable coronary artery disease (80%) and controls (85%).

5.4.5 *H. pylori* and molecular mimicry

**CagA antibodies**

To determine the link between CagA-positive strains and atherosclerosis, Franceschi *et al*. (2002) investigated whether anti-CagA antibodies cross-reacted with antigens of normal and atherosclerotic arteries. Immunohistochemical examination of eight umbilical
cord sections, 14 atherosclerotic artery sections and ten gastrointestinal tract sections showed that polyclonal anti-CagA antibodies cross-reacted with antigens of both normal and atherosclerotic blood vessels, suggesting that molecular mimicry may exist between CagA and vascular wall peptides (Franceschi et al., 2002). Based in part on these findings, Franceschi et al. (2009) recently explored the role of CagA-positive H. pylori strains in the pathogenesis of coronary instability. The study showed that, in patients with unstable angina, anti-CagA antibody titres were significantly higher than in patients with stable angina \((P < 0.02)\), normal coronary arteries \((P < 0.01)\) or in 50 age- and sex-matched healthy controls \((P < 0.02)\). Significantly, in all specimens obtained from patients with both stable and unstable angina, anti-CagA antibodies recognized antigens localized inside coronary atherosclerotic plaques. Based on these findings Franceschi et al. (2009) concluded that ‘in a subset of patients with unstable angina, an intense immune response against CagA-positive H. pylori strains might play a critical role in precipitating coronary instability mediated by antigen mimicry between CagA antigen and a protein contained in coronary atherosclerotic plaques’.

5.4.6 Conclusion

Despite the large number of studies investigating the possible link between H. pylori and cardiovascular disease, the relationship between H. pylori and cardiovascular disease remains in dispute. Similarly, the pathogenic mechanism by which this bacterium might contribute to atherosclerosis in the coronary arteries and thus induce IHD also remains contentious.

5.5 Asthma

5.5.1 Associations between H. pylori and asthma

Since the late 1960s a sharp increase in the global prevalence of asthma and allergy has occurred, particularly in children (Peat et al., 1994; Omran and Russell, 1996; Howarth, 1998; Eder et al., 2006). Asthma is considered one of the most common chronic diseases of the Western world (Codolo et al., 2008), with approximately 300 million people worldwide currently reported to have this condition (Braman, 2006). Given that asthma is associated with a high degree of morbidity and mortality (Braman, 2006), considerable research has been invested into determining the aetiology of this chronic condition, and in particular why such dramatic increases have occurred in the developed world.

One hypothesis posited to explain the increasing incidence of asthma is the ‘hygiene hypothesis’, which suggests that a lack of exposure to microorganisms in the early years of life predisposes to later development of allergic diseases, including asthma (Strachan, 1989). The scientific rationale for this hypothesis is based on the view that exposure to specific chronic microbial infections, at an
early age, results in the host developing an immune phenotype that reduces their susceptibility to allergic disease. That is, underexposure to infectious agents has the potential to lead to immature and pro-allergenic immune responses (Strachan, 1989; Liu, 2007).

A considerable number of studies investigating the aetiology of allergic disease have focused upon exposure to a range of microorganisms and parasites that cause chronic infections in man (Matricardi et al., 2000). Given that H. pylori chronically infects >50% of the world’s population, is acquired during childhood (Mitchell, 2001) and, if left untreated, causes lifelong inflammation, this makes H. pylori a potential candidate. Moreover, the observation that the increase in incidence of asthma and related allergic diseases over recent decades correlates with a decline in H. pylori prevalence in developed countries over a similar timeframe, led researchers to investigate the possible relationship between H. pylori and allergic disease.

In an early study investigating the possible association between anti-H. pylori and anti-CagA IgG antibody titres and food allergy, atopic asthma and inflammatory bowel disease (n = 30), Corrado et al. (1998) found significantly higher anti-H. pylori IgG titres in children with food allergy as compared with the other two groups. In contrast, the anti-CagA IgG titres did not differ significantly between the patients. As a result Corrado et al. (1998) hypothesized that virulence factors other than CagA may be involved in the pathogenesis of H. pylori infection in paediatric patients with food allergy.

In a later study, Matricardi and colleagues (2000) investigated markers of exposure to food-borne and orofaecal microbes (Toxoplasma gondii, H. pylori and hepatitis A virus) in 240 atopic and non-atopic Italian male cadets, aged 17–24 years. Exposure to both T. gondii and hepatitis A virus was inversely associated with atopy. A similar trend was observed with H. pylori but this did not reach significance, although the adjusted odds of atopy were shown to decrease with a gradient of exposure to H. pylori, T. gondii and hepatitis A virus (Matricardi et al., 2000). A nested case–control study conducted in the same year by Bodner et al. (2000) showed no association between exposure to hepatitis A, T. gondii or H. pylori and the development of wheezing symptoms or atopic status in adulthood. Similarly a study by Tsang et al. (2000) of 90 asthmatic Chinese patients with mild intermittent asthma, and 97 age-, sex- and occupation-matched healthy control subjects, found no significant difference in H. pylori seroprevalence in asthmatic patients with mild intermittent asthma and controls.

A further investigation of H. pylori and allergy was undertaken by Kosunen et al. (2002), who determined the prevalence of allergen-specific IgE antibodies to the four most common inhalant allergens causing allergic reactions in Finland (birch, timothy grass, cats and dogs) in two cross-sectional adult populations, using sera samples collected in 1973 and 1994 for which H. pylori serostatus was known. Between 1973 and 1994 a rise in specific IgE antibodies to the above allergens had occurred, with this increase being mainly limited to H. pylori-seronegative subjects. Interestingly, a particularly strong effect was noted in the 15–24-year age group, where the prevalence of IgE antibodies rose from 7.9% in 1973 to 29.7% in 1994 in H. pylori-seronegative subjects, as compared with 15.4% in 1973 to 20.0% in 1994 in H. pylori-seropositive subjects (Kosunen et al., 2002).

The first major study to show a strong association between H. pylori and asthma was conducted by Chen and Blaser (2007), who investigated whether acquisition of H. pylori was associated with a reduced subsequent risk of asthma and allergy using data drawn from 7663 adults who had been part of the Third National Health and Nutrition Examination Survey (NHANES III), conducted in the USA between 1988 and 1991. In this survey, all interviewed subjects were questioned as to whether they had ever been diagnosed with asthma or hay fever, the age at which they were first diagnosed, and whether they still had asthma or hay fever. Furthermore, information on a range of allergy symptoms in the previous year as well as potential exposures that may have elicited allergic symptoms was obtained. In all
subjects >20 years of age, *H. pylori* and CagA status was determined and, based on these results, subjects were categorized into three groups: (i) *H. pylori* - and CagA-positive; (ii) *H. pylori*-positive but CagA-negative; and (iii) *H. pylori*- and CagA-negative. In addition, immediate hypersensitivity reactions to ten allergens were investigated.

Overall, this showed that no association existed between the presence of cagA-positive or cagA-negative strains of *H. pylori* infection and current asthma status (Chen and Blaser, 2007). However, individuals who carried cagA-positive *H. pylori* strains had a decreased risk of ever having had asthma or rhinitis compared with those not infected with *H. pylori* (OR = 0.79; 95% CI 0.63, 0.99). When subjects with asthma were grouped into those diagnosed at <15 or >15 years of age, an even stronger inverse association was observed between *H. pylori* cagA positivity in the younger age group (OR = 0.63; 95% CI 0.43, 0.93) compared with those with adult onset (OR = 0.97; 95% CI 0.72, 1.32). Interestingly, a similar protective association was seen for rhinitis and skin sensitization due to moulds and pollens. Furthermore, infection with either cagA-positive or -negative strains of *H. pylori* was associated with a reduction in allergy symptoms, these associations being more commonly observed in younger individuals (Chen and Blaser, 2007). In contrast, an earlier study by Jun et al. (2005) failed to observe a significant difference in either anti-*H. pylori* or anti-*H. pylori* CagA IgG levels between patients with asthma and controls.

In 2008 Reibman et al. confirmed the inverse association between *H. pylori* cagA-positive strains and asthma reported by Chen and Blaser (2007). In this case-control study conducted in an ethnically diverse urban population resident in the USA, the relationship between asthma and both *H. pylori* and CagA status was examined in 318 adult patients (18–64 years old) diagnosed with asthma, and 208 controls. Although a trend towards an association between CagA status and asthma was observed, the crude odds for asthma associated with either *H. pylori*-positive/CagA-negative or CagA-positive status did not reach significance (Reibman et al., 2008). However, when the data were adjusted for confounding factors including age, race, Hispanic ethnicity, income and the genetic relatedness among some of the subjects, a significant inverse association was observed between CagA status and asthma (OR = 0.57; 95% CI 0.36, 0.89). Further analysis showed that CagA positivity was associated with an older age of asthma onset (Reibman et al., 2008).

In a second study Chen and Blaser (2008) investigated whether *H. pylori* colonization during early life was associated with the risk of childhood asthma. This cross-sectional study used data from 3327 participants aged <20 years, enrolled in the NHANES III study (1999–2000), to assess the association between *H. pylori* infection and childhood asthma. While an inverse association was found between *H. pylori* positivity and either current asthma or ever having had asthma in children aged 3–19 years, this did not reach statistical significance. In contrast, when the age of asthma onset was taken into consideration, a significant inverse association was observed in children in whom asthma onset had occurred at <5 years (OR = 0.58; 95% CI 0.38, 0.88). Stratification of the subjects by age further showed that, in children aged 3–13 years, *H. pylori* positivity was inversely associated with current asthma (OR = 0.41; 95% CI 0.24, 0.69) or ever having had asthma (OR = 0.49; 95% CI 0.30, 0.80) (Chen and Blaser, 2008).

However, a recent study by Dowd et al. (2009), which examined the association between the burden of common chronic infections (including *H. pylori*, cytomegalovirus, herpes simplex virus 1, hepatitis A and B virus) with asthma or chronic respiratory conditions in American children aged 6 years and older, failed to show a protective effect of infection by any of these organisms. Indeed, a higher burden of infection was associated with an increased likelihood of asthma, a finding clearly at odds with the hygiene hypothesis (Dowd et al., 2009).

### 5.5.2 Potential mechanisms

While, based on epidemiological studies, there is increasing evidence of an inverse association between *H. pylori* infection and
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Asthma, until recently a plausible biological explanation for this association had not been forthcoming. While some researchers suggested that the immune response to *H. pylori* may lead to changes in the immune environment that may protect against the development of asthma, it was not until 2006 when Amedei et al. (2006) provided evidence that, in vitro, the typical T-helper type 2 (Th2) responses observed in allergic asthmatic patients could be redirected towards a Th1 response by the *H. pylori* virulence factor, neutrophil-activating protein (HP-NAP). In that study, addition of HP-NAP to allergen-induced T-cell lines obtained from allergic asthmatic patients resulted in a substantial increase in interferon-γ (IFNγ)-producing T cells and a decrease in cells secreting interleukin (IL)-4. Based on these studies, Amedei et al. (2006) concluded that HP-NAP was able to inhibit the development of allergen-specific Th2 responses and to stimulate the production of IL-12 and IL-23 via the Toll-like receptor-2 (TLR2) pathway.

In a follow-up in vivo study, Codolo et al. (2008) used an ovalbumin (OVA)-induced mouse model of asthma to investigate whether HP-NAP might provide a novel therapeutic approach for redirecting Th2 to Th1 responses. Mice were first primed intraperitoneally (IP) with OVA, and then Th2 responses induced in their lungs by repeated OVA aerosol challenges. To determine the effect of HP-NAP on asthma development, mice were injected IP with either phosphate-buffered saline (PBS) or HP-NAP, simultaneously with the initial OVA sensitization. Eighteen days following initial priming and subsequent challenge with OVA, differential white blood cell counts were measured in lung washings (bronchoalveolar lavage (BAL)) collected from mice initially treated with OVA alone or OVA+PBS or OVA+HP-NAP. As expected, administration of OVA resulted in the recruitment of eosinophils in the bronchial airways of the mice and a subsequent increase in serum IgE levels (Codolo et al., 2008). However mice co-administered HP-NAP and OVA had significantly reduced numbers of BAL and airway eosinophils (P < 0.01), and OVA-induced airway eosinophilia was prevented. In contrast, the numbers of macrophages, neutrophils and lymphocytes in systemic HP-NAP-treated mice were similar to those of OVA-treated mice. Moreover, mucosal administration of HP-NAP also suppressed the development of OVA-induced asthma (Codolo et al., 2008). Finally they compared the potential immunomodulating activity of TLR2 and HP-NAP by measuring the Th2 cytokines, IL-4, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), in the BAL of wild-type and TLR2−/− mice. While a significant reduction in IL-4, IL-5 and GM-CSF was observed in BAL from wild-type mice following systemic and mucosal treatment with HP-NAP, no such reductions were observed in the TLR2−/− mice, suggesting that TLR2 expression is required for the beneficial effects of HP-NAP. These findings suggest that both systemic and mucosal administration of HP-NAP may be effective in preventing allergic asthma (Codolo et al., 2008; D’Elios et al., 2009).

In a recent paper, Cam et al. (2009) investigated the relationship between atopy and *H. pylori* infection in healthy schoolchildren followed for a period of 6 years. All children were tested using skin prick tests for reactivity to a range of allergens, including *Dermatophagoides pteronyssinus* (Der p1 – house dust mite), and *H. pylori* status was determined by 13C-urea breath test. The immunological interaction between atopy and *H. pylori* infection was then determined in ten *H. pylori*-positive atopic children, nine *H. pylori*-positive non-atopic children, seven *H. pylori*-negative atopic children and eight *H. pylori*-negative non-atopic children. Immunological interactions were based on IFNγ, IL-4, IL-10 and transforming growth factor-β (TGFβ) production by peripheral blood mononuclear cells (PBMC) obtained from these four groups following stimulation with Der p1 or *H. pylori* antigen. This study showed that *H. pylori*-induced IFNγ production by PBMC was significantly higher in *H. pylori*-positive children; however, when atopy was simultaneously present in *H. pylori*-infected cases, *H. pylori*-induced IFNγ production was reduced. In contrast IL-4 production was highest in non-infected atopic children, but significantly suppressed when *H. pylori*
infection was concurrently present in atopic children. IL-10 production was not modified by either *H. pylori* infection or the presence of atopy. Production of TGFβ was suppressed in atopic children irrespective of the presence of *H. pylori* infection. Based on these findings Cam et al. (2009) postulated, ‘*H. pylori* infection that primarily involves a Th1 immune response may suppress the Th2 pathway which is mainly associated with atopy’.

### 5.6 *H. pylori* and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), are idiopathic diseases of the gastrointestinal tract (Podolsky, 1991). Since the mid-1990s, an increasing number of studies have investigated the association between *H. pylori* infection and IBD. The rationale behind such studies relates to the observed correlation between the increased prevalence of IBD (Logan, 1998; Loftus, 2004; Gisbert, 2008; Thia et al., 2008) and the decreased prevalence of *H. pylori* (see Malaty, Chapter 1, this volume), as well as to recent studies suggesting that *H. pylori* colonization may lead to modulation of the immune response.

In the first study to examine the possible association between *H. pylori* infection and IBD, El-Omar and colleagues (1994) compared the *H. pylori* IgG status of 110 patients with IBD with that of 100 age- and sex-matched controls. Overall, 22% of IBD patients were shown to be *H. pylori*-positive compared with 52% of control patients. While this was an interesting result, post hoc analysis suggested that the lower prevalence of *H. pylori* infection in the IBD group may have been related to current or previous use of sulfasalazine (El-Omar et al., 1994). Although this latter finding was supported by a number of later studies (Pearce et al., 2000), others found no relationship between sulfasalazine use and the prevalence of *H. pylori* infection (Halme et al., 1996; Vare et al., 2001; Luther et al., 2009; Song et al., 2009).

Since El-Omar et al.’s initial study in 1994, an estimated 350+ papers or abstracts have reported on the possible link between *H. pylori* and IBD (Luther et al., 2009). For example, Vare et al. (2001) compared the sero-prevalence of *H. pylori* in 94 patients with CD, 185 with UC and 17 with intermediate colitis to that in 70 healthy age- and sex-matched controls. This showed an inverse association to exist between *H. pylori* prevalence and the presence of IBD, the overall prevalence in IBD patients (24%) being significantly lower than that in controls (37%). Interestingly the prevalence of *H. pylori* in CD patients (13%) was significantly lower than that in UC (30%), suggesting that *H. pylori* may be more likely to protect against CD. This stronger association between *H. pylori* and CD has been confirmed by many other studies (Halme et al., 1996; Wagtmans et al., 1997; Duggan et al., 1998; Pearce et al., 2000; Feeney et al., 2002; Song et al., 2009).

Unfortunately, as discussed previously in relation to other extragastric diseases, many studies investigating the possible association between *H. pylori* infection and IBD have significant limitations. Not only do they relate to study design, the number of subjects investigated and failure to adjust for confounding factors, but also in many studies the criteria laid down to establish the diagnosis of IBD are poor.

In 2009 an excellent systematic review and meta-analysis of published studies investigating the association between *H. pylori* infection and IBD was published, which has helped to clarify, to some degree, the possible association between *H. pylori* and IBD. In this review, Luther and colleagues (2009) conducted a literature search (Medline, EMBASE, bibliographies and meeting abstracts) and identified 369 articles on this topic. Following exclusion of a large number of these studies due to poor design, small sample size, poor or no controls, as well as suboptimal criteria for diagnosis of IBD or determination of *H. pylori* status, 29 studies were reviewed in detail. Of 23 studies that fitted their final inclusion criteria, 13 showed a statistically significant inverse correlation between *H. pylori* infection and IBD, the relative risk (RR) for these studies being less than 1. In an attempt to explain the observed heterogeneity in the 23 studies, multiple subgroup analyses were conducted, the data being divided according to the...
methods of *H. pylori* and IBD diagnosis, study location (eastern versus western hemisphere) and study population age (paediatric versus adult). While this subgrouping failed to explain the observed diversity in study outcomes, division of the dataset according to IBD type (CD or UC) showed a statistically significant reduction in the RR of *H. pylori* infection in CD patients diagnosed by *H. pylori* non-serological methods (RR = 0.71; 95% CI 0.58, 0.87) (Luther et al., 2009). These authors concluded that, although *H. pylori* infection may potentially have a protective effect for IBD development, and in particular CD, a number of factors might have impacted on their results, including the possibility that participants had been treated for *H. pylori* infection prior to entering the study, thus resulting in a falsely low *H. pylori* infection rate.

Given these limitations, they suggested that future studies should ideally be conducted at the time of IBD diagnosis, and that diagnosis of IBD should be based upon review of endoscopic and histological findings. In addition, they recommended that diagnosis of *H. pylori* infection should be based upon urea breath testing, rapid urease testing of gastric biopsies or histology, given that serological assays cannot determine whether a patient is currently infected with *H. pylori*. Given the possible protective benefits of *H. pylori* cagA-positive strains against other autoimmune diseases, they also suggested that CagA status should be investigated in future studies.

To date, there are limited data on the potential mechanism by which *H. pylori* may protect against IBD, although a number of studies have suggested that regulatory T cells (Tregs) may play an important role (see Robinson and Atherton, Chapter 6, this volume). Given that adoptive transfer of Tregs has been shown to prevent the development of and treat experimental colitis in a range of animal models (Read et al., 2000; Mottet et al., 2003), it may be that Tregs produced in response to *H. pylori* infection have a role in preventing the development of colitis. Clearly further studies investigating the potential mechanism by which *H. pylori* may protect against IBD are required.

### 5.7 Hepatobiliary and Pancreatic Diseases

Studies on the association between *Helicobacter* infection and hepatobiliary and pancreatic diseases have mainly focused on enterohepatic *Helicobacter* species. Of the work performed on *H. pylori*, detection of DNA in samples collected from diseased patients has been most frequently employed. Indeed, to date, only one study has reported the isolation of *H. pylori* from hepatobiliary samples, in this case from human liver (De Magalhaes Queiroz and Santos, 2001). This bacterial strain had the same morphology as *H. pylori*, was catalase-, oxidase- and urease-positive, and had >99% homology with *H. pylori* (De Magalhaes Queiroz and Santos, 2001).

#### 5.7.1 *H. pylori* and hepatobiliary disease

Several studies have reported the detection of *H. pylori* DNA in bile samples from patients suffering from a variety of hepatobiliary diseases (Lin et al., 1995; Myung et al., 2000). Interestingly in one study, the authors measured a significantly lower bile pH in patients who were positive for *H. pylori*, but could not find a pathogenic role for *H. pylori* in the formation of hepatolithiasis (Myung et al., 2000). In a study by Nilsson et al. (2000) in which 24 patients with either primary sclerosing cholangitis or primary biliary cirrhosis were examined, *H. pylori* DNA was detected in a significantly higher number of patients than in controls with normal livers (*P* < 0.05).

In 2002, Fukuda et al. proposed that ‘*Helicobacter* species may play a role in the pathogenesis of hepatobiliary cancer through an acceleration of biliary cell kinetics’. These authors reported a statistically significant difference between the detection of *Helicobacter* DNA in patients with and without hepatobiliary cancer (*P* = 0.03). In this study, however, of ten *Helicobacter* DNA-positive patients, only one had *H. pylori* DNA and that patient was also positive for DNA from another *Helicobacter* species (Fukuda et al., 2002), a finding that suggests that *H. pylori* per se was not associated with hepatobiliary cancer.
Further support for this conclusion comes from a study of 122 patients with and without non-malignant gallbladder diseases, which failed to find a significant difference in the detection rate of *H. pylori* DNA between patients and controls (Chen et al., 2003). In addition, no differences in blood biochemistry and liver function tests were observed between patients positive or negative for *Helicobacter* DNA. Chen et al. (2003) concluded from their study that *H. pylori* DNA is commonly present in the human gallbladder, and whether or not *H. pylori* plays a significant role in hepatobiliary diseases remains unclear.

Further studies have expanded the literature on *H. pylori* DNA detection in patients with hepatobiliary diseases (Huang et al., 2004; Stalke et al., 2005; Krasinskas et al., 2007; Pirouz et al., 2009), with two showing striking differences between patients and controls. For example, while *Helicobacter* 16S rDNA (of *H. pylori*- and *H. pullorum*-like organisms) was detected in, respectively, 61 and 68% of liver samples from patients with hepatitis C virus-positive cirrhosis with and without hepatocellular carcinoma (HCC), only 4.2% of liver samples obtained from controls and 3.5% of patients with non-cirrhotic chronic hepatitis C were shown to be positive (Rocha et al., 2005). In addition, Li et al. (2006) examined liver samples from 34 patients with HCC and 20 patients without primary liver carcinoma for *H. pylori* DNA. Remarkably, in this study, 65% of HCC samples tested positive for *H. pylori* DNA compared with none of the controls (Li et al., 2006).

Several mechanisms have been proposed to explain how *H. pylori* infection may lead to hepatobiliary diseases. To better understand the possible pathogenicity of this bacterium within the hepatobiliary system, a number of mouse colonization studies have been performed. For example, one study conducted in *Helicobacter*-free C57L/J mice examined whether or not *H. pylori* could promote gallstone formation, but found no evidence that *H. pylori* plays a role in this process (Maurer et al., 2006). However, a case of primary biliary cirrhosis in a C57BL/6 mouse infected with *H. pylori* has been reported, with the authors suggesting that an increase in vacuolating toxin resulting from *H. pylori* infection may play a role in the development of cirrhosis (Goo et al., 2008). More recently, Huang et al. (2009) studied the pathological changes in the livers and gallbladders of C57BL/6 mice infected with *H. pylori*. Of 20 mice inoculated with *H. pylori* three developed mild-to-moderate hepatitis but, more importantly, *H. pylori* was observed morphologically in four liver specimens and six gallbladders from infected mice. This indicates that *H. pylori* inoculated orally may reach the hepatobiliary system and cause inflammation (Huang et al., 2009).

The degradation of urea by *H. pylori* results in the formation of high levels of ammonia, which has been associated with the degree of encephalopathy (Sethar et al., 2004). Several studies have therefore focused on the role of ammonia present in patients’ blood and hepatic encephalopathy. For example, Wang et al. (2006) assessed the involvement of ammonia produced as a result of *H. pylori* infection in hepatic encephalopathy status and demonstrated that the bacterium was an important source of ammonia. The authors concluded that these findings support a possible role for the bacterium in the development of hepatencephalopathy in cirrhotic patients (Wang et al., 2006).

### 5.7.2 *H. pylori* and pancreatic disease

Studies investigating the relationship between *H. pylori* infection and pancreatic diseases have yielded contradictory results. For example, Kountouras et al. (2005) proposed that the organism might trigger autoimmune pancreatitis (AIP) through induction of autoimmunity and apoptosis. This hypothesis was derived from the finding that *H. pylori* is capable of mimicking host factors to induce irregularities in T-cell apoptosis, which ultimately contribute to extragastric tissue destruction (Kountouras et al., 2005). However, Chang et al. (2009) recently compared the *H. pylori* status of 40 patients with AIP and 113 patients with non-autoimmune chronic pancreatitis (NACP) and found that, although frequencies of gastric ulcers were significantly higher in patients with AIP than in those with NACP, *H. pylori* status could not explain the pathogenesis of AIP in these patients.
Lindkvist et al. (2008) studied the possible involvement of *H. pylori* infection in the increased risk of pancreatic cancer after previous studies had reported conflicting results. Patients with pancreatic cancer (*n* = 87) were compared with 263 age- and sex-matched controls selected from a total cohort of 33,346 subjects. The authors found no association between *H. pylori* seropositivity and risk of pancreatic cancer in the total cohort; however, adjustment for variables such as smoking and alcohol consumption suggested that patients with healthier lifestyles had an increased risk of pancreatic cancer if they were *H. pylori*-positive (Lindkvist et al., 2008).

### 5.8 Concluding Comment

Despite many thousands of studies investigating the role of *H. pylori* in a diverse range of extragastric diseases, in most cases the epidemiological data remain inconsistent. Plausible biological explanations for these putative associations are commonly not well formed, and evidence that eradication of *H. pylori* leads to clinical improvement is missing. Unless well-designed prospective studies that control for the many factors that can confound such studies are undertaken, the putative association between *H. pylori* infection and these extragastric manifestations is likely to remain unresolved.

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6 Helicobacter pylori-induced Acquired Immunity and Immunoregulation

K. Robinson* and J.C. Atherton

6.1 Introduction

Helicobacter pylori infections are commonly acquired during early childhood (Goodman and Correa, 1995) and colonization persists lifelong unless antibiotic treatment is administered, as natural clearance of the infection is rare (Xia and Talley, 1997). How the bacterium persists in the gastric mucosa of its host for decades remains incompletely understood. There is evidence to suggest that H. pylori has co-evolved with man and has adapted to manipulate and evade the immune response, aiding its survival, but also contributing to pathogenesis (Blaser and Atherton, 2004). Following uptake by antigen-presenting cells, probably in intestinal Peyer’s patches (Gewirtz and Sitaraman, 2007), the bacteria stimulate an acquired immune response consisting of high antibody titres and specific T cells which are detectable in both the gastric mucosa and peripheral blood (O’Keeffe and Moran, 2008). The components thought to be necessary for clearance of many bacterial infections, such as antibody, complement components and phagocytic cells (Mueller-Ortiz et al., 2004), can be detected in the gastric mucosa (Berstad et al., 1997), yet colonization persists regardless. The possibility that an ineffective or inappropriate type of immune response is provoked by the bacteria has been of recent research interest, especially since a marked regulatory T-cell response has been demonstrated (Lundgren et al., 2005b; Rad et al., 2006; Robinson et al., 2008). These suppressive cells are thought to reduce the inflammatory response and inhibit protective immune mechanisms (reviewed by Joosten and Ottenhoff, 2008). A recent hypothesis is that H. pylori-induced immune responses may also play a general regulatory role in preventing other immunological or inflammatory conditions, since inverse epidemiological associations have been noted between these and H. pylori prevalence (Blaser et al., 2008). This chapter reviews recent findings on the acquired immune response to H. pylori, its role in modulating gastroduodenal pathology and disease, and also discusses possible implications for the immune system in general and for other diseases.

6.2 B-cell and Antibody Responses

H. pylori stimulates the production of mucosal and systemic immunoglobulin (Ig) A and IgG antibodies, and this forms the basis for some commercial diagnostic tests (Kindermann et al., 2001). Traditionally, bacterial vaccines have depended upon the stimulation of a high-titre humoral response for protective efficacy, but

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the effect of antibody upon *H. pylori* colonization is unclear. *H. pylori* is susceptible to complement-mediated killing in the presence of serum in *vitro* (Gonzalez-Valencia et al., 1996; Berstad et al., 2001). IgG is detectable in the gastric juice of infected people (Meining et al., 2002), and *H. pylori* bacteria in the human gastric mucosa are coated in IgG, IgA and IgM (Wyatt et al., 1986), but the successful immunization of B-cell-deficient mice (Ermak et al., 1998; Sutton et al., 2000) indicates that antibodies are not essential for protection.

Human studies have found that the presence of anti-*H. pylori* IgA in maternal milk is associated with delayed colonization in infants, suggesting that IgA can block infection (Thomas et al., 2004). Others have tested the effects of orally administered bovine colostrum containing high levels of *Helicobacter*-specific IgA, showing protective effects in mouse models (Casswall et al., 2002; Marnila et al., 2003), but results from human volunteer studies were disappointing (Casswall et al., 1998). Intragastric administration of specific monoclonal IgA antibodies to mice provided protection against *Helicobacter felis* infection (Czinn et al., 1993), but others have shown that specific IgA and IgG may potentially promote bacterial colonization and inhibit protective immune mechanisms in mice (Akhnani et al., 2004b). Human studies have also shown that the presence of an elevated IgA response to infection is associated with a significantly increased risk of gastric cancer (Kosunen et al., 2005; Knekt et al., 2006). The humoral response to *H. pylori* may even play a role in pathogenesis by triggering autoimmune processes (D'Elios et al., 2004), where antibodies cross-react with host antigens such as Lewis blood-group antigens on gastric epithelial cells (Appelmelk et al., 1996) and the parietal cell H⁺,K⁺-ATPase (Amedei et al., 2003). Sera from approximately half of infected individuals contain parietal cell-reactive antibodies (Negrini et al., 1991), which could potentially contribute to local inflammation and tissue damage.

While B cells are usually involved in the positive induction of immune responses, a suppressive subset of CD1d<sup>-hi</sup>CD5<sup+</sup> regulatory B cells (Bregs) has recently been discovered (Yanaba et al., 2008). These form part of the early defence against infection via production of polyreactive IgM, but also suppress the responses of T cells and natural killer T (NKT) cells by secreting interleukin (IL)-10 and may promote the expansion of regulatory T cells (Tregs) (Bouaziz et al., 2008; Mauri and Ehrenstein, 2008). Bregs appear to be important in some bacterial infections (Majlessi et al., 2008); however, studies on *H. pylori* infection in B-cell knockout mice reported no significant increase in gastric pathology (Ermak et al., 1998; Garhart et al., 2003b), thus any role of Bregs in *H. pylori* infection remains speculative.

### 6.3 CD4<sup+</sup> T-cell Responses and Cross-talk with Innate Immunity

*In vitro* work has shown that *H. pylori* can utilize many different mechanisms for down-regulating T-cell activity. Lymphocyte cell cycle arrest can be induced by several virulence factors including cytotoxin-associated gene A protein (CagA) (Paziak-Domanska et al., 2000), proliferation-inhibiting protein (Knipp et al., 1996) and γ-glutamyl transpeptidase (Gerhard et al., 2005; Schmees et al., 2007). Vacuolating toxin (VacA) inhibits T-cell responses indirectly by blocking major histocompatibility complex class II-mediated antigen presentation (Molinari et al., 1998) and by direct suppression of T-cell activation (Boncristiano et al., 2003; Gebert et al., 2003; Torres et al., 2007). Furthermore, expression of the co-stimulatory molecule B7-H1 by gastric epithelial cells is elevated during *H. pylori* infection in *vivo*, and this molecule can suppress T-cell activity (Das et al., 2006).

Aside from inhibiting T-cell activity in general, the response to infection is greatly influenced by the type of T-cell response elicited. CD4<sup+</sup> T cells orchestrate the immune response in mammals: they direct the class and subclass of antibodies, dictate properties of professional phagocytes, and can up- or down-regulate the level of inflammation (Romagnani, 2006). Certain T-helper (Th) subsets are important in immunity to different types of infection and these are summarized in Fig. 6.1. Th1 cells help to eliminate
in intracellular bacteria and viruses, whereas Th2 cells are protective against intestinal helminth parasites. Th2 cells may develop into Th9 cells, which may also play a role in parasite immunity (Veldhoen et al., 2008). Th17 cells may be important in infections with fungi and extracellular bacteria (MacDonald and Maizels, 2008; Curtis and Way, 2009). Tregs comprise a suppressive subset, which prevent immune attack against self-antigens or innocuous environmental antigens (Belkaid, 2008; Costantino et al., 2008b) and also regulate inflammation. These T-cell subsets are discussed in more detail below.

During the development of Th cells from naive precursors, the cells are exposed to an array of signals, which direct them down particular differentiation pathways (Romagnani, 2006; Kaiko et al., 2008). Until recently it was thought that mature Th cells were terminally differentiated and subsets were fixed. In fact there is a degree of plasticity in Th cells as they may respond to signals such as innate cytokines and acquire the properties of a different subset (Rowell and Wilson, 2009; Wei et al., 2009). For example, Th2 cells may be converted into interferon-γ (IFNγ)-secreting Th1 cells in the presence of Th1-associated cytokines (Krawczyk et al., 2007). H. pylori has recently been shown to induce IL-12p40 and IL-18 expression by gastric epithelial cells and monocytes (Shimada et al., 2008; Yamai et al., 2008; Takeshima et al., 2009), thus it is possible that these cytokines could contribute to this conversion. Under the direction of transforming growth factor-β (TGFβ) and IFNγ, CD4+CD25− effector T cells can be induced to acquire the suppressive attributes of Tregs (Wang et al., 2006). High levels of these cytokines are present in the H. pylori-infected gastric mucosa. The balance between Tregs and Th17 cells is known to be influenced by the presence of TGFβ, IL-6 and retinoic acid; reduced levels of retinoic acid (a factor favouring Treg differentiation) are associated with increased pathology in the H. pylori-infected
gastric mucosa (Matsumoto et al., 2005a). Similarly, Th17 cells can shift towards a Th1 phenotype in the presence of reduced TGFβ and increased IL-12 and IL-23 (Lee et al., 2009), or be induced by IL-6 and TGFβ to express IL-10 (McGeachy et al., 2007).

Th1, Th2, Th17 and Treg cells have all been reported in the H. pylori-infected gastric mucosa (Bamford et al., 1998; D’Elios et al., 2003; Caruso et al., 2008; Robinson et al., 2008). Given new findings on Th subset plasticity, we should view these data with caution, especially where cells have been isolated from their natural innate cytokine environment and cultured for extended periods prior to phenotyping. In addition to the conversion of one subset into another, one must also consider that hybrid cell types exist, e.g. Th1 cells may secrete IL-10 (O’Garra and Vieira, 2007) and IL-17-producing FOXP3+ Tregs have recently been described in humans (Voo et al., 2009). Previously reported gastric IFNγ+ Th1 cells might also secrete IL-17 and may actually be Th17 cells (Annunziato et al., 2007). Also IL-10, thought to indicate a Treg response, could be derived from Th1 cells. In summary, the field of T-helper subset responses has recently become more complex, making it important that panels of markers for the different cell types are used in such studies.

6.3.1 T-helper subset responses

**T-helper 1**

In humans, a predominant IFNγ-producing CD4+ T-cell response is found in the H. pylori-infected gastric mucosa (Fan et al., 1994; D’Elios et al., 1997; Bamford et al., 1998), and is associated with the expression of proinflammatory cytokines such as tumour necrosis factor-α (TNFα), IL-12 and IL-18. When macrophages are activated in a Th1 cytokine environment they become ‘angry’. ‘Angry’ macrophages secrete proinflammatory factors and have enhanced bactericidal activity as compared with Th2-activated macrophages (Ma et al., 2003). The number of IFNγ-secreting cells in the infected human gastric mucosa correlates with both the severity of gastritis (Lehmann et al., 2002) and the presence of peptic ulcer disease (Robinson et al., 2008) (Fig. 6.2). H. pylori infection influences the levels of gastric hormones, reducing somatostatin release and
stimulating gastrin production. This inhibits Th2 responses and increases Th1 activity (Zavros and Merchant, 2005). C57BL/6 mice, which mount a strong Th1 response to H. pylori, develop severe gastritis when infected (Smythies et al., 2000; Garhart et al., 2002). A Th1 response is associated with reduced bacterial colonization density both in humans (Holck et al., 2003) and in mouse models (Lucas et al., 2001; Stoicov et al., 2004). An expression profiling study of the H. pylori-infected murine stomach found that genes associated with an IFNγ-dependent Th1 response were up-regulated (Vivas et al., 2008), and several groups have shown that a Th1 response is required for successful immunization (Eaton et al., 2001; Akhiani et al., 2002; Garhart et al., 2003b; Taylor et al., 2008).

A number of H. pylori virulence factors have been reported to promote Th1 responses. For example, administration of H. pylori neutrophil-activating protein (HP-NAP) to mice suppresses Th2 responses to intestinal parasites (Amedei et al., 2006; Del Prete et al., 2008) and down-regulates Th2-mediated allergy in a model for asthma (Codoto et al., 2008). The cag pathogenicity island is associated with increased Th1, IL-12 and IL-18 responses (Wang et al., 2007; Yamauchi et al., 2008; Takeshima et al., 2009), outer inflammatory protein A (OipA) stimulates IL-18 (Yamauchi et al., 2008) and VacA induces T cells to express IL-12p40 (Takeshima et al., 2009). The Th1 response is also influenced by the plasticity region locus jhp0947–jhp0949 (de Jonge et al., 2004), genomic DNA recombination (Robinson et al., 2005) and phase-variable expression of Lewis blood-group antigens via interaction with the cellular innate immune receptor, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (Bergman et al., 2004). The fact that so many features of H. pylori strongly stimulate host Th1 immunity indicates that this type of response is important for successful chronic colonization of the gastric mucosa by these bacteria.

**T-helper 2**

Although a predominant Th1 response has been reported in the infected human gastric mucosa, Th2 cells have also been detected (Serrano et al., 2007; Robinson et al., 2008). Initial vaccine studies in mice found that a Th2 response correlated with reduced bacterial loads (Mohammadi et al., 1997), although subsequent gene knockout studies found that IL4 is unnecessary for induction of protective immunity (Garhart et al., 2003b), whereas Th1-associated genes such as IL12 and IL18 are required (Garhart et al., 2003a; Akhiani et al., 2004a) (Fig. 6.2). Gastritis in mice is Th1 mediated and is more severe in IL-4-deficient mice, implying that a Th2 response suppresses inflammation (Smythies et al., 2000). Moreover, the co-administration of an intestinal parasite in order to skew the Th1/Th2 balance towards Th2 in mice resulted in reduced gastric pathology, but Helicobacter colonization densities were increased (Fox et al., 2000). Regarding human infections, several groups have shown that there is a shift from Th1 immunity in active gastritis, towards IL-4+ or IL-13+ Th2 responses in patients with gastric cancer or precancerous changes (Ren et al., 2001; Marotti et al., 2008). The significance of this is unclear.

A new T-helper subset (Th9), which secretes IL-9 and IL-10, has recently been discovered in the mouse (Tato and Cua, 2008). These cells are derived from Th2 cells under the influence of TGFβ1 and perform specialized functions in the gut, mediating protection from parasites (Veldhoen et al., 2008) and contributing to inflammation in a colitis model (Dardalhon et al., 2008). There are no data regarding Th9 cells in H. pylori infection, but elevated IL-9 and IL-10 expression in the human gastric mucosa is associated with both H. pylori-positive status and incidence of gastric adenocarcinoma (Ellmark et al., 2006).

**T-helper 17**

The presence of IL-17 in the Helicobacter-infected gastric mucosa was reported before the discovery of Th17 cells (Luzza et al., 2000). IL-17 is produced mainly by CD4+ (Th17) and CD8+ (Tc17) cells, and is also reportedly expressed by some γδ T cells and invariant NKT cells. Th17 cells express IL-17A and IL-17F, which have similar functions (Dong,
The role of IL-17A and IL-17F in immunity to extracellular infections involves activation of fibroblasts, endothelial and epithelial cells, and macrophages to secrete cytokines and chemokines, leading to the neutrophil accumulation. They also express antibacterial peptides and matrix metalloproteinases (Matsuzaki and Umemura, 2007; Iwakura et al., 2008). As neutrophilic gastritis is a prominent feature of human H. pylori infection, IL-17 and Th17 cells have become of particular interest.

Greatly reduced levels of neutrophil infiltration were reported in the gastric mucosa of H. pylori-infected IL-17-deficient mice (Shiomi et al., 2008). Neutrophil numbers in the infected human gastric mucosa correlate with IL-17 expression, with the highest concentrations being found at ulcer sites (Mizuno et al., 2005). Human gastric epithelial cells and lamina propria mononuclear cells express receptors for, and are capable of responding to, IL-17. Stimulation of MKN28 gastric adenocarcinoma cells with IL-17 induced activation of extracellular signal-regulated protein kinase 1/2 (ERK 1/2), resulting in activator protein-1 (AP-1) and nuclear factor (NF)–κB activation and IL-8 expression (Sebkova et al., 2004). Neutralization of IL-17 in mucosal mononuclear cell cultures markedly reduced the IL-8 response to H. pylori (Luzzà et al., 2000), implying that the Th17 response is key in inflammation. One study investigated associations of IL17A and IL17F genetic polymorphisms and the incidence of gastric disease, showing increased inflammation in H. pylori-infected individuals with the IL17F 7488T allele (Arisawa et al., 2007). A recent paper reported that IL-17A has anti-inflammatory effects on H. pylori-induced gastritis (Otani et al., 2009), where the administration of neutralizing anti-IL-17A antibody increased Th1 cytokine expression and gastritis severity in infected mice. Other groups have also reported anti-inflammatory properties of IL-17A, and that Th17 cells negatively regulate Th1 responses (Nakae et al., 2007; Ke et al., 2009). The Th17 response may therefore also limit gastric inflammation and pathology by suppressing Th1 cell differentiation.

IL-23 stimulates differentiation and expansion of Th17 cells, and is involved in several intestinal inflammatory conditions and cancer development (Tan et al., 2009). IL-23 is over-expressed in the H. pylori-infected human gastric mucosa, and both Th17 and Tc17 cells are present (Caruso et al., 2008). Th17 cells are present at higher levels than normal in the stomach and peripheral blood of patients with gastric cancer (Zhang, B. et al., 2008). Similar observations have been made with ovarian, renal and pancreatic malignancy (Kryczek et al., 2007) and increasing frequencies of Th17 cells correlated with advancing tumour progression. IL-17 (as with IL-23) is associated with reduced anti-tumour immunity while promoting angiogenesis and tumour growth (Numasaki et al., 2003; Langowski et al., 2006).

In addition to participating in the inflammatory response to H. pylori, two recent studies report a requirement for IL-17 in vaccine-induced protective immunity. The first publication demonstrated that intranasal immunization using a lysate of the H. pylori SS1 strain with cholera toxin adjuvant induced a CD4+ T-cell response characterized by IL-17 and IFNγ secretion upon their antigenic re-stimulation in vitro (DeLyria et al., 2009). When animals were subsequently infected, there was a more substantial IL-17 response, increased gastritis and reduced colonization density compared with unvaccinated mice. Antibody-mediated depletion of neutrophils inhibited clearance of the infection in vaccinated animals, indicating that neutrophils play a role in protective immunity. This result contrasts with a previous study, however, which showed that neutrophil depletion had no effect on vaccine-induced reductions of colonization (Velin et al., 2005). A second publication on the contribution of Th17 cells to vaccination-mediated bacterial clearance examined the effect of neutralizing IL-17 antibody treatment on H. felis infection in mice (Velin et al., 2009). This group also found a more dramatic influx of CD4+IL-17+ cells in the infected gastric mucosa, but found that anti-IL-17 antibody treatment (administered at the same time as immunization) inhibited clearance of the infection and reduced gastric inflammation. Both papers show that Th17 cells contribute greatly to protective immunity.
6.4 CD8+ Cytotoxic T Cells

Although most studies on cellular immunity to *H. pylori* have focused on CD4+ cells, increased numbers of CD8+ cytotoxic T cells (Tc) are also found in the gastric mucosa and blood of infected human subjects and the stomachs of infected mice (Stromberg et al., 2003; Rossi et al., 2004; Nurgalieva et al., 2005; Munoz et al., 2007). Increased numbers of activated CD8+ cells were found in the peripheral blood of children with duodenal ulcers (Figueiredo Soares et al., 2007). More severe gastritis, mediated by CD8+ T cells, is also observed in *H. pylori*-infected CD4+ cell-deficient mice (Fukui et al., 2007; Tan et al., 2008). Most human gastric mucosal Tc cells are memory cells, which could readily be activated by culture with *H. pylori*-exposed B cells (Azem et al., 2006). These cells may express IL-17 (Caruso et al., 2008), and CD8+ T cells in inflamed lesions of the infected human stomach have been shown to contain cytolytic granules and express the proinflammatory chemokine RANTES (Ohtani et al., 2004). Together these data indicate a potential role for CD8+ T cells in *H. pylori* inflammation and disease.

6.5 Regulatory T Cells

The term 'regulatory T cell' (Treg) refers to a cell type that actively controls or suppresses the function of other cells. They are usually CD4+; however, some CD8+ cells and even NKT cells may have regulatory activity (Coleman et al., 2007).Suppressive T cells were first described several decades ago (Gershon and Kondo, 1971), but the field fell into disrepute because of inconsistent findings and a lack of cellular markers to conduct rigorous studies (Germain, 2008). These cells, renamed ‘regulatory’ rather than ‘suppressor’ T cells, are now recognized as being extremely important as they can prevent inflammatory and immune-mediated disease conditions (e.g., autoimmunity, allergy, inflammatory bowel disease) (Larche, 2007; Costantino et al., 2008a; Izcue et al., 2009). Because of suppressive activity on antitumour immunity, Tregs also play an important role in cancer progression (Ghiringhelli et al., 2006; Kono et al., 2006; Gallimore and Godkin, 2008). Treg responses are even exploited by some infections (probably including *H. pylori*) to prevent their clearance (Belkaid, 2007; Coleman et al., 2007).

While several types of Treg have been described that express different profiles of markers (listed in Table 6.1), based on the location of their differentiation the two main categories are natural (nTregs) and inducible (iTregs). nTregs are produced in the thymus and due to their specificity for self antigens are believed to play a primary role in preventing autoimmune reactions. In contrast, iTregs are generated in the periphery from naïve T cells, similar to Th cells, and are generally thought to be specific for non-self antigens. Since *H. pylori* expresses a number of antigens with similarity to host molecules, both of these Treg types could be present in the infected gastric mucosa. Identification is difficult because of the diversity of Tregs and the lack of cellular markers that can distinguish these subsets. CD4+ Tregs commonly express high levels of CD25 (IL-2Rα) and the transcription factor FOXP3 (which represses effector genes and induces expression of suppressive mediators) (Wing et al., 2005; Coleman et al., 2007). Tregs act via a variety of mechanisms (reviewed by Vignali, 2008); for example, by secreting suppressive cytokines such as IL-10 and TGFβ (which may have bystander activity on antigenically unrelated effector cells), or in a contact-mediated fashion where a negative signal is delivered to another cell to suppress its activity.

*H. pylori* infection of mice stimulates a Treg response that regulates gastric inflammation and is thought to blunt protective immune mechanisms to maintain a chronic infection. Initial studies showed that Tregs suppress inflammatory and Th1 responses to *H. pylori* infection. Adoptive transfer of CD4+CD25-depleted lymph node cells into athymic nu/nu C57BL/6 mice, followed by infection with *H. pylori*, induced earlier and more severe gastritis compared with the recipients of lymph node cells including Tregs (Raghavan et al., 2003, 2004). Following on from this, other groups showed that *H. pylori* induces a high-level gastric CD25+FOXP3+...
**Table 6.1.** Main markers and suppressive factors expressed by regulatory T cells.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>Main identifying markers</strong>&lt;br&gt;FOXP3</td>
<td>Forkhead transcription factor; strong marker for murine CD4+CD25+ Tregs but not expressed by all human Tregs; required for murine Treg function; represses effector genes and regulates expression of Treg-associated genes</td>
</tr>
<tr>
<td>CD25^{high}</td>
<td>IL-2Rα chain; high Treg expression levels induce cytokine deprivation-mediated apoptosis in Teffs</td>
</tr>
<tr>
<td>GITR</td>
<td>Constitutively expressed by Tregs; protection from glucocorticoid-induced apoptosis; mediates survival during thymic generation of natural Tregs; interaction with its ligand abrogates Treg suppressive activity</td>
</tr>
<tr>
<td>CD127^{-}</td>
<td>IL-7R; absence correlates with expression of FOXP3 by Tregs</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Tregs are antigen-specific memory T cells</td>
</tr>
<tr>
<td><strong>Cytokines and secreted factors</strong>&lt;br&gt;IL-10</td>
<td>Suppressive cytokine; inhibits NF-κB activation; inhibits activity of Teffs and differentiation of naive T cells</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Suppressive cytokine; inhibits inflammation, activity of Teffs and differentiation of naive T cells</td>
</tr>
<tr>
<td>IL-35</td>
<td>Suppressive cytokine; inhibits inflammation and activity of Teffs; suppression of Th17 development</td>
</tr>
<tr>
<td>Granzymes A and B, perforin</td>
<td>Cytolytic molecules; kill target cells</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Binds type 1 purinergic adenosine A2A receptor on Teffs and inhibits activity by metabolic disruption</td>
</tr>
<tr>
<td>IDO</td>
<td>Induces conversion of tryptophan into pro-apoptotic metabolites</td>
</tr>
<tr>
<td><strong>Cell-associated molecules</strong>&lt;br&gt;CTLA-4 (CD152)</td>
<td>Constitutively expressed on CD4+CD25+ Tregs; interacts with CD80/86 on DCs and delivers a negative signal; induces IDO</td>
</tr>
<tr>
<td>LAG3 (CD223)</td>
<td>Binds MHC class II on immature DCs to suppress maturation and capacity to stimulate an immune response</td>
</tr>
<tr>
<td>Neuropilin-1 (Npl-1)</td>
<td>Constitutively expressed on CD4+CD25+ Tregs; expression linked with suppressive function</td>
</tr>
<tr>
<td>CD39 and CD73</td>
<td>Generate pericellular adenosine production</td>
</tr>
</tbody>
</table>

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; DC, dendritic cell; GITR, glucocorticoid-induced TNF receptor-related protein; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; LAG3, lymphocyte activation gene 3; MHC, major histocompatibility complex; NF, nuclear factor; Teff, effector T cell; TGF, transforming growth factor; TNF, tumour necrosis factor; Treg, regulatory T cell.

Treg response in mice (Kaparakis et al., 2006; Rad et al., 2006). In vivo depletion of Tregs by administration of an anti-CD25 monoclonal antibody resulted in more severe gastritis in infected C57BL/6 mice (Rad et al., 2006), a markedly diminished Th1 cytokine response in infected BALB/c mice (Kaparakis et al., 2006), and also enhanced clearance of infection following immunization with H. pylori-pulsed dendritic cells (Zhang, M. et al., 2008). In addition, CD25+ Tregs were shown to induce anergy in effector T cells during H. pylori infection (Stuller et al., 2008).

Human studies also found increased numbers of Tregs in H. pylori-infected gastric mucosal tissues (Lundgren et al., 2005a,b; Rad et al., 2006; Harris et al., 2008; Robinson et al., 2008). These cells suppressed memory T-cell responses to H. pylori (Lundgren et al., 2003), and an elevated Treg response is reported to be associated with higher colonization densities, lower-level expression of inflammatory cytokines and lower frequencies of Th1 and Th2 cells in gastric tissue (Robinson et al., 2008). Two studies have also shown that high-level Treg responses are found when peptic ulcer...
disease is absent, indicating that Tregs may protect against the development of disease (Harris et al., 2008; Robinson et al., 2008).

The Treg response to \textit{H. pylori} is described in more detail below.

6.5.1 Interleukin-10-secreting regulatory T cells

The importance of IL-10 secretion as a mechanism of action for \textit{H. pylori}-induced Tregs is clear from mouse models. Chronic infections cannot be established in IL-10-deficient mice, probably due to the development of a severe unregulated inflammatory response in these animals (Chen et al., 2001; Matsumoto et al., 2005b). Adoptively transferred Tregs from IL-10-deficient mice, unlike cells from wild-type animals, are unable to inhibit \textit{H. pylori}-induced gastritis (Lee et al., 2007). IL-10 is also highly expressed in the gastric mucosa and peripheral blood of \textit{H. pylori}-infected human subjects (Goll et al., 2007; Lundin et al., 2007; Serrano et al., 2007; Kandulski et al., 2008). Particularly high IL-10 responses were found in those colonized by \textit{cagA}+ strains (Wang et al., 2007; Robinson et al., 2008). Studies on \textit{IL10} gene polymorphisms have yielded contradictory results (see Sutton et al., Chapter 7, this volume), but low IL-10 producer genotypes have been associated with increased gastritis and premalignant pathology (Zambon et al., 2005; Achyut et al., 2008). A large proportion of the CD4\textsuperscript{+}CD25\textsuperscript{hi} Tregs in human gastric tissues express IL-10, and peptic ulcer disease more commonly occurs when there are low numbers of these cells (Robinson et al., 2008). \textit{IL10} mRNA levels also correlate inversely with IL-8 response and colonization densities (Robinson et al., 2008). Therefore, as in mouse models, the IL-10 Treg response influences persistence of \textit{H. pylori} and the level of inflammation induced.

6.5.2 TGF\textbeta-secreting regulatory T cells

TGF\textbeta1 is another Treg-associated cytokine with an important role in \textit{H. pylori} infection, since loss of TGF\textbeta signalling in infected mice leads to increased gastric inflammation and incidence of adenocarcinoma (Hahm et al., 2002). Moreover, infected human gastric mucosa also has elevated TGF\textbeta1 expression (Lindholm et al., 1998; Li and Li, 2006; Goll et al., 2007; Harris et al., 2008; Robinson et al., 2008). \textit{In vitro}, \textit{H. pylori} induces TGF\textbeta release from gastric epithelial cells and leukocytes (Wu et al., 2007), and gastric \textit{FOXP3} mRNA expression is positively correlated with the TGF\textbeta response. Colonization density is also positively correlated with TGF\textbeta expression (Kandulski et al., 2008). TGF\textbeta1\textsuperscript{+} cells in gastric tissues are predominantly CD25\textsuperscript{+} and FOXP3\textsuperscript{+}, i.e. Tregs. Increased numbers coincide with reduced levels of gastritis in children (Harris et al., 2008). Our own studies found that, in contrast to IL-10, low TGF\textbeta1 expression levels were not associated with the incidence of peptic ulceration (Robinson et al., 2008). This implies that, although TGF\textbeta1 inhibits inflammation, it is not sufficient to prevent \textit{H. pylori}-mediated ulceration in humans.

6.5.3 Other known regulatory T-cell mechanisms in \textit{H. pylori} infection

One of the best characterized contact-mediated mechanisms for Treg suppression involves the co-stimulatory molecule cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) where, following binding of CD80/86, a negative signal is delivered directly into dendritic cells to inhibit their maturation and function (Vignali, 2008). After transduction of this signal, dendritic-cell cytokine production is reduced, thereby inhibiting inflammation. The ability of dendritic cells to present antigens would also be impaired leading to a reduced capacity to stimulate T-cell responses. Increased CTLA-4\textsuperscript{+} cells have been detected in the \textit{H. pylori}-infected human gastric mucosa (Stromberg et al., 2003; Lundgren et al., 2005b), and blockade of CTLA-4 in \textit{H. pylori}-infected mice reverses T-cell anergy, resulting in increased inflammation and reduced bacterial loads (Watanabe et al., 2004; Anderson et al., 2006). Hyporesponsive T cells induced by intraperitoneal injection of bacteria into mice, which inhibited the
development of \textit{H. pylori}-induced gastritis, were also found to express higher levels of CTLA-4 (Watanabe \textit{et al.}, 2002). Human CTLA4 gene polymorphisms are associated with susceptibility to mucosa-associated lymphoid tissue (MALT) lymphoma (Cheng \textit{et al.}, 2006) and differential regulation of \textit{H. pylori}-induced indoleamine 2,3-dioxygenase (IDO) activity. Tregs induce IDO expression by dendritic cells in a CTLA-4/CD80/86-dependent manner, resulting in the catabolism of tryptophan into pro-apoptotic compounds and the inhibition of effector T-cell function (Mellor and Munn, 2004). \textit{TGFB1} polymorphisms also affect IDO (Raitala \textit{et al.}, 2007).

In addition, it has been shown that interaction of naive T cells with the programmed cell death ligand 1 (B7-H1) on gastric epithelial cells favoured their development into \textit{CD25^+FOXP3^+} Tregs. \textit{H. pylori} stimulates increased expression of B7-H1 (Beswick \textit{et al.}, 2007), possibly contributing to increased Tregs in the gastric mucosa.

Many other functional mechanisms for the inhibition or killing of effector cells have been attributed to Tregs (see Table 6.1), including metabolic disruption, perforin- and granzyme-mediated killing, IL-2 sequestration and secretion of IL-35 (reviewed by Vignali, 2008). However, as yet, there is no data on their involvement in \textit{H. pylori} immune responses.

### 6.5.4 Regulatory T cells and associations with \textit{H. pylori} disease

We have previously reported that low numbers of Tregs in the human gastric mucosa are associated with increased Th1 responses, inflammation and the occurrence of peptic ulcer disease in adults (Robinson \textit{et al.}, 2008), indicating that Tregs may protect against ulcer development. In contrast, another group showed that elevated Tregs in the duodenum are linked with the presence of duodenal ulcers (Stromberg \textit{et al.}, 2003). There are several differences between the two studies. In Robinson \textit{et al.} (2008), data from patients with gastric or duodenal ulceration were combined together into one group and compared with samples from patients without ulcers. Treg responses during gastric and duodenal ulceration were not significantly different, but the group sizes were small. In addition, gastric CD4^+CD25^{high} cells were quantified by flow cytometry, where levels of CD25 staining were higher than in activated CD4^+CD25^{low} cells. In contrast, Stromberg \textit{et al.} (2003) quantified CD4^+CD25^{low} cells, which would have included activated effector T cells with intermediate CD25 expression as well as Tregs. In this study, higher levels of CTLA-4^+ cells were also detected, which is indicative of a Treg response. In children, where \textit{H. pylori}-induced pathology is much less severe, Harris \textit{et al.} (2008) showed that the Treg response is of a higher level than that observed in adults. This may confirm that Tregs are protective against disease; however, firm conclusions are difficult from these sorts of association studies. There may be differences in the populations studied and also in sampling and analysis methods. Animal models do not tend to result in ulceration, which means that it is difficult to show whether Tregs prevent peptic ulcer disease or not.

However, mouse infection studies have firmly demonstrated that Tregs can reduce gastric inflammatory responses and this has been confirmed by co-culturing human gastric Tregs with gastric epithelial cells \textit{in vitro} (Stromberg \textit{et al.}, 2005). Because the development of gastric adenocarcinoma is strongly linked with a chronic inflammatory response, it would seem likely that Tregs are protective against this malignancy. Moreover, Tregs have been shown to prevent the induction of colonic adenocarcinoma in a mouse model by suppressing the \textit{Helicobacter hepaticus}-induced inflammatory response (Erdman \textit{et al.}, 2003). One suggestion for the wide variation in the worldwide incidence rates of gastric cancer is variability in the cellular immune response mounted to \textit{H. pylori} infection. This could be affected by immune responses generated against other commonly occurring infections. For example, in mice, a concurrent intestinal helminth infection modulated the Th1 response to \textit{H. felis} and reduced gastric premalignant pathology (Fox \textit{et al.}, 2000). Many intestinal helminths induce strong Treg responses and are now being tested as anti-inflammatory therapeutics (Maizels and Yazdanbakhsh, 2008).
Increased levels of Tregs have been detected in the blood and tumour tissues of patients with different types of cancer (including gastric), and these responses are associated with advancing cancer progression and a poor prognosis (Sasada et al., 2003; Kono et al., 2006; Miller et al., 2006). Some tumours secrete factors that expand Treg numbers and thus inhibit antitumour immunity (Ghiringhelli et al., 2006). Increased numbers of FOXP3+ Tregs have been found in gastric adenocarcinoma tissue compared with surrounding normal tissue (Enarsson et al., 2006). Also, systemic and mucosal Tregs from patients with gastric adenocarcinoma produced high levels of IL-10, which suppresses both the antitumour cytotoxic T-cell response in the stomach (Lundin et al., 2007) and the transendothelial migration of T cells in a cell contact-mediated manner (Enarsson et al., 2007).

We hypothesize that a strong Treg response is initially beneficial in inhibiting inflammation and carcinogenesis. However, if a tumour subsequently develops, then protective immune mechanisms are suppressed leading to more rapid cancer progression. This poses a problem if Tregs were to be targeted in an immunomodulatory strategy to prevent H. pylori disease.

6.6 H. pylori-mediated Immunomodulation of Other Diseases

6.6.1 Allergy

A number of epidemiological studies have found a protective association between H. pylori infection and the incidence of atopy, allergic rhinitis, dermatitis and asthma (Kosunen et al., 2002; Pessi et al., 2005; Chen and Blaser, 2007, 2008; Seiskari et al., 2007; see Mitchell et al., Chapter 5, this volume). A potential role for H. pylori has been proposed, whereby childhood exposure to certain microorganisms (including H. pylori) is needed for healthy development of the immune system (Rook and Brunet, 2005). The typical age of acquisition of this infection (during early childhood) coincides with the common age for asthma onset. Indeed, stronger protective associations against childhood rather than adult asthma have been reported (Chen and Blaser, 2007). Higher IL-10 responses have been found in younger children with H. pylori, which may perhaps protect against allergy (Oderda et al., 2007). Higher levels of IL10 mRNA and a stronger IL-10+ Treg response have been found in CagA+ compared with CagA- human gastric tissue samples (Hida et al., 1999; Robinson et al., 2008), perhaps explaining the stronger protective effects associated with CagA+ infections (Chen and Blaser, 2007). As increased numbers of Tregs are present in the blood of H. pylori-infected patients (Kenefeck et al., 2007; Wang et al., 2007), these cells could play a general immunosuppressive role.

As H. pylori infection induces a strong Th1 response (Bamford et al., 1998), any protective effects of these bacteria against asthma could potentially be mediated by skewing the Th subset balance away from Th2. Infection with cagA+ strains is reported to elicit increased human Th1 (Hida et al., 1999) and reduced Th2 responses (Orsini et al., 2003). One recent study found that the presence of H. pylori was associated with reduced Th2 responses and reduced risk of atopy (Cam et al., 2009). Significantly, administration of purified HP-NAP, a Th1-promoting H. pylori virulence factor, has been shown to inhibit allergy in a mouse model (Codolo et al., 2008).

6.6.2 Autoimmunity

Several groups have shown a significantly lower prevalence of H. pylori-specific antibodies among people with autoimmune diseases such as multiple sclerosis (Wender, 2003; Li et al., 2007) and systemic lupus erythematosus (Sawalha et al., 2004). This suggests that H. pylori infection may also protect against, or attenuate, autoimmune diseases. Indeed, it has even been suggested that H. pylori-containing nanoparticles could be used as a treatment for multiple sclerosis (Pezeshki et al., 2008). As yet there is no information regarding the mechanisms behind these protective associations, but Tregs are...
likely to be good candidates because they are key suppressors of autoimmune reactions.

### 6.7 Conclusion

Gaining a better understanding of the immune response to *H. pylori* is important. The recent discovery of new types of T cells and the phenomenon of T-helper subset plasticity means that many long-established dogmas now need revision. Detailed knowledge of the immune mechanisms required for clearance of the infection is vital to underpin effective vaccine development. The need for vaccines is growing, with increasing prevalence of antibiotic-resistant strains, especially in countries where gastric cancer is common (Wong et al., 2003).

Investigating the balance between the Treg response and proinflammatory Th1 or Th17 subsets may also reveal new indicators for who is most at risk of peptic ulceration or gastric cancer as a consequence of their infection. Only 10–15% of infected individuals have symptomatic disease, and infection may mediate protection against other immune and inflammatory diseases. Humans have co-evolved with *H. pylori* and are normally meant to have these bacteria in the stomach. It is possible that a *Helicobacter*-free person is missing various immunological stimuli needed for good health. Thus, a targeted approach to *H. pylori* management, focusing on people at greatest risk of developing *H. pylori*-associated disease, would appear sensible.

Many key questions remain. There have been no interventional human or animal studies to prove whether *H. pylori* protects against allergy and autoimmunity. If present, it is also unknown whether such effects would be diminished following *H. pylori* eradication. Further into the 21st century, it will be interesting to see whether *H. pylori* preparations can be used to treat or prevent such conditions. *H. pylori*-induced Treg responses may also potentially impact upon the disease sequelae of other infections, or even the effectiveness of some vaccines. It has been reported that a concurrent *H. pylori* infection can suppress virus-specific CD8+ T cells and prevent clearance of a viral infection in mice (Shirai et al., 1998), most likely via the stimulation of Tregs. We know that Tregs inhibit the efficacy of *H. pylori* vaccination (Zhang, M. et al., 2008); perhaps there are also deleterious effects on other immunizations that have not been identified. Indeed, a recent study in Bangladesh showed that intestinal parasite infections are responsible for some cholera vaccine failures (Harris et al., 2009). These issues, coupled with the fact that Tregs can increase the rate of cancer progression, indicate that immunity and the immunoregulatory responses to *H. pylori* should now be investigated in more detail. This will not only provide information on how to manage *H. pylori*-associated conditions, but could also have an important impact on extragastric diseases.

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7 Host Genetic Factors in Susceptibility and Resistance to *Helicobacter pylori* Pathogenesis

P. Sutton,* A.L. Every and S.N. Harbour

7.1 Introduction

In the years following the discovery of *Helicobacter pylori* as a common infection of the human stomach, there was great controversy regarding whether this infection was responsible for a range of disease states, including peptic (duodenal and gastric) ulcers and stomach cancer. A major reason for this controversy was the diverse pathologies that appeared to be associated with this infection. It was hard to understand why many individuals infected with *H. pylori* would be asymptomatic, while others would develop ulcers and yet others cancer. The fact that the majority of infected persons do not develop disease remains a key argument for those sceptical of a role for *H. pylori* in the aetiology of these diseases.

Over the last 20 years, however, the reason for the diversity of disease outcomes resulting from *H. pylori* infection has started to become clearer. Our understanding now indicates that the development of symptomatic disease in response to *H. pylori* is a result of the chronic inflammation that is typically present for decades following infection during childhood (see Malaty, Chapter 1, this volume). The site of inflammation is particularly important. Persons who develop *Helicobacter*-associated gastritis in the acid-secreting corpus region of the stomach are susceptible to developing gastric ulcers, as well as the most serious consequence of *H. pylori* infection, namely gastric adenocarcinoma (see Correa and Piazuelo, Chapter 3, this volume). The most important feature in development of this cancer is believed to be the loss of acid-secreting cells due to tissue changes resulting from chronic inflammation of the gastric corpus. In contrast, individuals who develop inflammation of the antrum and/or duodenum are prone to duodenal ulcers and, as the inflammation does not damage the acid-secreting regions of the stomach, appear protected against developing gastric adenocarcinoma.

Another key feature that appears to play an important role in determining which *H. pylori*-positive individuals will develop disease is the severity of the inflammation. The greater the severity of the inflammation resulting from *H. pylori* infection, the more chance there is for an associated disease to occur. It is logical that more severe inflammation increases the risk of tissue ulceration; similarly more severe inflammation in the corpus means more chance of destructive loss of acid secretion, thus increasing susceptibility to cancer progression.

A key question is what dictates the severity of *Helicobacter*-associated inflammation? Why is there a high prevalence of *H. pylori* infection but a low incidence of gastric cancer

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in India and Thailand, while Japan and some areas of China suffer much greater incidences of this malignancy (Singh and Ghoshal, 2006)? This is also not a simple matter, as it is clearly a result of a complex interaction of three broad areas (Fig. 7.1). Environmental factors, such as smoking, a high-salt diet and a low intake of fresh fruit and vegetables, clearly play a role. Bacterial genetic factors are certainly extremely important in pathogenesis and there is a quite remarkable degree of diversity in important H. pylori virulence factors (see Backert et al., Chapter 11, this volume). The third area is that of host genetic factors, i.e. variability in genes of the infected host that make an individual more likely to develop Helicobacter-associated disease. That is the subject of this chapter.

Information regarding the importance of host genetic factors has been obtained predominantly from two sources. A large number of studies have examined the association between human gene polymorphisms (i.e. differences in gene sequence that alter the functional activity of encoded proteins when expressed) and susceptibility to H. pylori infection and/or associated diseases, in particular gastric cancer. These findings are supported by observing the severity of inflammation and disease progression following Helicobacter infection of genetically modified mice that are deficient for key host genetic factors.

7.2 Human Polymorphism Studies Indicate that Host Variability in Immune-related Genes can Impact upon Susceptibility to H. pylori-associated Pathologies

Strong associations have been made between a number of human gene polymorphisms and susceptibility to Helicobacter-associated disease. However, this can be extremely complicated as there is also ethnic variation, with genes that appear to be of considerable significance in Caucasians being possibly irrelevant in an Asian population, and vice versa. The majority of the important host factors identified involve gene products that regulate or impact upon the host immune response to infection, in particular cytokines, as well as receptors of the host immune system that detect the presence of pathogens (Toll-like and nucleotide-binding oligomerization domain-like receptors). Other factors, such as mucins that limit access of the bacteria to the stomach surface, may also play an important role. A summary of known associations between human gene polymorphisms and H. pylori infection and disease state is presented in Table 7.1 and Fig. 7.2. The potentially most important findings are discussed further in the following text. It should be remembered that the statistical power of studies involving small numbers of patients is limited, and in these cases false negative associations between particular gene polymorphisms and disease are possible.

7.2.1 Cytokines produced by T-helper lymphocytes

The T-helper lymphocyte (Th cell) is the conductor of the host acquired (specific and memory) immune response to infection. This is achieved by the secretion of a range of cytokines that impact heavily upon and control the activities of other immune cells, in particular those involved in the inflammatory response to infection. Based on the cytokine
<table>
<thead>
<tr>
<th>Gene (polymorphism)</th>
<th>Association with <em>H. pylori</em> pathogenesis</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFNGR</strong> (IFNGR1 –56)</td>
<td>Increased risk of gastric cancer in Portuguese</td>
<td>Canedo et al. (2008b)</td>
</tr>
<tr>
<td><strong>HLA</strong> (DQA1*1012)</td>
<td>Reduced risk of pathology in Japanese</td>
<td>Azuma et al. (1998); Yoshitake et al. (1999)</td>
</tr>
<tr>
<td><strong>IL1B</strong> (–31, –511, RN*2)</td>
<td>Significant risk of gastric ulcers and gastric cancer; effects more obvious in Caucasian rather than Asian populations</td>
<td>El-Omar et al. (2000, 2003); Xuan et al. (2005)</td>
</tr>
<tr>
<td><strong>TNFA</strong> (–308, −857)</td>
<td>Increased risk of gastric ulcers and gastric cancer in Caucasian and some Asian populations</td>
<td>El-Omar et al. (2003); Machado et al. (2003); Sugimoto et al. (2007)</td>
</tr>
<tr>
<td><strong>IL6</strong> (–174)</td>
<td>Increased risk of gastric cancer in Brazilians, but not in Korean or Japanese populations</td>
<td>Hwang et al. (2003); Gatti et al. (2007)</td>
</tr>
<tr>
<td><strong>IL8</strong> (–251)</td>
<td>Increased risk of intestinal metaplasia and gastric cancer in Asian, but not Caucasian populations</td>
<td>Taguchi et al. (2005); Canedo et al. (2008a); Kim et al. (2008)</td>
</tr>
<tr>
<td><strong>IL10</strong> (–1082, −819, −592)</td>
<td>Unclear; possible increased risk of gastric cancer due to hyperinflammatory response</td>
<td>El-Omar et al. (2003); Garcia-Gonzalez et al. (2007)</td>
</tr>
<tr>
<td><strong>TGFB</strong> (+509, +869, +905)</td>
<td>Reduced risk of duodenal ulcers in Caucasians, but no association with gastric cancer</td>
<td>Garcia-Gonzalez et al. (2006)</td>
</tr>
<tr>
<td><strong>TLR4</strong> (+299)</td>
<td>Unclear; possible reduced risk of MALT lymphoma in Caucasians</td>
<td>Hellmig et al. (2005a); Hofner et al. (2007); Trejo-De La et al. (2008)</td>
</tr>
<tr>
<td><strong>CD14</strong> (−260)</td>
<td>Increased risk of gastric cancer and intestinal metaplasia</td>
<td>Zhao et al. (2007)</td>
</tr>
<tr>
<td><strong>TLR2</strong> (−196)</td>
<td>Unclear; possible increased risk of gastric cancer in Japanese</td>
<td>Tahara et al. (2007b); Moura et al. (2008)</td>
</tr>
<tr>
<td><strong>NOD1</strong> (G796A)</td>
<td>Increased risk of duodenal ulcer in Caucasians; unknown in other populations</td>
<td>Hofner et al. (2007)</td>
</tr>
<tr>
<td><strong>MUC1</strong> (short allele)</td>
<td>Increased risk of gastric adenocarcinoma and gastritis in Caucasians; unknown in others</td>
<td>Carvalho et al. (1997); Vinall et al. (2002)</td>
</tr>
<tr>
<td><strong>COX1</strong> (−1676)</td>
<td>Associated with gastric ulcers and functional dyspepsia in Japanese</td>
<td>Arisawa et al. (2007a, 2008a)</td>
</tr>
<tr>
<td><strong>COX2</strong> (−1195, −765)</td>
<td>Gastric cancer in Chinese (−1995) and gastric cancer and peptic ulcers in Indians (−765)</td>
<td>Liu et al. (2006); Saxena et al. (2008)</td>
</tr>
<tr>
<td><strong>MDR1</strong> (+3435)</td>
<td>Contradictory reports on MDR1 polymorphisms and gastric cancer in Japanese</td>
<td>Tahara et al. (2007a); Sugimoto et al. (2008)</td>
</tr>
<tr>
<td><strong>MBL</strong> (+54)</td>
<td>Increased risk of gastric cancer in Japanese but no association with gastritis in Polish</td>
<td>Bak-Romaniszyn et al. (2006); Wang et al. (2008)</td>
</tr>
<tr>
<td><strong>MIF</strong> (−173, −794)</td>
<td>Increased risk of mucosal atrophy in Japanese</td>
<td>Arisawa et al. (2008b)</td>
</tr>
<tr>
<td><strong>IL2</strong> (−330)</td>
<td>Increased risk of atrophic gastritis in Japanese but not Koreans</td>
<td>Togawa et al. (2005); Shin et al. (2008)</td>
</tr>
<tr>
<td><strong>IL17</strong> (−7488, −197A)</td>
<td>Increased inflammation during <em>H. pylori</em> infection, and increased risk of gastric cancer in Japanese</td>
<td>Arisawa et al. (2007b); Shibata et al. (2009)</td>
</tr>
<tr>
<td><strong>IL18</strong> (−137, −607)</td>
<td>Increased severity of gastritis in Japanese with possible effects on <em>H. pylori</em> eradication</td>
<td>Leung et al. (2006); Sakai et al. (2008)</td>
</tr>
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</table>

MALT, mucosa-associated lymphoid tissue.
Host Genetic Factors in Susceptibility and Resistance

profiles produced, Th cells are separated into distinct subpopulations. For example, Th1 cells produce proinflammatory cytokines including interferon-γ (IFNγ) and interleukin (IL)-12, while Th2 cells produce cytokines including IL-4 that suppress inflammation. More recently, Th17 cells (producing IL-17) have risen in prominence as important mediators of H. pylori-driven inflammation (Shiomi et al., 2008; DeLyria et al., 2009). The importance of Th cells in Helicobacter pathogenesis is reviewed in detail elsewhere (see Robinson and Atherton, Chapter 6, this volume). Here, we discuss how variability in host genes for T-helper cytokines may contribute to selecting individuals who progress to Helicobacter-associated pathologies.

**Interferon-γ**

IFNγ is an important cytokine, produced primarily by Th1 cells, that is a key mediator of the inflammatory Th1 immune response. It has been shown to be crucial for H. pylori-mediated immunopathology, which is dominated by IFNγ-producing Th1 cells in the gastric mucosa (Bamford et al., 1998). Studies using knockout mice have shown that, in the absence of IFNγ, Helicobacter gastric colonization is significantly increased but pathology scores are significantly decreased compared with wild-type mice (Eaton et al., 2001; Akhiani et al., 2002). While polymorphisms in the human IFNG gene (i.e. +874 (A/T)) have been found to increase cytokine production, studies investigating the significance of IFNG polymorphisms in German, Italian and Japanese populations have found no association with H. pylori-induced disease, including gastric cancer (Rad et al., 2004; Zambon et al., 2005; Seno et al., 2007).

The strongest associations with regard to Helicobacter pathogenesis have been made with the receptors of this cytokine (IFNGR1 and IFNGR2). Whole-genome scanning of Senegalese patients found the polymorphisms in IFNGR1 were strongly associated with high levels of anti-H. pylori serum antibodies (Thye et al., 2003). While a –56 (C/T) polymorphism potentially led to loss of receptor function, –1004 (A/C) and –1400 (T/C) polymorphisms both resulted in amino acid substitutions with the potential to affect IFNGR1 signalling (Thye et al., 2003; Canedo et al., 2008b). Further, a study in Portuguese patients found that the IFNGR1 –56 polymorphism was significantly associated with increased risk of gastric cancer. A polymorphism in IFNGR2 has been associated with increased risk of gastric cancer in Polish subjects, with the highest risk present in those carrying both the IFNGR2 and TNFA –308 polymorphisms (Hou et al., 2007).

**Interleukin-4**

IL-4, the key mediator of the Th2 immune response, is anti-inflammatory due to its inhibition of IFNγ and IL-2 production by...
Th1 cells (Tanaka et al., 1993). During *Helicobacter* infection, IL-4 levels decrease in the human gastric mucosa (Orsini et al., 2003). While most studies have found no association between IL4 polymorphisms and *H. pylori*-induced disease in Caucasian (El-Omar et al., 2003; Garcia-Gonzalez et al., 2007; Zambon et al., 2008) and Japanese (Togawa et al., 2005) patients, one study did find that Japanese carriers of both IL4 –984/–2983 AA/GA were significantly protected against gastric cancer (Seno et al., 2007), although the mechanism of protection is unknown.

**Major histocompatibility complex antigens**

Major histocompatibility complex (MHC) antigens are critical components of the acquired immune response to infection and MHC loci are among the most genetically variable in humans, with >1500 MHC class I allele and >5000 MHC class II allele combinations (Marsh et al., 2005).

As MHC class II antigens (HLA-DR, HLA-DP and HLA-DQ) are used by antigen-presenting cells (such as macrophages and dendritic cells) to present antigen to and activate Th cells, variability in these alleles can significantly influence the host immune response to infection. Therefore, MHC class II alleles were among the first components of the host response to *Helicobacter* infection to be examined. These studies revealed an ethnic bias in the importance of human leucocyte antigen (HLA) type on the host response to *Helicobacter*, with the most pronounced associations being observed in the high-risk Japanese population.

A number of Japanese studies have found that HLA-DQA*0102*, HLA-DQB1*0601* and HLA-DRB1*1502* are associated with a significantly reduced risk of *H. pylori*-induced atrophic gastritis, duodenal ulcer, gastric ulcer and gastric adenocarcinoma (Azuma et al., 1998; Yoshitake et al., 1999; Hirata et al., 2007), although a similar study found that HLA-DQB*0601*, along with HLA-DQA*0103*, conferred a significantly increased risk of *H. pylori*-associated mucosa-associated lymphoid tissue (MALT) lymphoma (Kawahara et al., 2005), suggesting that the HLA-DQB*0601* allele can affect different pathological outcomes. Other HLA alleles have been associated with an increased risk of *H. pylori* disease outcomes in the Japanese. HLA-DQA*0301* was significantly lower in uninfected controls compared with duodenal ulcer patients (Azuma et al., 1995) and HLA-DQB1*0401* was significantly higher in atrophic gastritis patients (Sakai et al., 1999), both indicating an increased risk of disease. HLA-DRB1*0401* has also been linked with increased risk of gastric cancer in the Japanese, although this was not associated with *H. pylori* infection (Ohtani et al., 2003).

In contrast, however, while associations have been made between HLA-DQB1*0301* and gastric cancer in American Caucasians (Lee et al., 1996), HLA-DQA1*0503* and gastric cancer in Mexicans (Garza-Gonzalez et al., 2004), and HLA-DQB*0602* and gastric cancer in Spanish Caucasians (Quintero et al., 2005), many studies on Caucasian patients have found no association between HLA molecules and gastric pathologies (Karhukorpi et al., 1999; Magnusson et al., 2001; Kunstmann et al., 2002; Perri et al., 2002). This has reinforced the belief that HLA molecules have a greater impact on host response to *Helicobacter* in the Japanese population compared with other ethnic groups.

**7.2.2 Inflammation-modulating cytokines produced by cells of the innate immune system**

**Interleukin-1β**

The best studied and potentially most important host gene is that encoding the cytokine IL-1β. IL-1β is a proinflammatory cytokine, produced by macrophages and epithelial cells, that is up-regulated by *H. pylori* infection (Noach et al., 1994; Jung et al., 1997). It induces T- and B-lymphocyte activation, as well as the production of other proinflammatory cytokines (Dinarello and Savage, 1989). However, perhaps the most important feature of IL-1β with regard to *Helicobacter* pathogenesis is its potent inhibition of gastric acid secretion, with reportedly 100-fold greater activity than proton pump inhibitors (Wolfe and Nompleggi, 1992; Beales and Calam, 1998). Low gastric acid production (hypo-
chlorhydria) is strongly associated with the development of gastric ulcers and gastric cancer. Suppression of gastric acid secretion by increased IL-1β production has significant effects on cancerous changes in the stomach. With lower stomach acid levels, bacterial toxins and by-products of inflammation that would normally be destroyed are accumulated (some of which are known mutagens), and bacteria that normally find the stomach inhospitable are able to colonize, leading to increased production of carcinogenic bacterial by-products.

IL-1β and its endogenous receptor antagonist IL-1Ra are encoded by the related genes IL1B and IL1RN. Three polymorphisms in the human IL1B gene have been reported, all representing C-T base transitions at positions –511, –31 and +3954 base pairs from the transcriptional start site (El-Omar et al., 2000). In addition, individuals who carry the less common allele 2 of the IL1RN gene (IL1RN*2) have a prolonged response to inflammatory stimuli, and this polymorphism has been associated with a number of conditions including ulcerative colitis, Crohn’s disease, alopecia, psoriasis and systemic lupus erythematosus (Witkin et al., 2002). The association of IL1B polymorphisms and gastric cancer has been studied in a number of different human populations, with some variable results.

Numerous studies have found associations between IL1B polymorphisms and gastric cancer in populations of European and African origin: IL1B –31, IL1B –511 and IL1RN*2 were independently associated with hypochlorhydria and increased frequency of atrophic gastritis, intestinal metaplasia and gastric cancer in Helicobacter-infected Scottish, Polish and German patients (El-Omar et al., 2000; Rad et al., 2003). While an increased risk of H. pylori-associated gastric cancer has been noted in American Caucasians with the IL1B –511 or IL1RN*2 polymorphism (El-Omar et al., 2003), a study of African American and Caucasian patients in the USA found that the IL1B +3954 polymorphism, but not IL1B –31, IL1B –511 and IL1RN*2 polymorphisms, was associated with increased risk of H. pylori-dependent multi-atrophic gastritis (Zabaleta et al., 2006). Mexicans with the IL1B –31 polymorphism alone or in combination with IL1RN*2 appear to have an increased risk of Helicobacter-associated gastric cancer (Sicinschi et al., 2006), while no association between IL1B polymorphisms and gastric cancer was found in Spanish Caucasian patients (Garcia-Gonzalez et al., 2007). Two studies investigated IL1B polymorphisms in the Portuguese, who have a high incidence of H. pylori infection and gastric cancer. One found that IL1B –511 and IL1RN*2 polymorphisms were independently associated with an increased risk of gastric cancer, with a substantial increase in gastric cancer risk in individuals carrying both polymorphisms (Machado et al., 2001). A second found an increased risk of gastric cancer in patients with IL1B –511 or both IL1B –511 and IL1RN*2 polymorphisms, but not IL1RN*2 alone (Figueiredo et al., 2002). While studies in central Italy, Costa Rica and Oman (areas of high gastric cancer prevalence) found no association between IL1B –31 and IL1B –511 polymorphisms and gastric cancer, carriers of the IL1RN*2 allele had an increased risk of gastric cancer (Alpizar-Alpizar et al., 2005; Palli et al., 2005; Al-Moundhi et al., 2006).

The story in Asia appears more complicated. Increasing evidence suggests that IL1B polymorphisms are less important to gastric cancer development in Japanese populations, with no correlation between IL1B polymorphisms and expression of the IL-1β cytokine in the stomach, the severity of H. pylori-induced inflammation, or atrophy (Kato et al., 2001; Xuan et al., 2005; Seno et al., 2007; Sugimoto et al., 2007). It has even been suggested that the IL1B –511 polymorphism may indicate less risk for gastric cancer in the Japanese (Ito et al., 2007), although a different study found H. pylori-infected Japanese patients with the IL1B –511 polymorphism had higher gastric pH, associated with more widespread infection and more severe inflammation (Furuta et al., 2002). Similarly in Korea (another country with a high prevalence of gastric cancer), studies have found no association between IL1B polymorphisms and H. pylori-induced pathologies, including gastric cancer (Chang et al., 2008; Kim et al., 2008; Shin et al., 2008), with the exception of a possible link with IL1RN*2 (Kim et al., 2006). In contrast, however, many studies in China
reflect the Caucasian findings, with \textit{IL1B} –511 and \textit{IL1RN}^{*2} polymorphisms associated with increased risk of \textit{H. pylori}-induced pathologies, including gastric cancer (Zeng et al., 2003; Chen et al., 2004; Leung et al., 2006; Li et al., 2007; Feng et al., 2008). One study from China did find that \textit{IL1B} –511 polymorphisms did not influence gastric acid secretion in \textit{H. pylori}-infected young healthy individuals, suggesting the association of this polymorphism with gastric cancer may be via an acid-independent mechanism (Hu et al., 2005).

Overall, these observations indicate a strong ethnic effect on the relative importance of IL-1β and \textit{Helicobacter} pathogenesis, with associations between \textit{IL1B} polymorphisms and gastric cancer depending upon the country and/or ethnic origin of the infected population. While the effect of IL-1β on acid secretion is clearly important in some populations, this variability likely reflects the interaction of a range of factors in this disease process, including bacterial virulence factors (see Backert et al., Chapter 11, this volume), lifestyle and environmental factors, as well as other host genes.

\textit{Tumour necrosis factor-α}

Another proinflammatory cytokine up-regulated by \textit{H. pylori} infection in humans is tumour necrosis factor-α (TNFα) (Crabtree et al., 1991). Produced predominantly by macrophages in response to bacterial products, TNFα activates nuclear factor-xB (NF-xB) (a transcription factor that triggers production of a range of inflammatory mediators) in a range of immune and non-immune cells, as well as attracting other immune cells to sites of inflammation. TNFα also inhibits gastric acid production (Beales and Calam, 1998) and TNFα-deficient mice develop significantly less severe gastritis than wild-type controls after \textit{H. pylori} infection (Yamamoto et al., 2004), suggesting an important role for TNFα in \textit{Helicobacter}-driven inflammation. Polymorphisms of the \textit{TNFA} gene have been associated with increased susceptibility or severity of a number of conditions including malaria (McGuire et al., 1994), Crohn’s disease (Negoro et al., 1999) and lymphoma (Fitzgibbon et al., 1999).

The \textit{TNFA} –308 polymorphism is associated with enhanced TNFα expression (Wilson et al., 1997; Gonzalez et al., 2003) and this polymorphism has been linked with an increased risk of peptic ulcers in German and Spanish Caucasians (Kunstmann et al., 1999; Lanas et al., 2001) and gastric cancer in Caucasian patients from Portugal, Poland and the USA (El-Omar et al., 2003; Machado et al., 2003; Hou et al., 2007). However, other reports found no association between \textit{TNFA} –308 polymorphism and increased cytokine production or increased \textit{H. pylori}-induced pathology (Rad et al., 2004; García-Gonzalez et al., 2007). Similarly, studies in Japanese and Korean patients found no association between \textit{TNFA} –308 polymorphism and \textit{H. pylori}-induced gastrroduodenal disease (Kim et al., 2006; Sugimoto et al., 2007). These differences are supported by a meta-analysis of 24 independent studies, which found a statistically significant association between \textit{TNFA} –308 polymorphism and gastric cancer only in Western populations (Gorouhi et al., 2008).

While the \textit{TNFA} –857 polymorphism has been associated with peptic ulcers and B-cell lymphoma in Italian and German patients (Hellmig et al., 2005b; Zambon et al., 2005), it was negatively associated with MALT lymphoma in Chinese patients (Wu et al., 2004). \textit{TNFA} –863 and \textit{TNFA} –1031 polymorphisms have been associated with higher TNFα levels and increased risk of peptic ulcers in Chinese patients (Lu, C.C. et al., 2005), and a study of \textit{H. pylori}-infected Japanese found that these polymorphisms, along with \textit{TNFA} –857, are independently associated with increased risk of gastric ulcers as well as gastric cancer, while simultaneous carriage of all three polymorphisms greatly increases risk of both pathologies (Sugimoto et al., 2007).

\textit{Interleukin-12}

IL-12 is a proinflammatory cytokine produced by dendritic cells and macrophages that induces IFNγ production and leads to the development of Th1 cells, the primary immune cell involved in \textit{Helicobacter}-mediated gastritis (Delios et al., 1997). While IL-12 is up-regulated in \textit{H. pylori} infection (Hida et al., 1999), IL-12 deficiency in mice has no effect
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on H. pylori colonization or resulting pathology (Akhiani et al., 2002).

IL-12 is a heterodimer, comprised of two subunits (IL-12p35 and IL-12p40) encoded by the genes IL12A and IL12B. Again, the importance of IL-12 polymorphisms is unclear, as one study in Italians identified IL12 polymorphisms (IL12A –504 and IL12B VNTR 9/11) that were significantly associated with gastric cancer (Navaglia et al., 2005), while studies in other European Caucasian (García-González et al., 2007; Hou et al., 2007) as well as Japanese (Seno et al., 2007) populations found no association between IL12 polymorphisms and risk of H. pylori-associated gastric cancer.

**Interleukin-6**

IL-6 is a multifunctional cytokine produced by many cells including T cells, macrophages and intestinal epithelial cells. It can act either to promote inflammation by inducing the production of acute-phase proteins such as C-reactive protein and serum amyloid A (Gabay, 2006), or as an anti-inflammatory cytokine by controlling the level of proinflammatory cytokines including TNFα, IFNγ and macrophage inflammatory protein 2 (MIP-2) (Xing et al., 1998). A number of polymorphisms in the IL6 gene have been identified; one notable finding was that the IL6 –174 polymorphism can lead to increased IL-6 production, which has been linked with chronic arthritis (Fishman et al., 1998).

IL-6 expression is up-regulated in the gastric mucosa of Helicobacter-infected patients (Crabtree et al., 1991) and is also increased in early gastric cancer tissue (Yamaoka et al., 2001). However the relevance of IL6 polymorphisms to Helicobacter disease outcome is unclear. While one study in Brazil found an association between IL6 –174 and gastric cancer (Gatti et al., 2007), other studies found no association between this polymorphism and Helicobacter disease outcome in Caucasian (El-Omar et al., 2003), Korean and Japanese (Hwang et al., 2003) populations.

**Interleukin-8**

IL-8 is a chemokine produced primarily by macrophages, but also by other cells (including bacterial-activated epithelial cells), that plays an important role in attracting neutrophils to sites of inflammation/infection, and activating them to release by degranulation a range of antibacterial and proinflammatory mediators (Ogra, 1999). IL-8 is up-regulated after H. pylori infection (Crabtree et al., 1994) and is potentially the most important cytokine produced by the host in response to H. pylori infection. The infiltration of neutrophils into the stomach mucosa in response to H. pylori infection (termed ‘active’ gastritis) is associated with more severe disease outcomes.

A single polymorphism in the IL8 gene, at position –251, is associated with increased IL-8 production (Taguchi et al., 2005). A number of studies have suggested that this IL8 polymorphism plays an important role in Helicobacter pathogenesis in Asian populations. The clearest association occurs in Japanese populations, with IL8 –251 being associated with increased risk of atrophic gastritis, gastric cancer and ulcers (Ohyauchi et al., 2005; Taguchi et al., 2005). In Korean and Chinese populations, the IL8 polymorphism has also been associated with an increased risk of gastric cancer, but not intestinal metaplasia or atrophic gastritis (Lee et al., 2005; Lu, W. et al., 2005; Leung et al., 2006; Kang et al., 2008; Ye et al., 2009).

The effect of this polymorphism on Helicobacter infection in non-Asians is unclear. While studies found an association between IL8 –251 and increased risk of duodenal ulcers and gastritis in Hungarian patients (Gyulai et al., 2007), no association was found with gastric cancer risk in Finnish (Kamangar et al., 2006) or Portuguese (Canedo et al., 2008a) subjects. This suggests the effect of the IL8 polymorphism may be ethnic specific.

**Interleukin-10**

Anti-inflammatory IL-10 is produced by a wide range of cells including monocytes, macrophages, mast cells, T and B lymphocytes, regulatory T cells and dendritic cells (Mosser and Zhang, 2008). IL-10 is a potent inhibitor of antigen presentation (limiting the acquired
immune response) as well as dendritic cell activation and maturation, thereby suppressing production of a range of important inflammatory cytokines including IL-1, IL-6, IL-12 and TNFα (Mosser and Zhang, 2008). IL-10 is up-regulated by Helicobacter infection in man (Karttunen et al., 1997) and given its wide-ranging effects on mediators of inflammation, it is perhaps not surprising that Helicobacter infection of IL-10-deficient mice results in increased severity of gastritis (Matsumoto et al., 2005).

Three main polymorphisms in the IL10 promoter have been identified (−1082 (G/A), −819 (C/T) and −592 (C/A)), which combine to form three main haplotypes: GCC (associated with increased IL-10 production), ACC and ATA (associated with reduced IL-10 production) (Eskdale et al., 1999; Rad et al., 2004). Once again, there are conflicting reports regarding IL10 polymorphisms and Helicobacter infection. One study found that American Caucasians with the IL10 ATA haplotype had an increased risk of H. pylori-induced gastric cancer (El-Omar et al., 2003). It is hypothesized that people carrying this low IL-10-producing haplotype are at increased risk of gastric cancer due to the increased inflammatory response resulting from reduced levels of this protective cytokine. IL10 polymorphisms have also been associated with increased risk of gastric cancer and intestinal metaplasia in Mexican and Korean patients (Sicinschi et al., 2006; Kim et al., 2008), gastritis in Indian patients (Achyut et al., 2008) and gastric cancer in a Chinese population (Lu, W. et al., 2005).

However, an equal number of studies have failed to find any association between IL10 polymorphisms and increased risk of H. pylori-induced gastric cancer (Rad et al., 2004; Hellmig et al., 2005c; Garcia-Gonzalez et al., 2007; Forte et al., 2008), Chinese (Leung et al., 2006) and Japanese (Saijo et al., 2007; Seno et al., 2007) patients.

Transforming growth factor-β

Transforming growth factor (TGFβ) is produced in three isoforms by many different cell types, including monocytes/macrophages, dendritic cells and lymphocytes. TGFβ is a pleiotropic cytokine with potent immunoregulatory properties, inhibiting both antigen presentation (Takeuchi et al., 1998) and lymphocyte proliferation (Letterio and Roberts, 1998; Gorelik and Flavell, 2000). It is chemotactic for monocytes, from which it can induce cytokine secretion. The role of TGFβ in regulatory T-cell development and function is complex, with TGFβ having been shown to be required for the induction, development and enhancement of regulatory T-cell function (Fantini et al., 2004). Enhanced expression of TGFβ has been observed in patients with gastric cancer and gastric ulcers (Ebert et al., 2000; Milani and Calabro, 2001) and administration of TGFβ can accelerate the healing of experimental gastric ulcers in animals (Ernst et al., 1996), suggesting TGFβ may play an important role in H. pylori pathogenesis.

Three main polymorphisms in the TGFB1 gene have been identified at +509, +869 and +915, the latter two leading to amino acid substitutions. A study on Spanish Caucasians found that the +869 polymorphism was associated with reduced risk of duodenal ulcer (Garcia-Gonzalez et al., 2006), and a second study on Russian Caucasians supported this by showing that carriage of all three TGFB1 polymorphisms was significantly reduced in those with duodenal ulcer (Polonikov et al., 2007). This cytokine appears to be most important in ulcer development, as studies on Caucasian and Japanese populations have found no association between TGFB1 polymorphisms and gastric cancer (Garcia-Gonzalez et al., 2007; Seno et al., 2007). However, one Chinese study found that polymorphisms in TGFB1 +509 and the TGFβ receptor TGFBRII were associated with a decreased risk of gastric cancer (Jin et al., 2007).

7.2.3 Impact of host variability in pathogen recognition receptors on H. pylori pathogenesis

The mammalian immune system possesses a range of pathogen detection receptors, including Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)
family members (see Kaparakis et al., Chapter 8, this volume). When activated, these receptors trigger an immune response to combat these infections, typically via the production of proinflammatory cytokines.

**Toll-like receptor 4 and CD14**

TLR4, expressed on the surface of many cells including monocytes/macrophages, dendritic cells and gastrointestinal epithelial cells, associates with CD14 (mainly on macrophages) and MD2 to form a receptor that detects lipopolysaccharide from Gram-negative bacteria. While *H. pylori* infection can up-regulate TLR4 expression (Asahi et al., 2007), the role of TLR4 in *Helicobacter* pathogenesis is controversial. While some in vitro studies found *H. pylori* can activate TLR4 on gastric cell lines as well as primary human cells (Su et al., 2003; Alvarez-Arellano et al., 2007), others found no TLR4 expression or activation in similar studies (Backhed et al., 2003; Smith et al., 2003). Results from in vitro experiments using mouse cells are similarly unclear (Mandell et al., 2004; Obonyo et al., 2007), although *Helicobacter felis* infection of mice with non-functional TLR4 did not induce gastritis (Sakagami et al., 1997), suggesting TLR4 may be important for *Helicobacter*-induced inflammation.

Several polymorphisms of human TLR4 have been identified, including an Asp/Gly replacement at +299 that has been shown to attenuate receptor signalling and diminish inflammation in certain disease models (Lorenz et al., 2002; Schmitt et al., 2002) and another at +399. Both of these polymorphisms have been associated with altered production of a range of inflammation-modulating cytokines in response to *Helicobacter* infection, including IL-1β, TNFα, IL-10 and IL-8 (Trejo-De La et al., 2008). However, while a study of German and Austrian Caucasian patients found the TLR4 +299 polymorphism was significantly associated with a reduced risk of MALT lymphoma (Hellmig et al., 2005a), no similar association was found in Hungarian (Hofner et al., 2007), Venezuelan (Kato et al., 2007), Mexican (Garza-Gonzalez et al., 2007) or Indian (Achyut et al., 2007) patients. Also, while a polymorphism at TLR4 +399 was associated with atrophy and metaplasia in an Indian population (Achyut et al., 2007), it was not associated with disease outcome in Hungarian, Mexican or American populations (Hofner et al., 2007; Trejo-De La et al., 2008; Murphy et al., 2009).

Two polymorphisms in the CD14 gene have been identified (positions –159 and –260). In Taiwanese, Japanese and Caucasian patients, CD14 –159 was not associated with gastric malignancy or dyspepsia (Wu et al., 2006; Tahara et al., 2008a; Hold et al., 2009) but was associated with a marginally increased risk of MALT lymphoma in Turkish patients (Türe-Ozdemir et al., 2008). However, the CD14 –260 polymorphism has been associated with an elevated risk of gastric carcinoma in Chinese (Zhao et al., 2007) and intestinal metaplasia in Venezuelan (Kato et al., 2007) patients.

**Toll-like receptor 2**

TLR2 is a cell surface receptor that recognizes a range of ligands, including bacterial glycolipids, lipopeptides and lipoproteins. Expression of TLR2 is widespread on immune cells, including monocytes/macrophages, dendritic cells, B cells and subsets of T cells, but is not believed to be expressed on the normal gastric epithelium. In vitro studies have shown that *Helicobacter* can activate TLR2 on cell lines and primary immune cells from mice and human subjects (Smith et al., 2003; Mandell et al., 2004; Alvarez-Arellano et al., 2007). A Japanese study found that a 22 bp deletion (−196 to −174) in the TLR2 gene that can affect promoter activity was present at a significantly higher frequency in gastric cancer patients (Tahara et al., 2007b), and was significantly associated with intestinal metaplasia in females and those over 60 years of age. However, no association was found between TLR2 polymorphisms and peptic ulcers in Japanese and Brazilian subjects (Moura et al., 2008; Tahara et al., 2008b).

**Nucleotide-binding oligomerization domain-1/2**

Intracellular NOD receptors sense intracellular bacteria and bacterial products. NOD1 is
expressed in gastric epithelial cells, as well as macrophages and dendritic cells (Hofner et al., 2007) and recognizes a muramyl peptidoglycan from Gram-negative bacteria (Girardin et al., 2003a). NOD2 is expressed in intestinal epithelial cells including Paneth cells and monocytes and recognizes a different muramyl (muramyl dipeptide) found in all bacteria (Girardin et al., 2003b). Helicobacter peptidoglycan introduced into gastric epithelial cells by a type IV secretion system (cag pathogenicity island) is recognized by NOD1, inducing a signal cascade ultimately resulting in activation of the important transcription factor NF-κB and the production of key proinflammatory cytokines (Viala et al., 2004). Both NOD1 and NOD2 are up-regulated in the gastric epithelium by H. pylori infection (Rosenstiel et al., 2006).

Few studies have evaluated NOD polymorphisms and Helicobacter-associated disease. Two studies in Caucasian patients found no association between NOD1 polymorphism and gastritis and gastric ulcers, but one did find a significant association between NOD1 G796A and duodenal ulcers (Rosenstiel et al., 2006; Hofner et al., 2007). The NOD2 polymorphism R702W (previously associated with increased risk of Crohn's disease; Cuthbert et al., 2002) has been associated with significantly increased risk of gastric lymphoma (Rosenstiel et al., 2006). No studies have been published from other high infection prevalence regions, so any ethnic effects of NOD1/NOD2 polymorphisms remain to be discovered.

7.2.4 The gastric epithelial mucin, MUC1

Mucins are a family of large complex glycoproteins that play an important role in protecting the mucosal surface against infection (Lindén et al., 2008). While some form gels and are key constituents of mucus, others are expressed by epithelial cells. MUC1, the main mucin expressed on the gastric epithelial surface, is an extremely long molecule, largely due to the presence of a heavily glycosylated, variable number of tandem repeat (VNTR) extracellular region comprising up to 120 repeat units of 60 nucleotides. Owing to its length, MUC1 extends above other cell surface molecules and is therefore most likely the first structure encountered by H. pylori approaching the epithelial surface.

Examination of human MUC1 gene sequences revealed a significant association between the possession of a short MUC1 allele and gastric cancer in Portuguese (Carvalho et al., 1997), H. pylori-induced gastritis in British (Vinall et al., 2002) and H. pylori-induced gastritis and intestinal metaplasia in Colombian (Silva et al., 2003) patients. Moreover, the in vitro binding of H. pylori is significantly decreased to human gastric cell lines with shorter MUC1 alleles (Costa et al., 2008), indicating that MUC1 plays an important role in limiting the attachment of H. pylori to the epithelial surface. MUC1 can limit attachment of H. pylori to the gastric epithelium both by acting as a releasable decoy and by steric hindrance (Lindén et al., 2009). The potential importance of MUC1 in Helicobacter pathogenesis was further demonstrated when infection of Muc1-deficient mice with H. pylori resulted in increased levels of colonization and more severe pathology compared with wild-type controls (McGuckin et al., 2007).

A short allele for MUC1 would translate into a short MUC1 molecule at the epithelial surface, and such a change could readily explain the increased association with H. pylori infection (Fig. 7.3).

7.2.5 The effect of gender on Helicobacter pylori infection and its pathological consequences

It is becoming increasingly recognized that simply being male is a risk factor for H. pylori infection and associated sequelae. In general, males are significantly more likely to develop H. pylori-associated pathologies, including gastric adenocarcinoma (Parkin et al., 2005) and peptic ulcer disease (World Health Organization, 2008). Globally, the burden due to peptic ulcer disease, resulting in loss of life and loss of productivity, is 3.3 million disability-adjusted life years (DALYs) in males, twice that of females at 1.67 million DALYs (World Health Organization, 2008).
Similarly, in 2004, it was estimated that 500 million males died from stomach cancer, compared with 300 million females (World Health Organization, 2008). This difference probably involves the influence of gender biology on *H. pylori* infection and associated pathologies. However, a highly complex interaction of social, economic and environmental gender effects (i.e. differences in smoking, diet, occupations and social interactions) is also likely to play a significant role. There is a surprising dearth of literature investigating the role of gender in susceptibility to *H. pylori*-associated pathologies, with the factors causing this disparate outcome in males versus females remaining poorly understood.

### Sexual dimorphism in *H. pylori* infection and pathology in human subjects

A meta-analysis published in 2006 recognized that several factors hamper our understanding of gender, *H. pylori* infection and disease outcomes (De Martel and Parsonnet, 2006). These include: (i) study populations not being representative of the general population;
failure to adjust for confounding factors such as socio-economic status; and (iii) failure to present gender data in the absence of statistically significant correlations. This meta-analysis of large population-based studies revealed that, on average, after adjusting for age and socio-economic status, men were infected with *H. pylori* 16% more often than women (De Martel and Parsonnet, 2006). As this meta-analysis included only one Chinese study, which found no sexual dimorphism in infection rates, it is difficult to reach a definitive conclusion of whether this is a global phenomenon. Interestingly, a concurrent, but limited analysis of paediatric studies indicated that the male predominance of infection appears to be confined to the adult population (De Martel and Parsonnet, 2006). This latter observation suggests that sex hormones, synthesized in great quantities from the onset of puberty, may contribute to this gender bias.

It is difficult to delineate the contribution of sex-specific factors on *H. pylori* colonization and subsequent development of pathology. Is the increased incidence of peptic ulcer disease and gastric cancer in males due to increased susceptibility to infection, or due to a qualitatively and quantitatively different response to infection? Indeed, sex-specific factors could potentially influence both susceptibility to infection and the response to infection.

While epidemiological studies recognize that gastric cancer is more prevalent in males than females, it is less clear whether gender influences the incidence of peptic ulcers. An investigation of gender, *H. pylori* infection and peptic ulcer disease in 55,000 dyspeptic Chinese patients found that male sex increased the risk of developing gastric and duodenal ulcers independently of *H. pylori* infection (Wu et al., 2008). The authors postulated that this reflects a protective effect of female sex hormones, as the sex difference dropped markedly in groups above 50 years of age, and was absent in the group aged 70 years or more. Broader epidemiological studies may reveal whether this is a global phenomenon. Such studies are, in part, confounded by sociological and environmental factors that contribute to gender effects. For example, the aforementioned Chinese study (Wu et al., 2008) does not adjust for smoking habits, known to also increase the risk of developing gastric cancer; in China, males are 67 times more likely to smoke than females (Pan and Hu, 2008).

**Gender effects observed from animal models**

Examination of gender effects on *H. pylori* infection in controlled animal studies provides an ideal means to investigate the influence of genetic and physiological sex-specific factors independently of socio-economic and environmental factors. While very few studies have used both male and female animals (mainly due to a tendency to only use female mice), experimental infections suggest that sex-specific factors do indeed influence colonization levels and development of pathology, although little is known about why sexual dimorphism exists.

Two studies have identified a gender difference in *H. pylori* colonization levels in mice. Colonization levels have been reported to be higher in males than females in C57BL/6 mice (Raghavan et al., 2002), as well as in BALB/c mice lacking the IL-4Rα cytokine receptor (Aebischer et al., 2001), highlighting a potential interaction between sex-specific factors and the immune response to infection. In contrast, *H. pylori* colonization of B6129SvEv mice was equivalent in males and females (Rogers et al., 2005).

Similarly, few reports have investigated gender and the development of *Helicobacter*-induced gastritis. Infected male mice that over-express gastrin developed more severe inflammation of the gastric corpus (with atrophy) than female mice after 5 months of infection (Fox et al., 2003). In contrast, infection of female C57BL/6 mice with *H. felis* (a relative of *H. pylori* that induces more severe gastritis in mice) induced more severe inflammation, with a marginal increase in colonization, than infection of male mice, but only 1 year post-infection (Court et al., 2003).

When infected with *H. pylori*, Mongolian gerbils can develop gastritis and gastric adenocarcinoma in a fashion akin to *H. pylori*-infected human subjects. Crabtree et al. (2004) found that female Mongolian gerbils develop more severe gastritis after 36 weeks of *H. pylori* infection, accompanied by increased
expression of IFN\(_\gamma\) and IL-12p40, again highlighting a potential interaction of sex-specific factors with the immune response to \textit{H. pylori}. While both male and female gerbils can develop adenocarcinoma (Zheng \textit{et al.}, 2004), it remains unclear whether there is a gender bias in the development of gastric cancer, and further studies are required.

Care must be taken extrapolating gender observations made in animal models to the human situation. With the exception of the hypergastrinaemic mice, more severe pathology is generally observed in female rodents, the reverse of that observed in human subjects. Despite this, animal studies may still represent the best means of investigating the influence of sex-specific factors on \textit{H. pylori} colonization and subsequent development of disease, as they allow for the control of socio-economic and environmental factors.

\textbf{How does gender contribute to \textit{H. pylori} infection and associated disease?}

It is well recognized that women in many parts of the world experience lower socio-economic status (a significant risk factor in \textit{Helicobacter} infection and pathogenesis), often with reduced access to health care, than their male counterparts. It is intriguing, therefore, that males are more prone to infection, peptic ulcer disease and gastric cancer than females (Green, 1992; Fish, 2008), suggesting this gender influence is potentially mediated by physiological factors.

Environmental factors, many of which may exhibit a gender bias, can also impact the outcome of \textit{H. pylori} infection. Smoking, for example, is globally four times more prevalent in males than females (Corrao \textit{et al.}, 2000). One study in Japanese men found that the risk of developing gastric cancer increased if they also smoked, with 50% of gastric cancer cases attributed to a combination of \textit{H. pylori} infection and smoking (Shikata \textit{et al.}, 2008). This increased the risk of developing gastric cancer 11-fold over either \textit{H. pylori} infection or smoking alone. Another Japanese study found that \textit{H. pylori} infection in men increased the risk of developing gastric cancer independently of smoking habits (Yamagata \textit{et al.}, 2000). These studies suggest that, while sex differences in smoking levels may contribute to increased progression to gastric cancer in Japanese males, it is not the only gender factor to play a role.

Evidence is accumulating that gender biology (including physiology and anatomy) has a strong influence on \textit{H. pylori} infection and pathogenesis. The most obvious physiological difference between males and females is the sex hormones. The predominant male hormone, testosterone, and the predominant female hormones, oestrogen and progesterone, can all influence immunity. It has long been recognized that inflammatory responses in men and women are disparate. Broadly speaking, males tend to develop more severe sequelae from infectious diseases, whereas females are more prone to autoimmune diseases (Whitacre, 2001). It is generally thought that females generate more robust cellular and humoral immune responses and thus are more resistant to certain infections than males. Consequently, however, they are more prone to autoimmune disease. The reasons for this are not well understood, but sex hormone receptors are expressed by various immune cells, including CD4\(^+\) and CD8\(^+\) T cells, B cells, natural killer cells and macrophages (Lang, 2004). However, the actions of oestrogen on immune cells are complex and could result from direct interaction with the receptor or indirectly as a result of interaction with other hormones such as androgens, progesterone, prolactin and gonadotrophin-releasing hormone, all of which are thought to possess immunomodulatory functions (Lang, 2004). Thus it is easily conceivable that sex hormones – directly and indirectly – can modify the response to \textit{H. pylori} infection (Sipponen and Correa, 2002). Indeed, considering the widespread expression of sex hormone receptors, sex hormones could alter the function of many cells and tissues that may change resistance to \textit{H. pylori} infection and could influence development of disease.

Another obvious gender difference is that females possess two X chromosomes, while males possess one X and one Y chromosome. Many Y chromosome genes, mostly involved in development of testes, are specific to males, but curiously little is known about them. Apart from the effects of sex hormones on the
immune response and other biological processes, little is known about the specific influence of the sex chromosomes on disease development. One elegant study used transgenic mice that were XY but lacking the testes-determining Sry gene, or XX with the Sry gene inserted on an autosome (Smith-Bouvier et al., 2008). Effectively, this allowed comparison of XX and XY mice on either a female or male hormonal background. They concluded that XX chromosome genes conferred greater susceptibility to experimental autoimmune disease, via a mechanism that was hormone independent. Several genes encoding immunomodulatory factors are present on the X chromosome, including those for CD40 ligand, Foxp3 and TLR-7, all important modulators of the inflammatory response. While dogma dictates that one X chromosome is inactivated, recent evidence suggests that X-inactivation can be incomplete (Carrel and Willard, 2005). Thus, a gene dosage effect of some of these genes could contribute to differential regulation in males and females of the inflammatory response to infections such as H. pylori.

Hence, while epidemiological data clearly show that gender plays an important role in host susceptibility to H. pylori-associated pathologies, our general ignorance of how sex-related factors interact with the host immune system and its response to infectious disease means no study has truly investigated the mechanism(s) behind this sexual dimorphism. Socio-economic and environmental factors certainly contribute to differential regulation in males and females of the inflammatory response to infections such as H. pylori.

7.3 Conclusion

In summary, host genetics clearly play an extremely important role in deciding which H. pylori-infected individuals will progress to develop associated pathologies. Variations in a range of host genes, particularly those that modify the scale of the inflammatory response to Helicobacter infection, can influence disease susceptibility. The overriding message emerging from the numerous studies investigating these genes, however, is that their relative importance can vary greatly between human populations. For example, polymorphisms in IL1B seem important for H. pylori pathogenesis in Caucasian populations, IL8 polymorphisms may be more relevant in Asians, while TNFA may be significant for both.

This variation may be due to a number of factors. First, a relevant gene polymorphism within a particular ethnic group may interact with other gene variants within that population, producing a combination that leads to either increased or decreased susceptibility. Additionally, it is well recognized that virulence factor expression by H. pylori is affected by geography, with some countries such as Japan having a high frequency of more virulent strains of these bacteria. Hence the importance of a host gene polymorphism may be affected by the type of H. pylori strain with which an individual is infected. Most likely it is a combination of both of these, with further contribution from social and environmental factors.

Future studies may help further dissect the complex interactions of host genes in Helicobacter pathogenesis, perhaps by examining ethnic groups that have grown up in different countries. An investigation of polymorphisms in, say, persons of Chinese origin born in Western countries with different social and environmental exposures may be informative. However, it is also recognized that H. pylori infections often pass down from mother to child, meaning infected Chinese in the USA are likely to still carry H. pylori strains of Chinese origin. Hence it is not straightforward to delineate the role of host genetics from bacterial and environmental factors. Despite these problems, such efforts are worthwhile. Currently, we are incapable of predicting the prognosis of H. pylori infection in an individual. Understanding the role of host genetics, within the context of other variables, would be invaluable for explaining the pathogenic process that occurs with H. pylori infection and would potentially provide indicators of disease susceptibility.
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Host Genetic Factors in Susceptibility and Resistance


Host Genetic Factors in Susceptibility and Resistance


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8 Innate Immune Initiators and Effectors in *Helicobacter pylori* Infection

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### 8.1 Introduction

Bacterial pathogens employ different strategies to induce responses in host cells. These strategies typically involve cell invasion, adhesion and/or secretion of bacterial products. During *Helicobacter pylori* infection, most of the bacteria occupy a predominantly extracellular niche within the gastric mucus layer of the human stomach (Chan et al., 1992; Dubois and Boren, 2007) and only 1% of *H. pylori* appear to be interacting directly with epithelial cells (Peterson, 1991; Ko et al., 1999). Despite the location of *H. pylori* within the gastric mucosa, the host develops a robust inflammatory response and a variety of diseases varying in severity (reviewed in Montecucco and Rappuoli, 2001; Peek and Blaser, 2002).

Due to the location of *H. pylori in vivo*, it is likely that interactions of these bacteria with epithelial cells represent a key step in the induction of host immune responses. Epithelial cells are not, however, of lymphoid origin, so how does *H. pylori* initiate an inflammatory response in these cells? Furthermore, what are the host and *H. pylori* signals mediating these responses and how do these products interact with one another to facilitate the development of a chronic inflammatory condition?

In this chapter we discuss the innate immune responses generated by the host in an attempt to control *H. pylori* infection. Specifically, we address the roles of host pathogen-recognition molecules (PRMs) in the identification of *H. pylori* bacteria within the gastric mucosa and the downstream signalling events that these molecules trigger. We also identify some of the key *H. pylori* products that facilitate the initiation of inflammation *in vivo*. Finally, we discuss how these processes result in the production of pro-inflammatory cytokines, ultimately leading to the development of gastritis within the host.

### 8.2 Innate Immune Initiators in Host Immune Responses to *H. pylori*

The last decade has seen a renaissance in the perceived importance of the innate immune system in host defence against microbial infection. Indeed, it is now recognized that the innate immune system is not only important for the initiation of broad host defence responses to microbial pathogens, but also in the development of adaptive immune responses to these microorganisms (Napolitani *et al.*, 2005; Pasare and Medzhitov, 2005; Fritz *et al.*, 2007). The initiation of innate immune responses to microbial pathogens is

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dependent on the recognition of conserved microbial structures or products by host PRMs, which are evolutionarily conserved across the plant and animal kingdoms (Akira et al., 2006). The key PRM signalling pathways for bacteria, in most cases, converge on the transcription factor nuclear factor-κB (NF-κB), a master regulator of genes encoding a broad range of inflammatory and immune products. Indeed, H. pylori is known to induce NF-κB activation both in vitro (Philpott et al., 2002; Viala et al., 2004; Brandt et al., 2005; Hirata et al., 2006a,b; Ferrero et al., 2008) and in vivo (Ferrero et al., 2008; Yanai et al., 2008).

Discussed below are the various PRMs that have been implicated in innate immune recognition of H. pylori, leading to NF-κB activation and the generation of proinflammatory responses to this pathogen.

### 8.2.1 The Toll-like receptor family

The first mammalian PRMs to be identified as playing a role in host defence against bacterial pathogens belonged to the Toll-like receptor (TLR) protein family (Akira et al., 2006). One of these proteins, TLR4, was shown to share structural similarities to the fungal defence protein, TOLL, in Drosophila (Medzhitov et al., 1997). Since the identification of TLR4 as the first known TLR, a total of 13 TLRs have been described, of which ten are expressed in humans (reviewed in Takeda and Akira, 2007; O'Neill, 2008). TLRs are expressed by multiple cells of the innate immune system, such as dendritic cells (DCs), macrophages and non-lymphoid cells such as epithelial cells (Takeda et al., 2003).

TLRs and the interleukin (IL)-1 receptors belong to a superfamily of receptors that shares conserved Toll/IL-1 receptor (TIR) domains that are vital to the transduction of signals in the cell (Medzhitov et al., 1997; O'Neill, 2008). TLRs are expressed by multiple cells of the innate immune system, such as dendritic cells (DCs), macrophages and non-lymphoid cells such as epithelial cells (Takeda et al., 2003).

As depicted in the representative diagram of TLR signalling (Fig. 8.1), microbial products mediate the dimerization of TLR molecules that, in turn, trigger the recruitment of adaptor proteins to the intracellular TIR domains (Rakoff-Nahoum and Medzhitov, 2009). These interactions result in a signalling cascade that facilitates the recruitment of one or more of the following TIR-containing adaptor proteins: myeloid differentiation primary response gene 88 (MyD88); MyD88 adapter-like protein (MAL) (also known as TIR domain-containing adaptor protein, TIRAP); TIR domain-containing adapter-inducing interferon-β (TRIF); or TRIF-related adaptor molecule (TRAM) (Fitzgerald et al., 2001; Kenny and O'Neill, 2008). The type of adaptor and co-adaptor molecules involved will vary depending on the specific TLR pathway that has been activated. For the present purposes, however, we discuss MyD88 and its interacting partners.

The recruitment of MyD88 to the TLR complex leads to the formation of a signalling scaffold involving the adaptor molecules IL-1 receptor-associated kinase-1 (IRAK1) and IRAK4, which interact with MyD88 via homophilic interactions involving death domains. These co-adaptor molecules are subsequently phosphorylated by IRAK4, leading to the activation and autophosphorylation of IRAK1. Both IRAK1 and IRAK4 then dissociate from the MyD88 complex (Brikos et al., 2007) and interact with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This results in TRAF6-mediated activation of the IκB kinase (IKK) complex, as well as phosphorylation of the mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. In turn, the IKK complex becomes phosphorylated, leading to the phosphorylation and degradation of the inhibitor of κB (IκB) proteins, thereby liberating transactivating
NF-κB complexes to translocate into the nucleus. These translocated NF-κB molecules then bind to conserved binding sites within the promoter regions of inflammatory or immune genes, resulting in the up-regulated expression of various proinflammatory mediators, such as the chemokine CXCL8 (or IL-8) (Rakoff-Nahoum and Medzhitov, 2009).
Toll-like receptor expression in epithelial cells within H. pylori-infected tissues

Although there are ten known human TLRs, only TLR2, -4, -5 and -9 are expressed by epithelial cells in the human stomach (Schmausser et al., 2004) and thus may be important for H. pylori recognition in vivo. Within healthy human gastric mucosa, TLR5 and TLR9 are present on the apical and basolateral surfaces; however, within inflamed gastric mucosa they are located only on the basolateral surfaces (Schmausser et al., 2004). Moreover, gastric levels of TLR4 and its co-receptor MD2 are increased in H. pylori-infected tissues (Ishihara et al., 2004; Schmausser et al., 2004).

Many early studies implicated TLR2, -4 and -5 in the recognition of H. pylori. Given the importance of epithelial cells in the host response to H. pylori infection, this discussion primarily focuses on PRM recognition in this cell type. It is important to note, however, that many of the studies in this area have been performed using epithelial cell lines transfected to over-express specific TLR molecules. The findings generated from such studies have been either confirmed or contested by subsequent investigations using established cell lines, primary cells and/or the genetic ‘knockout’ or ‘knockdown’ of specific PRMs in different cell types.

TLR2 and TLR4. TLR2 detects and responds to stimulation with a wide variety of microbial products, including lipoproteins from Gram-negative bacteria, lipoteichoic acid, Gram-positive peptidoglycan and fungal cell wall components (Takeuchi et al., 1999; Akira et al., 2006). In contrast, TLR4 has a very narrow specificity for Gram-negative lipopolysaccharide (LPS) (Poltorak et al., 1998). Nevertheless, some forms of Gram-negative LPS have been reported to signal preferentially via TLR2 (Akira et al., 2006). Indeed, from overexpression studies in TLR2/TLR4-deficient cells and/or gastric epithelial cells constitutively expressing TLR, it was reported that TLR2, and not TLR4, was involved in the recognition of H. pylori LPS (Smith et al., 2003; Lepper et al., 2005; Chaouche-Drider et al., 2009). It was even suggested that H. pylori LPS may act as an antagonist for TLR4 signalling (Lepper et al., 2005). The findings in favour of a role for TLR2 recognition of H. pylori LPS were indirectly supported by the work of Backhed and colleagues. These workers showed that H. pylori could induce CXCL8 responses in primary gastric epithelial cells that do not express TLR4, as well as in several epithelial cell lines that, for the most part, lack the CD14 co-receptor needed for TLR4 signalling (Backhed et al., 2003). It is noteworthy that H. pylori LPS has a 100-fold lower biological activity compared with LPS molecules of other Gram-negative bacteria, such as Escherichia coli or Salmonella enterica (Muotiala et al., 1992; Birkholz et al., 1993). Moreover, it was reported that H. pylori LPS, in contrast to that of E. coli, is poorly bound by LPS-binding protein (Cunningham et al., 1996). This would be expected to have an effect on the transfer of H. pylori LPS to CD14, and thus may explain the low TLR4 agonist activity of H. pylori LPS.

Some authors have nevertheless identified TLR4 as being important for the induction of proinflammatory responses by H. pylori LPS, both in guinea pig gastric pit cells and in transfected epithelial cells (Kawahara et al., 2001; Mandell et al., 2004). For example, human HEK293 epithelial cells, which do not express endogenous TLR2 or TLR4, became responsive to H. pylori LPS when transfected to express TLR4 (Mandell et al., 2004). The divergent results were attributed to the different amounts of LPS used in the experimental work (i.e. nanograms versus micrograms), and the purity of the H. pylori LPS preparations used, which lacked trace amounts of TLR2 agonist (Mandell et al., 2004). Consistent with this suggestion, highly purified preparations of H. pylori LPS induced cytokine responses in macrophages from TLR2-knockout animals, whereas TLR4-deficient cells were non-responsive (Mandell et al., 2004). Although these findings could be interpreted as confirming the role of TLR4 in H. pylori LPS recognition, it is also possible that the data simply reflect the reported differences in LPS responsiveness of macrophages and epithelial cells (Maeda et al., 2001; Su et al., 2003).

If there is still controversy regarding the identity of the specific TLR involved in the
recognition of *H. pylori* LPS, the situation for TLR recognition of live *H. pylori* bacteria seems slightly clearer with multiple reports describing a role for TLR2. For example, human HEK293 epithelial cells, stably transfected to express functional TLR2, recognized intact *H. pylori* whereas TLR4-transfected cells were non-responsive (Mandell et al., 2004; Torok et al., 2005). These findings highlight the difference in results one can obtain when studying *H. pylori* LPS-induced responses using purified *H. pylori* LPS compared with viable *H. pylori* bacteria. Using TLR neutralizing antibodies, it was also found that TLR2 in concert with TLR9 mediated prostaglandin E2 release and angiogenic responses by gastric epithelial cells in response to *H. pylori* stimulation (Chang et al., 2005). These findings were supported by experiments in which macrophages from TLR2-deficient mice were found to be non-responsive to stimulation with *Helicobacter* (Mandell et al., 2004; Obonyo et al., 2007). In contrast, macrophages from both wild-type and TLR4-deficient mice responded to *H. pylori* by the production of IL-6 and CCL2 (or monocyte chemotactic protein-1, MCP-1) (Mandell et al., 2004). Interestingly, however, another group reported that TLR4-deficient macrophages exhibited reduced responses to stimulation with *H. pylori* (Obonyo et al., 2007). The inter-study differences in the findings may be attributed to the origin of the macrophages used (peritoneal versus bone marrow derived, respectively) as well as the cytokines measured (IL-6 and MCP-1 versus IL-10 and IL-12, respectively) (Mandell et al., 2004; Obonyo et al., 2007).

Further evidence for the proposed role of TLR2 as the dominant TLR required for *H. pylori* recognition arises from mouse vaccination studies, which showed a significant reduction in the bacterial loads of TLR2-deficient mice vaccinated against *H. pylori* when compared with wild-type animals (Panthel et al., 2003). To our knowledge, similar animal studies have yet to be conducted with genetically constructed TLR4-deficient mice.

The bacterial components responsible for the effects of whole live *H. pylori* on TLR2 signalling have yet to be identified. Nevertheless, one potential candidate is the *H. pylori* neutrophil-activating protein (HP-NAP; see discussion in Section 8.3.1). HP-NAP is capable of stimulating neutrophils and monocytes to enhance their production of IL-12, thereby facilitating the differentiation of naive T-helper (Th) cells into the Th1 phenotype (D’Elios et al., 2007) (Fig. 8.2). Moreover, stimulation of TLR2-transfected HEK293 cells with HP-NAP activated NF-κB in a dose-dependent manner, whereas NF-κB activation was absent in HEK293 cells not expressing TLR2 (Amedei et al., 2006).

Despite the conflicting data regarding the role of TLR4 in epithelial and macrophage responses to *H. pylori* stimulation, there is evidence that TLR4 may play a partial role in initiating DCs to produce cytokines in response to *H. pylori* (Rad et al., 2009). Indeed, while *H. pylori* stimulation of bone marrow-derived DCs from wild-type mice resulted in the induction of IL-6, stimulation of DCs from both TLR2- or TLR4-deficient mice had a reduced level of IL-6 production, which was significantly reduced in TLR2-deficient mice (Rad et al., 2009). These findings suggest that TLR2, and to a much lower extent TLR4, are the predominant surface TLRs involved in the response of DCs to *H. pylori* lysates (Rad et al., 2009). Furthermore, two separate studies examining detailed immune responses to *H. pylori*, by murine chromosome mapping or microarray analysis, implicated the involvement of additional PRMs, including TLR9 (Anderson et al., 2007; Rad et al., 2009) and retinoic acid-inducible gene I (RIG-I), which recognize unmethylated CpG DNA and RNA, respectively (Rad et al., 2009).

TLR5. Several reports have identified the ability of bacterial flagellin to function as a major agonist of proinflammatory responses in human epithelial cells (Gewirtz et al., 2001a,b; Zeng et al., 2003). Bacterial flagellin is specifically recognized by TLR5 (Hayashi et al., 2001). Given that *H. pylori* expresses four to six polar, sheathed flagella (Eaton et al., 1996), it seemed reasonable to assume that TLR5 may play an important role in *H. pylori* recognition by epithelial cells. Indeed, two reports described the ability of TLR5 to recognize *H. pylori* flagellin and to initiate signalling in HEK293 cells over-expressing TLR5 (Smith et al., 2003; Torok et al., 2005). Other workers,
however, subsequently demonstrated that flagellin-responsive epithelial cell lines did not detect *H. pylori* flagellin, suggesting that this component of the bacterium has very low immunostimulatory ability and may thus be able to evade TLR5 recognition (Lee et al., 2003; Gewirtz et al., 2004). Furthermore, non-flagellated *H. pylori* mutant bacteria retained most, if not all, of their ability to induce CXCL8 production in flagellin-responsive cell lines (Lee et al., 2003; Gewirtz et al., 2004). It is now established that *H. pylori* flagellin molecules cannot be sensed by TLR5, because these molecules lack the conserved amino acids required for full TLR5 agonist activity (Andersen-Nissen et al., 2005). Interestingly,
enteric Helicobacter species (Chaouche-Drider et al., 2009), as well as species within the closely related Campylobacter genus (Andersen-Nissen et al., 2005), also seem to have evolved flagellins that are able to escape TLR5 detection.

### 8.2.2.2 The nucleotide-binding oligomerization domain-like receptor family

In addition to TLRs, host cells may also express one or more types of cytosolic PRMs. One of these protein families, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, has been shown to contain members that play very important roles in host defence against microbial pathogens, as well as in the recognition of ‘danger signals’ from within eukaryotic cells (reviewed in Ferrero, 2005; Fritz et al., 2006; Kaparakis et al., 2007).

#### Nucleotide-binding oligomerization domain proteins

The archetypal NLR, NOD1, is specifically involved in host defence against Gram-negative bacterial infection (Viala et al., 2004). NOD1 is comprised of three domains: a central nucleotide-binding oligomerization domain (NOD), a C-terminal LRR domain and a caspase-activated recruitment domain (CARD). The LRR domain in NOD1 is required for the sensing of Gram-negative peptidoglycan, whereas NOD2 is a sensor of all forms of bacterial peptidoglycan (reviewed in Ferrero, 2005; Fritz et al., 2006; Kaparakis et al., 2007). The NOD component of these proteins is important for protein self-oligomerization (Inohara and Nunez, 2003; Fritz et al., 2006), whereas the CARD facilitates the interaction of NOD1 with downstream signalling molecules through homophilic and heterophilic protein interactions (Inohara and Nunez, 2003). NOD1 has one CARD, whereas NOD2 has two of these regions in its C terminus. NOD1 is predominantly contained within epithelial cells and, to a lesser extent, within lamina propria monocytes, whereas NOD2 seems to be more abundant in cells of the monocyte/macrophage lineage (Bertin et al., 1999; Ogura et al., 2001; Hisamatsu et al., 2003).

Human NOD1 senses a specific structure that is most commonly found in Gram-negative bacterial peptidoglycan (Chamaillard et al., 2003; Girardin et al., 2003). This muropeptide structure is composed of a disaccharide moiety, N-acetyl glucosamine-N-acetyl muramic acid (GlcNAc-MurNAc), linked to a tripeptide of which the terminal amino acid is meso-diaminopimelate (mDAP) (Chamaillard et al., 2003; Girardin et al., 2003). Interestingly, human NOD1 was shown to specifically recognize this so-called GM-TriDAP structure, whereas murine NOD1 is most responsive to a tetrapeptide muropeptide containing l-alanine-d-glutamate-mDAP-d-alanine (GM-TetraDAP) (Magalhaes et al., 2005).

Although *H. pylori* is primarily an extracellular pathogen, it can activate NOD1 signalling via its type IV secretion system (T4SS), encoded by the cytotoxin-associated gene (cag) pathogenicity island (cagPAI) (Viala et al., 2004) (see Backert et al., Chapter 11, this volume). Specifically, *H. pylori* activates NOD1 by transfer of its peptidoglycan into the cytoplasm of non-phagocytic epithelial cells via a cagPAI-dependent mechanism (Viala et al., 2004). As in other models, *H. pylori*-mediated NOD1 signalling results in the induction of NF-kB activation and CXCL8 secretion in vitro. Interestingly, cagPAI-positive *H. pylori* strains are associated with higher levels of CXCL8 expression in vivo than cagPAI-negative strains (Crabtree et al., 1995; Peek et al., 1995), suggesting that NOD1 may play an important role in the development of the more severe immunopathology associated with cagPAI-positive strains (see the more detailed discussion below). Indeed, Western blot analyses of inflamed gastric tissues obtained from *H. pylori*-positive patients revealed that NOD1 synthesis was up-regulated in vivo during inflammatory conditions (Rosenstiel et al., 2006). Similarly, NOD2 protein levels were also increased in these tissues; moreover, NOD2 was reported to mediate responses to cagPAI-positive *H. pylori* in NOD2-transfected epithelial cells (Rosenstiel et al., 2006). The importance of NOD2 for *H. pylori* infection, however, awaits further investigation.
contrast, animal studies showed that Nod1-deficient mice were more susceptible than wild-type animals to infection by H. pylori strains harbouring a functional T4SS, but not to isogenic T4SS mutant bacteria (Viala et al., 2004). This suggests a role for NOD1 in host defence to infection by cagPAI-positive H. pylori bacteria.

NOD1 sensing of peptidoglycan initiates a signalling cascade that ultimately results in the up-regulation of NF-κB and the production of proinflammatory cytokines, including CXCL8 and its murine homologue, CXCL2 (or macrophage inflammatory protein-2, MIP-2) (Girardin et al., 2003; Viala et al., 2004). NOD1 activation was demonstrated to mediate the recruitment and oligomerization of the RIP-like interacting CLARP kinase (RICK) (Inohara et al., 2000; Girardin et al., 2001; Hasegawa et al., 2008), a member of the receptor-interacting protein kinase family (Meylan and Tschopp, 2005). RICK is also known as receptor-interacting protein (RIP)-2 or CARD-containing ICE-associated kinase (CARDIAK) (Thome et al., 1998). RICK was shown to interact with NOD1 (or NOD2) via its CARD region, resulting in the formation of a transient complex comprising NOD1 (or NOD2), RICK and the IKK complex (Inohara et al., 2000; Girardin et al., 2001; Hasegawa et al., 2008) (Fig. 8.1). Ultimately, this complex initiates a signalling cascade that results in the phosphorylation and degradation of the IKK complex. As for TLR signalling, the activation of the IKK complex promotes the degradation of the IκB proteins, which enables the dissociation of the NF-κB complex, consisting of p65 and p50 subunits. Moreover, the downstream proinflammatory responses of this signalling pathway, as measured by CXCL8 production, were found to be enhanced by a concomitant up-regulation of AP-1 (Allison et al., 2009).

As predicted by the model of NOD1 signalling presented above, H. pylori-mediated activation of this pathway results in the translocation to the nucleus of the transactivating NF-κB complex, consisting of p65 and p50 subunits. Therefore, the downstream proinflammatory responses of this signalling pathway, as measured by CXCL8 production, were found to be enhanced by a concomitant up-regulation of AP-1 (Allison et al., 2009). These data highlight the fact that although different infectious agents may activate the same PRM signalling cascades, the mechanisms by which they do so are likely to vary, as are the downstream effector responses regulated by these cascades. Despite these differences, many of the PRM signalling pathways merge to NF-κB. This transcription factor typically targets the genes encoding proinflammatory cytokines and chemokines (e.g. CXCL8, CXCL5 and CCL20), which function to recruit neutrophils and DCs (Kim et al., 2001; Tomimori et al., 2007) (Fig. 8.2).

In addition, PRMs may regulate the expression of various antimicrobial peptides. Indeed, NOD1 was shown to be essential for the up-regulated expression in epithelial cells of the gene (DEFB4) encoding the antimicrobial peptide, human β-defensin-2 (HBD-2), by cagPAI-positive H. pylori strains (Boughan et al., 2006; Grubman et al., 2010). In contrast, a NOD1/cagPAI-independent
mechanism was involved in *H. pylori* upregulated expression of *DEFB3*, which encodes another major member of the HBD family, HBD-3 (Boughan et al., 2006; Grubman et al., 2010). A more detailed discussion of these and other antimicrobial factors is provided in Section 8.4.2. Significantly, culture supernatants from *cag*PAI-positive *H. pylori*-stimulated AGS control cells, but not those in which NOD1 was stably ‘knocked down’ by siRNA, significantly reduced *H. pylori* numbers in a salt- and cathepsin L-dependent manner, suggestive of a role for HBD-2 in bacterial killing (Grubman et al., 2010). This finding was confirmed by the pre-treatment of *H. pylori*-stimulated cells with siRNA directed against the *DEFB4* gene (Grubman et al., 2010). Taken together, it appears that NOD1 may have a dual role in host defences against bacterial pathogens: first by inducing chemokine production that results in the recruitment of proinflammatory cells to the site of infection; and second by initiating the synthesis of antimicrobial peptides with direct activity against these microorganisms.

Although *H. pylori* is a potent activator of NOD1 signalling, it is also able to avoid detection by this PRM, and thus probably also NOD1-mediated clearance by the host. *H. pylori* can evade NOD1 detection through the actions of an amidase, AmiA, which mediates modifications of cell wall peptidoglycan; these modifications are characterized by a decrease in the quantities of GM-TriDAP and the concomitant accumulation of an N-acetyl-d-glucosaminyl-(1,4)-N-acetylMuramyl-L-Ala-d-Glu structure (Chaput et al., 2006). As *H. pylori* AmiA is also essential for the morphological transition of *H. pylori* from the rod to coccoid form, the coccoid forms are no longer detected by human NOD1 and are incapable of inducing NF-κB activation or CXCL8 secretion by gastric epithelial cells (Chaput et al., 2006). These findings are consistent with work in other infection models describing the ability of different bacterial pathogens to avoid detection by NOD1 or NOD2 through changes in their peptidoglycan structures (Lenz et al., 2003; Boneca et al., 2007; Wolfert et al., 2007).

### 8.2.3 NACHT-LRR-PYD-containing proteins

The NACHT-LRR-PYD-containing proteins (NALPs) represent another important group of proteins belonging to the NLR family. The name of these proteins derives from their conserved three-domain structure comprising: a central domain NACHT (an acronym for NAIP (neuronal apoptosis inhibitor protein), Ç2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from *Podospora anserine*) and TlP1 (telomerase-associated protein)); a C-terminal LRR region; and an N-terminal pyrin domain (PYD) (Martinon et al., 2002). NALPs are activated by microbial products including toxins (Gurcel et al., 2006) and a variety of danger-associated molecular patterns (Martinon et al., 2002; Fritz et al., 2006). Proteins of the NLR family regulate the activation of caspase-1 (Martinon and Tschopp, 2004), which is responsible for the maturation of proinflammatory cytokines. Both IL-1β and IL-18, which play key roles in host defence against infection, are synthesized as inactive cytoplasmic precursors and are proteolytically cleaved by caspase-1 to produce the biologically active mature forms (Thornberry et al., 1992).

Upon activation by their ligand, NALPs associate via their PYD domains with apoptosis-associated speck-like protein (ASC). ASC subsequently interacts with the CARD of procaspase-1, leading to the formation of a complex called the ‘inflammasome’ and the cleaving of IL-1β and IL-18 (Martinon et al., 2002, 2004). Although the role of NALPs in innate immune responses to *H. pylori* have yet to be explored in detail, it was found that ASC-deficient mice challenged with *H. pylori* exhibited higher bacterial loads and significantly lower levels of gastritis when compared with wild-type animals (Benoit et al., 2009). Moreover, ASC-deficient mice were incapable of producing any IL-1β or IL-18, and produced substantially less interferon-γ (IFNγ) in response to *H. pylori* infection compared with wild-type animals (Benoit et al., 2009). It is therefore possible that ASC functions to up-regulate IL-1β and IL-18 production, thereby enhancing the development of IFNγ-
dependent inflammatory responses and the reduction of gastric *H. pylori* loads in the host. These findings suggest that the inflammasome may thus play a role in host immune responses to *H. pylori*. Further studies are, however, required to confirm this possibility.

### 8.2.3 The impact of polymorphisms in genes encoding pathogen-recognition molecules on *H. pylori* disease

Given the importance of PRMs in host recognition and responses to pathogens, several studies have investigated whether PRM gene polymorphisms may contribute to the varying disease outcomes associated with *H. pylori* infection. Although the findings of such studies have thus far been largely inconclusive, there does seem to be a general trend between gene polymorphisms in certain TLR signalling molecules and the development of more severe gastritis.

A −159C/T promoter polymorphism in the gene encoding CD14 was associated with an increased risk of precancerous lesions in *H. pylori*-infected Caucasians (Hold *et al.*, 2009). A similar association with precancerous outcomes was found for a −1237T/C polymorphism in TLR9 (Hold *et al.*, 2009). However, no such associations were found between either of these gene polymorphisms and gastric adenocarcinoma risk in Caucasians (Hold *et al.*, 2009). Similarly, no association could be found between the −159C/T CD14 gene polymorphism and the risk for gastric carcinoma, or mucosa-associated lymphoid tissue (MALT) lymphoma, in a Taiwanese Chinese population (Wu *et al.*, 2006). Interestingly, however, a −196 to −174 deletion in the TLR2 promoter region was more frequently found in Japanese patients with gastric cancer (Tahara *et al.*, 2007, 2008). Moreover, the TLR2 −196 to −174 deletion allele also correlated with an increased risk of intestinal metaplasia, a precursor of gastric adenocarcinoma, but only in individuals over 60 years of age (Tahara *et al.*, 2008). On the other hand, no association was found between this polymorphism and the severity of neutrophil or mononuclear cell infiltrates (Tahara *et al.*, 2008). In contrast, Achyut and colleagues (2007) were not only able to show an association between a TLR4 gene polymorphism (T399I) and an increased risk for precancerous lesions among *H. pylori*-infected individuals from an Indian population, but also found a correlation with plasma cell infiltration, atrophy and intestinal metaplasia. Neither the T399I TLR4 polymorphism nor another common polymorphism (A299G) was found to increase the risk of duodenal ulcer (Hofner *et al.*, 2007). Similarly, no association was found between several NOD1 or NOD2 gene polymorphisms in *H. pylori*-related gastric ulcer, although a polymorphism in the NOD2 gene was associated with an increased risk of developing MALT lymphoma (Rosenstiel *et al.*, 2006). Taken together, the data suggest that several PRM gene polymorphisms may favour the development of precancerous lesions in *H. pylori*-infected subjects. Nevertheless, it is unlikely that a single polymorphism will be linked to gastric carcinogenesis risk (see Sutton *et al.*, Chapter 7, this volume). This probably reflects the multifactorial determinants (i.e. host, bacterial and environmental) that influence *H. pylori* disease severity (Peek and Blaser, 2002).

### 8.2.4 The role of triggering receptor expressed on myeloid cells in innate host responses to *H. pylori*

Receptor proteins belonging to the new family of molecules known as triggering receptor expressed on myeloid cells (TREM) have been reported to have diverse functions, including a role in innate immunity and host defence against bacterial infection (Sharif and Knapp, 2008). One of these proteins, TREM-1, was shown to be expressed on the surface of neutrophils, monocytes and macrophages (Sharif and Knapp, 2008). TREM-1 initiates a signal upon association with DAP12, an immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane adaptor protein (Bouchon *et al.*, 2000; Bleharski *et al.*, 2003). To date, the natural ligand that activates TREM-1 is unknown; however, stimulation of TREM-1 in neutrophils and monocytes leads to the secretion of CXCL8 and
TNFα (Bouchon et al., 2000). TREM-1 is expressed on gastric epithelium in vitro and in vivo in response to H. pylori stimulation (Schmausser et al., 2008). Interestingly, in vitro stimulation of gastric epithelial cells with CXCL8 or LPS alone could not up-regulate TREM-1; however, TREM-1 could be up-regulated by CagA-positive H. pylori (Schmausser et al., 2008). Furthermore, stimulation of gastric epithelial cells with an anti-TREM-1 antibody resulted in the up-regulation of CXCL8, suggesting that TREM-1 may amplify inflammation by up-regulating CXCL8 (Schmausser et al., 2008). Further investigations are required to identify the H. pylori factor that is responsible for TREM-1-induced chemokine production, as well as the role that TREM-1 plays in the innate immune response to H. pylori infection.

8.3 Bacterial Danger Signals that Initiate Innate Immune Responses in Host Cells

8.3.1 H. pylori neutrophil-activating protein

The influx of neutrophils into the gastric mucosa is characteristic of an acute H. pylori infection (Craig et al., 1992; Crabtree, 1996). During infection, H. pylori produces the 150 kDa protein HP-NAP, which can adhere to surrounding H. pylori facilitating their adherence to host epithelial cells (Namavar et al., 1998). However, HP-NAP also has a pro-inflammatory function as it can rapidly cross the endothelia and promote the adhesion of neutrophils to endothelial cells, facilitating the transendothelial migration of these cells into infected tissues (Polenghi et al., 2007). In addition, HP-NAP is able to further enhance the generation of gastritis by up-regulating the expression of β2-integin on the surface of endothelial cells, thereby promoting neutrophil migration (Montecucco and de Bernard, 2003), and by activating neutrophils to produce reactive oxygen radicals and chemokines such as CXCL8, CCL3 (or macrophage inflammatory protein-1α, MIP-1α) and CCL4 (or MIP-1β) (Wang et al., 2006; Polenghi et al., 2007). These factors are likely to contribute to the further recruitment of neutrophils, monocytes, DCs and lymphocytes to the gastric mucosa. The production of HP-NAP also appears to facilitate the development of a consistent maturation of DCs, as indicated by the elevated surface expression of the major histocompatibility complex (MHC) class II and the co-stimulatory molecules CD80 and CD86 (Amedei et al., 2006). Finally, HP-NAP enhances the development of inflammation by inducing the production of IL-12 and IL-23 by human monocytes, DCs and neutrophils, therefore promoting the development of the Th1 proinflammatory immune response (Amedei et al., 2006) that is typical of an H. pylori infection (Fig. 8.2).

8.3.2 Heat-shock protein 60

H. pylori heat-shock protein 60 (HSP60), a homologue of the E. coli GroEL chaperone, is an immunodominant antigen of H. pylori (Macchia et al., 1993; Suerbaum et al., 1994; Ferrero et al., 1995). This protein is secreted by H. pylori in vivo (Dunn et al., 1997) and is able to induce IL-6 production in macrophages (Gobert et al., 2004). HSP60 was identified by the screening of soluble H. pylori fractions, which had been isolated by ion exchange and size exclusion chromatography, for their ability to induce IL-6 secretion in RAW 264.7 macrophages via a TLR2- and MyD88-independent mechanism (Gobert et al., 2004). In contrast, TLR2/4 and MyD88 were reported to be important in CXCL8 responses to HSP60 in Kato III cells, a gastric epithelial cell line (Takenaka et al., 2004).

8.3.3 Cytotoxin-associated gene A protein

Cytotoxin-associated gene A protein (CagA), which is encoded by the cagPAI, is an important virulence determinant of H. pylori (Peek and Blaser, 2002). The ability of CagA to induce cytoskeletal changes and to cause damage to the gastric epithelial cell barrier is discussed elsewhere (see Backert et al.,
Chapter 11, this volume). Nevertheless, it should be noted that CagA also has a role in inducing CXCL8 production by epithelial cells (Brandt et al., 2005; Kim et al., 2006). Specifically, it was reported that the T4SS-dependent delivery of CagA into epithelial cells, or via the process of transfection, induced MAPK signalling and the secretion of CXCL8 (Brandt et al., 2005; Kim et al., 2006; Kwok et al., 2007). These responses occur after prolonged exposure of cells to CagA (i.e. at 36–48 h post-exposure) and appear to be induced via a MAPK-dependent but NOD1-independent mechanism (Brandt et al., 2005).

8.4 Innate Immune Effector Responses to H. pylori Infection

A variety of antimicrobial factors are produced in a rapid manner by host epithelial cells in response to a bacterial stimulus, thus functioning as a first line of defence against microbial pathogens. The various factors that are specifically produced by host epithelial cells in response to H. pylori infection are discussed below.

8.4.1 Lactoferrin

One of the earliest reported antimicrobials identified as being induced in response to H. pylori infection was lactoferrin. This 80 kDa iron-binding glycoprotein can be detected in most biological fluids of mammals (Lonnerdal and Iyer, 1995). Lactoferrin is synthesized by the cardiac and pyloric glandular epithelial cells of the stomach (Luqmani et al., 1991) and by innate immune cells such as neutrophils (Lonnerdal and Iyer, 1995), which secrete lactoferrin from secondary granules (Baggioni et al., 1970; Leffell and Spitznagel, 1972). Although lactoferrin is constitutively produced, its concentration can be elevated within secretions at sites of infection and inflammation in vivo. Therefore, lactoferrins are considered to be an important component of the host defence mechanism due to their function as anti-inflammatory and immunomodulatory molecules (Arnold et al., 1977; Ellison et al., 1988; Yamauchi et al., 1993).

In addition, lactoferrin can inhibit the growth of several human bacterial pathogens (Arnold et al., 1977), including H. pylori (Miehlke et al., 1996). There are two mechanisms whereby lactoferrin functions as an antimicrobial agent in vivo. First, due to its high iron-binding capacity, lactoferrin can deplete the bacterial environment of free iron, hence hindering growth of the organism (Weinberg, 1984). Second, the N terminus of lactoferrin has a domain, distinct from its iron-binding site, that functions as an antimicrobial against Gram-positive and Gram-negative bacteria (Bellamy et al., 1992). At physiological concentrations, lactoferrin directly damages the outer membrane permeability of Gram-negative bacteria by releasing LPS (Ellison et al., 1988). However, due to its iron-binding abilities, it had been suggested that lactoferrin may serve as a mechanism utilized by H. pylori to acquire iron from the environment and to stimulate bacterial growth (Husson et al., 1993). Research has identified that H. pylori growth in an iron-restricted environment induced the production of lactoferrin-binding proteins, which were able to bind human lactoferrin (Dhaenens et al., 1997). Hence, H. pylori may be increasing its chance of survival within a challenging environment by taking advantage of host innate immune peptides so as to enhance its growth in vivo. Nevertheless, given the antimicrobial activity of lactoferrin, it has been suggested that this protein may represent a host defence strategy against H. pylori infection (Miehlke et al., 1996; Dial et al., 1998). Indeed, examination of gastric biopsies revealed that the gastric concentrations of lactoferrin were significantly higher in individuals with H. pylori infection and were elevated in a gastritis-dependent manner (Nakao et al., 1997a,b).

The ability of lactoferrin to function as an antimicrobial against H. pylori has resulted in numerous efforts by researchers to utilize lactoferrins as a treatment for H. pylori-infected individuals, although the findings from such trials have thus far been inconclusive (Dial et al., 2000; Di Mario et al., 2003; Huynh et al., 2009). As many of the studies on gastric lactoferrin responses preceded the discovery of PRMs, it is not clear which, if
any, of these innate immune molecules regulate the production of this antimicrobial factor during *H. pylori* infection.

### 8.4.2 Defensins

More recently, a second group of antimicrobial peptides, termed defensins, have been studied in relation to *H. pylori* infection. Defensins can be divided into two main classes, α and β, according to their genetic and structural features (reviewed in Ganz, 2003). Microarray studies of *H. pylori*-infected human subjects and animals have reported *H. pylori*-mediated modulation of defensin gene expression (Walduck et al., 2004; Resnick et al., 2006; Hornsby et al., 2008).

#### α-Defensins

The α-defensins are produced and released by neutrophils, granulocytes and Paneth cells. These antimicrobial peptides may play a role in the local host response to *H. pylori* infection (Kocsis et al., 2009). The α-defensins include the human neutrophil peptides (HNPs), which are the major components of neutrophil-derived α-defensins. Studies have revealed significantly increased levels of HNP-1, -2 and -3 in the plasma and gastric juice of *H. pylori*-infected patients, which decline after eradication of *H. pylori* (Isomoto et al., 2004; Nishi et al., 2005), indicating that α-defensin production is part of the response to *H. pylori* infection.

**Human β-defensins**

Human β-defensins (HBDs) are cationic, cysteine-rich and disulfide-linked low-molecular-weight peptides with immunomodulatory properties required for host defence from bacterial pathogens (Ganz, 2003). While these peptides are endogenously produced by epithelial cells, their expression can be up-regulated during infection (Harder et al., 2000; O’Neill et al., 2000; Krisanaprakornkit et al., 2002). To date, six members of the HBD family have been identified (Zhao et al., 1996; Harder et al., 1997; Garcia et al., 2001a,b; Yamaguchi et al., 2002). As discussed below, the expression levels of genes encoding several of these defensins have been shown to increase during *H. pylori* infection.

**HUMAN β-DEFENSIN-1.** HBD-1 is constitutively expressed in several tissues and by various epithelial cells during normal homeostasis in *vivo*, including the gastric epithelium (Zhao et al., 1996; Valore et al., 1998). HBD-1 has antimicrobial activity against both Gram-positive and Gram-negative bacteria, and various isoforms of HBD-1 exist, although the individual role of each isoform is unclear (Valore et al., 1998). Unlike the other HBDs, HBD-1 is not significantly up-regulated during *H. pylori* infection in *vivo* (Bajaj-Elliott et al., 2002; Grubman et al., 2010). Hence, HBD-1 is not thought to play a key role as an antimicrobial during *H. pylori* infection.

**HUMAN β-DEFENSIN-2.** Basal HBD-2 expression is detected in the skin, lung, trachea, urogenital and gastrointestinal tracts of man and its expression is strongly regulated (Harder et al., 1997). Most significantly, this β-defensin has been shown to have a role in defending the host against Gram-negative bacterial and fungal infections at mucosal surfaces (Schroder and Harder, 1999). Indeed, recombinant HBD-2 is able to potently kill *H. pylori* in a dose- and salt-dependent manner (Hamanaka et al., 2001; Uehara et al., 2003). Several studies investigating the antimicrobial activity of recombinant HBD-2 have shown that complete inhibition of *H. pylori* growth is possible with as little HBD-2 as 70 µg/ml or 10⁻⁵ M (Hamanaka et al., 2001; George et al., 2003). Moreover, as discussed above, our own work has shown that HBD-2, which was released into the culture supernatants of gastric epithelial cells stimulated with *cag*PAI-positive *H. pylori*, is effective in killing *H. pylori* in *vitro* (Grubman et al., 2010). Initial studies showed that direct contact of *cag*PAI-positive *H. pylori* bacteria with gastric epithelial cells was essential for HBD-2 activation in response to this pathogen (Wada et al., 1999). However, paired isogenic parental and *cag*PAI mutant strains were not used, thus some form of bystander effect could not be excluded. It
was subsequently shown that expression of the HBD-2 gene, DEFB4, by epithelial cells was dependent on both the presence in H. pylori bacteria of a functional T4SS as well as the actions of NOD1 (Boughan et al., 2006; Grubman et al., 2010).

DEFB4 expression can be induced by treatment of cells with TNFα, IL-1 or by exposure to microorganisms such as H. pylori (Wada et al., 1999; O’Neil et al., 2000; Hamanaka et al., 2001). The HBD-2 promoter contains three binding sites for NF-κB and one binding site for AP-1 (Wada et al., 2001), indicating that stimulation of the NF-κB or MAPK pathway should result in the up-regulation of HBD-2. Given that H. pylori activates both pathways in a T4SS- and NOD1-dependent manner (Allison et al., 2009), it is therefore logical that this same mechanism should be implicated in the induction of HBD-2 expression by cagPAI-positive H. pylori bacteria.

HUMAN β-DEFENSIN-3. HBD-3 has been reported to be the β-defensin with the most potent activity against H. pylori. The heightened potency of this peptide has been attributed to the fact that it exists as a dimer, whereas all other HBDs exist as monomers (George et al., 2003). In contrast to other HBDs, HBD-3 possesses salt-insensitive antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria (Harder et al., 2001; Wu et al., 2003). HBD-3 can reduce the number of viable H. pylori by 95% at a concentration of 7 μg/ml (George et al., 2003) and can render H. pylori completely non-viable in as little as 1 h (Uehara et al., 2003). Experimental evidence has shown that maximal levels of HBD-3 in response to H. pylori infection were generated 12 h post-stimulation. This is different from HBD-2, which reaches maximal levels at 6–8 h post-infection (Bajaj-Elliott et al., 2002). Thus, it is possible that HBD-3 may have a more delayed effect during the acute phase of infection.

Similar to HBD-2, the expression levels of the gene encoding HBD-3, DEFB3, increased in response to H. pylori infection in gastric mucosal tissues and in gastric cancer cell lines (George et al., 2003; Kawauchi et al., 2006). This increase in DEFB3 expression may be linked to the HBD-3 promoter containing AP-1 and IFNγ binding sites (Jia et al., 2001). Nevertheless, both a functional T4SS and NOD1 seemed to be dispensable for the up-regulation of HBD-3 expression in gastric epithelial cell lines (Boughan et al., 2006; Grubman et al., 2010). H. pylori-mediated DEFB3 expression was found to be dependent on epidermal growth factor receptor (EGFR)-mediated ERK activation (Boughan et al., 2006).

HUMAN β-DEFENSIN-4. HBD-4 has antimicrobial activity against Gram-positive and Gram-negative bacteria (Garcia et al., 2001b). Interestingly, in contrast to other HBDs, the expression of the HBD-4 gene, DEFB104A, in gastric epithelial cells is not significantly affected by stimulation with proinflammatory cytokines, such as IL-1α, IL-6, INFγ or TNFα (Otte et al., 2009). Instead, it appears that p38 MAPK has a role in DEFB104A expression (Otte et al., 2009). Similar to other HBD genes, DEFB104A is expressed in the gastric mucosa during H. pylori infection, and HBD-4 levels are greater in patients infected with CagA-positive H. pylori strains compared with those colonized with CagA-negative bacteria (Otte et al., 2009). Nevertheless, the mechanism whereby CagA may regulate HBD-4 expression during H. pylori infection, including the PRM involved, has yet to be identified.

Cathelicidin LL-37

The cathelicidins comprise another group of mammalian cationic antibacterial proteins. To date, only a single cathelicidin has been identified in man: the human cationic antibacterial protein (hCAP18), which is an 18 kDa protein whose C-terminal 37-amino-acid peptide is termed LL-37 (Gennaro and Zanetti, 2000; Lehrer and Ganz, 2002). The antimicrobial function of LL-37 is based on its amphipathic α-helical structure, which is thought to integrate within or deform the membrane of bacteria, resulting in cell death (Oren et al., 1999). LL-37 has been shown to have bactericidal activity against Gram-positive and Gram-negative bacteria (Bals et
al., 1998; Travis et al., 2000). In concordance with other human antimicrobial peptides, LL-37 has LPS-binding and -neutralizing properties that function as a chemoattractant for monocytes, neutrophils and CD4+ T cells (Kirikae et al., 1998).

LL37 mRNA has recently been identified to be constitutively expressed on the surface and upper crypts of the gastric and intestinal epithelium (Hase et al., 2002, 2003). Moreover, using human gastric epithelial cells as a model, it was shown that epithelial LL-37 production was up-regulated in response to H. pylori infection via a cagPAI-dependent mechanism, but not to proinflammatory cytokines, nor to other activators of NF-κB. Surprisingly, a synergistic effect was observed for LL-37 and HBD-1 in H. pylori killing (Hase et al., 2003).

8.4.3 Inflammatory cells

H. pylori infection and its recognition by various PRMs results in elevated levels of proinflammatory chemokines that are produced by epithelial cells at the site of infection. The production of these chemokines facilitates the second stage in the innate immune response, which involves the recruitment of innate immune cells to the site of infection and the development of acute inflammation (Fig. 8.2). Neutrophils are recruited to the stomach, as are macrophages and DCs, by proinflammatory cytokines that are fundamental to the genesis of adaptive immune responses to H. pylori. In fact, the recruitment of DCs to the gastric mucosa occurs within 6 h post-infection, whereas after 5 days, these cells have migrated out of the tissue, possibly to draining lymph nodes (Kao et al., 2006). Thus, innate immune cells, such as DCs, can activate Th CD4+ cells and initiate the development of the adaptive immune response. For further detail regarding the H. pylori-specific adaptive immune response, see Robinson and Atherton, Chapter 6, this volume.

Interestingly, it was shown that NOD1 within epithelial cells regulates the development of an adaptive immune response to both inert agonists and infectious agents; of particular note, NOD1 was shown to be required for the development of the characteristic Th1 responses to cagPAI-positive H. pylori bacteria in mice (Fritz et al., 2007). In addition, Rad and colleagues (2007) reported that MyD88−/− mice challenged with H. pylori displayed a defect in the development of immunoglobulin (Ig)G2c/IgG1 antibody responses, indicative of a Th1 response. The mechanisms whereby PRMs regulate the development of Th responses to H. pylori infection have, however, yet to be fully characterized.

Macrophages, in unison with DCs, are responsible for the production of IL-12 and the recruitment of Th cells to the gastric mucosa (Haerle et al., 1997; Meyer et al., 2000) (Fig. 8.2). Macrophages further amplify the inflammatory response by their production of cytokines such as IL-1, TNFα and IL-6 in response to H. pylori antigens (Mai et al., 1991; Harris et al., 1998; Gobert et al., 2004). IL-6 production by macrophages is linked to responses initiated by TLR4, MAPK and NF-κB (Pathak et al., 2006), and is likely to facilitate the development of IFNγ-producing T cells (Meyer et al., 2000). The requirement for macrophages to initiate acute inflammation has been demonstrated in vivo (Kaparakis et al., 2008; Yanai et al., 2008). Transient macrophage depletion of H. pylori-infected mice or Mongolian gerbils by chlodronate treatment significantly reduced the gastric pathology, thus confirming the role of macrophages in the initiation of pathology. In addition, DCs were shown to produce IL-6, CXCL8, IL-10, IL-12, IL-1β and TNFα when treated with H. pylori or their products (Kranzer et al., 2004, 2005; Kao et al., 2006). The presence of a cagPAI was not required for the induction of cytokines responses in DCs (Kranzer et al., 2005; Kao et al., 2006). Importantly, MyD88-deficient DCs were found to be affected in their abilities to secrete proinflammatory cytokines and to up-regulate MHC and co-stimulatory molecules, suggesting that TLR recognition of H. pylori may be important for DC activation (Rad et al., 2007).

Consistent with these in vitro data, in vivo studies showed that MyD88−/− mice developed lower levels of gastric inflammation and increased bacterial loads in response to H. pylori challenge (Rad et al., 2007).
8.5 Conclusions

*Helicobacter pylori* causes chronic inflammation within the host, resulting in disease outcomes of varying severity. This pathogen produces several products or factors that have the potential to be recognized by host PRMs. Consistent with this observation, multiple PRMs have been implicated in innate immune responses to *H. pylori*. We propose that these PRMs are likely to act in a cell type-specific manner with epithelial cells relying primarily on NOD1 and possibly TLR2. It now seems clear, however, that epithelial cells do not use TLR5 to detect *H. pylori*, nor is it likely that TLR4 plays a significant role in epithelial cell responses to the bacterium under physiological conditions.

In contrast to the restricted PRM repertoire involved in epithelial cell responses to *H. pylori*, immune cells utilize multiple PRMs, with macrophages and DCs shown to respond to *H. pylori* via TLR2/4-dependent mechanisms and even TLR-independent pathways (Blehar et al., 2003; Gobert et al., 2004; Mandell et al., 2004; Rad et al., 2007, 2009). Whether there is any cross-talk or synergism between PRM signalling in different cell types remains to be determined. In any case, PRM ‘sensing’ of *H. pylori* within gastric tissues is clearly likely to play a significant role in the generation of proinflammatory cytokines by host cells. These cytokines enable inflammatory cells to be recruited to the site of infection, where they mature and progress throughout the periphery and secondary lymphoid tissues, thereby mediating the development of adaptive immune responses. We propose that the innate immune system, via its effects on these responses, is likely to influence the severity of disease outcomes induced by *H. pylori* infection.

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9 Helicobacter pylori Vaccines

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9.1 Introduction

Helicobacter pylori has been estimated to infect one-half of the world’s population. These infections are not readily cleared by host inflammatory and immune responses and may persist for life. While the majority of infected individuals do not exhibit overt signs of severe disease, 10–20% are at high risk of developing peptic ulcers or gastric cancers. Combined antimicrobial and anti-acid therapy can eradicate H. pylori infections, but not without significant side-effects or risks for the development of antibiotic-resistant strains (see Mégraud, Chapter 4, this volume). The infection is also so widespread that antimicrobial treatment is an impractical approach for controlling H. pylori-associated disease. In addition, animal model studies and anecdotal reports for human infections have shown that antimicrobial ‘cure’ of H. pylori infection does not prevent re-infection. For these reasons, vaccination has been suggested as an alternative approach for the control of H. pylori-associated disease. In addition, animal model studies and anecdotal reports for human infections have shown that the incidence of gastric cancer without having to identify specific subjects harbouring H. pylori infections (Rupnow et al., 2001).

9.2 Candidate Antigens for Immunization Against H. pylori

Small animal models, particularly the mouse, have been used extensively to identify both candidate vaccine antigens and adjuvant/delivery systems for an H. pylori vaccine (Nedrud, 2001). The initial studies demonstrating the efficacy of a mucosal vaccine against Helicobacter infection in mice were performed with whole-cell preparations or crude whole-cell lysates (Chen et al., 1993; Czinn et al., 1993). These preparations have continued to be used by several laboratories for the study of the protective immune response. However, many laboratories soon began investigating specific purified and recombinant proteins, with less potential for undesirable side-effects, as candidates for subunit vaccines in mice. These antigens are involved in virulence, adhesion and normal bacterial activity in the gastric mucosa and some of the proteins tested for efficacy in mice include urease subunits (Lee et al., 1995), cytotoxic-associated gene A protein (CagA).

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and vacuolating cytotoxin (VacA) (Ghiara et al., 1997), catalase (Radcliff et al., 1997), heat-shock protein A (GroES) (Ferrero et al., 1995), *H. pylori* adhesin A (Sutton et al., 2007) and neutrophil-activating protein (NAP) (Satin et al., 2000), as well as others. The rationale for selecting specific proteins includes blocking adhesion, preventing an important activity or neutralizing a virulence mechanism.

There is some debate on the merit of using certain proteins as antigens. Urease, for instance, despite its size and complexity, is not immunodominant in many infected subjects where it has been shown to induce only a weak antibody response (Leal-Herrera et al., 1999). CagA, one of the most immunogenic factors in the infected host, is not present in all strains of *H. pylori*. Three observations seem salient, however, when considering the construction of an effective subunit vaccine.

First, all of these proteins must induce strong immune responses when delivered as vaccine antigens in the context of an appropriate adjuvant, regardless of the immunogenicity displayed during chronic infection.

Secondly, almost every *H. pylori* protein that has been tested in mice in a challenge model against *H. pylori* or *Helicobacter felis* infection has been, in general, as efficacious as the others. Although biopharmaceutical companies may have utilized the published sequences of the *H. pylori* genome to evaluate vaccine candidates that subsequently proved to have poor efficacy, there does not seem to be a shortage of prospective antigens. The variety of candidate vaccine antigens is particularly instructive when considering antigens such as catalase (Radcliff et al., 1997) that are not present on the cell surface. The role of antibodies in limiting *H. pylori* infection or in contributing to vaccine-induced immunity remains controversial but, as discussed below, mice deficient in antibody production can be protectively immunized against *Helicobacter* infection (Ermak et al., 1998; Blanchard et al., 1999a; Gottwein et al., 2001; Akhiani et al., 2004). These observations, combined with a growing literature that reports bacterial loads inversely proportional to the degree of inflammation in the gastric mucosa (Garhart et al., 2002), suggest that a cell-mediated, proinflammatory response can be important for bacterial eradication. Thus, using effective adjuvants or delivery systems (see below) that can become re-stimulated upon challenge with an infectious organism to promote a protective gastritis may be one mechanism for protective immunity.

As the final effect of such an inflammatory response on the bacterium is non-specific, the location of those antigens on the bacterium becomes less important.

Finally, there is evidence that multiple antigens may be necessary for the development of a truly protective vaccine against *H. pylori*. This concept was first promoted by Ferrero et al. (1995) who noted that studies by other investigators with urease were yielding protection in approximately 80% of immunized mice. To address this problem, in the *H. felis* model, they performed oral immunizations with a combination of recombinant *H. pylori* heat-shock protein A (HspA) and urease in mice. The dual antigen immunization induced 100% protection from challenge with *H. felis*, whereas either component given individually induced only 80% protection. It is worth noting that the studies in non-human primates and human subjects described below, in which urease has been used either in its purified or expressed form, have been disappointing in terms of both efficacy and immunogenicity. Murine studies often report ‘protection’ as a significant reduction in bacterial load. Conversely, some studies in mice in which whole-cell lysates continue to be used as the immunogen do sometimes achieve complete protection as determined by the inability to grow *H. pylori* from gastric biopsies (Garhart et al., 2002). Additionally, a multivalent subunit vaccine that incorporates VacA, CagA and NAP, currently in development for use in humans, was shown to be protective in a therapeutic study using beagles as measured by immunohistochemical detection of *H. pylori* in histological sections (Rossi et al., 2004). Another multivalent vaccine consisting of urease B subunit (UreB), HspA and the surface-localized protein HpaA, delivered either orally with a mutant heat-labile toxin or parenterally with alum, induced greater protection against *H. pylori* in Mongolian gerbils than did mono- or bivalent vaccines (Wu et al., 2008).
9.3 Vaccine Adjuvants/Delivery Systems and Routes of Immunization

Because of the small size, low price and easy manipulation of mice, *H. felis* and then *H. pylori* mouse models became the models of choice for early stage *H. pylori* vaccine studies in the 1990s. Thus the first successful demonstrations of *Helicobacter* vaccine feasibility used these models, plus the germ-free piglet. Formalin-killed *H. pylori* delivered subcutaneously in incomplete Freund’s adjuvant to germ-free pigs (Eaton and Krakowka, 1992), live *H. felis* delivered intraperitoneally to mice (Chen et al., 1993) and *H. felis* lysate with cholera toxin (CT) adjuvant delivered by oral gavage (Chen et al., 1993; Czinn et al., 1993) were all able to reduce *Helicobacter* colonization in these first trials. Oral vaccination of mice then dominated subsequent vaccine trials in the 1990s as many investigators moved away from bacterial lysate and killed whole-cell vaccines towards purified and recombinant antigens (detailed above). Although CT and the closely related heat-labile toxin of *Escherichia coli* (LT) were then, and remain today, the ‘gold standards’ of mucosal adjuvants, it was recognized early on that CT and LT use in human subjects would not be practical because of the watery diarrhoea they induce. Similar to its use as a primary screen for vaccine antigens, the mouse has also been used to screen alternative adjuvants/delivery systems and routes of immunization for *H. pylori* vaccines. Table 9.1 lists published results of mouse immunization experiments using various adjuvants/delivery systems and routes of vaccination where a challenge versus *H. felis* or *H. pylori* was reported. Not included in the table are results where immunogenicity was evaluated in the absence of a bacterial challenge. The balance of this section highlights some of the results from the table. Many of the results obtained in the studies from the pre-clinical mouse models summarized here subsequently made their way into human and non-human primate vaccine trials, which are described in Section 9.6.

9.3.1 CT- and LT-based adjuvants

Because *H. pylori* is a gastrointestinal pathogen, most of the earliest vaccine studies involved oral immunization and the well-established mucosal adjuvants CT and LT. CT and LT are composed of non-toxic pentameric B (binding subunits), non-covalently linked to an enzymatically active A subunit that mediates toxicity (Spangler, 1992). The B subunit binds to ubiquitous gangliosides that are widely expressed on a variety of mammalian cell surfaces. The A subunit has been determined to be essential for intestinal adjuvanticity, as the use of recombinant B subunit was shown to lack oral adjuvant activity (Blanchard et al., 1998), although chemical coupling of an *H. pylori* antigen to an undefined B subunit was reported to yield a protective oral vaccine (Ruiz-Bustos et al., 2000), as was the parenteral delivery (avoids gut toxicity) of an LT or recombinant LTB subunit-adjuvanted *H. pylori* vaccine (Weltzin et al., 2000). Another approach to reduce the toxicity of CT- or LT-based adjuvants is to mutate the A subunit active site. This approach has been evaluated against several pathogenic microorganisms, including *H. pylori* (Ghiara et al., 1997; Marchetti et al., 1998; Cardenas-Freytag et al., 1999). Yet a third approach to eliminate the toxicity of CT/LT has been to replace the B subunit, which targets many cell types including intestinal epithelium, with another targeting motif. When the CTB subunit was replaced by two copies of the protein A immunoglobulin-binding domain (‘D’) it yields an adjuvant named CTA1-DD that was determined to be non-toxic and demonstrated to possess murine intranasal adjuvant activity that induced a reduction in bacterial loads when mice were challenged with *H. pylori* (Akhiani et al., 2006). This novel approach retains the enzymatically active portion of CT, which has been shown to be required for optimal adjuvant function, but eliminates toxicity by targeting this activity towards cells of the immune system rather than the epithelium.
Table 9.1. Adjuvant/delivery system and route of vaccination comparison for *Helicobacter pylori* vaccines in rodents.<sup>a</sup>

<table>
<thead>
<tr>
<th>Adjuvant/delivery system&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Route&lt;sup&gt;c&lt;/sup&gt;</th>
<th>*H. pylori antigen&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Protection&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>IN</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt; S</td>
<td>Garhart et al. (2002)</td>
</tr>
<tr>
<td>pCTB</td>
<td>Oral</td>
<td>Lysate</td>
<td>*H. felis&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Lee and Chen (1994)</td>
</tr>
<tr>
<td>rCTB</td>
<td>Oral</td>
<td>Lysate</td>
<td>*H. felis&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Blanchard et al. (1998)</td>
</tr>
<tr>
<td>CTB-couple</td>
<td>Oral</td>
<td>HSBP</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ruiz-Bustos et al. (2000)</td>
</tr>
<tr>
<td>LT</td>
<td>Oral</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Weltzin et al. (2000)</td>
</tr>
<tr>
<td>LTB</td>
<td>Oral</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>LT, LTB, No adjuvant</td>
<td>Subcutaneous</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Kleanthous et al. (2001)</td>
</tr>
<tr>
<td>LT</td>
<td>Oral, IN, rectal</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>Oral</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Michetti et al. (1994); Lee et al. (1995)</td>
</tr>
<tr>
<td>LTK63</td>
<td>Oral</td>
<td>CagA, VacA, urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ghia et al. (1997); Marchetti et al. (1998)</td>
</tr>
<tr>
<td>CTA1-DD</td>
<td>IN</td>
<td>Urease</td>
<td>*H. felis&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Weltzin et al. (1997)</td>
</tr>
<tr>
<td>Cpg</td>
<td>IN</td>
<td>Urease</td>
<td>*H. felis&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cpg</td>
<td>Oral</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Shi et al. (2005)</td>
</tr>
<tr>
<td>Cpg</td>
<td>Subcutaneous</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Sommer et al. (2004)</td>
</tr>
<tr>
<td>Cpg+CT</td>
<td>IN</td>
<td>Lysate</td>
<td>*H. felis&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Jianget al. (2003)</td>
</tr>
<tr>
<td>Cpg</td>
<td>Oral</td>
<td>No antigen</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Raghavan et al. (2003)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Oral</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Xie et al. (2007)</td>
</tr>
<tr>
<td>ISCOM™, ISCOMATRIX™&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Oral, IN, subcutaneous</td>
<td>Lysate, catalase</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Skene et al. (2008)</td>
</tr>
<tr>
<td>Mannan</td>
<td>Oral, IN, IP</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Skene (2009)</td>
</tr>
<tr>
<td>No adjuvant</td>
<td>Subcutaneous</td>
<td>Lysate, formalin whole cell</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Harbour et al. (2008)</td>
</tr>
<tr>
<td>No adjuvant</td>
<td>IP</td>
<td>Live <em>H. felis</em></td>
<td>*H. felis&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Chen et al. (1993)</td>
</tr>
<tr>
<td>LT</td>
<td>Oral</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Guy et al. (1998)</td>
</tr>
<tr>
<td>Saponin, Bay</td>
<td>Subcutaneous</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ermak et al. (1998)</td>
</tr>
<tr>
<td>LT</td>
<td>Oral</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ermak et al. (1998)</td>
</tr>
<tr>
<td>Alum, Bay</td>
<td>Subcutaneous</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>NO=alum (1&lt;sup&gt;st&lt;/sup&gt;–2&lt;sup&gt;nd&lt;/sup&gt;)</td>
<td>IN, subcutaneous</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>NO=Bay (1&lt;sup&gt;st&lt;/sup&gt;–2&lt;sup&gt;nd&lt;/sup&gt;)</td>
<td>IN, subcutaneous</td>
<td>Urease</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Alum</td>
<td>IP</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Gottwein et al. (2001); Eisenberg et al. (2003)</td>
</tr>
<tr>
<td>IFA</td>
<td>Subcutaneous</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CFA</td>
<td>IP, subcutaneous</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Alum, CFA</td>
<td>IP</td>
<td>Lysate</td>
<td>*H. felis&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Chionh et al. (2009)</td>
</tr>
<tr>
<td>M-cell targeting</td>
<td>Oral</td>
<td>Formalin whole cell</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Bacterial ghost</td>
<td>Oral</td>
<td>Lysate</td>
<td>*H. pylori&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Panthel et al. (2003b)</td>
</tr>
<tr>
<td>Bacterial ghost+CT</td>
<td>Oral</td>
<td>Bacterial ghost</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CT/outer membrane vesicles</td>
<td>Oral</td>
<td>Bacterial ghost</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Keenan et al. (2000)</td>
</tr>
<tr>
<td>Polio replicon</td>
<td>Oral</td>
<td>UreB</td>
<td>*H. pylori&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Smythies et al. (2005)</td>
</tr>
</tbody>
</table>
9.3.2 Other adjuvants

In parallel with advances in the adjuvant field in general, a number of additional adjuvants and combinations of adjuvants have been tested for their capacity to enhance *H. pylori*/*H. felis* immunogenicity and to improve vaccine performance in challenge experiments (Table 9.1). Included are CpG oligonucleotides, biodegradable microspheres, saponins, chitosan, muramyl dipeptide (MDP), and more established adjuvants such as alum, Freund’s complete and incomplete adjuvants, and no adjuvant at all. One popular approach has been the use of immunostimulatory CpG oligonucleotides, Toll-like receptor-9 agonists which have been used both parenterally and mucosally and are adept at stimulation of
T-helper 1 type (Th1) immune responses (Krieg, 2006). Because Th1 immunity appears to be important in *H. pylori* immunity (see Section 9.4 on mechanisms of vaccine-induced *H. pylori* immunity), several laboratories have investigated CpGs for *Helicobacter* vaccines (Table 9.1). In particular, when mice were immunized intranasally with *H. felis* lysate and a combination of CT and immunostimulatory CpGs, sterilizing immunity to *H. felis* was achieved (Jiang *et al.*, 2003). Another recent approach has been the use of ISCOMSTM (immune-stimulating complexes), which are saponin–cholesterol–phospholipid cage-like structures with incorporated antigen (Sjolander *et al.*, 1998). Empty cage-like structures (ISCOMATRIXSTM) can be mixed with antigen and also have an adjuvant function. These adjuvants are able to induce both antibody and cell-mediated immune responses (Sjolander *et al.*, 1998). When delivered intranasally or subcutaneously along with recombinant antigens (catalase or HpaA), they induced protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity equivalent to CT (Skene *et al.*, 2008).

### 9.3.3 Vector vaccines and DNA vaccines

Another popular approach for vaccination is to use a live attenuated vector or a DNA vaccine. Several laboratories have evaluated attenuated *Salmonella* bacteria as a vector for oral, intranasal or mucosal-prime, parenteral-boost vaccination protocols. While these studies have enjoyed moderately good success in the mouse (Table 9.1), recombinant *Salmonella* vaccines for *H. pylori* have been disappointing in human trials (see Section 9.6). Polio virus replicons that lack viral RNA but can carry heterologous epitopes have also been used successfully in a mouse *H. pylori* vaccine study (Smythies *et al.*, 2005). Plasmid DNA encoding vaccine antigens can be delivered either as naked DNA or by a vector such as *Salmonella*. Several DNA vaccines for *H. pylori* have been evaluated in mouse models and, in one trial, DNA genomic libraries were used to immunize mice and achieved up to 90% sterilizing immunity (Dzwonek *et al.*, 2004).

#### 9.3.4 Immunization routes

**Mucosal immunization**

*H. pylori* infection is recognized as a relatively non-invasive colonizer of the gastric mucosa and, as such, vaccine strategies have largely mimicked those employed for other enteric and mucosal bacterial infections. Immunizations therefore have predominantly been applied to optimize induction of immune responses at mucosal surfaces including oral/gastric, intranasal and rectal administrations, in combination with mucosal adjuvants or delivery vehicles. The earliest studies concentrated on oral immunization using CT or LT adjuvants. However, successful intranasal and rectal immunization has been reported by several groups (Weltzin *et al.*, 1997; Kleanthous *et al.*, 2001; Garhart *et al.*, 2003; Jiang *et al.*, 2003). Garhart and colleagues showed that intranasal immunization was much more effective than oral immunization and in fact could lead to undetectable levels of *H. pylori* in immunized mice (Garhart *et al.*, 2002). This may actually represent ‘sterilizing immunity’, which has been infrequently reported for *H. pylori* vaccines. A close examination of the literature reveals, however, several other instances where both intranasal and other mucosal vaccinations have yielded sterilizing immunity against *H. pylori*. One such case involved an *H. pylori* strain that colonized mice only at very low levels and rectal immunization with a recombinant antigen plus LT (Kleanthous *et al.*, 2001). An oral sonicate plus CT immunization study in gerbils also reported sterilizing immunity, but the magnitude of infection in unimmunized animals was not reported (Jeremy *et al.*, 2006). A small number of mice immunized orally with *H. pylori* bacterial ghosts also
appeared to achieve sterilizing immunity (Panthel et al., 2003b). Finally, as mentioned above, *H. pylori* DNA genomic library vaccines and intranasal lysozyme immunization with a combination of CT and CpG adjuvants also achieved sterilizing immunity in mice (Jiang et al., 2003; Dzwonek et al., 2004).

Thus, although many of the published *H. pylori* vaccination trials listed in Table 9.1 have reported ‘protection’ as a reduction in gastric *H. pylori* bacterial load of only ten- to 100-fold (sometimes even less) when compared with non-immunized challenged animals, it has been possible to achieve sterilizing immunity with several different types of immunization.

**Parenteral immunization**

Although major efforts have been expended in the development of mucosal *H. pylori* vaccines, there is also considerable evidence that *H. pylori* infection induces inflammation that recruits lymphocytes from the circulation. Thus, immunity to *H. pylori* is not, strictly speaking, limited to effector mechanisms of the mucosal immune system. The induction of protective immunity therefore may not require mucosal immunizations and may be possible through more traditional parenteral vaccination strategies. In retrospect, one of the very first mouse studies in which oral immunization was demonstrated to provide protective immunity to *H. felis* also provided evidence that parenteral immunization might serve as an alternative strategy for *Helicobacter* immunotherapy (Chen et al., 1993). Whereas the authors focused on oral immunization, five weekly doses of live *H. felis* into the peritoneum induced protection in 55% of challenged mice. Several additional studies in mice have confirmed the potential of parenteral immunization (Table 9.1).

For example, Ermak et al. (1998) demonstrated that subcutaneous immunization of mice with urease antigen in combination with alum induced significant protection as measured by urease activity in gastric biopsies, although protection was much reduced when determined by bacterial load. Guy et al. (1998) also employed subcutaneous immunization using several different experimental adjuvants, including a saponin derivative, a glyco-lipopeptide, a lipid-based formulation and a phosphopolymer. Each of these formulations induced reductions in bacterial load compared with non-immune controls, although only the saponin derivative and glyco-lipopeptide achieved significant protection. Weltzin et al. (2000) tested the mucosal adjuvant LT for its potential to work as an adjuvant when administered with urease subcutaneously, and were able to achieve protection that was equivalent to mice immunized with the previously established protocol for oral immunization. Our own group has also investigated the efficacy of systemic immunization in the mouse model, choosing aluminium hydroxide (alum) as the candidate adjuvant, as this adjuvant is the only one licensed for use in the USA. We demonstrated that a single injection of bacterial lysate plus alum was sufficient to induce protective immunity equivalent to oral vaccination when challenging with *H. pylori* (Gottwein et al., 2001). We then demonstrated that this immunization strategy could induce similar immunity when administered to neonatal mice less than 24 h old, thereby demonstrating the potential use of systemic immunization against *H. pylori* in children (Eisenberg et al., 2003). Parenteral vaccines for *H. pylori* immunity have been tested in non-human primates and more recently in early human clinical trials (see Section 9.6.2).

**9.4 What are the Mechanisms of Vaccine-induced *H. pylori* Immunity?**

**9.4.1 The role of antibodies**

*H. pylori* is generally assumed to be primarily an extracellular bacterium that should be susceptible to antibody effector mechanisms. This fact, along with observations in early human studies that gastric secretions from *H. pylori*-positive patients contained anti-*H. pylori* antibodies and antibody-coated *H. pylori* (Rathbone et al., 1986; Wyatt et al., 1986), as well as the fact that antibodies could be detected in gastric secretions of orally immunized animals (Czinn and Nedrud, 1991; Lee et al., 1995; Ferrero et al., 1997), led to the
hypothesis that anti-*Helicobacter* antibodies were responsible for vaccine-mediated protective immunity. Further support for antibodies came from passive oral monoclonal immunization studies and a ‘backpack tumour’ passive monoclonal antibody model, which reduced *H. felis/H. pylori* colonization in infected mice (Czinn *et al*., 1993; Blanchard *et al*., 1995; Keenan *et al*., 2000). In a similar manner, therapeutic administration of bovine colostrum preparations from *H. pylori*-immunized cattle, or a monoclonal antibody recognizing an *H. pylori* urease fragment, reduced colonization levels in *H. pylori*-infected adult mice (Casswall *et al*., 2002; Marnila *et al*., 2003; Hifumi *et al*., 2008). There is also considerable evidence that human breast milk can contain anti-*H. pylori* antibodies and that breastfeeding can delay or reduce the incidence of *H. pylori* infections in infants. This was first reported for Gambian subjects (Thomas *et al*., 1993, 2004) and the topic has recently been extensively reviewed (Chak *et al*., 2009). Combined, all of these studies are highly suggestive that anti-*H. pylori* antibodies can therapeutically reduce *H. pylori* infections and by inference could be an effector mechanism in prophylactic vaccination as well.

In contrast to the positive evidence for antibody-mediated vaccine protection from *H. pylori* infection, there is considerable evidence that prophylactic immunization does not require antibodies. First, we and others have observed that ‘protection’ does not always correlate with the magnitude of the anti-*Helicobacter* antibody response. A striking example of this was provided by Weltzin and colleagues in the late 1990s (Weltzin *et al*., 1997). In that study, mice were intranasally immunized with recombinant *H. pylori* urease 15 times in the absence of adjuvant, or four times in the presence of CT adjuvant, over 3 weeks. Mice immunized in the absence of adjuvant achieved equivalent or even higher levels of serum immunoglobulin (Ig) G and mucosal IgA anti-*H. pylori* urease antibodies than did mice immunized in the presence of adjuvant. When challenged with *H. felis*, however, only one of ten mice immunized in the absence of adjuvant was protected from infection, while nine of ten mice immunized in the presence of CT were protected. In addition, by 10 weeks post-challenge, non-immunized challenged mice also achieved equivalent levels of antibody yet remained infected (Weltzin *et al*., 1997). It is of course possible that immunization in the presence of a suitable adjuvant alters the specificity, affinity, capacity to agglutinate bacteria, or another critical characteristic of the induced antibody response. Indeed, we observed alterations in the specificity of the anti-*Helicobacter* antibody response in protectively immunized mice relative to infected but non-immunized mice and in a patient who spontaneously cleared his infection (Blanchard *et al*., 1999b,c) and recent evidence suggests that agglutinating antibodies may be protective (Skene, 2009).

To more directly address the role of antibodies in vaccine-mediated protection from *Helicobacter* infections, we and others utilized both IgA-deficient and pan-antibody-deficient µMT mice. In either oral or parenteral prophylactic immunization against both *H. pylori* and *H. felis* (Ermak *et al*., 1998; Blanchard *et al*., 1999a; Gottwein *et al*., 2001; Akhiani *et al*., 2004) or therapeutic oral immunization against *H. pylori* (Sutton *et al*., 2000), antibody-deficient mice were uniformly as well protected against *Helicobacter* infections as were wild-type animals. Moreover, protection could not be transferred by passive administration of serum antibodies from successfully immunized mice (Ermak *et al*., 1998). In addition, although not a vaccine study, human patients with IgA deficiency did not exhibit any apparent increase in *H. pylori* infection, based on seropositivity (Bogstedt *et al*., 1996). It has even been suggested that the presence of antibodies may facilitate the persistence of *H. pylori* in the stomachs of wild-type or interleukin (IL)-10-deficient mice (Akhiani *et al*., 2004, 2005). This conclusion was reached after observing that antibody-deficient mice had approximately 300-fold fewer bacteria colonizing their gastric mucosa than did wild-type animals, 8 weeks after infection with *H. pylori*. This reduction in bacterial load was accompanied by an increased gastritis score and an increased number of infiltrating CD4+ T cells (Akhiani *et al*., 2004). In a similar manner, IgA−/− × IL-10−/− double knockout mice displayed enhanced gastritis, enhanced gastric CD4+ T cell levels and reduced bacterial load.
(Akhiani et al., 2005) compared with IL-10−/− single knockout mice. Thus not only were antibodies not needed for prophylactic vaccine protection, but their presence may have actually impeded clearance of *H. pylori* in non-immunized mice.

Overall, the evidence suggests that antibodies can influence immunity to *H. pylori* in either a positive or a negative way, but that antibodies are not required for vaccine-mediated immunity to *H. pylori*. These conclusions should be tempered, however, by the fact that they are largely drawn from experiments in mice.

### 9.4.2 *H. pylori* vaccines and cell-mediated immunity

The absence of a requirement for antibody in vaccine-mediated protective immunity to *H. pylori* implicates some type of cell-mediated immunity. This was first suggested when adoptively transferred spleen cells from orally immunized mice, or an *H. felis*-specific CD4+ T-cell line, reduced the bacterial burden in naïve recipient mice challenged with *H. felis* (Mohammadi et al., 1997). A role for CD4+ T cells in mediating vaccine-mediated immunity was confirmed in experiments in which vaccinated major histocompatibility complex class II knockout mice (CD4+ T-cell-deficient) were not protected from *H. pylori* infection (Ermak et al., 1998; Pappo et al., 1999).

**Post-immunization gastritis**

Enhanced gastritis has also been frequently observed after *H. pylori* challenge of prophylactically immunized mice. This enhanced inflammation, termed ‘post-immunization gastritis’, was observed as early as 1994 by Michetti and colleagues, who protectively immunized mice with an *H. pylori* lysate or urease plus CT (Michetti et al., 1994). In a year-long kinetic study in mice, we observed that prophylactic immunization of mice did not yield immediate protection from infection, but rather clearance of gastric *H. pylori* began a few days after bacterial challenge and was coincident with the development of post-immunization gastritis (Garhart et al., 2002).

Enhancement of gastritis by vaccination against an organism where inflammation represents a major component of the disease is a significant worry. However, Garhart et al. (2002) showed that post-immunization gastritis can resolve over time to background or to levels no higher than observed in infected mice. It is worth noting that the uninfected stomach of both mice and man, unlike the lower gastrointestinal tract, generally lacks organized and diffuse mucosal lymphoid tissue or even white blood cells in general. Thus it may be that for bacterial clearance to occur, some level of white blood cell recruitment (inflammation) must occur and would naturally remain present until the bacteria are eradicated.

Post-immunization gastritis has been observed so frequently in mouse immunization experiments that it has become the accepted norm and experiments without post-immunization gastritis are considered exceptions. Two such exceptions have been reported recently. In the first, Mongolian gerbils were orally immunized three times with bacterial lysate plus CT before challenge with a homologous strain of *H. pylori*. Six weeks later bacterial load and gastric inflammation were measured. No *H. pylori* could be detected in the majority of immunized challenged gerbils and no post-immunization gastritis was observed (Jeremy et al., 2006). This is an exciting result that adds credence to the concept of *H. pylori* vaccination. A kinetic study of bacterial clearance and gastritis would be interesting in this model to see if any parallels with mouse studies exist, or to determine if perhaps some post-immunization gastritis does occur but resolves more quickly than in the mouse. The second exception occurred when BALB/c mice were orally immunized with killed whole bacteria targeted to M cells with a lectin, inducing protection against *H. pylori* challenge, without any gastritis (Chionh et al., 2009). Although BALB/c mice exhibit low levels of gastritis in response to *H. pylori* and *H. felis* infections (Mohammadi et al., 1996; Sakagami et al., 1996), this seems an unlikely reason for the absence of post-immunization gastritis in this experiment, as other laboratories have observed post-immunization gastritis in...
BALB/c mice (Michetti et al., 1994; J.G. Nedrud et al., unpublished). Perhaps alternative mechanisms of protective immunity such as the induction of non-inflammatory agglutinating antibodies (Skene, 2009) could explain these results. In spite of these exceptions, it does seem clear that, in many cases of prophylactic immunization, clearance of *H. pylori* from mice is associated with enhanced, but often transient, gastritis.

**Th1 immune responses**

Humans and animals both develop a Th1, interferon-γ (IFNγ)-producing T-cell predominant response to *H. pylori* infections (Svennerholm and Lundgren, 2007), and it is generally accepted that Th1 responses are most important for *Helicobacter* immunity (Taylor et al., 2008). The role of the signature Th1 cytokine, IFNγ, remains controversial. At least two laboratories have reported that IFNγ is critical for protective immunity in mice (Akhiani et al., 2002; Sayi et al., 2009), while other two laboratories have found it to be less important (Sawai et al., 1999; Garhart et al., 2003). It is not easy to resolve these contradictory results, although numerous laboratory to laboratory differences exist, including the antigens and adjuvants used, the route of immunization, assay time points and the end point used to define protective immunity.

One area on which most laboratories agree is the critical importance of IL-12p40 in anti-*Helicobacter* immunity. IL-12p40-deficient mice failed to achieve protective immune responses after either oral or intranasal immunization with *H. pylori* lysate plus CT adjuvant (Akhiani et al., 2002; Garhart et al., 2003). Because the p40 subunit is shared by IL-12 and IL-23, however, defective protective immunity in these animals could be due to the lack of IL-12, the lack of IL-23, or both. Panthel et al. (2003a) tested IL-12p35 null mice and observed mixed results depending on the genetic background of the mouse strain. IL-12-deficient p35<sup>−/−</sup> mice on the C57BL/6 background were only weakly colonized and exhibited a poor vaccine response, while p35<sup>−/−</sup> mice on the BALB/c background were colonized at 500% of wild-type BALB/c mice and exhibited a robust vaccine response (Panthel et al., 2003a). We have also examined p35<sup>−/−</sup> C57BL/6 mice and, contrary to Panthel, found good colonization but an inconsistent vaccine response from experiment to experiment (J.G. Nedrud, H. Ding et al., unpublished data). p19-deficient mice have not yet been evaluated, so it remains unclear whether IL-12, IL-23 or both play a role in vaccine-mediated protective immunity.

**IL-23 and IL-17**

The IL-23/IL-17 pathway has also been investigated for its potential role in *H. pylori* immunity. Originally associated with autoimmune inflammatory responses, an IL-23/IL-17 axis has been found to play a role in other inflammatory responses such as inflammatory bowel disease (reviewed in Maloy, 2008) and defence against pathogenic microorganisms (reviewed in Curtis and Way, 2009). Luzza and colleagues reported enhanced levels of both IL-17 mRNA and protein in gastric biopsies from *H. pylori*-infected patients relative to uninfected controls (Luzza et al., 2000). They also demonstrated that IL-17 production was involved in neutrophil recruitment via stimulation of IL-8. More recently, this group demonstrated that IL-23 was involved in the regulation of IL-17 production in biopsies from *H. pylori*-infected patients (Caruso et al., 2008). Results consistent with this were observed in *H. pylori*-infected wild-type mice, where neutrophil recruitment was shown to be blunted in IL-17 knockout animals, coinciding with a concomitant increase in bacterial load (Shiomi et al., 2008).

These results led other investigators to investigate the role of IL-17 in vaccine-mediated protection from *H. pylori*. One vaccine study demonstrated that T cells from mice immunized intranasally with *H. pylori* produced significant amounts of IL-17 when stimulated with *H. pylori*-pulsed antigen-presenting cells *in vitro* (DeLyria et al., 2009). Gastric tissue from these immunized mice also expressed significant amounts of mRNA for IL-17 and the neutrophil-activating chemokines LIX (lipopolysaccharide-induced CXC), KC (keratinocyte-derived) and MIP-2 (macrophage inflammatory protein-2) after *H. pylori* challenge. An influx of neutrophils...
was noted to coincide with a drop in \textit{H. pylori} numbers (protection). Depletion of gastric neutrophils from the immunized mice blunted this protective immunity. These results support a role for the IL-23→Th-17→CXC chemokines→neutrophil pathway of inflammation in vaccine-mediated protection (DeLyria \textit{et al.}, 2009). Anna Muller’s laboratory has also suggested that neutrophils may be important in vaccine-mediated protection of mice from \textit{H. pylori} (Sayi \textit{et al.}, 2009). In further work, however, we have shown that immunized IL-17 knockout mice and immunized IL-17 receptor knockout mice both exhibited wild-type levels of protection from \textit{H. pylori} challenge (E.S. DeLyria \textit{et al.}, unpublished data) arguing against a role for this cytokine in vaccine-mediated protection against \textit{H. pylori}. Another group of investigators used a neutralizing anti-IL-17 monoclonal antibody to demonstrate a blunting of vaccine efficacy in \textit{H. felis}-immunized wild-type animals (Velin \textit{et al.}, 2009). Thus, similar to the role for IFNγ detailed above, the role for IL-17 in protective \textit{Helicobacter} immunity remains unresolved. It may be noteworthy, however, that we have observed enhanced neutrophil recruitment into the gastric mucosa of immunized IL-17 receptor knockout mice, suggesting that compensating mechanisms for neutrophil recruitment may operate independently of IL-17 signalling (E.S. DeLyria \textit{et al.}, unpublished data).

### 9.4.3 The role of mast cells in vaccine-mediated \textit{H. pylori} immunity

Another granulocyte that has been studied as a possible effector cell in \textit{H. pylori} immunity is the mast cell, which has been identified in the inflammatory infiltrate of infected human subjects and mice (Mohammadi \textit{et al.}, 1996; Nakajima \textit{et al.}, 1997). The data, however, are conflicting. Whereas Velin \textit{et al.} (2005) utilized transgenic mast cell-deficient mice and observed that mast cells were essential for protective immunity against \textit{H. felis}, our own results employing an \textit{H. pylori} challenge and a different mast cell-deficient mouse strain demonstrated that the absence of mast cells resulted in only partial ablation of vaccine-induced protective immunity (Ding \textit{et al.}, 2009).

One unapparent but potentially important laboratory to laboratory difference in the results summarized above on the roles of IFNγ, IL-17, neutrophils and mast cells in protective \textit{H. pylori} immunity may be the resident microbiota within otherwise ‘identical’ or similar mouse strains within different animal facilities. Different populations of ‘normal flora’ have the capacity to differentially modulate host immune responses and, similarly, bacterial species can interact with each other to shape the host–microbe outcome (Ivanov \textit{et al.}, 2008; Mazmanian \textit{et al.}, 2008; Willing and Finlay, 2009). Indeed, the immune and inflammatory responses towards \textit{H. pylori} have been shown to be modulated by concurrent infections with intestinal helminths as well as by other \textit{Helicobacter} species that can be unapparent residents in the gastrointestinal tracts of different mouse colonies (Fox \textit{et al.}, 2000; Lemke \textit{et al.}, 2009).

### 9.4.4 RNA profiling and the role of adipokines in \textit{H. pylori} immunity

The discussion above highlights the difficulties in identifying the effector mechanisms that eradicate \textit{H. pylori} from the gastric mucosa. A relatively recent approach to this problem, microarray analysis of messenger RNA (RNA transcriptional profiling), has been used to identify up-regulated and down-regulated host genes in pathogenesis and vaccine studies for a large number of microorganisms, including \textit{H. pylori} immunization. Two of the published \textit{Helicobacter} host transcriptional profiling studies identified IFNγ and IFNγ-related genes as important in vaccine-mediated immunity against \textit{H. pylori} in mice (Rahn \textit{et al.}, 2004; Sayi \textit{et al.}, 2009). As discussed previously in this chapter, the importance of IFNγ for effective immunity against \textit{H. pylori} has been inconsistent among laboratories. However, to the degree that IFNγ promotes enhanced gastritis, it does appear to play an important but variable role in clearance of \textit{H. pylori} from immunized rodents. Three other studies have identified and expanded upon up-regulation of adipokines and related molecules as a
Adipokines are cytokines or cytokine-like hormones produced by adipose tissue. Leptin, one of the most highly studied adipokines, has been shown to regulate appetite, energy metabolism, insulin sensitivity, reproductive fertility and, importantly, T-cell immunity and inflammation (Cava and Matarese, 2004; Fantuzzi, 2005). It has recently been shown that leptin receptor (LepR)-deficient mice were not protected from \textit{H. pylori} challenge after oral vaccination with bacterial lysate and CT (Wehrens et al., 2008). Overall levels of gastritis were not reported, but similar numbers of T cells were shown to infiltrate the stomachs of immunized mice from both groups. Curiously, given the proposed role of post-immunization gastritis in protection, transcriptional profiling of gastric tissue from the non-protected LepR-negative mice showed higher levels of some immune and inflammation-related genes than from the protected LepR-positive mice. The authors suggested that this could represent higher numbers of proliferating regulatory T cells in the LepR-negative mice, since De Rosa and colleagues recently demonstrated enhanced proliferation of regulatory T cells in LepR-negative mice (De Rosa et al., 2007). It also seems likely that leptin signalling may have direct effects on T effector cells, since when CD4^+CD45^high effector T cells from LepR-negative mice were injected into SCID recipients, colitis was blunted when compared with recipients of CD4^+CD45^high cells from wild-type donors (Siegmund et al., 2004). The relationships among \textit{H. pylori} infection, gastritis, vaccine-mediated protective T-cell immunity and adipokines such as leptin are fascinating and complex, and await future research to unravel.

### 9.5 Bypassing Host Regulatory Immune Response to Induce Protective Immunity

While specific effector mechanisms that contribute to vaccine-induced \textit{H. pylori} eradication remain difficult to identify, it seems clear from experiments in multiple mouse models that an overall increase in the intensity of the gastric inflammatory response correlates with reductions in bacterial load (Garhart et al., 2002). In fact, most models in which mice have been shown to reduce or eradicate gastric \textit{H. pylori} infections respond to challenge with robust mucosal inflammation that is significantly more severe than that induced by chronic infection of wild-type mice. These models include IL-10^{−/−} mice (Berg et al., 1998; Eaton et al., 2001; Ismail et al., 2003; Matsumoto et al., 2005), NADPH phagocyte oxidase-deficient mice (Blanchard et al., 2003), SCID or rag^{−/−} immunodeficient mice reconstituted with splenic T cells from wild-type donor mice (Eaton et al., 1999; Stuller et al., 2008) and, as discussed above, mice immunized against \textit{H. pylori} by any number of strategies.

Such observations have led investigators to question whether the natural host immune and inflammatory response to \textit{H. pylori} infection might actually be down-regulated. Early studies performed to assess the T-cell recall response of \textit{H. pylori}-infected subjects showed that the host immune response to \textit{H. pylori} is suppressed. Lymphocytes isolated from blood or the gastric mucosa of infected patients have been demonstrated in numerous studies to respond no better than T cells from seronegative patients, as measured by \textit{H. pylori}-induced cytokine production and proliferation \textit{in vitro} (Blanchard et al., 2004). These observations were difficult to put into context knowing that infected subjects routinely displayed significant histological gastritis.

Recent advances in our understanding of regulatory T cells (Tregs) have facilitated investigation of the role suppression may play in \textit{H. pylori} pathogenesis (see Robinson and Atherton, Chapter 6, this volume). Studies in both mice and humans have revealed that \textit{H. pylori}-specific Tregs participate in the host response to infection and actively suppress immunity. Several models exist in which immune dysregulation results in reduced bacterial loads or even eradication of \textit{H. pylori}, suggesting that vaccine-mediated protection may also be due to the ability to induce active immunity instead of the regulatory response.
IL-10, an important product of both Tr1 and Treg cells, is known to down-regulate immunity (Couper et al., 2008). Perhaps more importantly, Rubtsov et al. (2008) have elegantly demonstrated that mice in which only cells expressing Foxp3 are deficient in IL-10 production develop inflammation in the colon and lungs in response to normal flora. Similarly, the NADPH phagocyte oxidase knockout mouse model, which can spontaneously clear H. felis and H. pylori infections (Blanchard et al., 2003), has now also been shown to be compromised in its ability to generate Treg cells (Romani et al., 2008). Reactive oxygen species are required to promote Treg development and, in the absence of functional phagocyte oxidase, an inflammatory effector T-cell response is favoured. Reconstitution of SCID mice with splenic T cells does not exclude Treg participation but any Treg cells that might be transferred fail to prevent newly activated T cells from promoting pronounced and protective gastritis. When transfer is limited specifically to memory cells, however, inflammation is limited and H. pylori continues to colonize (Eaton et al., 2001; Stuller et al., 2008). Ultimately, the ability of immunization to induce a protective immune response may be due to its ability to induce an active immune response in the absence of induced Treg cells, possibly due to the site of T-cell activation being lymph nodes instead of the mucosa where down-regulation is favoured.

In addition to the possible role of Tregs in inhibiting an effective immune response towards H. pylori, it has been suggested that H. pylori may impair the capacity of dendritic cells (DCs) to stimulate a T-cell response. Mitchell et al. (2007) demonstrated that, although H. pylori treatment of human DCs induced expression of the co-stimulatory molecules CD40 and CD86, the inhibitory member of the B7 family, PD-L1, was also induced. In addition they showed that prolonged (48 h) incubation of DCs, such as might be observed in chronic H. pylori infections, leads to DC ‘exhaustion’. The exhausted DCs produced low levels of IL-12 and IL-10 and had an impaired capacity to stimulate allogeneic T-cell IFNγ production when compared with DCs incubated with H. pylori for only 8 h (Mitchell et al., 2007). Thus it may be necessary for H. pylori vaccine strategies to effectively overcome not only Treg cells but also inhibitory effects on DCs.

9.6 Non-human Primate and Human Clinical Trials

9.6.1 Mucosal vaccines

Non-human primate models have also been used to obtain compelling evidence to proceed with clinical trials. Initially, a therapeutic strategy was employed in a colony of cynomolgus monkeys harbouring an endogenous Helicobacter heilmannii infection (Guy et al., 1997). Oral or intranasal immunizations with inactivated whole H. pylori plus recombinant urease plus LT were used. These strategies failed to reduce the bacterial load determined 1 month after immunization, but it was difficult to ascribe the lack of efficacy to the immunization strategy or to the use of H. pylori antigens to eradicate H. heilmannii. Subsequent studies have been performed with rhesus monkeys and models of natural H. pylori acquisition. In the first such study, young seronegative animals were given oral doses of either recombinant urease plus LT or placebo and then tested 10 months later for acquisition of H. pylori from the greater monkey population (Dubois et al., 1998). Eight of 26 (31%) immunized monkeys remained H. pylori-free compared with two of 29 (7%) animals receiving placebo. Another study using older, naturally infected rhesus monkeys to test the therapeutic potential of oral recombinant urease plus LT found no therapeutic activity (Lee et al., 1999b). These monkeys were then treated with antimicrobial therapy to further test the capacity of the vaccination to prevent re-infection. Treated monkeys received the oral vaccine again and were then challenged with a strain of H. pylori previously isolated from one of the monkeys in the study. Although all monkeys became experimentally infected, immunized monkeys had significantly fewer bacteria in the stomach compared with placebo-immunized animals. A separate study by the
same investigators used adult rhesus monkeys that had been treated with antimicrobial therapy to eradicate naturally acquired \textit{H. pylori} and then compared oral immunization with recombinant urease plus LT with either parenteral immunizations or a combination of oral and parenteral immunizations for protection against infectious challenge (Lee \textit{et al.}, 1999a). In this study, no protective efficacy was demonstrated by prophylactic oral immunization, although some success was achieved with parenteral and combination vaccines (discussed in Section 9.6.2). The most recent rhesus \textit{H. pylori} vaccine trial used animals that were removed from the colony at birth and hand reared to ensure absence of endogenous \textit{H. pylori} infection. This study attempted to compare oral and parenteral vaccination using recombinant urease. Because all immunized macaques became infected after challenge, the vaccines appeared to be a failure (Solnick \textit{et al.}, 2000). However, only one of two sham-immunized animals became infected after challenge, making a quantitative determination of possible vaccine efficacy impossible.

Results achieved in murine and non-human primate models have provided the impetus for performing clinical trials in human volunteers. In the first study of note investigators tested recombinant urease as a therapeutic vaccine in \textit{H. pylori}-positive volunteers (Michetti \textit{et al.}, 1999). Various doses of urease were given orally with either 5 or 10 µg LT in four weekly immunizations, with mixed results. No individuals eradicated \textit{H. pylori}, but reductions in bacterial load were measured in the group receiving the lowest dose of urease. Because bacteria were not eradicated, it is not surprising that gastric inflammation was not reduced. Unfortunately, the wild-type LT was not well tolerated by volunteers, with the 10 µg dose of LT being discontinued mid-study, and even the 5 µg dose inducing diarrhoea in many subjects. The results of this study highlight the complications associated with mucosal immunizations, an issue further illustrated by a subsequent study to evaluate limiting amounts of LT in combination with urease administered as a soluble vaccine or in capsules for enteric release (Banerjee \textit{et al.}, 2002). LT was given in doses as high as 2.5 µg and as low as 0.1 µg. Only those subjects receiving 2.5 µg responded immunologically to immunization, but this dose induced diarrhoea in 50% of the test subjects. The most recent human trial with urease evaluated rectal immunization as an alternative to oral immunization (Sougioulitzis \textit{et al.}, 2002). The vaccine, consisting of 60 mg urease and either 5 or 25 µg LT, was given as an enema to \textit{H. pylori}-negative volunteers, three times over the course of 28 days. LT was immunogenic in most subjects but few developed circulating antibodies to the urease protein and no meaningful T-cell recall response could be measured. The use of a soluble, mucosally delivered vaccine against \textit{H. pylori}, therefore, will require additional research into mucosal adjuvants, effective doses and routes of immunization (see Table 9.1 for alternative adjuvants which have been evaluated in mice but generally have not yet been approved for human use).

The second type of \textit{H. pylori} vaccine tested in human subjects has been inactivated whole-cell preparations. Such vaccines have the benefit of multivalency. Kotloff \textit{et al.} (2001) utilized a formalin-inactivated whole-cell vaccine given orally in combination with a mutant form of LT that has reduced susceptibility to trypsin and therefore should possess less toxicity. The vaccine was given to both infected and uninfected subjects. Antibody responses were observed only in infected individuals receiving the highest dose of vaccine. Conversely, \textit{in vitro} recall responses of blood leucocytes were observed only from vaccinated, but uninfected, volunteers. The efficacy of this vaccine as a therapeutic agent was tested in infected subjects given either whole-cell preparation plus mutant LT or one of several placebos. Colonization was assessed by the $^{13}$C-urea breath test and all subjects were shown to remain infected up to 7.5 months later, irrespective of vaccination. Because bacterial load was not determined, reductions in colonization levels could not be established. Similar to studies described above with wild-type LT, 28% of test subjects and 33% of placebo recipients developed diarrhoea in response to the mutant LT, suggesting that additional mutations or other strategies such as with CTA1-DD (see Section 9.3.1) may be necessary to develop an acceptable mucosal adjuvant from CT or LT.
The last types of mucosal vaccine tested for *H. pylori* in humans are the recombinant bacterial vector vaccines expressing *H. pylori* proteins. The vectors employed have been exclusively strains of *Salmonella*. Two initial studies to assess immunogenicity utilized avirulent strains of *Salmonella enterica* serovars Typhi or Typhimurium, expressing *H. pylori* urease (DiPetrillo et al., 1999; Angelakopoulos and Hohmann, 2000). Only one of the studies elicited any antibodies to urease subunits and this result was not uniform among subjects. Another group performed a series of trials with *Salmonella* vaccine strain Ty21a expressing both subunits of *H. pylori* urease. Their initial safety study demonstrated only weak responses in *H. pylori*-negative volunteers with none of the oral vaccine recipients generating antibodies to the urease subunits, although several subjects did develop T-cell memory to urease (Bumann et al., 2001). A follow-up study was performed to determine if prior exposure to the carrier vector might impact on the host response to the recombinant *H. pylori* vaccine strain (Metzger et al., 2004). Prior immunization with *Salmonella* vaccine strain Ty21a did not impact on the subsequent response to the vaccine, and subjects again failed to develop humoral immunity to urease. However, similar to the initial study, over half of the subjects developed a urease-specific cellular immune response. This vaccine was later used in a human challenge study in which *H. pylori*-negative volunteers were given multiple oral doses of the urease-expressing *Salmonella* strain or a second recombinant strain expressing the HP0231 antigen (Aebischer et al., 2008). Subjects were challenged with a previously characterized human challenge strain of *H. pylori* (Graham et al., 2004), 42 days after the final immunization. Subjects developed immune responses consistent with *H. pylori* infection, including cytokine production and histological gastritis. Immune responses to urease were not detectable until after challenge. The bacterial load was shown to decrease in eight vaccinated subjects, but a similar decrease was also observed in non-immunized controls. What may be instructive here, however, is that, regardless of immunization status, 69% of the subjects who did clear or reduce *H. pylori* colonization demonstrated T-cell reactivity *in vitro*, compared with only 13% of subjects who did not reduce bacterial load. This result offers partial confirmation of animal studies demonstrating a need for cell-mediated immune responses in *H. pylori* immunity and suggests that future efforts should proceed in this direction. Finally, although these live vector vaccines have met with limited success, they do have the advantage of being well tolerated by test subjects and seem to avoid the undesirable side-effects inherent to enterotoxin mucosal adjuvants.

### 9.6.2 Parenteral vaccines

Although many studies have reported on the use of non-human primates for *H. pylori* pathogenesis and vaccine development, only a few have evaluated systemic immunization strategies. The earliest study compared oral, parenteral and a combination of both oral and parenteral vaccination with whole killed *H. pylori* and urease as therapeutic immunization strategies against a pre-existing *H. heilmannii* infection in cynomolgus monkeys (Guy et al., 1997). Only limited protection was achieved, but parenteral and combination immunizations induced a higher proportion of animals with reduced bacterial load. A subsequent study in rhesus monkeys compared parental, oral and an oral prime followed by parental boost for efficacy against challenge with *H. pylori* (Lee et al., 1999a). In that study, parenteral immunization failed to induce immunity, although the combination strategy did induce a reduction in bacterial load to one-tenth that of sham-immunized animals. Finally, another study performed in rhesus monkeys employed prophylactic immunization to compare oral immunization to intramuscular immunization with urease in Bay adjuvant, but all animals became infected upon challenge and there were no differences in bacterial load (Solnick et al., 2000).

Rossi et al. (2004) used a canine model to test therapeutic vaccination of experimentally infected beagles. Their vaccine, which consisted of purified recombinant VacA, CagA and NAP in combination with aluminium...
hydroxide and administered by intramuscular injections, induced a reduction in bacterial colonization and, most encouragingly, gastritis. Results from the study provided the impetus for the same laboratory to test their vaccine formulation for safety and immunogenicity in human volunteers (Malfertheiner et al., 2008). Subjects were given three intramuscular immunizations with the multianti VacA, CagA and NAP proteins plus aluminium hydroxide. No untoward side-effects were noted beyond what might be observed at the site of injection for well-established aluminium hydroxide-based vaccines. The vaccine proved to be highly immunogenic, inducing strong antibody and cellular recall responses as measured by IFNγ production in vitro. Importantly, this response was detectable for several months and booster immunizations applied almost 2 years later induced a strong memory response. These promising results require further study to determine if this might ultimately be a practical approach for vaccination of human subjects against H. pylori.

9.7 Concluding Remarks

Many H. pylori vaccine formulations have now been tested in several animal models and in various early-stage clinical studies. Although success in terms of acceptable efficacy has not been achieved in humans, a survey of the efforts put forth since the mid-1990s is instructive. It bears noting that few vaccines exist for the large number of enteric infections that are the cause of significant morbidity and mortality worldwide, and many of the problems associated with developing a vaccine against H. pylori have confronted the immunological community for decades. Nevertheless, we now know that there is much utility in the use of a subunit vaccine that incorporates multiple antigens. It is also apparent that bacterial enterotoxins, while too toxic in their native form, may after further modification have the potential of inducing acceptable and safe immunity to H. pylori. The CTA1-DD construct may prove to be one such improvement.

Other strategies such as the use of attenuated bacterial vectors, though well tolerated, have exhibited generally disappointing immunogenicity in human H. pylori trials. A new generation of highly attenuated and highly immunogenic Salmonella vectors (see e.g. Kong et al., 2008; Curtiss et al., 2009) may improve the performance of these vectors for H. pylori vaccines. There may also be value in either exploring parenteral immunization further or, at the very least, exploring combination vaccines in which mucosal vaccination is administered along with intramuscular or subcutaneous injection. Most importantly, as difficult as it is to immunize a host against a gastrointestinal bacterial infection, it has been amply demonstrated in animal models, and observed within some subjects in several clinical studies, that it is in fact possible to positively influence the host immune response to H. pylori. Studies in the mouse model routinely achieve significant reductions in bacterial load and sterilizing immunity has even been noted in several reports. Encouragingly, a reduction in bacterial load was observed in one human therapeutic vaccine trial using recombinant urease with LT adjuvant (Michetti et al., 1999). In addition, in an H. pylori challenge human trial, a cell-mediated immune response was associated with clearance or reductions in bacterial load, even though these reductions occurred in both vaccinated and placebo-treated volunteers (Aebischer et al., 2008). Continued innovation in adjuvant and carrier technology, along with novel protocols for administration of prototype vaccines (Table 9.1), also offers the potential for improvements in vaccine efficacy and ultimately achieving the goal of inducing protective immune responses capable of eradicating H. pylori from the gastric mucosa.

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10 Lipopolysaccharides of *Helicobacter pylori*: Importance in Gastric Adaptation and Pathogenesis

A.P. Moran

10.1 Introduction

As in other Gram-negative bacteria, *Helicobacter* spp., including *Helicobacter pylori*, contain lipopolysaccharides (LPS) in the outer membrane of the cell envelope (Moran *et al.*, 1992; Moran, 1995a; Hynes *et al.*, 2004). In general, LPS play a central and essential role in the integrity of the cell envelope of Gram-negative bacteria but, despite this, due to their ability to adversely affect a broad range of host immunological and physiological properties, have been considered toxic and have been termed endotoxins (Rietschel *et al.*, 1990; Raetz and Whitfield, 2002; Caroff and Karibian, 2003). Nevertheless, these molecules do not simply reside in the outer membrane; they can be released by multiplying or disintegrating bacteria, as well as by blebbing of outer membrane vesicles from the bacterial cell surface as occurs in *H. pylori* (Hynes *et al.*, 2005; Keenan *et al.*, 2008). Thus, these phosphorylated lipoglycans, which in many Gram-negative bacteria possess potent immune-stimulating and immune-modulating properties, play a central role in the pathogenesis and virulence of these bacteria. In the case of *H. pylori*, although an important pathogenic factor, LPS particularly possesses properties influencing the severity and chronicity of this infection (Moran, 2007; Moran and Trent, 2008).

Like *Escherichia coli*, *H. pylori* is capable of producing high-molecular-mass (smooth-form) LPS composed of an outermost saccharide moiety, divided into the O-polysaccharide or O-specific chain (O-antigen) and core oligosaccharide regions, covalently linked to a lipid moiety, termed lipid A, that anchors the molecule in the outer leaflet of the outer membrane (Rietschel *et al.*, 1990; Moran *et al.*, 1992; Moran, 1995b; Caroff and Karibian, 2003) (Fig. 10.1A). This contrasts with the production of the LPS-related, but distinct, low-molecular-mass lipooligosaccharides by certain other mucosal pathogens, e.g. *Campylobacter jejuni*, *Neisseria* spp., *Haemophilus influenzae*, *Bordetella pertussis*, etc. (Rietschel *et al.*, 1990; Moran, 1995b, 2009) (Fig. 10.1B). The latter differ from LPS by the absence of the O-polysaccharide chain and exhibit greater structural variability in their outer core oligosaccharides. In general, each of the domains of LPS has differing structural and biological properties: the O-chain contributes to the antigenicity and serospecificity of the molecule; the core oligosaccharide influences permeation properties of the outer membrane, modulates the immune response to lipid A and can influence virulence; whereas the lipid A moiety interacts with immune receptors and endows the LPS molecule with a range of immunological and potent endotoxic properties (Rietschel *et al.*, 1990; Moran *et al.*, 1992; Moran, 1995b).
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Moran, 1995b; Caroff and Karibian, 2003). Clinical isolates of *H. pylori* produce smooth-form LPS with O-polysaccharide chains of relatively constant chain length compared with enterobacterial LPS (Moran et al., 1992; Moran, 1995a). Their length is determined by an enzymatic molecular ruler mechanism (Nilsson et al., 2006). As surface antigens (O-antigens), *H. pylori* LPS can induce a specific antibody response, and their structural variation forms the basis for serotyping and lectin typing schemes (Simoons-Smit et al., 1996; Hynes et al., 1999, 2002). However, numerous subcultures on conventional solid media can induce the production of low-molecular-mass, rough-form LPS lacking the O-polysaccharide chain in many strains (Moran, 1995a), which has proven a useful manipulation when studying the structure and properties of the LPS core (Moran, 1999). The ability of such strains to revert to smooth-form LPS production and the influence of environmental factors, such as pH, on LPS expression and antigenic phase variation has been reviewed previously (Moran, 2001b; Moran and Trent, 2008). Moreover, the genetics and characteristics of the enzymes required for biosynthesis of *H. pylori* LPS also have been detailed in a number of reviews (Berg et al., 1997; Moran, 2001a,b, 2008; Ma et al., 2006; Moran and Trent, 2008).

*H. pylori* has been proposed as a model for investigating and understanding the dynamics of bacterial persistence and parasitism in chronic infections (Blaser and Kirschner, 1999). Likewise, studies on the attributes of *H. pylori* LPS have produced novel insights into the structure and contributing properties of this class of molecule to chronic pathogenesis (Moran, 2007). Therefore, the present chapter discusses how the structure and properties of *H. pylori* LPS are adapted and modelled to fit the environmental niche of this bacterium, and the pathogenic consequences of these structures. Although the core oligosaccharide contributes to *H. pylori* pathogenesis (the subject of other reviews; see Moran 1996, 1999, 2001a,b), discussion here focuses on the lipid A moiety and O-antigen of this molecule, reflecting the intensive investigation of these portions of the *H. pylori* LPS molecule in recent years.

10.2 Low Immunological Activity of *H. pylori* Lipopolysaccharides

A striking feature of *H. pylori* LPS is the significantly lower endotoxic and immunological activities of these molecules, compared with enterobacterial LPS as the gold standard (reviewed extensively elsewhere; see Moran 1999, 2001a, 2007; Moran and Trent, 2008). The pyrogenicity and mitogenicity of *H. pylori* LPS is 1000-fold lower, lethal toxicity in mice 500-fold lower, and induction of various cytokines and chemokines 1000-fold lower than enterobacterial LPS (Muotiala et al., 1992; Pece et al., 1995). Priming of neutrophils to release toxic oxygen radicals, E-selectin expression on endothelial cells, induction of prostaglandin E2 and nitric oxide, natural killer-cell activity and abolition of suppressor T-cell activity are also all significantly lower (Baker et al., 1994; Nielsen et al., 1994; Moran, 1999, 2001a; Odenbreit et al., 2006). Similarly, cyclooxygenase-2 induction in gastric cells by
H. pylori LPS is negligible compared with that induced by *Escherichia coli* (Smith, G.V. et al., 2003).

Importantly, early studies that tested chemically modified *H. pylori* LPS-derived components in immunological assays indicated that the molecular basis for these low immunoactivities resided in the lipid A moiety, and is modulated by the saccharide moiety, and is modulated by the saccharide component of *H. pylori* LPS, particularly the core oligosaccharide. For instance, the phosphorylation pattern in lipid A influences cytokine production and induction of pro-coagulant activity from mononuclear leukocytes, as well as *Limulus* amoebocyte lysate activity; nevertheless, the core oligosaccharide modulates these effects (Pece et al., 1995; Semeraro et al., 1996). In contrast, the lipid A acylation pattern has been attributed responsibility for the lack of abolition of suppressor T-cell activity by *H. pylori* LPS (Baker et al., 1994) and the lipid A phosphorylation pattern is considered of lesser importance than acylation for reduced priming of neutrophils to release toxic oxygen radicals (Nielsen et al., 1994). Agreeing with the hypothesis that the structure of lipid A endows *H. pylori* LPS with low immunoactivities (Muotiala et al., 1992), detailed structural studies have found under-phosphorylation, under-acylation and substitution by long-chain fatty acids in this lipid A (Moran et al., 1992, 1997; Moran, 1995a; Suda et al., 2001) compared with enterobacterial-derived lipid A (Moran, 1998). Based on established structure–bioactivity relationships of lipid A molecules, the primary structural characteristics of *H. pylori* are likely to be reflected in reduced immunological activities (Rietschel et al., 1990, 1994).

10.3 *H. pylori* Lipid A Fine Structure and Biosynthesis

10.3.1 Structural analysis of *H. pylori* lipid A

Using two different strains, two independent research groups examined the fine structure of lipid A from *H. pylori* rough-form LPS. One study reported the presence of a tetra-acyl lipid A (Moran et al., 1997), whereas the other reported a tri-acyl lipid A with an identical phosphorylation pattern to the tetra-acyl form, but lacking acylation at position 3 of the lipid A backbone (Suda et al., 1997). A re-investigation by the latter group of the same strain found the tetra-acyl form of *H. pylori* lipid A (Suda et al., 2001) and thus, potentially, the tri-acyl lipid A may have arisen as a degradation product of the former during chemical isolation of the lipid A (Moran, 2001b). Hence, it may be concluded that the major molecular species in lipid A of *H. pylori* rough-form LPS is composed of a β-(1→6′)-linked α-glucosamine disaccharide backbone, acylated by (R)-3-hydroxyoctadecanoic acid (18:0(3-OH)) and (R)-3-hydroxyhexadecanoic acid (16:0(3-OH)) at positions 2 and 3, with (R)-3-(octadecanoyloxy)octadecanoic acid (3-(18:0-O)-18:0) at the 2′-position, and carries phosphate or phosphoethanolamine (PEtN) groups at position 1 (Moran et al., 1997) (Fig. 10.2B).

Likewise, this mono-phosphorylated tetra-acyl lipid A predominates in *H. pylori* smooth-form LPS, but there is a second, minor constituent consisting of a bis-phosphorylated hexa-acyl lipid A that is distinguished from tetra-acyl lipid A by carrying 3-(12:0-O)-16:0 or 3-(14:0-O)-16:0 at position 3′ and an extra phosphate group at position 4′ (Moran et al., 1997) (Fig. 10.2A). Overall, whether derived from rough- or smooth-form LPS, compared with the lipid A of *E. coli* (Fig. 10.3) the predominant lipid A molecular species of *H. pylori* lacks the usual 4′-phosphate group, as well as the 3′-ester-linked fatty acyl chains, thus containing only four rather than six fatty acids, which have longer chain length (16–18 carbons versus 12–14 carbons), and is derivatized with a PEtN residue at the C-1 position of the proximal glucosamine (Moran et al., 1997; Moran, 1998). However, because this bacterium can synthesize a bis-phosphorylated hexa-acyl lipid A, it can be deduced that *H. pylori* expresses enzymes (e.g. a 4′-phosphatase and 3′-acyloxyacyl deacylase) capable of remodelling, and hence modifying to a tetra-acyl form, its lipid A domain (Reynolds et al., 2006; Tran et al., 2006) after completion of the conserved hexa-acyl lipid A biosynthetic pathway (Tran et al., 2005; Moran and Trent, 2008).
Fig. 10.2. Structures of the minor hexa-acyl lipid A molecular species found in *Helicobacter pylori* smooth-form lipopolysaccharide (LPS) (A) and the predominating tetra-acyl lipid A species found in *H. pylori* rough- and smooth-form LPS (B) (Moran et al., 1997). Numbers in circles refer to the number of carbon atoms in the acyl chains. One 3-deoxy-d-manno-2-octulosonic acid residue, as occurs in the *H. pylori* core oligosaccharide, is shown attached to the 6'-position of lipid A. Compared with the hexa-acyl species (A), the tetra-acyl molecular species (B) lacks 4'-phosphate and is substituted at position 1 by phosphoethanolamine.
The constitutive lipid A biosynthesis pathway is highly conserved, occurs on the cytoplasmic surface of the inner membrane, and generally a single copy of each *lpx* gene can be found in the genomes of virtually all Gram-negative bacteria (reviewed in Raetz et al., 2007). Similarly, bioinformatic analysis of the *H. pylori* 26695 and J99 genomes indicates that the enzymatic machinery for lipid A biosynthesis is conserved in *H. pylori* (Moran and Trent, 2008).

Most enterobacterial lipid A structures are characterized by primary-linked acyl chains of 14 carbons (14:0(3-OH)) in length (Rietschel *et al*., 1990, 1994; Moran, 1995b; Raetz and Whitfield, 2002; Trent *et al*., 2006; Raetz *et al*., 2007). The occurrence of acyl chains of longer length in *H. pylori* compared with *E. coli* lipid A can be attributed to substrate preferences of the primary and secondary acyltransferases. Since the primary-linked hydroxylated acyl chains of *H. pylori* lipid A are either 16 carbons or 18 carbons in length (Fig. 10.2), the early acyltransferases of the *H. pylori* lipid A pathway, LpxA and LpxD, utilize

**Fig. 10.3.** Structure of the major lipid A species of *Escherichia coli* (Rietschel *et al*., 1990; Raetz *et al*., 2007). Numbers in circles indicate the number of carbon atoms in the acyl chains. This lipid A is composed of a hexa-acylated α-glucosamine disaccharide that is substituted at the 1- and 4’-positions with phosphate groups and at C-6’ with the 3-deoxy-α-manno-2-octulosonic acid residues of the core oligosaccharide. The phosphate groups may be substituted with ethanolamine phosphate (C-1) and 4-amino-4-deoxy-α-arabinopyranose (C-4’).
substrates bearing longer fatty acyl chains when compared with enzymes expressed by most Gram-negative bacteria (see Fig. 10.3 for comparison). The major tetra-acyl lipid A molecular species of H. pylori contains a single secondary acyl chain of 18 carbons in length (18:0) located at the 2′-position (Moran et al., 1997; Suda et al., 1997) (Fig. 10.2B), which, again, is longer than the secondary acyl chains found in E. coli lipid A. The minor hexa-acyl lipid A molecular species (Fig. 10.2A) bears two secondary acyl chains (at positions 2′ and 3′) and the primary 16:0(3-OH) chain at the 3′-position is substituted with either a dodecanoyl (12:0) or tetradecanoyl (14:0) group. Only a single secondary acyltransferase, encoded by HP0280 or JHP0265 (Tomb et al., 1997; Alm et al., 1999), was initially identifiable in the sequenced H. pylori genomes of 26695 and J99. This led to the inference that either the HP0280-encoded protein is a bifunctional acyltransferase or a further unknown secondary acyltransferase functions to acylate H. pylori lipid A. Biochemical characterization of this protein using an in vitro assay system has confirmed it to be a monofunctional octa-decanoyl (18:0) acyltransferase (Stead et al., 2008). The enzyme utilizes an acyl–acyl carrier protein (acyl-ACP) as its donor, requires 3-deoxy-β-manno-2-octulosonic acid (Kdo) residues attached to the lipid A precursor for activity, and transfers 18:0 to the primary 18:0(3-OH) chain at the 2′-position of H. pylori lipid A. Therefore, this acyltransferase functions similarly to E. coli LpxL (Clementz et al., 1996) except for acyl chain preference. Additionally, studies assaying H. pylori membranes with key lipid A precursors have detected a second acyltransferase, a 3′-secondary acyltransferase, that can utilize 12:0 and 14:0 acyl-ACPs (Stead et al., 2008), and hence functions in a similar manner to E. coli LpxM (Clementz et al., 1996). Thus, the latter enzyme transfers 12:0 or 14:0 residues to the primary 16:0(3-OH) chain at position 3′ of H. pylori lipid A. Concerning the phosphorylation pattern, lpxH and lpxK are essential genes in E. coli encoding the UDP-2,3-diacylglycosamine pyrophosphatase (Babinski et al., 2002) and the lipid A 4′-kinase (Garrett et al., 1997), required for 1- and 4′-phosphorylation, respectively. Of note, only distant orthologues of LpxH and LpxK are present in H. pylori (Moran and Trent, 2008). Nevertheless, the bacterium has the necessary machinery to synthesize a completely phosphorylated hexa-acyl lipid A, although this represents a minor molecular species in the outer membrane.

Transfer of Kdo residues, which link the core and O-antigen carbohydrate domains to the lipid A moiety (Rietschel et al., 1990; Raetz and Whitfield, 2002) (see Fig. 10.3), is catalysed by the glycosyltransferase WaaA (formerly KdtA) in E. coli and Salmonella, where WaaA is a bifunctional enzyme transferring two sugars to the tetra-acylated lipid A precursor known as Lipid IVα (Clementz and Raetz, 1991; Belunis et al., 1995). However, not all Gram-negative bacteria contain two Kdo sugars (Isobe et al., 1999; Brabetz et al., 2000) and structural studies have shown that the inner core region of H. pylori LPS contains a single Kdo sugar (Aspinall et al., 1994, 1995, 1996, 1997, 1999; Knirel et al., 1999; Monteiro et al., 2000; Moran et al., 2002a), which at first would suggest that H. pylori WaaA is a monofunctional transferase. Importantly, Stead et al. (2005) demonstrated that H. pylori WaaA transfers two Kdo residues to the disaccharide backbone of lipid A, but also reported the occurrence of a Kdo-trimming enzyme, a Kdo hydrolase, that was predicted to function following MsbA-dependent transport (Zhou et al., 1998; Doerrler, 2006) across the inner membrane. Why the bacterium transfers two Kdo residues, but then removes the second, is unclear; it is possible that the charge of the second sugar is necessary for proper functional transport or that secondary acylation of H. pylori lipid A precursors may require the presence of two Kdo sugars (Moran and Trent, 2008). After assembly and transport of the H. pylori core–lipid A, removal of the outer Kdo residue may benefit the organism by reducing the net negative charge on the bacterial surface, thereby promoting resistance to antimicrobial peptides. After transport across the inner membrane, a number of bacterial species modify the Kdo–lipid A domain of their LPS by modification systems that are found on the periplasmic side of the inner membrane or at the extracellular surface (Raetz et al., 2007). Thus, there is a physical
H. pylori lipid A contains a PEtN group directly linked to the C-1 hydroxyl group of the proximal glucosamine (Moran et al., 1997; Suda et al., 1997) (Fig. 10.2B), which arises from the removal of the phosphate group at position 1 of lipid A, followed by the addition of PEtN to form a glycosidic phosphodiester linkage, and is catalysed by the proteins encoded by HP0021 and HP0022, respectively (Tran et al., 2004, 2006; Moran and Trent, 2008). This modification of the 1-position of H. pylori lipid A occurs on the periplasmic side of the inner membrane. Although separated by only 10 bp of DNA sequence, the genes encoding these enzymes appear to form a typical prokaryotic operon as they are unidirectionally transcribed. The HP0021-encoded protein, also known as LpxE, has distant sequence similarity to E. coli PgpB, a phosphatidyl glycerolphosphatase (Icho and Raetz, 1983; Icho, 1988), but is highly selective for the 1-phosphate group of lipid A (Wang et al., 2004). HP0022, an orthologue of eptA, is homologous to the lipid A PEtN transferases identified in other Gram-negative pathogens including E. coli, Salmonella enterica serovar Typhimurium and Neisseria meningitidis (Tran et al., 2004), but the H. pylori EptA contrasts in activity with the EptA of other bacteria, which modifies the lipid A phosphate group directly (Trent and Raetz, 2002; Lee et al., 2004). Disruption of lpxE (JHP0019) in H. pylori J99 results in the production of a lipid A bearing a single phosphate group at the 1-position, since lack of LpxE function results in loss of PEtN transfer as H. pylori EptA (HP0022-encoded) requires prior dephosphorylation for activity (Tran et al., 2006). Unexpectedly, it has been observed that disruption of lpxE in H. pylori 26695 (HP0021) results in the synthesis of lipid A with two phosphate groups and six acyl chains (Tran et al., 2006). The structure is identical to the minor lipid A species previously reported (Fig. 10.2A), thereby confirming the findings of Moran et al. (1997).

The highly conserved lipid A kinase, LpxK, phosphorylates the 4′-hydroxyl group of the disaccharide backbone in the constitutive biosynthetic pathway (Garrett et al., 1997) but in some microorganisms, including H. pylori, the 4′-phosphate group is removed subsequently due to phosphatase activity (see Moran and Trent, 2008). Although a 4′-phosphatase, LpxF, has been identified in Francisella tularensis (Wang et al., 2006), and is localized to the inner membrane and removes the 4′-phosphate group from lipid A after transport across the inner membrane, there is no identifiable homologue in the genome of H. pylori J99 or 26695. Attempts to clone the structural gene encoding Helicobacter LpxF have been unsuccessful (Moran and Trent, 2008). Thus, collectively the findings indicate that dephosphorylation of the 1- and 4′-positions of H. pylori lipid A occurs within the periplasmic region of the bacterial cell.

The final modification of the H. pylori lipid A domain is the removal of the 3′-acyloxyacyl residue, resulting in the production of a tetra-acylated lipid A (consistent with the structural findings above), which is catalysed by the outer membrane protein encoded by HP0694 (JHP0634), also known as LpxR (Rutten et al., 2009). Inactivation of HP0694 by insertion of a selectable marker results in the production of a hexa-acylated lipid A structure, but does not inhibit modification of the phosphate groups. Although LpxR resides in the outer membrane of Salmonella under normal growth conditions, the enzyme remains dormant (Reynolds et al., 2006), whereas the LpxR of H. pylori is constitutively active since H. pylori lipid A is not substituted at the 3′-position (Fig. 10.2B). Despite these findings, the factors that promote enzymatic activation of H. pylori LpxR or function of the deacetylase to date remain unknown.

Synthesis of the Kdo–lipid A domain of LPS is followed by the attachment of the core oligosaccharide and transport of the core–lipid A across the inner membrane (Raetz and Whitfield, 2002). For this process, MsbA, a conserved ABC transporter that is also found in H. pylori, serves as the lipid A flippase (Zhou et al., 1998; Doerrler, 2006). The O-chain of H. pylori is then attached on the periplasmic side of the inner membrane to form whole LPS. Additional steps are required for translocation of LPS to the outer leaflet of the outer membrane, but are not yet clearly defined (Doerrler, 2006).
10.3.3 Molecular and supramolecular basis for the low immunological activities of H. pylori lipid A

A structural comparison of the predominant H. pylori lipid A molecular species with that of E. coli (Moran, 1998) shows the absence of 4′-phosphate and the occurrence of four, rather than six, fatty acids whose average chain length is longer (16–18 carbons versus 12–14 carbons) in H. pylori lipid A. These structural differences in H. pylori lipid A are consistent with the established structure–bioactivity relationships for low immunological activities of lipid A (Rietschel et al., 1990, 1994). Moreover, natural and synthetic H. pylori lipid A, with the reported underacylation and under-phosphorylation patterns, have been shown to have low endotoxic potency and immunological activities (Suda et al., 2001; Ogawa et al., 2003) like H. pylori LPS, as discussed above. Importantly, these novel attributes not only impact upon the established primary structure–bioactivity relationships but also upon the supramolecular conformation of H. pylori lipid A (Schromm et al., 2000) and other biophysical properties of lipid A that have been correlated previously with lower bioactivity (Seydel et al., 2000). These include a higher phase transition temperature and a lower inclination angle of the lipid A diglucosamine backbone to the membrane plane than those encountered with enterobacterial lipid A (Moran et al., 2005), which would influence interaction with immune receptors.

10.4 Relevance of Low Lipopolysaccharide and Lipid A Immunoactivities to H. pylori Pathogenesis: a Model

As seen with other chronic bacterial infections or colonizing commensal bacteria, the induction of low immunological responsiveness may aid the prolongation of H. pylori infection and aid chronicity (Lee and Moran, 1994). It has been hypothesized that H. pylori LPS, and its lipid A component in particular, have evolved their present structure, on the one hand, to fulfill their role in producing a functional macromolecular matrix for bacterial interaction with its environment and, on the other, to reduce the immune response to these essential molecules of the H. pylori outer membrane (Muotiala et al., 1992; Moran, 1995a, 1996). Thus, in producing a functional macromolecular matrix relevant to the microbial niche of H. pylori, the lipid A component, which is embedded in the outer membrane, would retain essential structural features to maintain structural integrity of the outer membrane, but undergo modification to allow survival within the specific microbial niche.

An example supporting this view concerns resistance to cationic antimicrobial peptides (CAMPs) produced by the innate immune system (see Kaparakis et al., Chapter 8, this volume). Of note, expression of human cathelicidin LL-37 (Hase et al., 2003) and human β-defensin 2 (Wada et al., 1999; Hamanaka et al., 2001) is up-regulated in the gastric mucosa of H. pylori-infected patients. Furthermore, H. pylori itself produces a ceropin-like peptide, Hp(2-20), to which it is resistant, that is derived from the amino-terminal part of its ribosomes protein L1 (Putsep et al., 1999). In order to persist in the human gastric mucosa, H. pylori must be able to resist the action of CAMPs. Importantly, in some other Gram-negative bacteria, ceropin, as an example of a CAMP, has been shown to bind to bis-phosphorylated lipid A (De Lucca et al., 1995), and masking the negative charge of their phosphate groups on lipid A with positively charged amine-containing substituents (e.g. ethanolamine and 4-amino-4-deoxy-L-arabinose) promotes CAMP resistance. As lipid A phosphate groups are necessary for binding of cationic peptides (Trent et al., 2006), the predominant lipid A molecular species, lacking 4′-phosphate and with PEtN substitution at C-1 of the H. pylori lipid A backbone, should promote resistance to CAMP during host infection. Experimentally, wild-type strains of H. pylori are inherently resistant to polymyxin, a CAMP, but disruption of lpxE (JHP0019) in H. pylori J99 results in the production of a lipid A bearing a single phosphate group at the 1-position, thereby producing a 25-fold...
In addition, the interactions of \textit{H. pylori} LPS via its lipid A component with a variety of immune recognition molecules and receptors, for example LPS-binding protein, CD14 and Toll-like receptors (TLRs), have consistently been reported to be low (reviewed in Moran, 2001a, 2007; Moran and Trent, 2008). Whether \textit{H. pylori} LPS and lipid A act as TLR4 agonists (see Kaparakis \textit{et al.}, Chapter 8, this volume), as classically seen with the majority of LPS of other Gram-negative bacteria, or as TLR2 agonists has been the focus of much discussion (Smith, M.F. \textit{et al.}, 2003; Mandell \textit{et al.}, 2004; Smith \textit{et al.}, 2006; Moran, 2007; Triantafilou \textit{et al.}, 2007; Uno \textit{et al.}, 2007; Moran and Trent, 2008). In any case, upon initial infection of the TLR4-expressing but TLR2-deficient gastric mucosa (Ortega-Cava \textit{et al.}, 2003; Mandell \textit{et al.}, 2004), \textit{H. pylori} is weakly recognized or unrecognized by TLR4 and may escape detection and elimination by the immune response initially. Consistent with this evasion of immune detection is the observation that, although functional TLR5 is expressed in the adult stomach (Schmausser \textit{et al.}, 2004), and TLR5 is considered to bind and respond to bacterial flagellins, \textit{H. pylori} flagellins FlaA and FlaB induce a very low activation of TLR5-mediated responses (Lee \textit{et al.}, 2003).

With progression of the immune response in long-term infection, a substantial inflammatory cytokine response to \textit{H. pylori} may develop only after the infiltration of TLR2-expressing granulocytes and monocytes into the infected gastric mucosa. \textit{H. pylori} can activate mononuclear cells by an LPS-independent, as well as a TLR4-independent mechanism (Mai \textit{et al.}, 1991), and hence non-LPS component(s) of the bacterium are the major inflammation-activating molecule(s). In general, because bacterial lipoproteins, lipopeptides and lipoteichoic acids are considered TLR2 ligands, the numerous putative lipoproteins encoded in the \textit{H. pylori} genome (Doig \textit{et al.}, 1999) potentially represent candidate TLR2 ligands (Moran, 2007). An \textit{H. pylori} heat-shock protein (HSP60) has been implicated in activation of TLR2 (Takenaka \textit{et al.}, 2004), but this protein has also been reported to induce interleukin (IL)-6 production by macrophages via a TLR2-independent mechanism (Gobert \textit{et al.}, 2004). Additionally, recognition of \textit{H. pylori}, which is considered to be predominantly non-invasive, by epithelial cells has been shown to be mediated by Nod1, an intracellular pattern-recognition molecule with specificity for peptidoglycan-derived muuropeptides, which are delivered intracellularly by the type IV secretion system-encoding \textit{cag} pathogenicity island (PAI) (Viala \textit{et al.}, 2004). This may be one mechanism by which \textit{cag}PAI-positive strains elicit a more vigorous inflammatory response and, in part, explain why these strains are associated with more aggressive disease symptoms, such as gastric cancer.

Moreover, as a consequence of enzymatic degradation of LPS by human phagocytes, some LPS and/or lipid A partially modified structures can be excreted by exocytosis. Such compounds retaining some immunological activity could play a role as subliminal, low-grade, persistent stimuli involved in \textit{H. pylori} pathogenesis (Jirillo \textit{et al.}, 1999; Moran, 1999). Notably, \textit{H. pylori} LPS can induce nitric oxide synthase in an \textit{in vivo} animal model, thereby contributing to intestinal damage (Lamarque \textit{et al.}, 2000; Kiss \textit{et al.}, 2001), and also influence gastric motor function (Quintana \textit{et al.}, 2005). Although controversial, whether, during long-term chronic infection of the gut mucosa by \textit{H. pylori}, low-grade LPS/lipid A stimuli contribute to extragastric infection sequelae remains an interesting question (Moran, 1999, 2007; Grebowska \textit{et al.} 2006) (see Mitchell \textit{et al.}, Chapter 5, this volume).

### 10.5 Lewis Antigen Expression

A further striking feature of \textit{H. pylori} LPS is the expression of Lewis and blood-group mimicry in the O-antigen component of this molecule (Moran, 2008). The Lewis (Le) antigens are biochemically related to the ABH blood-group antigens of humans that are formed by the sequential addition of \textit{l}-fucose, \textit{d}-galactose and \textit{N}-acetyl\textit{d}-galactosamine to the backbone carbohydrate chains of both lipids and proteins (Fig. 10.4). The structural basis of type 1 and type 2 backbone chains, as
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Fig. 10.4. Structure of the saccharide component of lipopolysaccharide of the \textit{Helicobacter pylori} type strain NCTC 11637 (A) and structures of the Lewis antigens expressed in \textit{H. pylori} strains (B) (for details see Aspinall \textit{et al.}, 1996; Moran, 2008). Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, \textit{N}-acetyl-glucosamine; \textit{dd}-Hep, \textit{d}-glycero-\textit{d}-manno-heptose; \textit{ld}-Hep, \textit{l}-glycero-\textit{d}-manno-heptose; Kdo, 3-deoxy-\textit{d}-manno-2-octulosonic acid; \textit{P}, phosphate; Neu5Ac, \textit{N}-acetyl-neuraminic acid (sialic acid).
well as Le and related antigens (e.g. H-1 and H-2), have been detailed elsewhere (Moran, 2001b, 2008). The mammalian genes controlling the expression and secretion of these molecules have been described (see Green, 1989). Of note, the terms secretor and non-secretor indicate the capacity of an individual to secrete such substances; secretors produce ABH, Le\textsuperscript{a} and Le\textsuperscript{b}, whereas non-secretors produce Le\textsuperscript{c} and Le\textsuperscript{d} in their saliva (Sakamoto et al., 1984).

Expression of Le\textsuperscript{a} or Le\textsuperscript{b} antigens is a common property of \textit{H. pylori} strains, as 80–90% of isolates from various geographical regions worldwide, which have been screened using anti-Le antibodies as probes, express these antigens (Moran, 2008). Nevertheless, serological analysis can underestimate Le\textsuperscript{a} and Le\textsuperscript{b} expression in a population of strains (Hynes and Moran, 2000), as some strains that are non-typeable with anti-Le antibodies have been shown to express these antigens when examined in structural studies (Knirel et al., 1999). The genetic determination of Le antigens and their biosynthetic pathways in \textit{H. pylori} have been extensively reviewed (Moran, 2001b, 2008; Ma et al., 2006; Moran and Trent, 2008). Collectively, factors affecting Le antigen expression in \textit{H. pylori}, which can thereby influence the biological impact of this molecular mimicry, include regulation of fucosyltransferase (FucT) genes through slipped-strand mispairing, the activity and expression levels of the functional enzymes, the preferences of the expressed enzyme for distinctive acceptor molecules, and the availability of activated sugar intermediates (Moran, 2008). With the accessibility of the crystal structure of \textit{H. pylori} FucTs, further insights should be gained into the molecular recognition and functioning of these enzymes (Sun et al., 2007).

Structurally, the O-chains of \textit{H. pylori} clinical isolates have a poly-N-acetyl-lactosamine (LacNAc) chain decorated with multiple lateral α-fucose residues forming internal Le\textsuperscript{a} units with terminal Le\textsuperscript{a} or Le\textsuperscript{b} units (Fig. 10.4) or, in some strains, with additional α-glucose or galactose residues (Aspinall et al., 1994, 1995, 1996, 1997, 1999; Knirel et al., 1999; Monteiro et al., 2000; Monteiro, 2001; Moran, 2001a,b, 2008; Moran et al., 2002a). Also, Le\textsuperscript{a}, Le\textsuperscript{b}; sialyl-Le\textsuperscript{a} and H-1 antigens have been structurally described in other strains, as well as the related blood groups A and B, but occur in association with Le\textsuperscript{a} and LacNAc chains (Moran, 2008). Overall, a mosaicism of Le antigen and blood group expression can occur in the same O-antigen, and hence, along with some variability in the core oligosaccharide of LPS, give rise to antigenic diversity that can be detected by antibody and lectin probing (Simoons-Smit et al., 1996; Hynes et al., 1999, 2002). On the other hand, \textit{H. pylori} strains that do not express Le antigens (Kocharova et al., 2000; Senchenkova et al., 2001; Monteiro et al., 2001; Britton et al., 2005) (Fig. 10.5) have been isolated from clinically asymptomatic individuals. The absence of Le antigen mimicry in these strains, and animal studies in which a genetically modified \textit{H. pylori} strain lacking Le antigen expression failed to induce gastritis compared with the parental strain (Eaton et al., 2004), are consistent with the view that Le antigen-expressing LPS contributes directly to disease development.

The biological and pathogenic roles of Le antigens expressed in the O-antigen of \textit{H. pylori} LPS have been the subject of recent reviews (Moran, 2008, 2009; Moran and Trent, 2008). Briefly, as extensively reviewed, \textit{H. pylori} Le expression has been implicated in evading the immune response upon initial infection and in influencing bacterial colonization and adhesion (see Lindén et al., Chapter 12, this volume). Furthermore and controversially, as chronic infection progresses, Le expression has been suggested to contribute to gastric autoimmunity leading to gastric atrophy (reviewed in Moran, 2008, 2009). As mentioned in Section 10.1, the discussion here focuses on the role of \textit{H. pylori}-expressed Le antigens in fitting the environmental niche of this bacterium.

### 10.6 Role of \textit{H. pylori}-expressed Lewis Antigens in Gastric Adaptation

In the human stomach, Le\textsuperscript{a} and Le\textsuperscript{b} are mainly expressed on the surface and foveolar epithelia, whereas Le\textsuperscript{a} and Le\textsuperscript{b} are predominantly...
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expressed by the mucous, chief and parietal cells of the gastric glands (Kobayashi et al., 1993), although the physical separation of Le antigens along the gastric pits is not always obvious (Murata et al., 1992). More specifically, in non-secretors the surface and foveolar epithelia express Leα, whereas these epithelia express Leβ and some Leγ in secretors; but irrespective of secretor status of the individual, the glandular epithelium lacks type 1 antigens (Leα and Leβ) and expresses type 2 antigens (Leα and Leγ), but in non-secretors Leγ expression is weaker in the glands (Sakamoto et al., 1989). Hence a variety of Le antigens are expressed within the ecological niche of *H. pylori*. On the basis of this known Le antigen expression in the gastric mucosa, it has been proposed that bacterial molecular mimicry and its adaptation could provide an escape for *H. pylori* from the humoral response by preventing the formation of anti-bacterial antibodies because of cross-reaction with the host (Wirth et al., 1997; Appelmelk et al., 2000).

In particular, an initial study that examined the relationship between infecting *H. pylori* isolates and gastric Le expression in the human host, as determined by erythrocyte Le(a,b) phenotype (i.e. secretor status), reported that the relative expression of Leα or Leγ by *H. pylori* strains corresponded to Le(a+,-b-) and Le(a-,-b+) blood group phenotypes, respectively, of the hosts from whom the individual strains were isolated (Wirth et al., 1997). Thus, it was inferred that because Leα and Leβ are isoforms of Leα and Leγ, and surface and foveolar epithelia express Leα in non-secretors and Leβ in secretors, that the correlations observed were a form of adaptation or selection of *H. pylori* strains to the gastric mucosa of the individual host (Wirth et al., 1997). A second study, performed in an identical manner but in a different patient population, did not find a correlation between Leα/Leγ on colonizing *H. pylori* isolates and host Le expression (Heneghan et al., 2000). Likewise, another study, which used the different approach of directly examining Le

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(A) 
\[\rightarrow 2\)-α-D-Man3CMe-(1→3)-α-L-Rha-(1→3)-α-D-Rha-α-(1→\]

(B) 
\[\rightarrow 2\)-α-DD-Hep-(1→3)-α-DD-Hep-(1→3)-α-DD-Hep-(1→\]

(C) 
\[\rightarrow 3\)-α-DD-Hep-(1,\]
\[\rightarrow 2\)-α-DD-Hep-(1→\]
\[\rightarrow 3\)-α-DD-Hep-(1→2)-α-DD-Hep-(1→3)-α-DD-Hep-(1→\]

\[\rightarrow 3\)-α-DD-Hep-(1→)],_n\]
\[\rightarrow 2\)-α-DD-Hep-(1→ [ 2\)-α-DD-Hep-(1→ ]_n\]

(D) 
\[\rightarrow 2\)-α-D-Glc-(1→3)-α-D-Glc-(1→\]

Fig. 10.5. Structures of the non-Lewis mimicking units of O-chains of *Helicobacter pylori* strains: (A) D1, D3 and D6; (B) D2, D4 and D5; (C) Hp1C2, Hp12C2, Hp62C, Hp7A, Hp77C and HpP1J; (D) serotype O2 (for details see Kochanov et al., 2000; Monteiro et al., 2001; Senchenkov et al., 2001; Britton et al., 2005). Abbreviations: Glc, glucose; dd-Hep, d-glycero-d-manno-heptose; Man3CMe, 3-C-methylmannose; Rha, rhamnose.
expression on gastric epithelial cells of infected human subjects, did not find a correlation (Taylor et al., 1998). Nevertheless, the discrepancies between these patient studies may reflect characteristics of the human populations under investigation and the study procedures employed (Moran, 1999; Broutet et al., 2002).

Of considerable importance is that, in the human study population where the correlation was observed, 26% of individuals were determined to be of the recessive Le phenotype, Le(a−,b−), based on erythrocyte testing (Wirth et al., 1997). This is higher than that usually observed for Caucasian or European populations, from which the two other study populations were drawn (Taylor et al., 1998; Heneghan et al., 2000). Importantly, using salivary testing rather than erythrocyte typing would have allowed the distribution of this phenotypically recessive group of patients to their true secretor/non-secretor phenotype and, based on theoretical calculations, would have influenced the correlative results (Heneghan et al., 1998). Also, in the two other studies, no patients of a recessive phenotype were observed (Taylor et al., 1998; Heneghan et al., 2000). Finally, despite high anti-LPS antibody titres (Appelmelk et al., 1996; Heneghan et al., 1996), no data are available as to whether the H. pylori-infected monkeys formed antibodies against Leα and Leγ that would have provided a selective pressure (Appelmelk et al., 2000). However, the use of transgenic animals as an H. pylori infection model, for example Leb−-expressing mice (Guruge et al., 1998), could help to more effectively resolve the question of whether host Le phenotype affects bacterial Le expression.

Of note, it has been demonstrated that H. pylori strains expressing Leα and those expressing Leγ can be isolated from the same host (Wirth et al., 1999), and that extensive diversity in expression of Leα and Leγ in H. pylori O-chains can occur over time and in different regions of the human stomach (Nilsson et al., 2006). Moreover, Nilsson et al. (2008) detected genetic modifications in H. pylori fucT genes that could be attributable to recombination events within and between these genes that creates diversity and which, together with phase variation, contributes to divergent LPS expression within an H. pylori colonizing community. The extent of this variation would apparently contradict the hypothesis of bacterial Le antigen adaptation to that of gastric Le expression as determined simply by the secretor status of the host (Wirth et al., 1997).

Notwithstanding this, the diversity of Le expression by H. pylori subclonal isolates in one host (Nilsson et al., 2006) may reflect an ability, and potential, of the bacterium to adapt to differing micro-niches and environmental conditions within the human stomach (Moran et al., 2002a; Keenan et al., 2008). For example, pH may vary locally in the stomach and has been shown to influence relative Leα/Leγ expression by H. pylori (Moran et al., 2002a). The pH may vary between the differing regions of the stomach (i.e. the antrum versus the corpus where the stomach’s acid-secreting cells are located), as well as varying across the microbial niches of the mucosa (i.e. pH 1–3 on the luminal surface of gastric mucus, about pH 4–5 within the mucus and pH 7 on the epithelial cell surface). Likewise, dietary iron can vary as can accessible iron for H. pylori acquisition (Keenan et al., 2004), which in turn affects pathogenesis of H. pylori.
and can influence the quantitative expression of LPS and the nature of Le antigen mimicry occurring in \textit{H. pylori} O-antigens (Keenan et al., 2008).

10.7 \textit{H. pylori}-expressed Lewis Antigens and Modulation of the Inflammatory Response

Contributing to the chronicity of \textit{H. pylori} infection by modulation of the immune response, strain-to-strain variability in Le expression can influence the interaction of \textit{H. pylori} LPS with the cellular innate immune receptor, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), a C-type lectin (Bergman et al., 2004). This binding contributes to a changed T-cell polarization after innate immune activation (Bergman et al., 2006). In the effector phase of an immune response, different T-cell subsets, called T-helper 1 (Th1) and T-helper 2 (Th2) cells, expand; Th1 cells promote proinflammatory cell-mediated immunity whereas Th2 cells promote humoral immunity that induces B cells to produce antibodies (O’Keeffe and Moran, 2008). Dendritic cells, in response to \textit{H. pylori}, secrete a range of cytokines, but preferentially IL-12 that induces a Th1 response, and also lesser amounts of IL-6 and IL-10 (Guiney et al., 2003). Since Le antigen expression is subject to phase variation (Appelmelk et al., 1999; Ma et al., 2006; Moran, 2008), a significant proportion of Le-negative variants can occur within a population of \textit{H. pylori} cells. Importantly, Le-negative variants of \textit{H. pylori} escape binding by dendritic cells and induce a strong Th1-cell response (Bergman et al., 2004). On the other hand, \textit{H. pylori} variants that express Le$^\alpha$/Le$^\gamma$ can bind to DC-SIGN on dendritic cells and enhance the production of IL-10, which promotes a Th2-cell response and blocking of Th1-cell activation (Bergman et al., 2006). Hence, a polarized Th1 effect can change to a mixed Th1/Th2-cell response through the extent of Le antigen–DC-SIGN interaction (Bergman et al., 2004). This modulation of the host response allows a switch from an acute infection response to one that allows development of chronic infection, as the humoral response and antibody production are not associated with \textit{H. pylori} eradication or protection (O’Keeffe and Moran, 2008). A similar alteration in the T-cell response, although based on over-expression of IL-10 but induced by polymeric Le$^\alpha$, has been observed in infection by eggs of the parasitic worm \textit{Schistosoma mansoni} (Velupillai and Harn, 1994; Moran et al., 1996).

Additionally, in patients with more aggressive inflammation and pathologies, the expression of both Le$^\alpha$ and Le$^\delta$ by \textit{H. pylori} may aid persistence of proinflammatory \textit{cagA}-positive strains (Wirth et al., 1996), i.e. strains carrying the \textit{cagPAI}-encoding type IV secretion system (see Backert et al., Chapter 11, this volume). This hypothesis was based on observations in a sample human population that \textit{H. pylori} isolates positive for Le$^\alpha$/Le$^\gamma$ were predominantly \textit{cagA}-positive, and that a genetically engineered \textit{cagA}-negative strain had diminished expression of Le$^\delta$ (Wirth et al., 1996). In contrast, two other studies drawn from a different human study population did not find an association between bacterial \textit{cagA} status and Le$^\alpha$/Le$^\gamma$ expression (Marshall et al., 1999; Heneghan et al., 2000). The discrepancy between these findings has been attributed to adaptation of \textit{H. pylori} strains with differing properties to the different human populations studied (Moran, 1999), a conclusion that was later supported in a pan-European study examining \textit{cagA} status and Le expression of isolates in differing ethnic populations (Broutet et al., 2002). Thus, Le$^\alpha$/Le$^\gamma$ expression by \textit{H. pylori} might aid persistence of more aggressive strains depending on the human host population.

10.8 Conclusions

\textit{H. pylori} has a well-established ability to evade and even subvert innate and adaptive immune responses during long-term infection and, as has been discussed (Moran, 2007, 2008), certain properties of \textit{H. pylori} LPS, particularly those associated with the O-chain and lipid A components, contribute to this. In addition, the properties of \textit{H. pylori} LPS,
which collectively contribute to both chronicity and disease development, conform with the proposal of *H. pylori* as a model for investigating and understanding the dynamics of bacterial persistence and parasitism in chronic infections (Blaser and Kirschner, 1999). In addition to the biological properties detailed above, a number of other bioactivities that have been attributed to *H. pylori* LPS and which influence pathogenesis, such as mucin production and quality, epithelial cell–mucin binding, trefoil factor binding, acid secretion, leptin induction and apoptosis, remain less well characterized (see Brzozowski *et al.*, 2000; Moran 2001b; Durkin *et al.*, 2006; Reeves *et al.*, 2008). The molecular structures within *H. pylori* LPS that are potentially responsible for these properties, and hence the relevant structure–bioactivity relationships, require establishment for unequivocal acceptance but could give further insights into the role of *H. pylori* LPS in pathogenesis.

Finally, despite being at an early stage of investigation, the comparative structural and biological characterization of LPS from different members of the genus *Helicobacter*, whether they colonize the gastric or hepatointestinal environments (Hynes *et al.*, 2004; Moran *et al.*, 2004; Sterzenbach *et al.*, 2007), is likely to yield new insights into the role of LPS in infection in general, and of *Helicobacter* pathogenesis in particular, in various compartments of the gut.

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11 Virulence Factors of *Helicobacter pylori*

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11.1 Introduction: Gastric Biology of *Helicobacter pylori* Infections

11.1.1 The interplay of multiple virulence and pathogenicity factors

The stomach has long been considered a sterile environment due to its low pH. The discovery of *H. pylori* in gastric biopsies by Barry Marshall and Robin Warren, a quarter of a century ago, radically changed this view and had fundamental consequences for our current understanding and treatment of gastric diseases (Marshall and Warren, 1984). This bacterium lives near the surface of the human gastric mucosa and is one of the most successful microbial pathogens. Half of the world’s population carries *H. pylori*, causing chronic gastritis in all infected human subjects, and more severe gastric disease in 10–15% of those infected (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002; Sepulveda and Graham, 2002; Suerbaum and Josenhans, 2007). Infections commonly occur in early childhood and can persist lifelong in the absence of antimicrobial therapy. Although *H. pylori* infection is consistently associated with an intense cellular inflammatory response that is initiated by the innate and adaptive immune systems, the bacteria are not eliminated. Several mechanisms of evasion have been identified, and *H. pylori* has become a paradigm for a persistent bacterium (Monack et al., 2004; Cover and Blanke, 2005; Rieder et al., 2005b; Wunder et al., 2006; Wilson and Crabtree, 2007). Studies have revealed not only the ability of *H. pylori* to colonize individual hosts for many decades, but also that this bacterium has coexisted with man for a very long time through history. Genetic studies indicate that *H. pylori* spread during human migrations from east Africa around 58,000 years ago (Linz et al., 2007). Because of this long period of co-evolution, it has been proposed that this colonization may have been beneficial for the human carrier and hence provided a selective advantage (Blaser and Atherton, 2004). In the modern world, however, infections with *H. pylori* exert a heavy toll of morbidity and mortality as a consequence of peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and, the most dangerous complication, gastric adenocarcinoma (Peek and Blaser, 2002; Sepulveda and Graham, 2002; Kusters et al., 2006; Correa and Houghton, 2007).

The clinical outcome of *H. pylori* infection is determined by a complex scenario of host–pathogen interactions (Blaser and Atherton, 2004; Peek and Crabtree, 2006).
the last few years, the cellular and molecular mechanisms used by *H. pylori* to subvert host defences have been investigated intensively (Fig. 11.1). Disease outcome is dependent on multiple parameters including both the bacterial genotype and genetic predisposition of the host as well as environmental factors. *H. pylori* isolates are surprisingly diverse in both their genome sequences and their pathogenicity. A myriad of bacterial factors have been shown to influence pathogenesis caused by *H. pylori* and can be classified as virulence factors and pathogenicity-associated factors. While virulence factors, by definition, have direct toxic or other damaging effects, pathogenicity factors are elements without direct damaging activities but which are also important, e.g. for establishment of successful bacterial colonization. There are two classical secreted virulence factors in *H. pylori*: the vacuolating cytotoxin (VacA) and the CagA protein encoded by the *cag* (cytotoxin-associated genes) pathogenicity island (*cag*PAI). Much of the research interest worldwide is focused on the CagA effector protein because *cag*-positive *H. pylori* strains are associated with the development of more severe gastric diseases. Other known pathogenicity-associated phenotypes include flagella-driven motility in the stomach mucus layer, acid neutralization via urease (UreA, UreB and accessory proteins), adhesion to gastric epithelial cells mediated by several adhesins (see Lindén et al., Chapter 12, this volume) and adherence of neutrophils to endothelial cells and chemotactic activities induced by the neutrophil-activating protein NapA (Graham and Yamaoka, 2000; Montecucco and Rappuoli, 2001; Aspholm et al., 2006; Dubois and Borén, 2007; Suerbaum and Josenhans, 2007) (Fig. 11.1). In contrast to many other bacteria, *H. pylori* lipopolysaccharide (LPS) has very weak proinflammatory activity, and was recently shown to bind trefoil peptides in the gastric lumen, which probably promotes colonization (Reeves et al., 2008). However, *H. pylori* induces proinflammatory responses by the concerted signalling activities of peptidoglycan, outer inflammatory protein A (OipA) and CagA, which activate the transcription factor nuclear factor-κB (NF-κB) (Graham and Yamaoka, 2000; Viala et al., 2004; Brandt et al., 2005). There are also numerous other marker genes for disease development such as *dupA* (duodenal ulcer promoting gene A) or *iceA* (induced by contact with the epithelium gene A), but their molecular functions and biological relevance are as yet unknown (Lu et al., 2005). In addition, specific polymorphisms in human genes involved in proinflammatory and immunoregulatory processes, such as those encoding interleukin (IL)-1β, IL-8, tumour necrosis factor-α (TNFα), NOD (nucleotide oligomerization domain) and others, have also been linked to an increased risk of developing gastric disease including cancer (Peek and Blaser, 2002; Amieva and El-Omar, 2008; see Sutton et al., Chapter 7, this volume). Moreover, *cag*PAI-positive *H. pylori* can induce the expression of a DNA-editing enzyme (AID) in host cells, resulting in the accumulation of mutations in the tumour suppressor TP53 (Matsumoto et al., 2007). Thus, induction of AID might be a mechanism whereby gene mutations accumulate during *H. pylori*-associated gastric carcinogenesis.

### 11.1.2 Animal and *in vitro* infection models

Recent functional studies in animal and cell culture models have provided compelling evidence for the importance of VacA, CagA and the *cag*PAI in *H. pylori* pathogenesis (Covacci and Rappuoli, 2000; Cover and Blanke, 2005; Rieder et al., 2005b; Backert and Meyer, 2006; Hatakeyama, 2008). A range of mouse, Mongolian gerbil and rhesus monkey models have proven useful for studying *H. pylori*-induced pathology *in vivo* (Hirayama et al., 1996; Watanabe et al., 1998; Ogura et al., 2000; Peek and Crabtree, 2006; Hornsby et al., 2008). For example, mice deficient in protein tyrosine phosphatase receptor type Z (Ptprz, also called PTP-ζ or RPTPβ) do not show mucosal damage and ulceration by VacA (Fujikawa et al., 2003). The gerbil is another useful model, as gastric pathology resulting from *H. pylori* infection is similar to that in humans and this animal has been used to evaluate virulence and colonization factors as
well as host responses (Kavermann et al., 2003; Franco et al., 2005; Rieder et al., 2005a). Much effort has been put into identifying the mechanism of *H. pylori*-associated carcinogenesis in gerbils and mice. A first direct causal link between CagA and oncogenesis in vivo was identified by the generation of transgenic C57BL/6J mice expressing CagA in the absence of *H. pylori*, some of which developed gastric polyps and adenocarcinomas of the stomach and small intestine (Ohnishi et al., 2008). Each animal model has distinct advantages and disadvantages and can, therefore, be considered as complementary systems (Rogers and Fox, 2004; Solnick et al., 2004; Rogers and Houghton, 2009).

In addition to these in vivo models, the use of several in vitro cell culture systems has proven very efficient for studying signalling cascades of relevance to disease development.

Fig. 11.1. Model for *Helicobacter pylori*-induced gastric damage, inflammation and persistence. The interplay between target cells and a variety of pathogenicity factors modulates multiple host responses leading to both chronic gastritis and persistent colonization. (1) *H. pylori* use their flagella to enter the mucous layer and secrete urease for pH buffering. (2) Several adhesins such as BabA/B, SabA, AlpA/B, HopZ and OipA mediate binding to gastric epithelial cells. (3) After adherence, *H. pylori* can translocate effector molecules such as CagA and peptidoglycan into the host cell. Peptidoglycan binds to the intracellular receptor NOD1 and activates transcription factor NF-κB to induce IL-8 release (Viala et al., 2004). *H. pylori* is also able to occasionally enter host cells (Kwok et al., 2002). Injected CagA can trigger several signalling cascades including the activation of proinflammatory transcription factor NF-κB, actin rearrangements and disruption of cell-to-cell junctions. (4) Disruption of cell junctions by CagA and cellular effects induced by VacA and urease may contribute to disruption of the epithelial barrier. Internalization of VacA into the cells leads to the formation of large vacuoles and gastric damage, a hallmark of the ulceration process. In addition, host cell functions are subverted by LPS, which can bind to trefoil peptides in the lumen, and the CGT enzyme allows host cholesterol glycosylation. (5) Injection of CagA appears to be dependent on integrins, which are receptors on the basolateral side of the epithelium. (6) VacA can also trigger apoptosis when recruited to the mitochondria to induce the release of cytochrome c. These processes may cause the disruption of epithelial barrier functions, leading to leakage of nutrients into the gastric lumen and to numerous bacterial pathogenicity factors crossing the epithelial layer. (7) *H. pylori* antigens can activate macrophages to release several proinflammatory cytokines, such as IL-8, IL-6, IL-1 and IL-12. This and the aforementioned induction of IL-8 by epithelial cells constitute crucial determinants for *H. pylori*-triggered inflammatory responses. These cytokines attract immune cells such as PMNs to infiltrate from the bloodstream into the gastric mucosa. Bacterial NapA seems to play a role in this process (Brisielt et al., 2005). (8) A number of indicated *H. pylori* factors have been shown to play a role in oxidative defence and are particularly important for bacterial survival when *H. pylori* comes in contact with PMNs. Antiphagocytosis, delayed phagocytosis and megasome formation are also observed. (9) The IL-12 micro-environment induced by activated macrophages is particularly important for shifting the CD4+ helper response into a prominent Th1 type. Pathogenicity factors such as urease and as yet unidentified cagPAI components appear to enhance the recruitment of T cells from the bloodstream (Enarsson et al., 2005). The production of TNFα, IFNγ and IL-1 from T cells and macrophages amplifies the VacA-induced apoptosis of epithelial cells. (10) *H. pylori* modulates the immune response by blocking T-cell proliferation using VacA based on down-regulation of IL-2 and IL-2R transcription (Boncristiano et al., 2003; Gebert et al., 2003). Inhibition of T-cell proliferation also occurs by the activities of CGT and GGT. Each of these activities is believed to contribute to the persistence and eventually to development of gastric disease. Abbreviations: AlpA/B, adherence-associated lipoproteins A and B; BabA/B, blood group antigen-binding adhesins A and B; CagA, cytoxin-associated gene A protein; CD4+ cells, cluster of differentiation 4-positive cells; CGT, cholesterol-glucosyltransferase; F-actin, filamentous actin; GGT, γ-glutamyl transpeptidase; Hop, *Helicobacter* outer-membrane porin; IFNγ, interferon-γ; IL, interleukin, IL-2R, interleukin-2 receptor; LPS, lipopolysaccharide; Mφ, macrophage; NapA, neutrophil-activating protein A; NF-κB, nuclear factor-κB; NO, nitric oxide; NOD1, nucleotide oligomerization domain protein 1; OipA, outer inflammatory protein A; PMN, polymorphonuclear neutrophil; RocF, protein with arginase activity; SabA, sialic acid-binding adhesin A; SD, superoxide dismutase; T1, cell, T-helper cell; TNFα, tumour necrosis factor-α; VacA, vacuolating cytotoxin. (Adapted from Backert et al. (2006) with kind permission from Elsevier.)
In particular, gastric epithelial and colonic cell lines (e.g. AGS, Caco2, KATO-III, AZ-521, HEP-2, MKN28 and MKN45), primary gastric epithelial cells and professional phagocytes, including human polymorphonuclear neutrophils (PMNs) and human or murine macrophage cell lines (e.g. JoskM, THP-1, U937, RAW264.7 and J774A.1), have been utilized. A series of well-studied phenotypic responses elicited during infection of both gastric epithelial cells and phagocytes in vitro is shown in Fig. 11.2. This includes induction of cell scattering, cell motility and dramatic cytoskeletal modifications in gastric epithelial cells resulting in the so-called ‘hummingbird’ or elongation phenotype, where the *cag*PAI and CagA have been shown to play a role (see Section 11.3.7). In addition, the induction of vacuolation and apoptosis can also be induced by VacA in these cells (see Sections 11.2.1 and 11.2.7). Each of these phenotypes probably contributes to *H. pylori*-induced disruption of epithelial barrier functions in vivo (Fig. 11.1). Such lesions are regularly seen in biopsies from patients with severe gastric disease, and suggest that *H. pylori* has direct contact with a variety of immune cells. Thus, long-term survival of the bacterium in the human stomach requires mechanisms to evade killing by the host immune system. Indeed, a multitude of such mechanisms have been identified using in vitro cell systems. For example, *H. pylori* actively retards uptake by macrophages and subsequently persists within large vacuoles called megasomes that result from phagosome fusion (Allen et al., 2000). Regulation of local actin polymerization and phagocytosis by atypical protein kinase C (PKC)-ζ plays a role in the delay of bacterial uptake (Allen and Allgood, 2002). In studies using human blood monocytes and PMNs, *H. pylori* has been shown to actively block its own phagocytosis (Ramarao et al., 2000b). Each of these phenotypes depends on the presence of a functional *cag*PAI and an unknown factor which appears not to be CagA. VacA also exhibits immune-suppressive effects (see Section 11.2.8) and arginase (encoded by *rocF*) that is expressed by *H. pylori* can down-regulate eukaryotic nitric oxide (NO) production (Gobert et al., 2001). The antimicrobial effect of NO is an essential part of innate immunity, thus down-regulation of NO may allow *H. pylori* to survive (Fig. 11.1). Other described pathogenicity-related factors include catalase or superoxide dismutase, with proposed functions in the oxidative defence of *H. pylori*, as well as cholesterol-α-glucosyltransferase and γ-glutamyl transpeptidase, which inhibit T-cell proliferation (Ramarao et al., 2000a; Wunder et al., 2006; Schmees et al., 2007; Carpenter et al., 2009). In addition, infection of JoskM and U937 cells with *cag*PAI-positive *H. pylori* is accompanied by the formation of large homotypic cell aggregates (Fig. 11.2). This occurs through up-regulation and recruitment of ICAM-1 (intracellular adhesion molecule-1) to the cell surface of infected cells, which then mediates aggregation via its ligand LFA-1 (lymphocyte function-associated molecule-1), a signalling pathway that may regulate cell–cell interactions, inflammatory responses and also inhibit bacterial uptake (Moese et al., 2002). In the following sections we describe in detail the epidemiology, function and signalling activities of the two major virulence constituents of *H. pylori*, VacA and CagA.

### 11.2 The Vacuolating Cytotoxin, VacA

#### 11.2.1 Discovery of VacA

Soon after the discovery of *H. pylori*, Leunk and co-workers (1988) reported that broth-culture supernatants of approximately 55% of *H. pylori* isolates contained a proteinaceous cytotoxin that induced cytoplasmic vacuolation in eukaryotic cells in vitro. Two other early studies indicated that the prevalence of infection with toxin-producing *H. pylori* is higher among infected persons with peptic ulceration than among infected persons with gastritis alone (Figura et al., 1989; Goossens et al., 1992). Multiple cell types from man and animal species were susceptible to its toxic activity. Subsequently, a protein with vacuolating activity was purified from *H. pylori* broth-culture supernatants, suggesting that it was secreted as a soluble toxin (Cover and Blaser, 1992). Under denaturing conditions, the molecular mass of the purified cytotoxin was ~87 kDa and the non-denatured form was estimated to be about 972 kDa in size.
suggesting that VacA can assemble into large oligomeric structures (Cover and Blaser, 1992). Antiserum raised against the purified cytotoxin completely neutralized vacuolating activity. Similarly, cytotoxin activity was neutralized by serum immunoglobulin (Ig) G antibodies from a subset of \textit{H. pylori}-infected persons, but not by serum IgG from uninfected persons. The gene encoding the vacuolating cytotoxin was termed \textit{vacA} and has been cloned from several strains (Cover \textit{et al}., 1990; Phadnis \textit{et al}., 1994; Schmitt and Haas, 1994; Telford \textit{et al}., 1994). The derived protein sequence of VacA does not show striking sequence similarity to any other known bacterial or eukaryotic protein, but exhibits short stretches homologous to ion channel or transport proteins (Cover and Blaser, 1992). However, the structural organization of VacA closely resembles the IgA protease type of exoproteins produced by pathogenic \textit{Neisseria} and \textit{Haemophilus} (Schmitt and Haas, 1994) that are today known as the autotransporter or type-V secretion family of proteins (Backert and Meyer, 2006). In response to acidic pH, VacA undergoes a structural change that increases its activity and renders it resistant to proteolysis by pepsin (de Bernard \textit{et al}., 1995). Thus, VacA is ideally suited to activity in the acidic gastric milieu.

\textbf{Fig. 11.2.} Phenotypic responses during infection of AGS gastric epithelial cells and immune cells with \textit{Helicobacter pylori} \textit{in vitro}. Infection of cultured AGS cells with \textit{H. pylori} results in different phenotypes that can be observed in a time- and strain-dependent manner. First, AGS infected with cytotoxin-associated genes pathogenicity island (cagPAI)-positive \textit{H. pylori} exhibit a strong motility response (after 1–2 h) followed by elongation of the cells (after 3–4 h) (Moese \textit{et al}., 2004). Vacuole formation can be observed during infection with toxin-producing \textit{H. pylori} (after 3–6 h). Vaculating cytotoxin (VacA) is also implicated in triggering apoptosis of infected epithelial cells (after 24–48 h). Infection of phagocytes by cagPAI-positive \textit{H. pylori} can induce delayed phagocytosis (Allen \textit{et al}., 2000) or anti-phagocytosis (Ramarao \textit{et al}., 2000b) and homotypic aggregation of the infected cells (Moese \textit{et al}., 2002). Bars, 5 µm. (Adapted from Backert \textit{et al}., 2006) with kind permission from Elsevier and Blackwell Publishing. The pictures for delayed phagocytosis and megasome formation were originally published by Allen \textit{et al}., (2000) and were kindly provided by Lee-Ann Allen with permission of \textit{The Journal of Experimental Medicine}.)
11.2.2 VacA expression, secretion, maturation and allelic variation

Although \( \text{vacA} \) is predicted to encode a potential 135–145 kDa protein depending on the strain, VacA proteins of this size have never been reported (Cover and Blanke, 2005). Like other classical autotransporters, VacA undergoes cleavage during the transport via bacterial membranes (Fig. 11.3A), and VacA contains a 33-amino-acid signal sequence. Three VacA protein spots were identified by two-dimensional gel electrophoresis (2-DE) in the TIGR strain 26695. The most dominant VacA species have apparent molecular masses of approximately 95.7 kDa and 87.9 kDa, found both in the \( \text{H. pylori} \) membrane and in culture supernatant fractions (Bumann et al., 2002; Backert et al., 2005). In addition, a 10.5 kDa VacA spot was found in the secreted protein fraction (Bumann et al., 2002). Mapping data of the identified peptides are consistent with a model where the 95.7 kDa VacA protein is the secreted toxin, which is then cleaved into an 87.9 kDa mature protein (p88) and a 10.5 kDa passenger domain (p10, also called the \( \alpha \)-protein) (Fig. 11.3A). The exact cleavage site between p98 and the
β-domain was detected between amino acids 991 and 992 (Bumann et al., 2002). The processed β-domain of the autotransporter domain was not detected by 2-DE but has been observed in total cell lysates (Telford et al., 1994). However, the mature, secreted p88 subunit can undergo further limited proteolytic cleavage to yield two fragments, p33 and p55 (Telford et al., 1994; Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder et al., 2005b) (Fig. 11.3A, bottom). The latter two processing products are considered to represent two functional domains or subunits of VacA. Cell binding is mediated by the p33 fragment of mature VacA (Reyrat et al., 1999) while both p33 and p55 have diverse functions (see Sections 11.2.3 to 11.2.9).

The vacA gene is present among virtually all H. pylori strains worldwide, but exhibits considerable sequence variation. In some studies, VacA and its subunits have slightly different sizes, but we will use the p33 and p55 nomenclature throughout this chapter. The basis of this sequence variation was found in several vacA alleles that have been identified in the signal region (genotypes s1 and s2) and in the mid-region (genotypes m1 and m2), occurring in all possible combinations (Fig. 11.3B). VacA molecules of the s2 genotype are inactive in assays for vacuolating cytotoxicity, genotype s1/m1 produces an extensive vacuolation phenotype in a large variety of cell types, and genotype s1/m2 produces detectable vacuolation in a more restricted range of

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**Fig. 11.3.** Processing, allelic variation and molecular structure of vacuolating cytotoxin (VacA). (A) The amino-terminal signal sequence (SS) and carboxy-terminal domain are cleaved from the 135–145 kDa VacA protoxin to yield a 98 kDa secreted toxin (p98) that is transported into the extracellular space via an autotransporter mechanism (Cover and Blanke, 2005). Proteins of this autotransporter family are able to mediate their own secretion without the need of additional bacterial proteins. The 33 kDa carboxy-terminal β-barrel domain of VacA is predicted to insert into the outer membrane and to form a channel through which the mature VacA toxin is secreted. The secreted toxin can be processed into the mature toxin (p88) and p10. The mature, secreted p88 subunit can undergo further limited proteolytic cleavage to yield two fragments, p33 and p55 (Telford et al., 1994; Blaser and Atherton, 2004; Cover and Blanke, 2005), believed to represent two functional domains or subunits of VacA. (B) A high level of sequence diversity occurs among vacA alleles from different H. pylori strains. The vacA gene is a polymorphic mosaic with two possible signal regions (designated s1 and s2) and two possible mid-regions (designated m1 and m2). Mosaic forms of vacA are thought to arise via homologous recombination among vacA alleles from different strains and different combinations can occur as indicated. The s1 signal region is fully active, but the s2 region encodes a protein with a different signal-peptide cleavage site, resulting in a short amino-terminal extension to the mature toxin that blocks vacuolating activity and attenuates pore-forming activity. The mid-region encodes a cell-binding site, but the m2 type binds to and induces the vacuolation phenotype in fewer cell lines in vitro. (C) Crystal structure of the p55 subunit. p55 adopts a β-helix structure composed of three parallel β-sheets connected by loops of varying length and structure (Gangwer et al., 2007). The α1-helix is contained within one of the loops to exemplify how it caps the end of the β-helix. The carboxy-terminal domain has a mixture of α/β secondary structure elements and contains a disulfide bond, not previously observed in an autotransporter passenger domain structure. The carboxy-terminus of the β-helix shown to the right is capped by a β-hairpin from the carboxy-terminal domain and the α1-helix located in one of the long helix loops. This view represents a rotation of the molecule on the left side by -90° into the plane of the page. (D) Docking p55 into a 19 Å cryo-EM (electron microscopy) map of the VacA dodecamer results in a model for oligomerization. Twelve p55 subunits are shown docked into a 19 Å cryo-EM map of a VacA dodecamer (El-Bez et al., 2005). Gangwer et al. (2007) proposed an oligomerization model in which p33 interacts with the amino-terminal portion of p55 from the neighbouring subunit. Regions of contact between p33 and p55 are depicted with dashed lines (right structure). From EM images, the shape of a VacA hexamer (inset, left) is similar to the shape of a single layer within the dodecamer (El-Bez et al., 2005). The rod-like shape of the p88 VacA monomer (inset, right) supports a model in which the β-helix observed in p55 will extend into p33. (Parts C and D were originally published in Gangwer et al. (2007) and were kindly provided by Borden Lacy with permission of the National Academy of Sciences, USA.)
cell types in agreement with its location in p55 being the VacA binding domain (Montecucco and Rappuoli, 2001; Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder et al., 2005b). Most of the research has focused on the most interactive (vacuolating) type, s1/m1. In addition, two intermediate region variants (i1 and i2) have been identified as important determinants of VacA toxicity (Rhead et al., 2007).

### 11.2.3 Molecular structure of VacA

The secreted p88 toxin can assemble into water-soluble oligomeric structures (Lupetti et al., 1996) and can insert into planar lipid bilayers to form anion-selective membrane channels (Czajkowsky et al., 1999; Iwamoto et al., 1999; Tombola et al., 1999). These structures resemble ‘flowers’ in which a central ring is surrounded by peripheral ‘petals’ (Fig. 11.3D). The first reported high-resolution images were 19 Å cryo-EM (electron microscopy) maps of VacA dodecamers (El-Bez et al., 2005), and atomic-force microscopy and electrophysiological studies suggest that membrane-associated VacA channels are single-layered structures (Czajkowsky et al., 1999; Iwamoto et al., 1999). When the p33 and p55 subunits are expressed independently and then mixed, p33 and p55 physically interact and reconstitute vacuolating activity (Ye et al., 1999; Torres et al., 2004, 2005). The p33 domain contains a hydrophobic sequence (residues 6–27) involved in pore formation (Vinion-Dubiel et al., 1999; McClain et al., 2003), whereas p55 contains one or more cell-binding domains (Reyrat et al., 1999; Wang and Wang, 2000). The 2.4 Å crystal structure of p55, which has an important role in mediating VacA binding to host cells, has recently been reported (Gangwer et al., 2007). The structure is predominantly a right-handed parallel β-helix, a feature that is characteristic of autotransporter passenger domains but unique among known bacterial toxins (Fig. 11.3C). Notable features of p55 include disruption of β-sheet contacts that result in five β-helix subdomains and a carboxy-terminal domain which contains a disulfide bond. Interestingly, p55 contains the m1 and m2 alleles, and investigation of protein sequences from unrelated *H. pylori* strains allowed the identification of structural features of the VacA surface that may be important for interactions with host receptors (Gangwer et al., 2007).

### 11.2.4 VacA targets multiple host surface molecules of epithelial and immune cells

In an effort to understand how VacA contributes to *H. pylori* colonization of the stomach and the development of gastroduodenal disease, the cellular effects of VacA have been investigated both *in vivo* and *in vitro*. A model for the complex interactions of VacA with multiple cell types and downstream signalling events is shown in Fig. 11.4 and interaction partners are summarized in Table 11.1. Like many bacterial toxins, VacA interacts with the plasma membrane of susceptible cells as a first step in intoxication (Cover and Blanke, 2005). Subsequent to binding, VacA is internalized by a pinocytic-like mechanism (Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder et al., 2005b). A remarkable feature of VacA is that it can target multiple cell-surface components *in vitro* or on epithelial cells, including RPTPβ (receptor protein-tyrosine phosphatase β) (Yahiro et al., 1999; Fujikawa et al., 2003), RPTPa (Yahiro et al., 2003), fibronectin (Hennig et al., 2005), the epidermal growth factor (EGF) receptor (Seto et al., 1998), heparin sulfate (Utt et al., 2001), various lipids (Moll et al., 1995; Molinari et al., 1998b; Czajkowsky et al., 1999) and sphingomyelin (Gupta et al., 2008), as well as CD18 (β2-integrin) on T cells (Sewald et al., 2008). Determining which of these factors is/are required for internalization and subversion of cellular signalling has been a challenging topic of research and is still not fully elucidated.

The role of RPTPs for VacA-dependent activities has been studied in great detail. For example, VacA causes gastric injury in wild-type mice but not in RPTPβ-knockout mice, which indicates that interaction of VacA with RPTPβ on the surface of gastric epithelial cells is mechanistically important in VacA-induced gastric injury (Fujikawa et al., 2003).
### Table 11.1. Interaction partners of vacuolating cytotoxin (VacA) and proposed roles in *Helicobacter pylori* infection.

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<th>Interaction partner</th>
<th>Proposed function</th>
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<th>Applied methods</th>
<th>Reference(s)</th>
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<tr>
<td>Anionic lipid bilayers</td>
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<td>Binding in vitro</td>
<td>AFM</td>
<td>Czajkowsky et al. (1999)</td>
</tr>
<tr>
<td>CD18 (β₂-integrin)</td>
<td>Receptor on T cells</td>
<td>Infection in vitro</td>
<td>IF, live cell imaging, use of knockout T cells, flow cytometry</td>
<td>Sewald et al. (2008)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Receptor on epithelial cells</td>
<td>Infection in vitro</td>
<td>IP, antibody blocking</td>
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<tr>
<td>Fibronectin</td>
<td>Binding of <em>H. pylori</em> to host cells</td>
<td>Binding in vitro</td>
<td>Cell adhesion and binding assays</td>
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</tr>
<tr>
<td>Glycosphingolipids</td>
<td>Binding partner for VacA internalization</td>
<td>Binding in vitro</td>
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<tr>
<td>Heparin sulfate</td>
<td>Receptor/co-receptor on epithelial cells</td>
<td>Binding in vitro</td>
<td>SPR-based biosensor studies</td>
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<tr>
<td>Lipid vesicles</td>
<td>Binding and host cell entry of the toxin</td>
<td>Binding in vitro</td>
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<tr>
<td>Lipid rafts</td>
<td>Putative docking and entering sites on cells</td>
<td>Treatment of cells in vitro</td>
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<tr>
<td>Phospholipids</td>
<td>Binding and host cell entry of the toxin</td>
<td>Binding in vitro</td>
<td>Photolabelling and ANS-binding studies</td>
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<tr>
<td>RPTPα</td>
<td>Receptor on epithelial cells</td>
<td>Infection and treatment of cells in vitro</td>
<td>IP, MS, antisense silencing</td>
<td>Yahiru et al. (2003); De Guzman et al. (2005)</td>
</tr>
<tr>
<td>RPTPβ</td>
<td>Receptor on epithelial cells, receptor for Git1β phosphorylation</td>
<td>Infection of mice and cells in vitro</td>
<td>IP, IF, siRNA, flow cytometry, use of knockout mice</td>
<td>Yahiru et al. (1999); Padilla et al. (2000); Fujikawa et al. (2003)</td>
</tr>
<tr>
<td>RACK-1</td>
<td>Not determined</td>
<td>Binding in vitro</td>
<td>Y2H, PD</td>
<td>Hennig et al. (2001)</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>Receptor on epithelial cells</td>
<td>Binding and treatment of cells in vitro</td>
<td>Binding studies, flow cytometry, ELISA, treatment with sphingomyelinase</td>
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</tr>
<tr>
<td>VIP54</td>
<td>Interaction between intermediate filaments and late endosomes</td>
<td>Infection in vitro</td>
<td>Y2H, IP, IF</td>
<td>de Bernard et al. (2000)</td>
</tr>
</tbody>
</table>

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*Abbreviations: EGFR, epidermal growth factor receptor; RACK-1, receptor for activated C-kinase 1; RPTPα/β, receptor protein-tyrosine phosphatase α/β; VIP54, VacA-interacting protein of 54 kDa.*

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*Abbreviations: AFM, atomic force microscopy; ANS, 1-anilino-8-naphthalene sulfonate; IF, co-localization by immunofluorescence; IP, co-immunoprecipitation; MCD, methyl-β-cyclodextrin; MS, mass spectrometry; PD, pull-down experiments; siRNA, silencing RNA knockdown; SPR, surface plasmon resonance; Y2H, yeast two-hybrid screening.*
Interestingly, VacA can still induce vacuolation in cultured RPTPβ−/− gastric epithelial cells and in G401 human kidney cells, which do not express RPTPβ (Fujikawa et al., 2003; Yahiro et al., 2003), but down-regulation of RPTPβ by silencing oligonucleotides in HL-60 cells inhibited VacA-induced vacuolation (Padilla et al., 2000). These data indicate that RPTPβ has an important role in the process of VacA-induced signalling but not necessarily vacuolation per se. VacA has also been shown to induce tyrosine phosphorylation of the G-protein-coupled receptor-kinase-interacting protein-1 (Git1) (Fujikawa et al., 2003) and lipid raft-dependent signalling (Nakayama et al., 2006). In line with these observations, VacA was shown to bind to a QTTQP motif (positions 747–751) in the extracellular domain of RPTPβ which was also necessary for inducing Git1 phosphorylation (Yahiro et al., 2003). Another reported consequence of VacA binding to RPTPβ is detachment of primary murine gastric epithelial cells from matrices (Fujikawa et al., 2003). In addition to the RPTPs, VacA has been shown to interact with lipid preparations and artificial membranes, but the specificity and importance of VacA–lipid interactions for toxin function was unknown for a long time. Recently, evidence was provided that sphingomyelin modulates the sensitivity of epithelial cells to VacA (Gupta et al., 2008). Sphingomyelin is a key structural component of the cell membrane and, in particular, lipid rafts, while at the same time serving a functional role as the parent compound of several lipid mediators. Sphingomyelin was shown to be important for VacA binding to cells and its association with lipid rafts on the plasma membrane, suggesting that sphingomyelin functions as a receptor for VacA on epithelial cells (Gupta et al., 2008). However, VacA...
interacts not only with gastric epithelial cells but also with immune cells including macrophages, B cells and T cells (Fig. 11.4). For example, VacA efficiently enters activated, migrating primary human T lymphocytes by binding to the CD18 receptor and exploiting the recycling of LFA-1 (Sewald et al., 2008). LFA-1-deficient Jurkat T cells were resistant to vacuolation, and genetic complementation restored sensitivity to VacA. Interestingly, VacA targeted human but not murine CD18 for cell entry, consistent with the species-specific adaptation of *H. pylori* (Sewald et al., 2008).

### 11.2.5 VacA-dependent effects on endosomal maturation and vacuolation of epithelial cells

The first identified effect of internalized VacA was its ability to induce cell vacuolation in many cell types (Leunk et al., 1988). A current model for vacuole formation proposes that VacA binds to the plasma membrane of target cells, is internalized, forms anion-selective channels in endosomal membranes and the vacuoles arise due to swelling of endosomal compartments (Fig. 11.4, middle). VacA-induced vacuoles are hybrid compartments of late endosomal origin containing some lysosomal markers (Molinari et al., 1997). Vacuole production depends on the presence and activity of a number of cellular factors including V-ATPase (Papini et al., 1993) and three GTPases, Rab7 (Papini et al., 1997), Rac1 (Hotchin et al., 2000) and dynamin (Suzuki et al., 2001). Protein kinase PIKfyve is also necessary for vacuole formation, and microinjection of its substrate PIP2 (phosphatidylinositol-3,5-bisphosphate) is sufficient to induce vacuoles (Ikonomov et al., 2002), whereas involvement of the SNARE protein syntaxin-7 is more controversial (de Bernard et al., 2002; Suzuki et al., 2003). When expressed intracellularly, the minimum portion of VacA required for cell-vacuolating activity comprises the entire p33 domain and about 110 amino acids from the amino terminus of p55 (de Bernard et al., 1998; Ye et al., 1999). Vacuolating activity also depends on VacA dimer or oligomerization (Willhite et al., 2002; Ye and Blanke, 2002) and requires the hydrophobic amino terminus (~30 amino acids) containing three tandem GXXXG motifs, which are transmembrane dimerization sequences (McClain et al., 2003; Kim et al., 2004). These requirements indicate that channel formation is a prerequisite for vacuolation, which is also supported by the fact that pharmacological anion-channel inhibitors block vacuole formation (Szabó et al., 1999).

### 11.2.6 VacA-induced effects on epithelial monolayer permeability

One of the characteristics of functional epithelia is the development of transepithelial electrical resistance (TER), a measurable indicator of epithelial integrity. In polarized host cell monolayers, VacA was shown to decrease TER significantly (Papini et al., 1998). This effect was observed in several different types of polarized epithelial cells including MDCK, T84 and EPH4. Interestingly, this effect was independent of VacA’s vacuolating activity as also observed with *vacA m2* alleles (Pelicic et al., 1999). It was proposed that selective permeabilization of epithelial monolayers by VacA results in the release of molecules such as Fe³⁺, Ni²⁺, sugars and amino acids, which might support the growth of *H. pylori* within the gastric mucus layer (Papini et al., 1998; Pelicic et al., 1999). As yet, the mechanism by which VacA alters paracellular permeability is not well understood. It has also been noted that VacA increases the transepithelial flux of certain molecules such as urea – the substrate of *H. pylori* urease (Tombola et al., 2001). The release of urea from cells, however, is attributed to the formation of VacA channels in the plasma membrane. Further studies have indicated that disruption of cell–cell junctions in polarized epithelia by *H. pylori* is not dependent on VacA as a sole factor, but also requires injected CagA, which targets both tight and adherens junctions (see Section 11.3.8). More complexity arises from another recent study showing that *H. pylori* induced a progressive loss of barrier function in MKN28 gastric epithelial cells and hypergastrinaemic INS-GAS mice, which was attenuated by inactivation of the *ureB* gene, but not *vacA* or...
Virulence Factors of *H. pylori* genes (Wroblewski *et al*., 2009). Interestingly, in that study *H. pylori* induced the deregulation of specific tight-junction proteins, including occludin internalization and phosphorylation of MLC (myosin regulatory light chain) by MLC kinase. Thus, epithelial barrier disruption by *H. pylori* is highly complex, requiring multiple bacterial factors and signalling pathways (Wessler and Backert, 2008).

### 11.2.7 Internalized VacA targets mitochondria to induce apoptotic cell death

Infection with *H. pylori* has been associated with both increased and reduced levels of apoptosis in the gastric epithelium (Blaser and Atherton, 2004). In *vitro*, *H. pylori* reproducibly stimulates apoptosis in infected gastric epithelial cells, and this has also been seen in tissues isolated from infected patients or animal models (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). Early studies indicated that purified VacA induced mitochondrial damage, indicated by a dramatic decrease in cellular ATP levels (Kimura *et al*., 1999). In transfected HEp-2 cells, p33 was found to localize specifically to mitochondria, whereas p55 was cytosolic (Galmiche *et al*., 2000). When purified VacA was incubated with purified mitochondria, p33 (but not p55) was translocated into these organelles. Transient expression of p33–green fluorescent protein (GFP) or VacA–GFP in HeLa cells induced the release of cytochrome c from mitochondria and activated caspase-3, as determined by the cleavage of poly (ADP-ribose) polymerase (PARP). PARP cleavage was antagonized specifically by co-transfection of DNA encoding Bcl-2, known to block mitochondria-dependent apoptotic signals (Galmiche *et al*., 2000). These findings are supported by *in vitro* infection studies using wild-type and vacA mutant *H. pylori* strains (Kuck *et al*., 2001; Cover *et al*., 2003), which show that the s1/m1 type of VacA induces high levels of apoptosis, while s2/m1 toxin or a vacA mutant lacking the hydrophobic region near the amino terminus does not.

These results indicate that VacA induces gastric epithelial cell apoptosis and suggest that differences in levels of gastric mucosal epithelial apoptosis among *H. pylori*-infected persons may result from strain-dependent variations in VacA structure. However, for gastric cell apoptosis *in vivo*, several other parameters are likely to be important. Normally, rapid self-renewal of gut epithelia, which occurs by a balance of progenitor proliferation and pit-cell apoptosis, serves as a host defence mechanism to limit bacterial colonization. It was demonstrated that *H. pylori* inhibits the apoptotic loss of pit cells in Mongolian gerbils (Mimuro *et al*., 2007). Suppression of apoptosis contributed to pit hyperplasia and persistent bacterial colonization of the stomach, and this was mediated by CagA, which stimulated the pro-survival mitogen-activated protein (MAP) kinase members ERK1/2 and anti-apoptotic protein MCL-1 in the gastric pits. Thus, CagA counteracts VacA effects and activates host cell survival and anti-apoptotic pathways to overcome self-renewal of the gastric epithelium and help sustain *H. pylori* infection (Mimuro *et al*., 2007).

### 11.2.8 VacA exhibits suppressive effects on immune cells

Research since the late 1990s has provided compelling evidence that, during evolution, *H. pylori* has accumulated multiple activities to suppress immune cell functions. Some of these functions have been attributed to VacA (Fig. 11.4). A very early study demonstrated that VacA can inhibit processing and presentation of antigenic peptides to human CD4+ T cells (Molinari *et al*., 1998a) and suggested for the first time that VacA may contribute to the persistence of *H. pylori* by interfering with protective immunity (Molinari *et al*., 1998a). In infected professional phagocytes, such as human THP-1 and mouse RAW 264.7 macrophage cell lines, wild-type *H. pylori* displayed an enhanced survival compared with vacA deletion mutants (Zheng and Jones, 2003). Thus, it appears that VacA arrests phagosome maturation by recruiting and retaining tryptophan aspartate-containing coat protein (TACO or coronin-1). However, increased survival of isogenic vacA mutants was not seen in two other reports when freshly
isolated human monocytes were infected (Ramarao et al., 2000b; Rittig et al., 2003). These differences may be due to different vacA alleles, infective dose and/or cell types used in the different studies.

Infection or co-incubation of purified VacA with T lymphocytes yields multiple effects. VacA specifically blocks the antigen-dependent proliferation of Jurkat T cells by interfering with IL-2-mediated signalling (Boncristiano et al., 2003; Gebert et al., 2003). As discussed above, H. pylori targets CD18 (β2-integrin) as a VacA receptor on human T lymphocytes. After entry, VacA inhibits Ca\(^{2+}\) mobilization and, subsequently, down-regulation of the activity of the Ca\(^{2+}\)-dependent phosphatase calcineurin. This in turn inhibits activation of the transcription factor NFAT (nuclear factor of activated T cells). Thus, NFAT target genes such as those encoding IL-2 and the high-affinity IL-2 receptor (IL-2Rα) are not expressed (Fig. 11.4). VacA, however, exerts a different effect on primary human CD4\(^+\) T cells, whose proliferation is inhibited through the T-cell receptor CD28, and eventually by other VacA activities (Sundrud et al., 2004). In the latter study, VacA was shown to suppress IL-2-induced cell-cycle progression and proliferation of primary T cells without affecting NFAT. Thus, VacA may inhibit the clonal expansion of T cells that have been activated by bacterial antigens, thereby allowing H. pylori to evade the adaptive immune response, resulting in chronic infection.

11.2.9 Other VacA-induced effects on cellular signal transduction pathways

Besides the VacA-induced activities described above, several other effects have been reported. Yeast two-hybrid studies and other experiments have demonstrated VacA’s potential for specific binding to RACK-1 (receptor for activated C-kinase 1) (Hennig et al., 2001) or to the intermediate filament-binding protein VIP54 (de Bernard et al., 2000), but its importance for infection remains unknown. Moreover, purified VacA or VacA-containing culture supernatants exhibit suppressive effects on proliferation, migration or wound healing of gastric epithelial cells in vitro (Rici et al., 1996; Pai et al., 1998, 2000; Tabel et al., 2003). In these studies it was shown that VacA can block the activity of externally added EGF, suggesting that VacA interferes with EGF receptor (EGFR) signalling. Indeed, VacA preparations or infection with VacA-expressing H. pylori exhibit(s) suppressive effects on phosphorylation of EGFR (Pai et al., 1998; Tegtmeyer et al., 2009) and HER2/Neu, another member of the EGFR family (Tegtmeyer et al., 2009). Immunofluorescence data showed internalized EGFR, possibly due to endocytosis, in infections with VacA-expressing H. pylori, providing a possible mechanism for how significant portions of EGFR are inactivated by VacA (Tegtmeyer et al., 2009). Interestingly, RPTPα but not vacuolation per se plays a role in this process. Further investigation is thus necessary to reveal which mechanism is involved in VacA-dependent inactivation of EGFR and HER2/Neu.

The effect of VacA on endosomal and lysosomal functions has also been studied by following procathepsin D maturation and EGF degradation in HeLa cells exposed to the toxin. VacA inhibited the conversion of procathepsin D (53 kDa) into both the intermediate (47 kDa) and the mature (31 kDa) form, and intracellular degradation of EGF was also suppressed (Satin et al., 1997). This suggests that VacA can also impair the degradative power of late endosomes and lysosomes. Further studies indicated that addition of VacA to AZ-521 cells activates two classes of MAP kinases (p38 and ERK1/2) and the activating transcription factor 2 (ATF-2) signalling pathway (Nakayama et al., 2004). VacA has also been reported to induce activation of p38 in several other types of cells (Boncristiano et al., 2003). Pharmacological inhibition of p38 kinase activity, however, did not block the vacuolation phenotype nor mitochondrial damage, which indicates that VacA-induced activation of the p38/ATF-2 signalling cascade is independent of endosomal and mitochondrial pathways of VacA (Nakayama et al., 2004). In another cell culture model system, RBL-2H3 mast cells, binding of VacA results in a rapid change in cytosolic calcium concentrations (de Bernard et al., 2005). This is accompanied by stimulation of proinflammatory cytokines, including TNFα
and IL-6, resulting in chemotaxis and degranulation of these mast cells (Supajatura et al., 2002; de Bernard et al., 2005). Finally, VacA treatment stimulates expression of cyclooxygenase-2 (COX-2), a proinflammatory enzyme in neutrophils and macrophages (Boncristiano et al., 2003). Thus, VacA is a remarkable bacterial virulence factor with multiple activities on epithelial and immune cells.

11.3 The Cytotoxin-associated Genes Pathogenicity Island (cagPAI)

11.3.1 Discovery of CagA and cagPAI genes

CagA was discovered in the early 1990s by pioneering work in the labs of Martin Blaser (Cover et al., 1990), Jean Crabtree (Crabtree et al., 1991) and Antonello Covacci (Covacci et al., 1993). Initially, a strong association between serological responses to CagA and peptic ulcer disease was reported (Cover et al., 1990), which led to cloning of the cagA gene (Covacci et al., 1993; Tummuru et al., 1993) as well as identification of adjacent cagPAI genes and their role in inflammation (Tummuru et al., 1995). One of these genes was picB (cagE) with homology to VirB4 family proteins of a putative type IV secretion system (T4SS) (Tummuru et al., 1995). A few years later the entire cagPAI was sequenced from different H. pylori isolates and found to represent a 40 kb DNA insertion element, which carries up to 32 genes flanked by 31 bp direct repeats (Censini et al., 1996; Akopyants et al., 1998). The cagPAI was acquired by a horizontal DNA transfer event from an as yet unknown ancestor and integrated into the chromosomal glutamate racemase gene. CagA is recognized as a marker for the cagPAI region, which is found in more virulent strains but is typically missing in less virulent H. pylori isolates (see Correa and Piazuelo, Chapter 3, this volume). Although the function of CagA and the cagPAI remained unknown for several years, a major breakthrough in the study of CagA came when five groups independently reported that the cagPAI encoded a T4SS which injects CagA into target cells (Covacci and Rappuoli, 2000).
antibody recognition that may allow immune evasion (Aras et al., 2003a). The T4SS needle base is covered with VirB7 (CagT) and VirB9 (CagW) proteins (Rohde et al., 2003; Tanaka et al., 2003). Immunogold-staining indicates the presence of CagA at the tip of the needle, providing the first direct evidence that CagA can be delivered through these surface appendages, a finding not yet shown for any other T4SS (Kwok et al., 2007). Delivery of CagA is proposed to proceed in an energy-dependent manner driven by the NTPases VirD4, VirB4 and VirB11 (Fig. 11.5).

11.3.3 Function of the type IV secretion system: the receptor hypothesis

For several years it was believed that CagA was randomly injected into epithelial cells. This is obviously not the case, as integrin receptors are required for injection of CagA (Kwok et al., 2007). An important role is played by CagL, a T4SS-pilus-covering protein and VirB5 orthologue (Backert et al., 2008), which acts as a specialized adhesion bridging the T4SS to β₁-integrin on target cells (Kwok et al., 2007). In addition, it was found that CagL activates the host kinases FAK (focal adhesion kinase) and Src ((sarcoma) intracellular tyrosine kinase) to ensure phosphorylation of CagA directly at the injection site. This was the first study to directly demonstrate that a bacterial T4SS protein targets a host receptor for its function. Binding of CagL to integrins induces local membrane ruffling, indicative of a general effect on membrane dynamics, and is the first T4SS effect on host cells. Once the T4SS injection needle is constructed, H. pylori modulates the actin cytoskeleton, transcriptional responses and cell–cell junctions. Interestingly, CagL contains an arginine–glycine–aspartate (RGD) motif that mediates contact of the T4SS to integrin-α₅β₁ (Kwok et al., 2007). CagL can also bind in an RGD-independent manner to another integrin member (αvβ5) and fibronectin (Urman, 2007). Thus, CagL can trigger signalling by RGD-dependent and RGD-independent pathways. Furthermore, integrins are not found at the apical membrane but at the basal membrane of polarized cells. This suggests a sophisticated control mechanism through which H. pylori injects CagA (Kwok et al., 2007). The basal injection model of CagA can also explain why H. pylori does not cause more damage and may only inject virulence proteins into target cells under certain circumstances. A likely scenario based on these findings is that cagPAI-independent factors such as the known adhesins BabA/B, SabA, HopZ and AlpA/B as well as VacA and OipA loosen local intercellular epithelial junctions before a limited number of bacteria gain access to the inner cell membrane and subsequently, utilize the CagL secretion machinery to deliver CagA to the target cell membrane and activate Src tyrosine kinase for CagA phosphorylation.

Fig. 11.5. Model for the assembled type IV secretion system (T4SS), cryo-EM (electron microscopy) structure of a T4SS core and crystal structure of the Helicobacter pylori VirB11 ATPase complex. (A) Hypothetical model of the T4SS machinery. The T4SS is a multicomponent protein complex spanning the inner and outer membranes of H. pylori. Current knowledge of T4SS functions and cellular localization of its components is shown in a simplified manner. The coupling protein VirD4 and structural components (VirB1 to VirB11) are typically required for secretion and are positioned according to their proposed functions. This transporter enables secretion of substrates (CagA, peptidoglycan) from the bacterial cytoplasm directly into the cytoplasm of infected host cells. The CagL protein interacts with integrin receptors to deliver CagA across the host cell membrane and to activate the Src tyrosine kinase for CagA phosphorylation. (B) The cryo-EM structure of the TraN/VirB7, TraO/VirB9 and TraF/VirB10 core complex from the T4SS of plasmid pMK101 (Fronzes et al., 2009). Side view (top left) and cutaway side view (top right) are shown. (C) Top view (view from outside the cell) on the left side and bottom view (view from the cytoplasm) on the right side. (D) Ribbon diagrams of the H. pylori VirB11 (HP0525), a hexameric traffic ATPase, from two different views (Yeo et al., 2000). Abbreviations: CagA/F/L, proteins encoded by the cag (cytotoxin-associated genes) pathogenicity island; FAK, focal adhesion kinase; RGD, arginine–glycine–aspartate motif; Src (short for sarcoma), intracellular tyrosine kinase. (Part A was adapted from Backert and Selbach (2008) with kind permission from Blackwell Publishing. Parts B to D were kindly provided by Remi Fronzes and Gabriel Waksman with kind permission of Science and Elsevier.)
access to integrins and inject CagA (Wessler and Backert, 2008). In line with this hypothesis, inactivation of the oipA gene also has an effect on FAK activity (Tabassam et al., 2008). The importance of β₁-integrin for H. pylori-induced host cell motility and elongation (Kwok et al., 2007) and bacterial adherence and invasion (Su et al., 1999) was also reported, and this signalling is associated with the phosphorylation of paxillin and the MAP kinase JNK (Jun N-terminal kinase) (Snider et al., 2008).

11.3.4 Injection of CagA requires accessory proteins

As mentioned above, the cagPAI contains up to 32 genes encoding 11 VirB proteins and VirD4 as well as several auxiliary factors. Mutagenesis of all other cag genes revealed that they are fully, partially or not required for injection of CagA (Covacci and Rappuoli, 2000; Fischer et al., 2001). While the roles of most of the T4SS-specific accessory factors are unknown, the function of CagF and CagD was recently elucidated. CagF is a chaperone-like protein that binds close to the carboxy-terminal secretion signal of the CagA effector protein and is crucial for its translocation through the T4SS (Couturier et al., 2006; Pattis et al., 2007). CagD serves as a multifunctional component of the T4SS that may be involved in CagA secretion at the inner membrane and may localize outside the bacteria to promote additional effects on the host cell (Cendron et al., 2009).

11.3.5 Crystal structures of several cagPAI proteins

A major contribution to our current understanding of T4SS transporters in bacteria came from resolution of a 15 Å crystal structure of a T4SS core from IncN plasmid pKM101 (Fronzes et al., 2009). This core complex is composed of three proteins (VirB7, VirB9 and VirB10), each present in 14 copies and forming a 1.1 MDa, two-chambered, double membrane-spanning channel (Fig. 11.5B and C). The structure is double-walled, with each component apparently forming a major part of the channel. The complex is open on the cytoplasmic side and constricted on the extracellular side. In addition to this major finding, the crystal structures of four H. pylori cagPAI proteins have been solved. The structure of VirB11 reveals a hexameric ring complexed with the regulator protein HP1451 and functions as a gating molecule at the inner membrane (Hare et al., 2007), which is proposed to cycle through closed and open forms as regulated by ATP binding/hydrolysis (Fig. 11.5D). The crystal structures of CagZ, a 23 kDa protein involved in the translocation of CagA, and CagS, a 23 kDa protein coded by a well-conserved cagPAI gene whose function remains elusive, have also been reported (Cendron et al., 2004, 2007). In addition, the structural characterization of CagD revealed that this protein is a covalent dimer in which each monomer folds as a single domain that is composed of five β-strands and three α-helices (Cendron et al., 2009). Finally, the structure of CagL was modelled based on the crystal structure available from another VirB5 orthologue, TraC from pKM101 (Backert et al., 2008). CagL forms a three α-helical bundle plus an exposed domain carrying the RGD motif available for binding of integrins. This is concordant with the published circular dichroism spectrum of CagL, which revealed 65% helical sequences (Kwok et al., 2007).

11.3.6 Phosphorylation of CagA by Src and Abl oncoproteins

As CagA proteins vary in size from about 120 to 145 kDa and exhibit no sequence homology to any pro- or eukaryotic proteins, the evolutionary origin of CagA is unknown. However, one important clue that identified CagA as an injected effector protein was the observation that it undergoes tyrosine phosphorylation (CagAPY) by host cell kinases (Covacci and Rappuoli, 2000). The phosphorylation sites are Glu–Pro–Ile–Tyr–Ala (EPIYA) motifs in the carboxy-terminal half of CagA (Backert et al., 2001b; Stein et al., 2002). Four distinct EPIYA sites have been described, EPIYA-A, -B,
-C and -D, each of which is conserved in sequence. Interestingly, while EPIYA-A and EPIYA-B are present in strains throughout the world, EPIYA-C is predominantly found in strains from Western countries (e.g. Europe, North America and Australia), while EPIYA-D is predominantly found in strains from Eastern countries (e.g. Japan, Korea and China). These EPIYA repeats are flanked by repetitive DNA sequences involved in recombination that could explain the variability in the number of these motifs in CagA variants (Aras et al., 2003b; Kim et al., 2006), as well as strain-specific differences in pathogenicity (Hatakeyama, 2008). Phosphorylation of CagA appears to be highly dynamic in infected cells (Tammer et al., 2007) and 2-DE analyses of CagA protein species from different strains showed that only two EPIYA motifs can be phosphorylated at a given time, both separately and simultaneously (Backert et al., 2001a). The EPIYA region also contains a multimerization motif (Ren et al., 2006) and represents a membrane-targeting signal (Higashi et al., 2005), although in another study CagA-EPIYA deletion mutants still localized to the membrane (Mimuro et al., 2002). Perhaps significantly, the kinases responsible for phosphorylation of CagA are known oncoproteins. Src family kinases (SFKs) that control actin cytoskeletal processes, proliferation and differentiation of normal cells are also key players in carcinogenesis, and have been found to phosphorylate CagA (Selbach et al., 2002; Stein et al., 2002). Following this discovery, Abl kinase members c-Abl and Arg were identified to directly phosphorylate CagA (Poppe et al., 2007; Tammer et al., 2007). Interestingly, *H. pylori* controls the activity of SFKs and Abl in a very specific and time-dependent manner. While Src is activated only during the initial stages of infection (0.5–2 h) and then is rapidly inactivated, Abl is continuously activated by *H. pylori* with strongly enhanced activities at later time points (2–8 h), supporting a model for the successive phosphorylation of CagA by Src and Abl (Tammer et al., 2007). Upon injection or transfection, CagA can then interact with multiple host cell factors in both a phosphorylation-dependent and -independent manner (Table 11.2).

### 11.3.7 Intracellular functions of CagA: phosphorylation-dependent signalling

An important hallmark of eukaryotic phosphotyrosine signalling is the recruitment of proteins with specific phosphotyrosine-binding modules such as Src homology 2 (SH2) domains. To date, nine SH2-domain-containing host cell proteins are known to bind CagA in a phosphorylation-dependent manner: the tyrosine phosphatases Shp1 and Shp2, the tyrosine kinase Csk, phosphoinositide-3 kinase (PI3K), Ras GTPase activating protein (Ras-GAP), and the adaptor proteins Crk, Grb2 and Grb7 (Higashi et al., 2002; Tsutsumi et al., 2003; Suzuki et al., 2005; Selbach et al., 2009). All nine interaction partners play complex roles in *H. pylori*-induced actin cytoskeletal rearrangements, scattering and elongation of infected host cells in culture, a scenario involving multiple signalling events as summarized in Fig. 11.6.

Gastric epithelial cells cultured *in vitro* with *H. pylori* elongate and scatter, a morphology referred to as the ‘hummingbird’ phenotype (Segal et al., 1999). This phenotype results from two successive events: (i) the induction of cell motility leading to scattering; and (ii) cell elongation (Moese et al., 2004). CagA<sup>PY</sup> can induce cellular elongation by triggering a cell retraction defect in focal adhesions, but this is still not completely elucidated (Bourzac et al., 2007). Transfection studies showed that CagA<sup>PY</sup>-Shp2 interaction contributes to cell elongation by direct dephosphorylation and inactivation of FAK (Tsutsumi et al., 2006) and by stimulating the Rap1→B-Raf→ERK signalling pathway (Higashi et al., 2004), respectively. CagA<sup>PY</sup>-induced cell elongation also involves tyrosine dephosphorylation of the actin-binding proteins cortactin, ezrin and vinculin (Selbach et al., 2003, 2004; Selbach and Backert, 2005; Moese et al., 2007). The phosphatases involved in this scenario remain unknown. Theoretically, interaction of CagA<sup>PY</sup> with the tyrosine phosphatase Shp2 could explain the latter events, but cortactin dephosphorylation does not require Shp2 (Selbach et al., 2003). Instead, CagA<sup>PY</sup> can inhibit Src both directly and via recruitment of Csk, a negative regulator of Src (Selbach et al., 2003; Tsutsumi et al., 2003). As Src is the
Table 11.2. Proteins that interact with cytotoxin-associated gene A protein (CagA) and proposed roles in *Helicobacter pylori* infections.

<table>
<thead>
<tr>
<th>Interaction partner&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proposed function&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Requirement of CagA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Experimental evidence</th>
<th>Applied methods&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference(s)</th>
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<tr>
<td>Abl</td>
<td>Phosphorylation of CagA and CrkII adapter proteins</td>
<td>n.d.&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Infection, transfection of CagA</td>
<td>IP, IF</td>
<td>Brandt <em>et al.</em> (2007); Poppe <em>et al.</em> (2007); Tammer <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>c-Met&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cell scattering, activation of PI3K, ERK, β-catenin and NF-κB</td>
<td>No</td>
<td>Infection</td>
<td>IP, IF</td>
<td>Churin <em>et al.</em> (2003); Suzuki <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>CrkI, CrkII, CrkL</td>
<td>Cell scattering, loss of AJs, MAPK signalling</td>
<td>Yes</td>
<td>Infection, transfection of CagA</td>
<td>IP</td>
<td>Suzuki <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Destabilization of AJs, β-catenin signalling</td>
<td>No</td>
<td>Proteomics</td>
<td>SILAC/MS</td>
<td>Selbach <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Grb2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Cell scattering, activation of Ras-MAPK signalling</td>
<td>No</td>
<td>Transfection of CagA</td>
<td>PD, IP, IF</td>
<td>Mimuro <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Grb7</td>
<td>Unknown</td>
<td>Yes</td>
<td>Proteomics</td>
<td>SILAC/MS</td>
<td>Selbach <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>PAR1</td>
<td>Inhibition of PAR1 activity, disruption of apical junctions</td>
<td>No</td>
<td>Transfection of CagA</td>
<td>IP, IF</td>
<td>Saadat <em>et al.</em> (2007); Zeaiter <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Unknown</td>
<td>Yes</td>
<td>Proteomics</td>
<td>SILAC/MS</td>
<td>Selbach <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>PLC-γ&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Cell scattering</td>
<td>No</td>
<td>Infection</td>
<td>IP</td>
<td>Churin <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>Ras-GAP</td>
<td>Unknown</td>
<td>Yes</td>
<td>Proteomics</td>
<td>SILAC/MS</td>
<td>Selbach <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Shp1</td>
<td>Unknown</td>
<td>Yes</td>
<td>Proteomics</td>
<td>SILAC/MS</td>
<td>Selbach <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Shp2</td>
<td>Cell scattering, activation of ERK</td>
<td>Yes</td>
<td>Transfection of CagA</td>
<td>IP</td>
<td>Higashi et al. (2002, 2004)</td>
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<tr>
<td>Tyrosine dephosphorylation of FAK</td>
<td>Yes</td>
<td>Transfection of CagA</td>
<td>IP</td>
<td>Tsutsumi et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>ZO-1, JAM</td>
<td>Disruption of lateral junctions and cell motility</td>
<td>No</td>
<td>Infection, transfection of CagA</td>
<td>IF</td>
<td>Amieva et al. (2003); Bagnoli et al. (2005)</td>
</tr>
</tbody>
</table>

Abbreviations: Abl, Abelson non-receptor tyrosine kinase; c-Met, hepatocyte growth factor receptor; Crk, SH2/SH3-domain-containing adapter protein; Csk, carboxy-terminal Src kinase; Grb, growth factor receptor-bound protein; JAM, junctional adhesion molecule; PAR1, partitioning-defective 1 kinase; PI3K, phosphoinositide-3 kinase; PLC-γ, phospholipase c gamma; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homologue; Ras-GAP, Ras GTPase activating protein; Shp1/2, SH2-domain-containing protein-tyrosine phosphatase-1/2; Src (oncogene of the Rous sarcoma virus), intracellular tyrosine kinase; ZO-1, zonula occludens 1.

Abbreviations: AJ, adherens junction; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; NF-κB, nuclear factor-κB; Ras-MAPK, Ras mitogen-activated protein kinase.

Abbreviations: IF, co-localization by immunofluorescence; IP, co-immunoprecipitation; PD, pull-down experiments; SILAC/MS, stable isotope labelling with amino acids in cell culture combined with mass spectrometry of bound proteins.

Activation of CagA-induced NF-κB activation and interleukin-8 secretion was not observed when CagA was co-transfected with dominant-negative c-Met in AGS cells (Brandt et al., 2005).

An interaction of non-phosphorylated CagA or CagA<sup>PY</sup> with Grb2 was reported (Mimuro et al. 2002; Selbach et al., 2009) but none of these interactions was found by Tsutsumi et al. (2003) and Churin et al. (2003) using transfected CagA.

PLC-γ IP revealed a strong background signal for non-injected CagA in a translocation-defective virb11 mutant.

n.d., not determined.
Fig. 11.6. A model for the role of *Helicobacter pylori* type IV secretion system (T4SS) effectors CagL, peptidoglycan and CagA in host cell interaction and signal transduction. T4SS effectors alter different cellular processes in gastric epithelial cells. During infection, *H. pylori* injects effector molecules into gastric epithelial cells associated with integrin \( \alpha_5 \beta_1 \) receptor (1), induces activation of receptor tyrosine kinases EGFR, HER2/Neu, c-Met and receptor DAF (2) and activation of intracellular tyrosine kinases Src and Abl which phosphorylate injected CagA (3). Phosphorylated CagA can influence GTPase activation (4), cell motility (5) and host cell elongation *in vitro* (6). Injected or transfected CagA modulates various
primary kinase activated by CagA, inhibition of Src by CagAPY generates a negative feedback loop that carefully controls the amount of intracellular CagAPY. Similarly, Src inhibition could occur via Shp2 and FAK (Fig. 11.6). Cortactin, ezrin and vinculin are all Src substrates, and Src inactivation causes dephosphorylation of these proteins that is essential for the elongation phenotype (Fig. 11.6). Interaction of CagAPY with CrkII can activate Rac1 via Dock180 (Suzuki et al., 2005). Activation of Abl by CagAPY amplifies this effect as it enhances phosphorylation of the Abl substrate CrkII (Brandt et al., 2007; Poppe et al., 2007; Tammer et al., 2007). Finally, a recent systematic proteomics-based approach verified most but not all of the latter CagAPY-interacting partners and even identified additional binding partners including Grb2, Grb7, Shp1, Ras-GAP and PI3K, but their specific role in signaling needs to be clarified in future studies (Selbach et al., 2009). Taken together, CagAPY interferes with a surprisingly high number of cellular factors and signalling cascades to induce cell scattering, elongation and likely other responses.

11.3.8 Intracellular functions of CagA: phosphorylation-independent signalling

Intracellular CagA functions do not always depend on its tyrosine phosphorylation. The cell adhesion protein E-cadherin, the hepatocyte growth factor receptor c-Met, the phospholipase PLC-γ, the adaptor protein Grb2 and the kinase PAR1 have all been reported to interact with CagA in its non-phosphorylated form (Mimuro et al., 2002; Churin et al., 2003; Murata-Kamiya et al., 2007; Saadat et al., 2007; Zeaiter et al., 2008), and phosphorylation-independent CagA interactions induce proinflammatory and mitogenic responses as well as the disruption of cell–cell junctions and loss of cell polarity (Fig. 11.6). Tight and adherens junctions are essential for the integrity of the gastric epithelium. CagA interferes with these intercellular junctions via several pathways. Injected CagA associates with the epithelial tight-junction scaffolding protein ZO-1 (zonula occludens 1) and the transmembrane protein JAM (junctional adhesion molecule), causing an ectopic assembly of tight-junction components at sites of bacterial

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**Abbreviations:** Abl, Abelson non-receptor tyrosine kinase; AJ, adherens junction; AP-1, activator protein 1 (a transcription factor); CagA/L, proteins encoded by the cag (cytotoxin-associated genes) pathogenicity island; c-Fos, transcription factor; c-Met, hepatocyte growth factor receptor; Crk, SH2/SH3-domain-containing adapter protein; Csk, carboxy-terminal Src kinase; cyclin D1, cell-cycle regulator; DAF, decay-accelerating factor; Dock180, dedicator of cytokinesis (a 180 kDa guanine exchange factor for GTPase Rac1); EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound protein 2; F-actin, filamentous actin; G-actin, globular actin; H-Ras, member of the Ras superfamily of GTPases; IL-8, interleukin-8; JAM, junctional adhesion molecule; JNK, Jun N-terminal kinase; MCL-1, myeloid cell leukaemia 1 protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; N-WASP, neural Wiskott–Aldrich syndrome protein; NF-κB, nuclear factor-κB; NOD1, nucleotide oligomerization domain protein 1; P, phosphate group; p38 MAP kinase, 38 kDa mitogen-activated protein kinase; PAR1, partitioning-defective 1 kinase; PKC-δ, protein kinase C-δ; PI3K, phosphoinositide-3 kinase; PTPase, phosphatidylinositol phosphatase; Rac1, Ras substrate of C3 toxin; Raf, serine/threonine kinase; Rap, serine/threonine kinase; Shp2, SH2-domain-containing protein-tyrosine phosphatase 2; SOS, son of sevenless (a guanine exchange factor for Ras); Src kinase, oncogene of the Rous sarcoma virus; SRF, serum response factor; T, tight junction; WAVE, WASP family Verprolin-homologous protein; ZO-1, zonula occludens 1 (a tight junction protein). For more details see text. (Adapted from Backert and Selbach (2008) with kind permission from Blackwell Publishing.)
attachment (Amieva et al., 2003; Bagnoli et al., 2005). Very recently PAR1, a central regulator of cell polarity, was found to play a role in this process. CagA directly binds PAR1 and inhibits its kinase activity to promote loss of cell polarity (Saadat et al., 2007; Zeaiter et al., 2008). CagA was also reported to interact with the transmembrane cell–cell junction protein E-cadherin (Murata-Kamiya et al., 2007). However, many of the more recent findings are based on vector-based expression of CagA constructs. Since integrins as T4SS receptors are protected by the junctions, the crucial question arises about the in vivo relevance of these processes for H. pylori pathogenesis. Thus, more detailed studies are necessary to clarify the intracellular function of CagA in infection models.

H. pylori infection also affects host cell gene transcription, and recent data have established a direct role of CagA in signalling to the nucleus (Fig. 11.6). The first direct evidence came from microarray studies investigating host cell gene expression after infection of target cells with wild-type H. pylori and cagA mutants (Guillemin et al., 2002; El-Etr et al., 2004). Subsequently, CagA was found to potentiate the activation of proinflammatory transcription factor NF-κB via the Ras→Raf→MEK→ERK pathway (Brandt et al., 2005). This process appears to involve binding of CagA to Grb2, an upstream effector of the small GTPase H-Ras (Mimuro et al., 2007). Very recently PAR1, a central regulator of cell polarity, was found to play a role in this process. CagA directly binds PAR1 and inhibits its kinase activity to promote loss of cell polarity (Saadat et al., 2007; Zeaiter et al., 2008). CagA was also reported to interact with the transmembrane cell–cell junction protein E-cadherin (Murata-Kamiya et al., 2007). However, many of the more recent findings are based on vector-based expression of CagA constructs. Since integrins as T4SS receptors are protected by the junctions, the crucial question arises about the in vivo relevance of these processes for H. pylori pathogenesis. Thus, more detailed studies are necessary to clarify the intracellular function of CagA in infection models.

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11.3.9 Type IV secretion system-dependent but CagA-independent cellular signalling

Until recently, CagA was the only known effector protein to be translocated through the T4SS. Early reports indicated that VirB structural components of the T4SS but not CagA itself were required for the induction of proinflammatory signalling (Tummuru et al., 1995; Censini et al., 1996) including activation of NF-κB (Sharma et al., 1998), AP-1 and the proto-oncogenes c-Fos and c-Jun (Meyer-ter-Vehn et al., 2000). This suggested that the T4SS might inject factors in addition to CagA. The T4SS was also found to activate the Rho-GTPases Rac1 and Cdc42 (Churin et al., 2001), and to stimulate EGFR, ERK1/2 and expression of the early growth response gene Egr1 (Keates et al., 2005) independently of CagA. Despite intensive efforts including a
systematic mutagenesis of all cagPAI genes, the hypothetical additional effector remained unknown (Fischer et al., 2001). A possible candidate, however, was identified when Viala and co-workers (2004) reported that proinflammatory signalling of H. pylori involved NOD1, an intracellular pathogen-recognition molecule with specificity for Gram-negative peptidoglycan. These observations suggest that T4SS-dependent delivery of peptidoglycan is responsible for activation of NOD1→NF-κB-dependent proinflammatory responses such as secretion of IL-8 (Viala et al., 2004) or β-defensin-2 (Boughan et al., 2006).

Finally, there are other cellular phenotypes that depend on a functional cagT4SS but which are independent of CagA. These include anti-phagocytic effects on professional phagocytes (Ramarao et al., 2000b) and the homotypic aggregation phenotype of macrophage-like cells (Moese et al., 2002). In addition, megasome formation has been observed with cagPAI-positive but not cagPAI-negative H. pylori strains (Allen et al., 2000). H. pylori has been also described to bind decay-accelerating factor (DAF) as a mediator of gastric inflammation, but the responsible bacterial factor remains elusive (O’Brien et al., 2006). Whether peptidoglycan is involved in targeting DAF, activation of EGFR, Rho-GTPases and Egr-1 is unclear and remains a pressing question. Alternatively, CagL-induced integrin signalling might also play a role in some of the latter activities.

11.4 Summary and Outlook

H. pylori is the most common chronic bacterial infection in the world and, although asymptomatic in the majority of infected subjects, it is also the cause of significant human disease. In the nearly 30 years since H. pylori was first discovered, a complete paradigm shift has occurred in our clinical approaches to a number of gastric diseases. From a medical point of view, H. pylori is a remarkable pathogen responsible for significant morbidity and mortality worldwide. The interest in this field is underlined by more than 27,000 scientific publications that have appeared in PubMed since its discovery. However, H. pylori infection occurs in approximately half of the world population, with disease being an exception rather than the rule. Understanding how this organism interacts with its host is essential for formulating an effective strategy to deal with its most important clinical consequences. Study of host–bacterial interactions and bacterial virulence factors has provided important insights for clinical practice. Research on VacA, CagA and the T4SS has provided us with many fundamental insights into H. pylori biology and function, but many open questions remain. It is now clear that each of these factors plays a central role in pathogenesis and several of the involved mechanisms have already been elucidated. However, much remains to be elucidated regarding how and when VacA and CagA are transported into the host. Both proteins have emerged as extremely versatile effector proteins that interfere with multiple host cell functions. It will be of interest to determine if the T4SS translocates other substrates in addition to CagA. Transfer of peptidoglycan is an intriguing possibility but, to date, there are no confirmatory studies. Finally, the evolutionary advantage of the T4SS for H. pylori needs to be investigated. Potentially, reduction of gastric epithelial cell turnover could be an adaptive advantage for H. pylori, but this should be studied in more detail. It appears that H. pylori’s virulence factors will continue to be a fascinating and rewarding research subject in the future.

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Out of the text, the following key points can be extracted:

- **VacA** (Vacuolating Cytotoxin) is a key virulence factor of *Helicobacter pylori*.
- *CagA* protein is important for the interaction with host cell signaling pathways.
- The *CagA* protein can activate focal adhesion kinase (FAK) and Src kinases, leading to pro-inflammatory cytokine production.
- The interaction of *CagA* with the SHP-2 phosphatase is crucial for the formation of host cell vacuoles.
- *Helicobacter pylori* produces a cytotoxin, VacA, which can cause cellular vacuolation.
- **OipA** is a protein produced by *Helicobacter pylori* which plays a role in the fibroblast growth factor receptor (FGFR) signaling pathway.
- The *CagA* protein has been implicated in the induction of pro-inflammatory cytokines and mast cell activation.
- The *CagA* protein can also interact with the EGF receptor and Erk1/2 kinase, leading to cell scattering and elongation.

These points illustrate the complex interactions between *Helicobacter pylori* and the host cell, highlighting the role of *CagA* and *VacA* in the pathogenesis of *H. pylori* infection.
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12 Helicobacter pylori Adhesion to the Gastric Surface

S.K. Lindén,* A. Arnlqvist, S. Teneberg and A.P. Moran

12.1 Introduction

To colonize mucosal surfaces and invade the host, microbes commonly exploit host cell structures. Bacterial adhesion to host cells is thought to be mediated by hydrophobic interactions, cation bridging (i.e. divalent cations countering the repulsion of the negatively charged surfaces of bacteria and host) and receptor-ligand binding. One of the most extensively studied mechanisms of adhesion is the binding of lectins to corresponding glycosylated receptors. Bacteria may have multiple adhesins with different carbohydrate specificities, and modulation of surface receptor density, kinetic parameters or topographical distributions of these receptors on cell membranes regulates adhesion. While individual adhesin–receptor binding is usually of low affinity, clustering of adhesins and receptors can cause multivalency effects resulting in strong attachment. Fimbriae (or pili), outer membrane proteins and cell wall components, for example lipopolysaccharides (LPSs), may all function as adhesins (Salyers and Whitt, 1994). Adhesion can affect bacteria by stimulation/inhibition of growth, as well as induction of other adhesive structures and proteins required for invasion, whereas the effects of adhesion on host cells can be altered morphology, fluid loss, induction of cytokine release, up-regulation of adhesion molecules and apoptosis. The first part of this chapter discusses the adhesins and other structures that Helicobacter pylori uses to adhere, as well as how these adhesins are regulated. The second part describes the host structures to which H. pylori binds.

12.2 H. pylori Adhesins and Mode of Adhesion

The genome of H. pylori codes for a large number of outer membrane proteins (Tomb et al., 1997) and at least five different lectin-like adhesins have been implicated in H. pylori adherence. Different adhesins may mediate adherence to various niches in the stomach or in other potential reservoirs, such as the oral cavity.

The blood group-binding adhesin (BabA) recognizes fucosylated structures such as Lewis (Le) b (Leb) and H-type-1 antigen (Borén et al., 1993). The majority of H. pylori strains investigated carry the babA gene (Ilver et al., 1998; Gerhard et al., 1999) and colonization with H. pylori strains expressing the BabA adhesin correlates with development of severe gastric disease (Ilver et al., 1998; Gerhard et al., 1999; Prinz et al., 2001; Yamaoka et al., 2006). Studies investigating the babA

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gene as a pathogenic factor have produced contradicting results. A plausible explanation for this is that, while some of these studies are based solely on PCR amplification of the babA gene (present in the majority of strains), others have included assays that distinguish BabA-expressing strains from BabA-non-expressing strains, such as reverse-transcription PCR that measures babA mRNA expression, immunoblot that detects expressed BabA protein or a measurement of Leβ-receptor binding activity. The potential expressed BabA protein or a measurement of Leβ-receptor binding activity. The potential correlation of BabA as a pathogenic factor has been recently discussed (Fujimoto et al., 2007).

The sialic acid-binding adhesin (SabA) is encoded by the JHP662 gene (Mahdavi et al., 2002). SabA recognizes sialyl-Leα and sialyl-dimeric-Leα structures, and 43% of the SabA-positive strains also bind sialyl-Leα. Of 95 Swedish clinical isolates tested, 39% bound sialyl-Leα, and the prevalence of this adhesin was higher in cytotoxin-associated gene product A (CagA)-positive than in CagA-negative strains (Mahdavi et al., 2002). Most (80%) SabA-positive strains are also BabA-positive. Strains with a SabA-positive and BabA-negative phenotype adhere to inflamed tissue, but not to healthy gastric mucosa (Mahdavi et al., 2002), which is perhaps explained by the observation that sialyl-Le antigens are preferentially expressed in inflamed gastric mucosa, with only minute levels present in healthy epithelium (Madrid et al., 1990; Mahdavi et al., 2002; Lindén et al., 2008a). Furthermore, SabA expression is associated with gastric cancer, intestinal metaplasia and corpus atrophy (Yamaoka et al., 2006).

The H. pylori neutrophil-activating protein (NAP) binds to sulfated carbohydrate structures such as sulfo-Leα, sulfated glycosphingolipids, the high-molecular-mass salivary mucin and the Leα, Leβ and H-type-1 structures as well as to sulfo-Leα (Huesca et al., 1996; Teneberg et al., 1997; Namavar et al., 1998). H. pylori NAP induces neutrophil adhesion to endothelial cells, and has been demonstrated to be an immunomodulating agent (Polenghi et al., 2007; Wang et al., 2008).

Adherence-associated lipoprotein A and B (AlpA and AlpB) have a function in adherence as mutations/deletions in AlpA or AlpB reduce binding to gastric sections (Odenbreit et al., 1999). Although no receptor for AlpA/B has been identified, it has been demonstrated that AlpA and AlpB aid H. pylori in colonizing the gerbil stomach (De Jonge et al., 2004).

Furthermore, H. pylori has the ability to bind to additional host structures via mechanisms that do not involve the above adhesins, although the H. pylori gene(s) for these have not been identified. One example is the low pH-binding mode, an activity present in all H. pylori strains investigated, that confers adherence to charged, preferably sialylated structures at acidic pH (Lindén et al., 2004a, 2008c). H. pylori can also bind to heparan sulfate and dextran sulfate (Utt and Wadström, 1997). Additionally, a subset of H. pylori strains possess a saliva-specific adhesion mode, allowing attachment to structures present on salivary mucins, but not to any of the mucins produced in either a healthy or a gastritis-affected stomach (Lindén et al., 2008c). This indicates that this binding mode may be especially adapted for the oral cavity, possibly because of the presence of sulfated structures that are present on oral mucins but absent on gastric mucins.

### 12.2.1 Regulatory mechanisms to alter adherence properties

To achieve successful establishment of persistent bacterial infection, optimal expression of virulence factors in concordance with the continuously changing environment is essential. To balance expression of gene activities, trans-acting elements that affect transcription via positive and negative regulatory proteins constitute common mechanisms for many bacterial species. In Gram-negative bacteria, alternative sigma factors play an important role in the regulation of gene expression, but in H. pylori, only the housekeeping sigma factors of the RNA polymerase (i.e. σ80, σ54 and σ28) have been found (Tomb et al., 1997). In addition, bioinformatic analysis of the two first H. pylori genomes sequenced (26695 and J99) has suggested that only a few regulatory networks operate and that only a limited number of two-component signal transduction systems are present (Alm et al., 1999).
Bacterial fitness is a key factor for establishment of a persistent infection, but this requires the generation of populations with high genetic diversity. A multitude of pathogenic bacteria use phase variation mechanisms to quickly turn genes on and off. Since phase shift variation enables a frequent and reversible on/off shift, and ensures the occurrence of dynamic populations with numerous clones with variable phenotypes, subclones of any given phenotype will always be present and ready to adapt to continuous alterations in the environment during persistent \textit{H. pylori} infection (Kuipers et al., 2000; Blaser and Berg, 2001; Aras et al., 2003; Nilsson et al., 2008). Populations with high diversity are also beneficial for evasion of the host immune response. Phase variation can occur via a number of mechanisms including slipped-strand mispairing (SSM), where mutations in a stretch of repetitive DNA sequences bring the open reading frame in and out of frame, DNA methylation and DNA rearrangements (e.g. by homologous recombination). The remainder of this section describes current knowledge of how expression of \textit{H. pylori} adhesins is regulated via phase variation.

Phase variation of BabA via homologous recombination

Annotation of the two first sequenced \textit{H. pylori} genomes revealed the presence of a large family of genes that encodes for outer membrane proteins, termed HOPs. This family shares extensive homologies in their N- and C-terminal regions (Alm et al., 2000). The HOPs constitute porins, adhesins like BabA and SabA, and proteins associated with adherence such as AlpA, AlpB and OipA (outer inflammatory protein A). The localization of hop genes on the chromosome varies between strains. The babA gene and the highly related babB gene are located in reversed loci in \textit{H. pylori} strains 26695 and J99 (Tomb et al., 1997; Alm et al., 1999; Oh et al., 2006). Thus, in strain J99 the babA gene is placed in the same locus as the 26695 babB gene and the J99 babB gene is placed in the same locus as the 26695 babA gene. The third sequenced HPAG1 strain carries babA in the same locus as strain 26695 (Oh et al., 2006). A third gene with high homology to both babA and babB, called babC, is present in strains 26695 and HPAG1, albeit in different loci (Colbeck et al., 2006). Thus, the genomic locations of the babA, babB and babC genes are different between strains 26695, J99 and HPAG1 (Alm et al., 1999; Oh et al., 2006). Further studies of the localization of the babA gene in additional strains have indeed confirmed that the localization of the three bab genes varies. The majority (54–57%) of the babA genes are placed in the locus corresponding to the 26695 babA locus. The second most common babA localization corresponds to the 26695 babB locus and the third commonest corresponds to the 26695 babC locus (Ilver et al., 1998; Colbeck et al., 2006; Hennig et al., 2006). In addition, the babA gene has occasionally been found in another, as yet unidentified locus. Most strains carry one babA gene but far from all express the BabA adhesin (Ilver et al., 1998; Yamaoka et al., 2002; Aspholm-Hurtig et al., 2004).

Several studies have highlighted BabA to be subject to phase variation via homologous recombination. Pride and Blaser (2002) first described the occurrence of babA/B chimeric genes, which were suggested to be the result of homologous recombination, i.e. translocation of the babB gene into the babA locus and a subsequent turn-off of BabA expression. Similar chimeras were later described by Solnick and co-workers (2004), who experimentally infected rhesus macaques with a BabA-expressing/Le^b-binding strain. Most of the recovered output clones had lost BabA expression and subsequent Le^b-binding properties. In some of the BabA down-regulated clones, the change in phenotype was due to translocation of the babB gene into the babA locus, which resulted in a BabA/B protein that was devoid of Le^b-binding activity (Solnick et al., 2004).

Although most strains carry the babA gene, it is not always expressed (Ilver et al., 1998). In such BabA-negative, Le^b-non-binding strains, occasional Le^b-binding cells have been observed at an average frequency of 1:75,000. The switch of some clones into a more virulent phenotype, i.e. by the acquisition of Le^b binding, has been modelled in a defined genetic system. Strain CCUG 17875 carries two babA genes: babA2 that is expressed
and babA1 that is silent. A babA2-knockout mutant that only carried the silent babA gene was used to analyse the underlying mechanism of gain of Leb\(^b\) binding. Clones of the Leb\(^b\)-binding phenotype were shown to carry a babB/A chimeric gene. A copy of the silent babA gene had been translocated into the babB expression locus via homologous recombination (Bäckström et al., 2004). The isolated Leb\(^b\)-binding clones exhibited the same Leb\(^b\)-binding affinity as the CCUG 17875 wild-type, although expression of the BabB/A protein was lower than BabA of CCUG 17875. This was probably due to a weaker promoter in the babB expression locus and, therefore, it was suggested that translocation via homologous recombination may not only be a mechanism to change expression from on to off and vice versa, but also to change the expression levels (Bäckström et al., 2004). Variation in BabA expression levels has been studied in a series of strains (Hennig et al., 2004; Fujimoto et al., 2007), but the impact of BabA high-expressing versus BabA low-expressing strains in disease outcome is not yet clear.

**Phase variation of BabA via slipped-strand mispairing**

Phase variation can also occur via SSM, which is a mutation process that occurs during DNA replication. In this process, DNA strands are misaligned, sometimes resulting in an inability to replicate nucleotide repeat motifs properly due to genes being in or out of frame. Comparison of the 26695 and J99 genome sequences indicated the presence of repeat motifs in the promoter region of some hop genes, indicating that phase variation mechanisms could control expression of these genes. In *H. pylori*, 46 genes with homopolymeric tracts and/or dinucleotide repeats, typical for genes regulated by SSM, have been noted and among them are some hop genes, including babA, babB, sabA and sabB (Salaun et al., 2004). BabA was first demonstrated to be subjected to phase variation by Solnick and co-workers (2004). Other studies have described babA also to carry CT repeats (Colbeck et al., 2006; Hennig et al., 2006). The babA genes with CT repeats appear to occur more often when babA is located in the 26695 babB locus and is thus very similar to a chimeric babB/A gene. Phase shift variation and switch of adhesin-expressing phenotype resulting from SSM occurs at a higher frequency than shifts caused by homologous recombination (Pride and Blaser, 2002; Bäckström et al., 2004).

In addition to on and off regulation via homologous recombination and phase shift variation, gene expression may also be affected by point mutations (in locations other than polynucleotide tracts) that bring the gene in or out of frame. The frequency of such events is, however, lower than for SSM mechanisms (Hennig et al., 2006).

**Phase variation of SabA expression**

An instability in the sialyl-Le\(^b\)-binding ability of *H. pylori* strains together with the finding that its cognate adhesin, SabA, carries a CT repeat motif has been described (Mahdavi et al., 2002). These changes in sialyl-Le\(^b\)-binding phenotype were related to modifications in the number of CT repeats of the sabA gene that were later found to bring the sabA gene in and out of frame (Sheu et al., 2006; Yamaoka et al., 2006). Sheu and co-workers (2006) also identified that a high proportion of *H. pylori*-infected individuals carry strains where the sabA gene lacks CT repeats. Thus, it is tempting to speculate that the sabA gene and the highly related sabB gene are translocated and exchange locations, similar to that observed with the babA and the babB genes, even though there are currently no data that confirm such a hypothesis.

**pH and effects on SabA expression**

Reduced acid secretion resulting from chronic *H. pylori* infection is strongly associated with an increased susceptibility to gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). Infection with *H. pylori* strains expressing SabA has been correlated to decreased acid secretion in patients (Yamaoka et al., 2006), suggesting a very important role for this adhesin in disease progression. Global gene expression analyses with cDNA arrays have found that sabA mRNA levels are down-regulated in response to acidic conditions (Merrell et al., 2003). The same repressing
Further emphasizing the role of Le x in adhesion in antral gastric biopsies (Edwards mediated, at least in part, adhesion to human bacterially labelled latex beads bearing polymeric Lex recognition phenomena, fluorescently volume). Preliminary evidence (Osaki 2008, 2009; see Moran, Chapter 10, this volume). A more recent study has suggested that the acid responsiveness of SabA expression is controlled via the arsRS operon (Goodwin et al., 2008). A J99arsS derivative, which exhibits increased sabA mRNA levels compared with the J99 wild-type strain, showed increased binding to AGS cells compared with wild-type J99 at neutral pH. Adherence to host cells was not analysed under acidic conditions. In addition, a J99arsSΔsabA strain still bound AGS cells at similar levels as the J99 wild-type at neutral pH. This indicates that additional adhesins are involved in mediating binding of strain J99 to AGS cells.

12.2.2 H. pylori lipopolysaccharide-mediated adhesion

O-antigen in H. pylori binding

The O-antigen components of LPSs from many H. pylori strains express Le antigens, particularly Leα, Leβ, Leγ and Leδ (Moran, 2008, 2009; see Moran, Chapter 10, this volume). Preliminary evidence (Osaki et al., 1998) showed that an anti-H. pylori LPS monoclonal antibody, later shown to be anti-Leα specific (Gerhardt et al., 2001), was capable of inhibiting the adhesion of H. pylori to human gastric cancer (MKN45) cells. Using fluorescently labelled bacteria, it was demonstrated that polymeric Leα expression by H. pylori mediated, at least in part, adhesion to human antral gastric biopsies (Edwards et al., 2000). Further emphasizing the role of Leα in adhesion-recognition phenomena, fluorescently labelled latex beads bearing polymeric Leα demonstrated the same tropic binding to the apical surface of mucosal epithelial cells and to cells lining the gastric pits, as fluorescently labelled H. pylori bacteria (Edwards et al., 2000). As noted elsewhere in this chapter, H. pylori adhesion to the mucosa can be mediated by a number of lectin-like adhesins, but the expression of polymeric Leα by H. pylori is considered to play a distinct adhesion role based upon findings with knockout mutants in binding studies (Mahdavi et al., 2003). Collectively, these results predict the existence of a molecule or receptor in the gastric epithelia, or a gastric Leα-binding lectin, that recognizes polymeric Leα borne on H. pylori.

Consistent with a role of Leα-mediated adhesion in colonization, an isogenic H. pylori galE mutant, with affected galactose incorporation and possessing a truncated LPS without an O-antigen, was unable to colonize a number of mouse strains compared with the Leα-expressing parental strain (Moran et al., 2000). Likewise, knockout mutation of a β(1,4)-galactosyltransferase gene, which affects synthesis of the O-antigen backbone, resulted in less efficient colonization of the murine stomach (Logan et al., 2000). Inactivation of rfbM, which encodes a GDP-α-mannose pyrophosphorylase required for GDP-fucose synthesis, resulted in a mutant with an i-antigen-expressing O-antigen lacking fucose, which exhibited reduced mouse colonization (Moran et al., 2000) and ablated interaction with the human gastric mucosa of biopsy specimens in situ (Edwards et al., 2000). Although one study reported no significant changes in mouse colonization by H. pylori mutants with ablated Leα/Leδ expression (Takata et al., 2002), that study used C3H/HeJ (TLR4−/−) mice, so-called LPS non-responder mice, which may not be the most appropriate animal model of infection, since H. pylori LPS may induce Toll-like receptor (TLR) 4 signalling (see Kaparakis et al., Chapter 8, this volume). In another study using a more appropriate model with LPS-responsive mice, however, a mutated H. pylori strain with a double knockout in both α-(1→3)-fucosyltransferases did not colonize, in contrast to the Leα/Leδ-expressing parental strain (Appelmelk et al., 2000). Taken together, these data indicate that Leα expression, rather than O-antigen occurrence alone, is required for colonization.

Initially, it was suggested that H. pylori-expressed Leα might interact with the host via
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a homotypic (Le⁺–Le⁺) interaction, as occurs in the Ca²⁺-dependent homotypic interaction in embryogenesis and tumour cell–cell interaction. However, while soluble Le⁺ can bind to liposome-incorporated Le⁺, attempts to show an interaction between soluble Le⁺ and H. pylori have been unsuccessful (Ilver et al., 1998). Also, anti-Le⁺ immunoglobulin (Ig) M monoclonal antibodies increased the adherence of H. pylori to AGS cells when tested in vitro by forming an interaction bridge between bacterial-borne Le⁺ and that on the host cells (Sheu et al., 2007). Although anti-Le⁺ IgM antibodies may be produced naturally in humans (Chmiela et al., 1999), whether these occur in the local gastric environment of H. pylori remains an open question. On the other hand, Fowler et al. (2006) identified the galactoside-binding lectin galectin-3 as the gastric receptor for H. pylori Le⁺. Expression of galectin-3 was found to be up-regulated by gastric epithelial cells following adhesion of H. pylori and subsequently influenced the host response to infection. Overall, the lectin-based interaction of galectin-3, rather than a homotypic interaction, appears the most likely mechanism for recognition of H. pylori-expressed Le⁺ by the gastric epithelium.

Consistent with an adhesion role, high expression of Le⁺ by H. pylori strains has been correlated with a higher colonization density in chronic gastritis patients than that by strains with weaker expression (Heneghan et al., 2000). By assisting bacterial adhesion and interaction with the gastric mucosa, like other adhesins, bacterial Le⁺ expression may enhance delivery of secreted products into the gastric mucosa (Rieder et al., 1997; Moran, 1999), such as those delivered through the type IV secretion system encoded by the cytotrans-associates genes (cag) pathogenicity island (see Backert et al., Chapter 11, this volume). This would in turn promote chemotaxis and leucocyte infiltration, as observed in an adherence-dependent mouse model of H. pylori-induced disease where Le⁺ expression correlated with inflammation (Guruge et al., 1998). It is important to consider the range of pH that exists within mucus and on the gastric cell surface, i.e. from pH 2 on the luminal side of the gastric mucus layer to almost pH 7 on the cell surface, and that environmental pH influences Le⁺ expression by H. pylori (Moran et al., 2002). Optimal expression of Le⁺ by H. pylori occurs at neutral pH, as found at the gastric epithelial cell surface, but reduced expression occurs at lower pH, as in the mucus layer, thereby modulating expression of the Le⁺-adhesin and allowing free-swimming H. pylori in the mucus layer. Potentially, bacteria in the mucus layer may act as a reservoir for continued infection of the mucosa. The observed on/off switching of fucosyltransferase activities, and resulting phase variation in Le⁺ expression (Appelmelk et al., 1999), would be consistent with this mechanism of adhesin molecular modulation (Moran, 2008).

Additionally, H. pylori binds surfactant protein D (SP-D), which is up-regulated in the vicinity of H. pylori during infection in vivo (Murray et al., 2002). H. pylori LPS, by interaction with the O-antigen moiety, mediates binding of SP-D, resulting in bacterial immobilization and aggregation (Murray et al., 2002; Khamri et al., 2005). To evade this potentially important mechanism of innate immune recognition of H. pylori, escape variants can arise within the bacterial cell population with modifications in O-antigen glycosylation, including greater expression of polymeric Le⁺, which has lesser affinity for SP-D and thereby decreases their interaction with SP-D (Khamri et al., 2005; Moran et al., 2005b). Notably, in vivo SP-D-binding organisms predominate in mucus, rather than on the epithelial cell surface, where escape variants predominate and expression of polymeric Le⁺ is optimized (Khamri et al., 2005).

Lipopolysaccharide core and H. pylori binding

The core of LPS from H. pylori has also been implicated in adhesion. Together with a 25 kDa sialic acid-specific lectin on H. pylori (Valkonen et al., 1997), which is produced in vivo (Moran et al., 2005a), the LPS core mediates binding to the extracellular matrix glycoprotein laminin. Although independent of Le antigen agglutination of red blood cells, depending on whether H. pylori strains are haemagglutinating or poorly haemagglutinating, two differing mechanisms of interaction
of the LPS core with amino acid sequences within laminin have been observed: a phosphorylated core structure mediates the interaction of haemagglutinating strains, whereas a non-phosphorylated structure is involved in poorly haemagglutinating strains (Valkonen et al., 1994). Despite this variation, the 25 kDa protein adhesin of *H. pylori* that recognizes the sialylated sugar chain of laminin is conserved in both haemagglutinating and poorly haemagglutinating strains (Valkonen et al., 1997). Thus, the binding of laminin by *H. pylori* illustrates a novel dual-recognition system, whereby sugars in the core of LPS interact with a peptide region in laminin and the 25 kDa adhesin of *H. pylori*, with lectin-like properties, recognizes a sialylated trisaccharide in laminin (Moran, 1996; Valkonen et al., 1997). Initial binding to laminin is believed to be mediated by the LPS core structures, and subsequently by the 25 kDa lectin (Valkonen et al., 1997; Moran, 1999). Despite a number of studies showing that *H. pylori* strains exhibit high-affinity binding to laminin, this interaction is likely to be a secondary event after initial colonization of the mucosa (see Moran, 1999). Nevertheless, as a proportion of colonizing *H. pylori* bacteria (5%) are observed associated with intercellular junctions, laminin binding may, in part, explain the association with this microniche. Moreover, this binding to laminin may have pathological consequences since *H. pylori* LPS interferes with the interaction between laminin and a 67 kDa protein receptor (integrin) on gastric epithelial cells (Slomiany et al., 1991), thus potentially contributing to disruption of gastric mucosal integrity.

### 12.3 *H. pylori* Interaction with Host Structures

#### 12.3.1 The first barrier – mucus

The first barrier *H. pylori* encounters when colonizing the stomach is the highly hydrated mucus gel that covers the mucosal surface and protects the epithelial cells against chemical, enzymatic, microbial and mechanical insult. In addition, mucus acts as an ‘unstirred layer’ in which bicarbonate ions secreted by the surface epithelium counteract protons diffusing from the lumen into the gel, causing the pH in the mucus layer to range from acidic in the lumen to neutral at the cell surface. The mucus gel is formed by high-molecular-mass oligomeric glycoproteins (mucins) and protection is reinforced by a number of ‘defence factors’ (such as defensins, trefoil factors and secretory IgA) trapped in the gel matrix. The mucins are produced by cells in the epithelial surface and/or by glands located in the submucosal connective tissues, and secretion occurs via both constitutive and regulated pathways. The constitutive pathway continuously secretes a small amount of mucin to maintain the mucus layer, whereas the regulated pathway affords a massive discharge of mucus as a response to environmental and/or pathophysiological stimuli. Stimulated mucin release occurs rapidly and is accompanied by a 100-fold or so expansion of secretory granules (reviewed in Lindén et al., 2008b). In the stomach, MUC5AC and MUC6 are the major secreted mucins, the former being produced by the surface epithelium and the latter by the glands (De Bolos et al., 1995).

**Mucin polymorphism and glycosylation**

Each mucin gene contains unique tandem repeat (TR) motifs coding for regions with a high density of serine, threonine and proline. The TR varies in length between mucins, and there is a genetic polymorphism in the number of repeats referred to as the variable number of tandem repeats (VNTR) polymorphism. VNTR polymorphisms cause mucin size to differ between individuals. The serine and threonine residues can be O-glycosylated and more than 50% (often 70–80%) of the mucin molecular mass is composed of carbohydrate. Each mucin carries the order of 100 different oligosaccharide structures (Klein et al., 1993). These carbohydrate chains are often clustered into highly glycosylated domains, giving the mucin a ‘bottle-brush’ appearance. The massive O-glycosylation of the mucins protects them from proteolytic enzymes and induces a relatively extended conformation (Jentoft, 1990). This extended conformation
MUC5AC mucin, whereas Le antigens are produced), whereas the Lex and Ley antigens appear on the surface epithelium (where MUC5AC is expressed), whereas the Lea and Leb blood-group antigens mainly appear in the peripheral region. The H1 structure is made by the secretor gene product; the majority of individuals (80% of Caucasians, all South American Indians and Orientals) carry this structure and are thus referred to as ‘secretors’ (Oriol et al., 1986; Oriol, 1995). Individuals may also express the Lewis gene (90% of the Caucasian population) and, provided that they are also secretors, will then express the Leb structure on the H1 antigen (Oriol et al., 1986; Oriol, 1995). If they are non-secretors, Lea will be expressed on type-1 chains (Oriol et al., 1986; Oriol, 1995). The terminal structures of mucin oligosaccharides are highly heterogeneous, and vary between/within species and even with tissue location within a single individual. The Lea and Leb blood-group antigens mainly appear on the surface epithelium (where MUC5AC is produced), whereas the Lea and Leb antigens are expressed in mucous, chief and parietal cells of the glands, co-localized with MUC6 (Murata et al., 1992; De Bolos et al., 1995). The Lea and Leb structures can be found on the MUC5AC mucin, whereas Lea and Leb structures are found on the MUC6 mucin (Lindén et al., 2002; Nordman et al., 2002). The mucins from a healthy human stomach express low levels of sialic acid and sulfate and are, therefore, predominantly neutral.

In gastric precancerous lesions and cancer, altered expression of MUC5AC, MUC6, MUC2 and MUC5B has been described (Buisine et al., 2000), with MUC2 being a marker for intestinal metaplasia, a precursor of gastric adenocarcinoma. In H. pylori-infected adults, aberrant MUC6 expression in the surface epithelium has been observed (Byrd et al., 1997). Mucins appear to be the major carrier of altered glycosylation in carcinomas (Saez et al., 2001) and incomplete glycosylation, leading to expression of Tn and T antigens, and/or sialylation/sulfation are common (Saez et al., 2001).

**H. pylori and the mucus layer**

*H. pylori* is well adapted to the mucus niche, having long, whip-like flagella facilitating locomotion through the mucus layer. Flagella, urease, adhesins and genes encoding proteins with a predicted function in chemotaxis are essential for *H. pylori* colonization of laboratory animals (Kavermann et al., 2003). *H. pylori* has been detected in the oral cavity of individuals with and without gastric *H. pylori* infection (Song et al., 2000a; Dowsett and Kowolik, 2003) and more than one strain may exist in the stomach and saliva simultaneously (Song et al., 2000a,b; Wang et al., 2002). Although oral infection could be transient (Czesnikiewicz-Guzik et al., 2004), the oral cavity may serve as a reservoir and portal of entry. Mother–child and sibling–sibling transmission have been indicated as the primary pathways of *H. pylori* infection/colonization, and the incidence of *H. pylori* is high in regions where mothers chew the food for their children (Kivi et al., 2003). While a limited number of studies have suggested that, clinically, the oral cavity may represent a possible source of *H. pylori* re-infection in individuals in whom gastric *H. pylori* has been eradicated due the fact that *H. pylori* eradication therapy is less effective for the mouth than for the stomach (Miyabayashi et al., 2000; Gebara et al., 2006), this view is controversial. Colonization of the oral cavity may provide the bacterium multiple chances to colonize the stomach. Individuals are likely to be frequently challenged by *H. pylori*, but may only be vulnerable to infection when protective factors are not functioning optimally. Significantly, 1 to 2 l of saliva, which has
excellent *H. pylori*-binding ability, are swallowed every day (Lindén et al., 2008c).

In both the oral and gastric compartment, *H. pylori* are found in the ‘stationary’ niche of the dental/gingival plaque and gastric epithelial cell surface, as well as in the constantly shedding mucus secretions (Czesnikiewicz-Guzik et al., 2004; Lindén et al., 2004a, 2009). It is reasonable to believe that it is more beneficial for the bacterium to adhere in the ‘stationary’ niche where they are protected against being flushed away. In contrast, it is in the host’s interest to inhibit *H. pylori* interactions with the cell surface, as adhesion is probably of significant importance for efficient delivery of key virulence factors such as the vacuolating cytotoxin (see Backert et al., Chapter 11, this volume). Further, *in vitro* studies show that *H. pylori* requires direct contact with the AGS cell line to induce an invasive phenotype (Prinz et al., 2001). The secreted mucins are likely to function as decoys for *H. pylori* interaction/binding (Lindén et al., 2004a), as they are produced in large amounts and constantly wash the mucosal surfaces. Patients with primary Sjogren’s syndrome, which is characterized by autoimmune destruction of exocrine glands and low levels of saliva, have a higher prevalence of *H. pylori* infection, supporting the view that the saliva/mucus layers play a role in protection against this bacterium (El Miedany et al., 2005). Furthermore, in the human-like rhesus monkey infection model of *H. pylori*, animals with mucins that more effectively bound *H. pylori* had a lower density of *H. pylori* in the stomach (Lindén et al., 2008a). Of note, *H. pylori* LPS decreases mucin synthesis at least *in vitro* (Slomiany and Slomiany, 2006), and the BabA and SabA adhesins are known to undergo phase variation and change expression during infection (Mahdavi et al., 2002; Solnick et al., 2004), events that may aid *H. pylori* to evade this host defence.

**H. pylori binding to mucins along the oro-gastric infection route**

The highly diverse mucin glycosylation motifs present in the oro-gastric tract present multiple potential binding motifs for *H. pylori*. Binding to mucins occurs via four distinct modes of adhesion: (i) BabA to fucosylated structures; (ii) SabA to sialylated structures; (iii) a charge/low pH-dependent mechanism; and (iv) via an adhesin specific for salivary structures (Lindén et al., 2008c) (see Fig. 12.1). No binding to mucins has been detected to occur via AlpAB (Lindén et al., 2008c). *H. pylori* binding to mucins differs substantially with the anatomical site, mucin type, pH, gastritis status and *H. pylori* strain (Lindén et al., 2008c).

Salivary mucins display a range of ligands to which *H. pylori* can bind via a range of factors including BabA, SabA, the charge/low pH-dependent mechanism and the saliva-specific adhesin (Lindén et al., 2008c). However, as the pH in the oral cavity niche is predominantly neutral, mucin binding is likely to occur at this site mainly via the SabA, BabA and saliva-specific adhesin. *In vitro* binding studies demonstrate that SabA is the adhesin that interacts with the most ligands in saliva (Lindén et al., 2008c). The ligands for *H. pylori* adhesion are present on MUC5B, MUC7 and the salivary agglutinin (Lindén et al., 2008c).

From the oral cavity, saliva containing *H. pylori* is swallowed and ends up in the lumen of the gastric compartment. In the gastric lumen, the mucins MUC5B, MUC7 and MUC5AC, as well as salivary agglutinin, are present and display ligands for the SabA, BabA, saliva-specific adhesin, and additionally for the charge/low pH-dependent mechanism (Lindén et al., 2008c). In this niche, the pH varies due to circadian variations in acid secretion, food and drug intake, as well as disease and stress; all adhesins thus have the potential to be functional at different times/stages of infection. *In vitro* binding studies demonstrate that, at acidic pH, the charge/low pH-dependent mechanism is the most efficient at binding *H. pylori*, whereas at a more neutral pH the SabA-dependent mechanism may be more efficient (Lindén et al., 2008c).

In the adherent gastric mucus layer, the pH ranges from acidic in the lumen to neutral at the cell surface. On the mucins present in this layer in healthy individuals, only ligands for BabA and a very small number of ligands for the charge/low pH-dependent mechanism of binding are present (Lindén et al., 2002, 2008a).
H. pylori binding to MUC5AC occurs at neutral pH and requires Le\(^b\) to be displayed on the mucin (Lindén et al., 2002).

In the adherent mucus layer of gastritis-affected stomachs, the ligands for BabA on MUC5AC remain constant, while there is an increase in the ligands for SabA and the charge/pH-dependent mechanism of binding (Lindén et al., 2008c). H. pylori-infected individuals have increased sialylation of their gastric epithelial cells, which return to normal levels following successful treatment (Ota et al., 1998; Mahdavi et al., 2002). The gastric glycosylation pattern in the rhesus monkey closely mimics that of man (Lindén et al., 2004b) and persistent H. pylori infections in these animals up-regulate sialyl-Le\(^a\) expression, thereby providing receptors that can be used for H. pylori adherence to the gastric surface epithelium (Mahdavi et al., 2002; Lindén et al., 2008a). A strong increase in sialylated Le antigens, as well as SabA-binding
ability, occurs within 1 week of *H. pylori* infection in the rhesus monkey (Lindén et al., 2008a). The majority of the changes that occur following acute infection in this model return to baseline levels by 10 months post-infection, indicating that this adhesin may play a more important role in early rather than chronic infection (Lindén et al., 2008a).

The BabA-positive strains thus bind to mucins from saliva, gastric juice and the adherent gastric mucus layer, demonstrating that this adhesin is the least dependent on anatomical site of the four different binding properties important in mucin binding (Lindén et al., 2008c). However, BabA binding is dependent on the blood group of the host (Lindén et al., 2002), and MUC5AC is also produced in several glycoforms within each individual, affecting the ability of this mucin to interact with BabA due to the steric presentation of the glycans involved (Lindén et al., 2002). BabA, the salivary-binding mode, and possibly also SabA, show their strongest mucin binding at neutral pH. Hence, these adhesins would bind tightly to ligands in the oral cavity and gastric epithelium, facilitating colonization of those niches. However, bacteria swallowed with saliva, or shed from the adherent gastric mucus layer, that arrive in the gastric lumen would be exposed to low pH. This reduced pH would reduce adhesin binding strength, releasing the bacteria from mucins and allowing them to swim through the mucus layer towards the protected niche of the gastric epithelium compatible with *H. pylori* survival. However, when highly charged moieties on mucins are present in the vicinity of these bacteria the low pH-binding mode may take over the *H. pylori*-binding role, thereby acting as a potential host mechanism for aiding in the removal of bacteria from the stomach (Lindén et al., 2004a, 2008c).

Underneath the mucus layer, the cells present a dense forest of highly diverse glycoproteins and glycolipids, which form the glycocalyx. The membrane-associated mucins that are also part of this glycocalyx are larger than most other membrane-bound glycoproteins. Due to their size, cell surface mucins are therefore likely to be the first point of direct contact between host tissue and organisms that penetrate the secreted mucus layer. MUC1 is the most highly expressed cell surface mucin in the stomach (Packer et al., 2004). MUC1 can carry the Le^b^, sialyl-Le^a^ and sialyl-Le^b^ structures and bind *H. pylori* via the BabA and SabA structures (Lindén et al., 2009). MUC1 protects the gastric epithelial cells by steric hindrance as well as by acting as a releasable decoy (Lindén et al., 2009). Mice deficient in Muc1 are more susceptible to infection by *H. pylori* (McGuckin et al., 2007). Moreover, short VNTR alleles for MUC1 are associated with increased risk of *H. pylori*-induced gastritis and gastric cancer (see Sutton et al., Chapter 7, this volume).

### 12.3.2 *H. pylori* binding to host glycosphingolipids

Below the mucus layer is another group of molecules to which *H. pylori* can adhere: the glycosphingolipids. Adherence of *H. pylori* to glycosphingolipids located in the plasma membrane of epithelial cells is likely to confer a close bacterial–epithelial cell association due to the small size of the extracellular part of the molecule. However, little is known about the events resulting from *H. pylori* binding to glycosphingolipids. The first indication that they may be important came early in the history of *H. pylori*, when it was reported that these bacteria bind to the two major acid glycosphingolipids of the human stomach, i.e. the GM_3_ ganglioside [α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-Glc-(1→1)-Cer] and sulfatide [(SO_3_→3)-β-Gal-(1→1)-Cer] (Saitoh et al., 1991). Since then, a large number of *H. pylori*-binding glycosphingolipids have been reported (summarized in Table 12.1). In some cases the binding-active carbohydrate sequences are also found on glycoproteins, as in the Le^b^ sequence [α-Fuc-(1→2)-β-Gal-(1→3) [α-Fuc-(1→4)-β-GlcNAc], the sialyl-neolacto sequence [α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-GlcNAc] and the neolacto sequence [β-Gal-(1→4)-β-GlcNAc], while in other cases the carbohydrate sequences have hitherto only been identified in glycosphingolipids, such as the lactose sequence [β-Gal-(1→4)-Glc] of lactosylceramide and the α-Gal-(1→4)-Glc sequence of galabiosylceramide. In the following text (and Table 12.1), the various
H. pylori glycosphingolipid-binding activities and their corresponding bacterial carbohydrate-binding proteins are described.

**Sulfatide**
While binding of *H. pylori* to both the GM₃ ganglioside and sulfatide has been reported (Saitoh et al., 1991), binding to GM₃ was later disputed when only sulfatide binding was observed upon examination of binding of *H. pylori* to Kato III cells (Kamisago et al., 1996). The heat-shock protein HSP70 has been proposed to be the adhesin involved in binding of *H. pylori* to sulfatide (Huesca et al., 1996, 1998). Another candidate is *H. pylori* NAP, which binds to sulfated glycoconjugates and sulfated gangliotetraosylceramide ([SO₃₋→3]-β-Gal-(1→3)-[β-GlcNAc-(1→3)]β-Gal(1→4)-[β-Gal-(1→4)]β-Glc-(1→1)-Cer (Teneberg et al., 1997). However, NAP also binds to sulfated oligosaccharides, e.g., (SO₃₋→3)-Gal, (SO₃₋→3)-GlcNAc and sulfated Lea, of human high-molecular-mass salivary mucins (Namavar et al., 1998).

**Neolactoseries gangliosides**
*H. pylori* was initially demonstrated to bind sialylated glycoconjugates and gangliosides (Evans et al., 1988; Miller-Podraza et al., 1997). Subsequently, the *H. pylori* adhesin SabA was identified and a novel high-affinity receptor for this adhesin was isolated from a human gallbladder adenocarcinoma and characterized as sialyl-dimeric-Leα (Mahdavi et al., 2002). The structural requirements for SabA-mediated *H. pylori* ganglioside binding were defined: N-acetyl-lactosamine (LacNAc)-based gangliosides with terminal α-(2→3)-linked Neu5Ac were recognized by wild-type SabA-expressing bacteria, whereas no binding to gangliosides or LacNAc-based gangliosides with terminal α-Neu5Ac-(2→6), α-NeuGc-(2→3) or α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3) occurred (Roche et al., 2004). The minimal epitope required for SabA-mediated *H. pylori* binding to gangliosides is α-Neu5Ac-(2→3)-Gal (Hirmo et al., 1996; Johansson and Karlsson, 1998; Roche et al., 2004). Nevertheless, comparative binding studies (Roche et al., 2004) have shown that the binding affinity is increased by: (i) increased length of LacNAc core chain, whereby this effect is most likely caused by an improved accessibility of the α-Neu5Ac-(2→3)-Gal head group when presented on a longer core chain; (ii) branches of the carbohydrate chain, i.e. a divalent presentation of

### Table 12.1. *Helicobacter pylori*-binding glycosphingolipids.

<table>
<thead>
<tr>
<th>Glycosphingolipid</th>
<th>Ligand</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfatide</td>
<td>HSP70, NAP</td>
<td>Saitoh <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Neolactoseries gangliosides</td>
<td>SabA</td>
<td>Evans <em>et al.</em> (1988); Hirmo <em>et al.</em> (1996); Miller-Podraza <em>et al.</em> (1997); Mahdavi <em>et al.</em> (2002); Roche <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>Ganglioseries glycosphingolipids</td>
<td>Not defined</td>
<td>Lingwood <em>et al.</em> (1992); Angstrom <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Fucosylated blood-group antigen-carrying glycosphingolipids</td>
<td>BabA</td>
<td>Borén <em>et al.</em> (1993); Aspholm-Hurtig <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-GlcNAc-terminated glycosphingolipids</td>
<td>NAP</td>
<td>Teneberg <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>Not defined</td>
<td>Angstrom <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>Galactosylceramide/glucosylceramide</td>
<td>Not defined</td>
<td>Abul-Milh <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>Lactotetraosylceramide</td>
<td>Not defined</td>
<td>Teneberg <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Neolactoseries glycosphingolipids</td>
<td>Not defined</td>
<td>Miller-Podraza <em>et al.</em> (2005, 2009)</td>
</tr>
<tr>
<td>Ganglioseries gangliosides</td>
<td>HP0721</td>
<td>Bennett and Roberts (2005)</td>
</tr>
<tr>
<td>Galabioacerosylceramide</td>
<td>VacA</td>
<td>Roche <em>et al.</em> (2007)</td>
</tr>
</tbody>
</table>

*Abbreviations: Hsp70, heat-shock protein 70; NAP, neutrophil-activating protein; SabA, sialic acid-binding adhesion; BabA, blood group-binding adhesion; VacA, vacuolating cytotoxin.*
the binding epitope; and (iii) fucose substitution of the LacNAc core chain, whereby the fucose residues may either interact with the carbohydrate-binding site of the SabA adhesin or affect the conformation of the ganglioside, providing an optimal presentation of the α-Neu5Ac(2→3)-Gal epitope.

Neutrophil infiltration and activity is believed to play an important role in gastritis severity and disease progression. Several of the H. pylori-binding gangliosides, for example α-sialyl-(2→3)-neolactotetraosylceramide, α-sialyl-(2→3)-neolactoctaheexasylceramide, α-sialyl-(2→3)-neolactoctaoctasylceramide, the VIM-2 ganglioside and sialyl-dimeric-Leα glycosphingolipid, are present on human neutrophils (Stroud et al., 1996), suggesting that the ganglioside-binding capacity may be involved in neutrophil activation. Indeed, the H. pylori-induced neutrophil oxidative burst reaction can be inhibited by pre-incubation with sialylated oligosaccharides (Teneberg et al., 1997). The relative contributions of SabA and NAP in neutrophil activation have been investigated using a battery of H. pylori deletion mutants (Unemo et al., 2005). Mutant and wild-type strains lacking SabA were devoid of neutrophil-activating capacity, while the absence of either NAP or the Leβ-binding BabA had no effect on neutrophil activation. Thus, SabA-mediated binding of H. pylori bacterial cells to sialylated neutrophil receptors can play an important initial role in the adherence and phagocytosis of the bacterium, and the induction of the neutrophil oxidative burst reaction.

$\alpha$-Neu5Ac(2→3)-β-Gal(1→4)-β-GlcNAc(1→3)-β-Gal(1→4)-β-Glc-(1→1)-Cer and α-Neu5Ac(2→3)-neolactooctaosylceramide [α-Neu5Ac(2→3)-β-Gal(1→4)-β-GlcNAc(1→3)-β-Gal(1→4)-β-GlcNAc(1→3)-β-Gal(1→4)-β-Glc-(1→1)-Cer]. A terminal α-NeuGc(2→3) was also tolerated, i.e. NAP also bound to α-NeuGc(2→3)-neolactooctaosylceramide and α-NeuGc(2→3)-neolactoctaohexaosylceramide, in contrast to the SabA adhesin which requires a terminal α-Neu5Ac(2→3) for binding.

Fucosylated blood-group antigen-carrying glycosphingolipids

In the initial study where the binding of H. pylori to the H5-type-1/Leb determinants was defined, no binding to glycosphingolipids carrying these determinants was observed (Borén et al., 1993). Nevertheless, subsequent studies have shown that BabA-expressing H. pylori strains bind to glycosphingolipids with Leβ-related carbohydrate epitopes, with the same recognition patterns as found with neoglycoproteins (Aspholm-Hurtig et al., 2004).

Lactosylceramide

Many H. pylori strains, as well as other bacteria including both pathogens and commensals (Karlsson, 1989), bind to lactosylceramide [β-Gal(1→4)-β-Glc-(1→1)-Cer] and isoglobotriaosylceramide [α-Gal(1→3)β-Gal(1→4)-β-Glc-(1→1)-Cer] with a concomitant binding to gangliotetraosylceramide [β-Gal(1→3)-β-GalNAc(1→4)-β-Gal(1→4)-β-Glc-(1→1)-Cer] (Angstrom et al., 1998). Interestingly, a putative glycosphingolipid-binding motif in H. pylori adhesin A (HpaA) was recently identified by structure similarity searches (Fantini et al., 2006). A synthetic peptide corresponding to this motif bound to lactosylceramide, but not to globotriaosylceramide, consistent with the glycosphingolipid-binding pattern of whole H. pylori bacterial cells (Angstrom et al., 1998). The identification of this glycosphingolipid-binding motif may allow further dissection of the lactosylceramide-binding properties of
H. pylori, and also other lactosylceramide-binding bacteria, in the future.

**Additional glycolipid structures to which H. pylori binds**

H. pylori has been demonstrated to bind to a range of additional glycolipid structures, for which the bacterial adhesin has not been identified, such as galactosylceramide with sphingosine and both hydroxy and non-hydroxy fatty acids (Abul-Milh et al., 2001), the neolacto [β-Gal-(1→4)-β-GlcNAc] core structure (Miller-Podraza et al., 2005, 2009) and lactotetraosylceramide [β-Gal(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-Glc-(1→1)-Cer] (Mahdavi et al., 2002; Teneberg et al., 2002). A novel sialic acid-binding protein of H. pylori (HP0721) was recently isolated (Bennett and Roberts, 2005) and bound to the GM₂ ganglioside [α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-Glc-(1→1)-Cer], the gangliosides GM₃, [β-Gal(1→3)-β-GalNAc-(1→4) [α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer] and GD₃ [α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4) [α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer], and also to gangliotetraosylceramide [β-Gal-(1→3)-β-GlcNAc-(1→4)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer, i.e. a non-sialylated glycolipid. Unfortunately no negative reference glycosphingolipid was included in this assay. Since H. pylori cells have previously been shown not to recognize the GM₃ ganglioside, or gangliosides based on the ganglio core structure (Kamisago et al., 1996; Miller-Podraza et al., 1997; Roche et al., 2004), the role of the sialic acid binding of HP0721 needs to be clarified.

**Vacuolating cytotoxin binding to galabiaosylceramide**

Human gastric glycosphingolipids recognized by the important H. pylori virulence factor vacuolating cytotoxin (VacA) have been characterized as galactosylceramide [β-Gal-(1→1)-Cer] and galabioosylceramide [α-Gal-(1→4)-β-Gal-(1→1)-Cer] (Roche et al., 2007). Comparison of the binding preferences of VacA using reference glycosphingolipids from other sources showed the additional recognition of glucosylceramide [β-Glc-(1→1)-Cer], lactosylceramide [β-Gal-(1→4)-β-Glc-(1→1)-Cer] and globotriaosylceramide [α-Gal-(1→4)-β-Gal-(1→4)-β-Glc-(1→1)-Cer]. No binding to the glycosphingolipids recognized by the VacA holotoxin was obtained with a mutant toxin with a deletion of the 37 kDa fragment of VacA (p58 molecule), indicating that carbohydrate recognition is mediated by the 37 kDa moiety (Roche et al., 2007). Since both toxin domains are required for binding and internalization of VacA, and also for its vacuolating cytotoxic effect (Torres et al., 2005), this suggests a scenario where target cell specificity is obtained by an initial binding of the toxin to high-affinity protein receptors via the p58 molecule, followed by an interaction via the p37 domain to short-chain glycosphingolipids. Binding to mono- and di-glycosylceramides would provide VacA with a membrane-close attachment that may facilitate the internalization of the toxin.

**12.4 Conclusions**

The presence of multiple binding abilities of H. pylori implies that adherence is a multistep process, and that different adhesins mediate adherence to different sites along the colonization axis and during the varying inflammatory status of the host. The personal repertoire of glycans on mucins and glycolipids and transient changes in mucin glycosylation in response to infection or stress (Bosch et al., 2003), as well as circadian variations in acidity in the gastric lumen and H. pylori adherence repertoire, all affect binding. The continuous secretion of mucins which can bind H. pylori via BabA, SabA and the low pH-binding modes are likely to have a protective function, interfering with the bacterial aim of binding to the gastric cell surface. Similarly, SP-D interaction with H. pylori LPS can also play an important protective role, but this may be subverted by the production of escape variants. Competition between binding to the cell surface-bound structures such as glycolipids and cell-associated mucins, the adherent gastric mucus layer, the mixture of sloughed-off gastric mucins and swallowed saliva in
the gastric juice in the lumen of the stomach is likely to influence the ability of the bacteria to colonize this region.

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13 Helicobacteromics – Genomics of a Highly Variable, Well-adapted and Persistent Pathogen

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13.1 From the Landmarks of Helicobacter pylori Research to ‘Helicobacteromics’

Helicobacter pylori-related diseases represent a common problem that many individuals encounter during their lifetime. The discovery of H. pylori led to a major breakthrough in gastroenterology. While reports on spiral bacteria in the stomach date back to the 19th century (Bizzozero, 1893; Konjetzny, 1923), many severe inflammatory diseases of the upper intestinal tract remained mostly incurable before the discovery of H. pylori by Marshall and Warren (http://nobelprize.org; Marshall and Warren, 1984; Marshall et al., 1985). The corresponding novel and revolutionary insights offered the possibility to treat gastric diseases by antimicrobial therapy and provided a novel understanding of mechanisms underlying gastritis, ulcerations, gastric cancer and lymphoma (Suerbaum and Michetti, 2002; Ahmed and Sechi, 2005; Cover and Blaser, 2009).

In the last decade, scientific investigation of H. pylori and infection-related diseases was greatly advanced by the release of genome sequences of several H. pylori strains (Tomb et al., 1997; Alm et al., 1999; Baltrus et al., 2009b; http://www.genomesonline.org), which opened the way to study and compare complete gene contents of individual strains. The results of such studies demonstrate a high degree of overall genetic instability (micro-evolution) within individual hosts. The rapid and ongoing process of functional and comparative genomics has revealed novel aspects of microbial biology and evolution, as well as of pathogenicity and gastric adaptation. In this chapter on ‘Helicobacteromics’, we focus on genomic and proteomic aspects of H. pylori and highlight the role of this novel information in the study of host-adaptation strategies and pathogen evolution (reviewed in de Reuse and Bereswill, 2007). Population genomics of H. pylori strains from different locations worldwide have metamorphosed epidemiology and provided astonishing information on the mechanisms that affect host adaptation and persistence (reviewed by Linz and Schuster, 2007). In addition, Helicobacteromics has also shed new light on the forces that shape the evolutionary history of bacterial pathogenesis and virulence acquisition, in some cases through co-evolution with the host. Even more spectacular is the genome information obtained from H. pylori strains worldwide that has been used to successfully retrace the migration of ancient human populations. More generally, multiple genomic sequences provide insights into the evolutionary processes that have shaped the H. pylori genome evolution and generated its unusual genetic instability. Analyses of genome plasticity and gene pools have led to
the definition of the core genome (genes in common to all sequenced strains) and the pan genome (the sum of the core and of dispensable genes shared by all sequenced strains). The overwhelming quantity of novel genetic information could not have resulted in answers to biologically relevant questions without a concomitant revolution in the development of bioinformatic approaches and high-throughput experimental technologies (functional genomics). This chapter also covers these different aspects and novel trends in Helicobacteromics.

By taking individual aspects of different disease manifestations as examples, we discuss the evolutionary forces that accompany the complex host interactions in the light of bacterial ecology. Most important frameworks of host adaptation are illustrated by the specificity for the human host and the lifelong persistence of this infection. Other paragraphs summarize how H. pylori has evolved through several mechanisms, with horizontal gene transfer and DNA recombination playing a major role. The resulting genetic drift has equipped H. pylori strains with different pathogenic potential and adaptation strategies. Some have acquired the capacity to rapidly adapt to changing environments in order to enhance the spectrum of sites within the stomach that can be infected. In conclusion, the combination of topics dealing with pathogenesis and evolution provides the reader with a global view of current and future trends in Helicobacteromics. To complete insights into this research focus, we discuss possible mechanisms involved in colonization of the restricted and hostile natural reservoir (reviewed by Kusters et al., 2006) including inter-species transmission, genetic diversity and regulatory pathways.

13.2 Comparative Genomics in H. pylori Research

13.2.1 Overall genetic variability – a key to host specificity and gastric adaptation

In the gastric mucosa, H. pylori is continuously faced with harsh conditions and a vigorous immune response. The need for adaptation to this extreme and variable microenvironment (van Vliet et al., 2001) is thought to drive the inter-strain genetic variation observed in the H. pylori population worldwide. Point mutations, insertions and deletions of genes (Blaser, 1994) and intergenic regions (Bereswill et al., 2000a) present in all H. pylori isolates provide flexible mechanisms to subvert host immunity (Cooke et al., 2005) and to adapt to unfavourable conditions (van Vliet et al., 2001). The genome displays a high degree of plasticity (Jiang et al., 1996; reviewed in Baltrus et al., 2009a). The inter-strain diversity of H. pylori is extended by plasmids (Hofreuter and Haas, 2002). Genetic recombination is promoted by active DNA import (Karnholz et al., 2006), which is a driving force of environmental adaptation (Baltrus et al., 2008). However, detailed molecular mechanisms mediating genetic variability remain to be elucidated and are currently being intensively investigated (reviewed in Baltrus et al., 2009a). As H. pylori encounters multiple forms of stress in the gastric environment that promote DNA damage, genome-wide analysis of the presence or absence of orthologues of DNA recombination and repair enzymes may provide clues towards understanding the microbial diversity related to host and niche. A recent study reported that H. pylori carries all biochemical functions that are associated with genome dynamics, namely DNA recombination and horizontal gene transfer, but lacks some of the most important enzymes involved in post-replication and DNA repair (Ambur et al., 2009). However, an understanding of the precise molecular functions of the enzymes participating in DNA metabolism and the possible future role for these enzymes as targets for therapeutic intervention await detailed investigations.

The release of the genome sequences of H. pylori strains 26695 and J99 (Tomb et al., 1997; Alm et al., 1999) offered the basis for the first investigation of genomic instability and macro-evolution. At that time, about one-third of the approximately 1600 genes predicted in the 1.6 Mbp genome were considered to be H. pylori-specific due to the absence of homologues in other organisms (Marais et al., 1999; Boneca et al., 2003). The fact that even these two genomes displayed a high degree of diversity in relation...
to insertions, deletions, overall genome structure and plasticity led to the proposal that the *H. pylori* population represents a ‘quasi-species’ (Covacci and Rappuoli, 1998) with a panmictic structure due to free recombination (Suerbaum et al., 1998; Suerbaum and Achtman, 2004). The repertoire of mechanisms by which *H. pylori* can generate genetic variability is completed by sequence changes affecting phase-variable genes in which mutations can ‘turn on’ or ‘shut off’ gene expression (De Vries et al., 2001). Comprehensive analysis of homopolymeric tracts and dinucleotide repeats in phase-variable gene subsets deduced from strains 26695 and J99 has revealed nucleotide variations that can be present or absent in these strains (Salaun et al., 2004). A recent global genomic investigation of single-sequence repeats (SSR) in pathogenic bacteria revealed that *H. pylori* encodes a number of SSR-associated proteins (Guo and Mrázek, 2008). The SSR-associated genes encode an ATP-binding protein (*mrp*), a rod shape-determining protein (*mreB*), a hypothetical protein (HP0059) and the cytotoxin-associated genes (*cag*) product, CagA. The authors conclude that location of longer SSRs near genes encoding membrane proteins and virulence genes is consistent with their possible role in antigenic variation.

The fact that 6–7% of genes in the first two sequenced *H. pylori* genomes were strain-specific (Alm et al., 1999) provided the first evidence for the presence of a variable gene pool, which is indicative of horizontal gene transfer between *H. pylori* strains. Because these strain-specific genes could be involved in gastric adaptation during co-evolution, this flexible gene pool has been extensively investigated in many strains worldwide. *In silico* analysis of the two *H. pylori* genomes (Garcia-Vallve et al., 2002; Saunders et al., 2005) revealed that both housekeeping genes and virulence genes are transferred among *H. pylori* strains. Divergent codon usage and GC content provided evidence that 69 genes were acquired from other species. Genetic variability and DNA exchange among *H. pylori* strains was further studied by microarray analysis. The results demonstrated that the gene content of *H. pylori* isolates from the same (Israel et al., 2001) and different (Salama et al., 2000) individuals displayed between 3 and 22% variability, respectively. Based on global gene distribution in 15 unrelated *H. pylori* strains obtained from different geographical origins (Salama et al., 2000), the core genome has been estimated to consist of 1280 genes, including those coding for central (housekeeping), conserved and essential functions (Salama et al., 2000). More than 300 genes are not homogeneously distributed and many of the dispensable genes are located in the so-called ‘plasticity zones’ and in the *cag* pathogenicity island (PAI). Other variable genes are involved in the synthesis of surface structures and in DNA modification or transposition. It is striking that many genes in the flexible pool encode *H. pylori*-specific proteins or conserved proteins of as yet unknown functions.

The extreme host specificity of *H. pylori* offers the possibility to use genome comparisons of related bacterial species to identify the genes responsible for adaptation to the human host. The availability of genomes of closely related species such as *Helicobacter hepaticus* (Suerbaum et al., 2003), *Campylobacter jejuni* (Parkhill et al., 2000) and *Wolinella succinogenes* (Baar et al., 2003) offered the opportunity to extend our knowledge regarding *H. pylori* evolution (Eppinger et al., 2004). A comprehensive analysis of the genomes available from these four different *Campylobacterales* species revealed that *H. pylori* and two of these other host-adapted pathogens have lost regulatory and environmental sensing circuits, while in the non-pathogenic commensal *W. succinogenes* (Eppinger et al., 2004) these are still conserved. The finding that *Wolinella* also contains more complete metabolic pathways supports the conclusion that the *Helicobacter* species analysed so far fulfil the paradigm of reductive evolution (as a consequence of host adaptation). In addition, comparison of the *Campylobacterales* genomes with the genetic information of the sequence databases has allowed the identification of genes and molecular signatures that are unique to members of the epsilon proteobacteria (Gupta, 2006). Whole-genome clustering of *H. pylori* and *C. jejuni* has demonstrated that 648 *H. pylori* genes are species-specific (Janssen et al., 2001). The fact that 95% of the 162 *H. pylori* genes displaying interstrain variability are absent in other bacteria...
indicates that genes of the flexible gene pool are exchanged among Helicobacter species, but are not transferred to bacteria of other genera.

Based on recent genome comparisons of \textit{H. pylori} and \textit{Helicobacter acinonychis}, which colonizes felines, it was postulated that a host jump occurred about 200,000 years ago, at which time \textit{H. pylori} was transferred from humans to cats (Eppinger \textit{et al}., 2006). Extended microarray analysis of the gene distribution and multilocus sequence typing (MLST) of seven core genes of 56 \textit{H. pylori} strains (Gressmann \textit{et al}., 2005) revealed that 25\% of genes that are common to both 26695 and J99 were missing in at least one isolate, and that 21\% of genes were absent or variable in \textit{H. acinonychis}. In addition, the authors of that study concluded that there was a core genome of 1111 genes and predicted that the \textit{cag} PAI genes were acquired ‘\textit{en bloc}’ after speciation. Variable genes are small, possess unusual GC content and encode mostly proteins of unknown function or outer membrane proteins. Many proteins of unknown function and transposases were predicted to have been acquired prior to speciation (Gressmann \textit{et al}., 2005). The fascinating insights and novel hypotheses provided by genome-wide analysis of \textit{H. pylori} are continuously further expanded and verified by the ongoing release of novel genome sequences of additional \textit{H. pylori} strains (Baltrus \textit{et al}., 2009b) and closely related \textit{Helicobacter} species, such as \textit{Helicobacter canadensis} (Loman \textit{et al}., 2009). A complete and actual listing is available on the Internet (http://www.genomesonline.org).

13.2.2 Diversification of \textit{H. pylori} strains in individual hosts

Changes in outer membrane proteins may represent a dynamic response in the \textit{H. pylori} surface structure that facilitates adherence to the gastric epithelium in individual human hosts, as well as promoting chronic infection. This conclusion is supported by the extensive genotypic diversity displayed by individual human \textit{H. pylori} isolates, as well as within strains colonizing individual patients at different time points, independent of disease development. As gene loss or gain occurring in a single host might play an important role in gastric adaptation, the resulting microevolution is a driving force for genetic diversity of \textit{H. pylori}. The gene content of \textit{H. pylori} strains isolated sequentially from single patients during persistent infection has confirmed that gene loss and acquisition of exogenous DNA occurs (Israel \textit{et al}., 2001). Furthermore, multilocus sequence analysis of ten genes in paired \textit{H. pylori} isolates from 26 different individuals (Falush \textit{et al}., 2001) has shown that point mutations occur in the stomach of a single host, and that mostly small mosaic DNA segments with a median size of 417 bp are exchanged. Calculations of mutation and recombination frequencies with respect to insert sizes revealed that genetic diversity displayed by the panmictic population structure is a result of continuous DNA exchange between parental strains (without mutations) and daughter strains, which have accumulated mutations. This finding was supported by gene content analysis of isolates taken from single patients at different time points, which demonstrated that the great majority of genetic changes were caused by homologous recombination, indicating that the adaptation of \textit{H. pylori} to the host is more frequently mediated by sequence changes acquired by recombination events, rather than loss or gain of genes (Kraft \textit{et al}., 2006).

13.2.3 Worldwide co-evolution and spreading with the human host

The rapid progress in sequencing technologies and related \textit{in silico} software tools for sequence analysis has paved the way for investigations focused upon the gene content and genetic diversity of \textit{H. pylori} populations worldwide. There is strong evidence that \textit{H. pylori} has colonized humans for more than 100,000 years (Covacci \textit{et al}., 1999) and that there is a significant correlation between \textit{H. pylori} genotypes and prehistoric human migrations (reviewed in Suerbaum and Achtman, 2004; Linz and Schuster, 2007; Suerbaum and Josenhans, 2007; Moodley and Linz, 2009). MLST analysis of selected core
genes in *H. pylori* isolates from ethnic subpopulations of people worldwide has established that the global *H. pylori* population can be subdivided into seven genetically distinct subpopulations, which derived their gene pools from ancestral populations that arose in Africa, Central Asia and East Asia (Falush et al., 2003a). The optimization of the mathematical basis for the use of multilocus genotype data (Falush et al., 2003b) has revealed close associations between *H. pylori* subtypes and human subpopulations in one continent or even ethnic subgroups within small geographic regions (Wirth et al., 2004). Thus, *H. pylori* subtypes can be used to trace human migrations during history, and the spreading of distinct *H. pylori* subtypes could be attributed to prehistoric and modern migratory fluxes (Suerbaum and Achtmann, 2004; Linz et al., 2007). Our view of the speciation and evolution biology of *H. pylori* has been further expanded by analysis of isolates from human subpopulations and ethnic subgroups in India (Devi et al., 2007), South-east Asia, Australia and the Pacific (Moodley et al., 2009). The genotyping of *H. pylori* isolates in India revealed that the Indian and European *H. pylori* isolates share a common ancestral origin (Devi et al., 2007). Moreover, the newly discovered *H. pylori* genotypes allowed the differentiation of human migrations to New Guinea, Australia and the Polynesian islands (Moodley et al., 2009). These results verified again that distinct local *H. pylori* subpopulations are shaped by complex human migrations and the mixing or separation of local ethnic subpopulations. In another study, *H. pylori* isolates from Malaysia could be assigned to previously identified *H. pylori* ancestral populations, hpEastAsia, hpAsia2 and hpEurope (Tay et al., 2009). Most interestingly, the results allowed the identification of a novel *H. pylori* subpopulation, hpIndia, within hpAsia2. Sequence comparisons with Chinese and Indian isolates revealed that the majority of the Malay and Indian *H. pylori* isolates share the same origin, while the Malaysian and Chinese *H. pylori* populations are distinctive. This suggests that the Malay population gained the pathogen only recently by ethnological mixing with other subpopulations (Tay et al., 2009).

### 13.2.4 Horizontal gene transfer – the driving force of strain diversity?

Lifelong persistence and intra-familial transmission of *H. pylori* support the view that genetic material is continuously exchanged among different generations of the bacterium via horizontal gene transfer. The importance of horizontal gene transfer in the variability of *H. pylori* has been underlined by the finding that clonal lineages of *H. pylori* populations coexist in individuals and evolve separately from one another (Ahmed and Sechi, 2005). Continuous horizontal gene transfer between strains of different generations is thought to be causative for: (i) genome plasticity (Baltrus et al., 2009a); (ii) evolution of distinct sets of *H. pylori* genotypes in individual human hosts and populations; (iii) the completely diverse and panmictic genetic population structure; and (iv) the association of defined *H. pylori* genotypes with particular geographic regions. The definitive coexistence of distinct sequence variants, and transfer from one *H. pylori* population in a given local area to another *H. pylori* population in another geographic region via human migration, is clearly driven by the high potential of *H. pylori* for DNA uptake and recombination. However, this makes the association of particular genotypes with specific geographic locations even more difficult. Hence, while polymorphisms within the *H. pylori* genome can serve as useful markers for studying ancient human migrations, these types of analyses are certainly complicated by the mixing of *H. pylori* strains from migrated and native populations (Ahmed and Sechi, 2005).

### 13.2.5 *H. pylori* microevolution and disease progression

The identification of flexible genome areas, plasticity zones, operons and single genes associated with severe pathologies represents a major and ongoing challenge in *H. pylori* research. Molecular analyses during the pre-genomic era established that the sequences of virulence-associated genes coding for the vacuolating cytotoxin VacA (Atherton et al.,...
1995) and the CagA protein (Blaser, 1994) varied considerably among strains. In addition, at that time, specific genotypes of VacA were associated with ulcer development (Atherton et al., 1995) and the presence of the cagPAI promoting injection of the CagA protein into host cells correlated with pronounced inflammation and more severe pathologies (see Backert et al., Chapter 11, this volume). The rhesus macaque model has greatly contributed to the elucidation of genomic changes in H. pylori that occur early during experimental infection (Solnick et al., 2004). Microarray analysis demonstrated that H. pylori recovered from infected macaques carried deletions in the locus coding for BabA, an adhesin that mediates attachment of H. pylori to gastric epithelia. In some isolates the babA gene was not expressed or was replaced by babB, which encodes a related protein. The absence of babA and duplication of babB has also been observed in H. pylori isolates derived from human clinical samples, suggesting that this gene conversion is of relevance to the human host and might reflect diverging selective pressures for adhesion either across hosts or within an individual (Colbeck et al., 2006).

Persistent colonization of the human stomach is associated with asymptomatic gastric inflammation (gastritis) and an increased risk of duodenal ulceration, gastric ulceration and non-cardia gastric cancer. Results from genome analysis of H. pylori isolates from patients suffering from different diseases (for a review, see Linz and Schuster, 2007) suggest that the genome content accounts for the development of different pathologies (Romo-González et al., 2009). The comparative investigation of genome sequences revealed that H. pylori strains isolated from patients with gastric cancer or gastric ulcer disease are most closely related to strains of East Asian or European origin, respectively (McClain et al., 2009). Among the core genes determined, a subset of alleles was found to be highly divergent in the East Asian strain, encoding proteins that exhibited <90% amino acid sequence identity compared with corresponding proteins in the strains of other geographic origin. Unique strain-specific genes were identified in each of the newly sequenced strains, and a set of strain-specific genes was shared among H. pylori strains associated with gastric cancer or malignant processes.

Although H. pylori causes an initial acute superficial gastritis which over time develops into a chronic gastritis, in some infected individuals this will progress to chronic atrophic gastritis (ChAG). This pathology is characterized by diminished numbers of acid-producing parietal cells and an increased risk for the development of gastric adenocarcinoma. The complete genome analysis of the first H. pylori isolate from a patient with ChAG gave further insights into gene sets that could be involved in causing this specific disease outcome (Oh et al., 2006). Whole-genome analysis of additional H. pylori isolates collected from an individual who progressed from ChAG to gastric adenocarcinoma revealed a gene signature shared among ChAG strains, as well as genes that may have been lost or gained during progression to adenocarcinoma. Many of these genes encode components of metal uptake and utilization pathways, outer membrane proteins and virulence factors, indicating that the bacteria adapt effectively to environmental changes during ChAG disease progression. Taken together, these data provide insight into the diversity that exists among H. pylori strains from different clinical manifestations. Highly divergent alleles and strain-specific genes identified in this study may represent useful biomarkers for analysing geographic partitioning of H. pylori and for identifying strains capable of inducing malignant or premalignant gastric lesions.

13.2.6 Novel proteomics tools identify biomarkers of disease progression

More recently, proteomic tools have been used for the identification of novel diagnostic H. pylori biomarkers for predicting disease progression (Wu et al., 2008). The current disease paradigm suggests that the final outcome of H. pylori infection is strongly dependent on host genetics and bacterial virulence, both playing important roles in modulating disease development. By using high-throughput platforms, the authors
identified several crucial proteins that have pathogenic and prognostic potential. For example, antibodies to alkaline hydroperoxide reductase and the co-chaperonin GroES of *H. pylori* could be utilized for identifying patients at high risk of disease complications after *H. pylori* infection. In addition, "immunoproteomics" has provided global information about antigens relevant in different disease states, and thus might be suitable for identifying novel vaccine candidates or serological markers. For example, the humoral immune response to *H. pylori* was used for diagnostic purposes and as a basis for vaccine development (reviewed by Bernardini et al., 2007). In another study, the analysis of protein patterns of *H. pylori* strains isolated from Korean and Colombian patients suffering from duodenal ulcer or gastric cancer by high-throughput surface-enhanced laser desorption/ionization time-of-flight mass spectrometry identified 18 statistically significant candidate biomarkers discriminating between the two clinical outcomes (Khoder et al., 2009). Three biomarker proteins were purified and identified as the neutrophil-activating protein NapA, an RNA-binding protein and a DNA-binding histone-like protein. These novel biomarkers might be of use for predicting the evolution to gastric cancer in *H. pylori*-infected patients.

Such proteomic technologies are predicted to facilitate biomarker identification that in the future may have the potential to provide antigens for use in vaccines for preventing *H. pylori* infection. Together with appropriate clinical phenotyping and genotyping, this information will clearly enhance our understanding of disease progression/pathogenesis and lead to more precise prediction of variable disease outcomes.

### 13.3 Functional Genomics to Study the Adaptation of *H. pylori* to the Gastric Environment: Transcriptome Studies

The stomach is the unique niche colonized by *H. pylori*, where it proliferates without competition, as no other microbe is able to permanently inhabit this hostile environment. To multiply and persist in the gastric environment, *H. pylori* has developed specific molecular strategies and factors to deal with adverse and unstable environmental conditions such as acidity, fluctuating nutrient availability, changes in oxygen tension and the intense immune response of the host. Despite these variable environments, *H. pylori* is a particularly successful pathogen, being able to proliferate in its host over many decades. In addition, there is a link between the *H. pylori* adaptive response and its pathogenicity, as illustrated by the roles of the strongly regulated urease virulence factor. In bacteria, transcriptional regulation is an initial and rapid response to changing environments and stresses. Since two complete genome sequences of *H. pylori* were already available at the end of the 20th century, profiling of gene expression using DNA arrays was the approach of choice to investigate the regulons and the stimulons of this pathogen. A regulon represents the group of genes regulated by a given transcriptional regulator and a stimulon, a set of genes that are transcriptionally regulated in response to a perturbation of the environment. Accordingly, genome-wide transcriptional profiling using a DNA array aims to define the changes in the transcriptome of an organism either exposed to a modification of its environment/growth conditions or that carries a mutation. Numerous transcriptomic studies have been carried out for *H. pylori* (Table 13.1) leading to the conclusion that *H. pylori* has a unique and particularly complex adaptation potential through modulation of gene expression. In the following sections we discuss mainly the currently known functions regulated under these conditions; however, this is not to say that a large number of other as yet unknown genes are not also regulated.

#### 13.3.1 Transcriptomics to characterize the *H. pylori* stimulons *in vitro*

In the case of *H. pylori*, a major effort has concentrated on examining its response to conditions mimicking those encountered during host colonization.
### Table 13.1. Studies using whole-genome transcriptional profiling of *Helicobacter pylori*.

<table>
<thead>
<tr>
<th>Condition or protein factor tested</th>
<th>Experimental procedure</th>
<th>Strain(s) used</th>
<th>Total number of regulated genes</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>H. pylori transcriptome in conditions mimicking those encountered in the human host</strong></td>
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<tr>
<td>In response to acidity</td>
<td>Growth on plates (48 h) at pH 5.5 versus pH 7</td>
<td>26695</td>
<td>84</td>
<td>Ang et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>Exposure to pH 4 versus pH 7 for 30 min</td>
<td>26695</td>
<td>11</td>
<td>Allan et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>Exposure to pH 5 of a liquid culture grown at pH 7. Time course from 0.5 to 2 h</td>
<td>G27</td>
<td>118</td>
<td>Merrell et al. (2003a)</td>
</tr>
<tr>
<td></td>
<td>Exposure to pH 4.5, 5.5, 6.2, 7.4 with and without 5 mM urea for 30 min</td>
<td>26695</td>
<td>300</td>
<td>Wen et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Exponential growth in liquid medium at pH 5 and pH 7</td>
<td>26695</td>
<td>101</td>
<td>Bury-Moné et al. (2004)</td>
</tr>
<tr>
<td>As a function of growth phase</td>
<td>Growth in liquid medium from exponential to stationary phase. Time course from 0 to 50 h</td>
<td>SS1</td>
<td>325</td>
<td>Thompson et al. (2003)</td>
</tr>
<tr>
<td>In response to iron starvation</td>
<td>Iron chelation or add-back of bacteria in exponential and stationary phase. Time course from 0 to 100 min</td>
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<td>183</td>
<td>Merrell et al. (2003b)</td>
</tr>
<tr>
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<td>Bacteria attached to AGS cells over a 4 h period versus in the same conditions without cells</td>
<td>69a</td>
<td>43</td>
<td>Kim et al. (2004)</td>
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<tr>
<td>In the gerbil stomach</td>
<td>Colonization during 10 days versus <em>in vitro</em> growth</td>
<td>69a</td>
<td>Not provided</td>
<td>Scott et al. (2007)</td>
</tr>
<tr>
<td><strong>H. pylori transcriptome in mutants</strong></td>
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<tr>
<td>NikR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WT versus ΔnikR mutant, in the presence of nickel</td>
<td>SS1</td>
<td>42</td>
<td>Contreras et al. (2003)</td>
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<tr>
<td>Fur&lt;sup&gt;b&lt;/sup&gt;</td>
<td>WT versus Δfur mutant in iron-restricted or iron-replete conditions grown for 20 h</td>
<td>26695</td>
<td>97 iron-responsive regulation</td>
<td>Ernst et al. (2005a)</td>
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<td></td>
<td></td>
<td></td>
<td>43 Fur-dependent regulation</td>
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<td></td>
<td>WT versus Δfur mutant, exponential and stationary growth phase</td>
<td>G27</td>
<td>29</td>
<td>Gancz et al. (2006)</td>
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<tr>
<td></td>
<td>WT versus Δfur mutant, exponential and stationary growth phase</td>
<td>G27</td>
<td>26 in exponential phase</td>
<td>Danielli et al. (2006)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>90 in stationary phase</td>
<td></td>
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<tr>
<td>ArsRS&lt;sup&gt;c&lt;/sup&gt; (HP166–HP165)</td>
<td>WT versus a HP165 mutant</td>
<td>26695 and B128</td>
<td>7</td>
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<tr>
<td>σ&lt;sup&gt;28&lt;/sup&gt;, FlgM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>WT versus mutants</td>
<td>N6</td>
<td>NA</td>
<td>Josenhans et al. (2002)</td>
</tr>
<tr>
<td>Condition or protein factor tested</td>
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</tr>
<tr>
<td>H. pylori transcriptome in mutants</td>
<td>WT versus mutants and mutants versus mutants</td>
<td>N6 and 88-3887 (a motile variant of 26695)</td>
<td>NA</td>
<td>Niehus et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>NikR&lt;sup&gt;a&lt;/sup&gt; WT versus ∆nikR mutant, in the presence of nickel</td>
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<tr>
<td></td>
<td>Fur&lt;sup&gt;b&lt;/sup&gt; WT versus ∆fur mutant, exposure of a liquid culture grown at pH 7 to pH 5. Time course from 0.5 to 1.5 h</td>
<td>G27</td>
<td>95</td>
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<tr>
<td></td>
<td>NikR&lt;sup&gt;a&lt;/sup&gt; and Fur&lt;sup&gt;b&lt;/sup&gt; ∆nikR-∆fur mutant during growth at pH 5 versus pH 7</td>
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<td>36</td>
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<tr>
<td>ArsR&lt;sup&gt;c&lt;/sup&gt;Sc (HP166–HP165)</td>
<td>WT versus ∆arsS mutant, exposure to pH 5 for 1 h</td>
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<tr>
<td>ArsR&lt;sup&gt;c&lt;/sup&gt;Sc (HP166–HP165)</td>
<td>WT versus ∆arsS mutant, exposure to pH 5 for 75 min</td>
<td>J99</td>
<td>68 on 101 considered genes</td>
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</tr>
<tr>
<td>CrdRS&lt;sup&gt;g&lt;/sup&gt; (HP1364)</td>
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<td>J99</td>
<td>63 on 101 considered genes</td>
<td>Loh and Cover (2006)</td>
</tr>
<tr>
<td>FlgS&lt;sup&gt;d&lt;/sup&gt; (HP0244)</td>
<td>WT versus a flgS mutant, exposure to pH 7.4, 4.5 and 2.5 with 10 mM urea</td>
<td>26695</td>
<td>NA</td>
<td>Wen et al. (2009)</td>
</tr>
</tbody>
</table>

*WT, wild type; NA, not applicable.

<sup>a</sup>NikR (HP1338): nickel-responsive regulator.

<sup>b</sup>Fur (HP1027): iron-responsive regulator.

<sup>c</sup>ArsR-S (HP166–HP165): two-component system involved in acid-responsive signalling. ArsR: response regulator, ArsS: sensor histidine kinase.

<sup>d</sup>σ<sub>28</sub> (FliA, HP1032), FlgM (anti-σ<sub>28</sub>, HP1122), σ<sub>54</sub> (RpoN, HP0714), FlgRS (HP0703–HP0244), FlhF (HP1035), FlhA (HP1041), HP0958, FliK (HP0906): regulators of the flagellar genes expression or components of the flagellar machinery.

<sup>e</sup>HP1021: orphan response regulator.

<sup>f</sup>HrcA (HP0111), HspR (HP1025): heat shock transcriptional regulators.

Response to acidity

One of the most remarkable properties of H. pylori is its unusual capacity to persistently colonize an extremely acidic niche. While this gastric pathogen is able to survive acidity in the stomach lumen in the initial phase of infection (median pH of 1.4), persistent colonization mainly establishes within the mucus layer where a less acidic pH prevails (Schreiber et al., 2004). Adhesion to the epithelial cells at a pH close to neutrality only occurs in about 20% of the H. pylori population and this process is thought to be dynamic, with bacteria continually attaching to and detaching from the epithelial cell layer. In this environment, H. pylori is frequently exposed to a fluctuating pH to which it needs to adapt rapidly in an appropriate and well-coordinated way. As a consequence, it became rapidly clear that the response of H. pylori to pH is much more complicated than that of other urease-negative gastrointestinal pathogens, such as Escherichia coli or Salmonella enteritidis.

Given this, low pH was the first environmental condition to be extensively investigated by transcriptional profiling of H. pylori (Allan et al., 2001; Ang et al., 2001; Merrell et al., 2003a; Wen et al., 2003; Bury-Moné et al., 2004; Table 13.1). The poor overlap between the results of these five transcriptomic studies in both the nature and the total number of regulated genes is probably related to the variety of experimental setups with shorter or longer exposure times to different acidic pH values, different culture media supplemented or not with urea, and also different H. pylori strains (Table 13.1). Strikingly, two studies (Merrell et al., 2003a; Bury-Moné et al., 2004) – although performed under different conditions – reported a similar number of acid-regulated genes (118 and 101), of which 22% were in common. Despite the differences, some common features can be extracted from these studies. First, these results clearly indicate that H. pylori modifies gene expression at low pH in order to induce mechanisms of protection against protons. It was previously established that ammonia production is the major strategy for acid resistance in H. pylori, mainly by its highly active and abundant urease enzyme that hydrolyses urea to produce ammonia and bicarbonate. Accordingly, most studies observed acid-induced expression of H. pylori urease operons and of two other ammonia-producing enzymes, the AmiE amidase and AmiF formamidase, that we previously characterized (Skouloubis et al., 1997, 2001). Furthermore, an acid protection strategy of H. pylori is suggested by the repeated observation of down-regulation in the expression of membrane proteins including transporters, permeases and outer membrane proteins. A common theme is also the enhanced expression of genes related to motility, including structural proteins of the flagellar apparatus and motor-related proteins. Stimulation of H. pylori motility by acid was experimentally demonstrated (Merrell et al., 2003a) and might be indicative of a strategy to escape acidity or of a pH-driven response directing the bacteria through the mucus pH gradient to a suitable site for multiplication or adhesion, as previously suggested (Schreiber et al., 2004). Two studies have indicated reduced expression of the VacA cytotoxin and the SabA adhesin at acidic as compared with neutral pH (Merrell et al., 2003a; Bury-Moné et al., 2004). This regulation, confirmed at the protein level for VacA using comparative two-dimensional gel electrophoresis (2-D GE) (Jungblut et al., 2000), is thought to optimize the production of virulence factors in the neutral pH environment found near the gastric epithelial cells.

Moreover, our investigations of the response of H. pylori to exponential growth at pH 5 have unravelled a novel category of acid-regulated genes, encoding proteins related to metal metabolism or regulated by metal-ion availability (Bury-Moné et al., 2004). Solubility of nickel, iron and other metal ions is known to be strongly enhanced by acid, and the role of the Fur iron-responsive regulator in the H. pylori acid resistance had already been reported (Bijlsma et al., 2002). In addition, urease activity depends on a nickel cofactor, underlining that acid resistance of H. pylori is also tightly linked to nickel metabolism. As a protective mechanism against the toxic side-effects of metal ions, we found enhanced expression of metal storage proteins and diminished expression of metal-ion transport systems under acidic conditions (Bury-Moné et al., 2004).
Finally, these transcriptome studies also revealed three major regulators of the acid response network in *H. pylori* that have been further examined: the two metal regulators Fur and NikR, responsive to iron and to nickel, respectively (van Vliet *et al.*, 2003; Bury-Moné *et al.*, 2004), and the HP165–HP166 genes that encode a two-component regulatory system (TCS) (Bury-Moné *et al.*, 2004; Wen *et al.*, 2003, 2006) designated ArsRS and further analysed by the group of D. Beier (Pflock *et al.*, 2004, 2005) (see below).

### Growth phase-dependent regulation

The growth phase-regulated genes of *H. pylori* have been identified in a time-course transcriptome study (Thompson *et al.*, 2003). A major switch in gene expression was observed during the transition between late logarithmic and stationary phase, with the expression of several genes related to virulence being modified (Thompson *et al.*, 2003). During this switch, genes encoding CagA, the NapA bacterioferritin and the FlaA flagellin were induced, while genes encoding proteins involved in iron homeostasis were regulated as if the bacteria were submitted to iron overload. The regulatory mechanisms governing the entry into stationary phase remain a black box in the case of *H. pylori*, particularly since there is no known homologue of the alternative sigma factor RpoS that is central to this switch in *E. coli*. HrcA, a heat-shock regulator found to be up-regulated during the logarithmic–stationary switch, might be a potential first candidate to play a role in the growth phase switch (Thompson *et al.*, 2003).

### Response to iron availability

During persistent stomach colonization and as a function of diet, *H. pylori* experiences periods of both iron overload and starvation. In addition, low pH will render iron more soluble. The human host sequesters this iron to prevent oxidative stress and to limit bacterial proliferation. Indeed, while iron is essential for several bacterial functions, it is toxic if its intracellular concentration becomes too high. Thus, iron homeostasis consists of a fine-tuned balance between uptake, efflux, utilization and storage. A time-course transcriptomic study was performed in response to iron starvation in both the exponential and stationary phase (Merrell *et al.*, 2003b). Of the 183 differentially regulated genes, only 30 were common between the two growth phases. The observation of a phase-dependent expression of the global ferric uptake regulator protein Fur, with enhanced production in the stationary phase (Danielli *et al.*, 2006), might partially explain this observation. Regulated genes comprised those involved in iron sequestration, storage and transport. Among them was *pfr*, which encodes ferritin, previously shown to be repressed by the Fur regulator (Bereswill *et al.*, 1998) in the absence of iron (Bereswill *et al.*, 2000b). This unusual function of Fur has been independently confirmed and the binding site of iron-free Fur on the *pfr* gene promoter has subsequently been identified (Delany *et al.*, 2002). In contrast, in the study of Merrell *et al.* (2003b), the *H. pylori* iron-binding protein neutrophil-activating protein (NAP) and the putative iron outer membrane transporters FrpB1 and FecA1/2/3 were induced in the presence of Fur and iron. In addition, a number of virulence factors were affected as a function of iron. Both VacA and NapA were induced by iron starvation and motility was affected in a complex growth phase-dependent manner. The two amidase genes (*amiE* and *amiF*) were induced by iron starvation in both growth phases, while a weaker induction of the *ureAB* genes was measured only in the stationary phase (Merrell *et al.*, 2003b).

### Response to epithelial cell attachment and to colonization in an animal model

To gain insight into the *H. pylori* response to adhesion to gastric epithelial cells, Kim *et al.* (2004) compared the global gene expression of *H. pylori* after attachment to AGS gastric cancer cells with that of bacteria incubated in the cell culture medium. Only 43 genes presented differential expression, including the down-regulation of genes related to motility that was compatible with close adhesion.

In an attempt to directly appraise the response of *H. pylori* to infection, the transcriptome of *H. pylori* after infection of Mongolian...
gerbils for 10 days was compared with that of 
*H. pylori* grown in vitro at pH 7.4 (Scott et al., 2007). From a considerable number of genes
presenting differential expression, some conclusions could be drawn. As expected for an acidic niche,
up-regulation of many acid acclimatization genes was measured, including the urease genes, *rocF*
coding for arginase, *hp1186* encoding the α-carbonic anhydrase (Marcus et al., 2005; Bury-Moné et al.,
2008) and those of the two amidases *amiE* and *amiF*. Transcription of two groups of motility and
chemotaxis genes was also enhanced, as well as nine genes of the *cag*PAI including CagA.
Strikingly, several of these genes were under the control of the ArsRS acid-responsive regulator system.
This type of study is crucial to understand the behaviour of a pathogen in its host,
although it remains technically challenging as only small amounts of RNA can be recovered from *in vivo*-infecting bacteria.

13.3.2 Transcriptomics to characterize *H. pylori* regulons using mutant strains

Results from whole-genome expression profiling have led to novel hypotheses on processes or mechanisms that are switched on in response to environmental changes. Generally, such switches are mediated by transcriptional regulators that act on multiple targets in a hierarchically organized way. All the genes controlled by a given regulator constitute its regulon, which can be defined by comparing the transcriptome of a wild-type strain with that of an isogenic mutant deficient in the regulator of interest, with or without an environmental stimulus.

*H. pylori* has a very limited number of global regulators including sigma factors with the housekeeping σ80, and two alternative sigma factors, σ54 (RpoN) and σ28 (FliA). It also possesses few transcriptional regulators, including the NikR and Fur metalloregulators that respond to nickel and iron, respectively, the carbon storage regulator CsrA, and two heat-shock regulators HspR and HrcA. In comparison to other bacteria, the number of TCSs is also dramatically reduced in *H. pylori*. Most enterobacteria possess more than 30 such systems. The TCSs are specialized in translating environmental stimuli into transcriptional regulation and usually comprise a histidine kinase sensor and a response regulator. Apart from two orphan response regulators (HP1043 and HP1021), *H. pylori* possess three complete TCSs that have been well characterized: HP1365–HP1364 (CrdRS; Waidner et al., 2005), HP166–HP165 (AsrRS; Pflock et al., 2004, 2005, 2006a) and HP703–HP244 (FlgRS; Niehus et al., 2004), involved in the response to copper ions, to acidity, and in flagellar gene expression as well as the response to acidity, respectively. A few other annotated transcriptional regulators have not yet been characterized. The low number of TCSs in *H. pylori* is proposed to be related to its unique environmental niche and small genome size, which is perhaps the result of reductive evolution in the course of co-evolution and host adaptation.

**Regulation of the flagellar system**

Flagellar motility is essential for colonization of the stomach by *H. pylori*. In several bacteria, the flagellar system is controlled by a complex transcriptional hierarchy acting on a large number of genes and by sophisticated feedback regulation mechanisms. In *H. pylori*, about 40 genes are related to motility. The flagellar transcriptional network has been investigated by DNA arrays using different mutants (Niehus et al., 2004). The role of the two sigma factors recruited for the expression of flagellar genes, σ54 (RpoN) and σ28 (FliA) with its cognate anti-sigma FlgM (Colland et al., 2001; Josenhans et al., 2002), have been studied in particular detail. Transcriptome analysis showed that negative feedback regulation of the FliA regulon was dependent on FlgM, which was, in addition, involved in FlhA– but not FlhF-dependent feedback control of the RpoN regulon (Niehus et al., 2004). Examination of the regulatory function of FlhA, encoding a flagellar basal body protein, revealed that it controls a large number of genes including those of FliA and RpoN regulons. In addition, analysis of the role of the FlgRS TCS supported the function of FlgR as an exclusive RpoN-associated regulator (Niehus et al., 2004). These investigations emphasized the complexity of this
system and revealed more unique features of *H. pylori*, such as the σ28 and σ28-independent expression of chemotaxis and flagellar motor genes, as well as the absence of a true master regulator for flagellar regulation.

A recent transcriptome study (Douillard et al., 2008) evaluated the role of HP0958 (a novel RpoN chaperone) using a mutant of HP0958, previously shown to have impaired motility (Ryan et al., 2005). A total of 44 genes were found to be differentially expressed in the HP0958 mutant, including the majority of the RpoN-dependent flagellar genes. Most other flagellar genes were not significantly differentially expressed in the HP0958 mutant. Interestingly, HP0958 was found to post-transcriptionally regulate the flaA gene encoding the major flagellin protein, by a mechanism that remains to be defined (Douillard et al., 2008). In addition, the effect of deleting the flaK gene (encoding a protein that controls the hook length during flagellar assembly) on the transcriptome of *H. pylori* was evaluated (Douillard et al., 2009). Again, only transcription of the genes under control of RpoN was differentially regulated in the mutant, suggesting specific and localized FliK-dependent feedback on the RpoN regulon (Douillard et al., 2009).

**The Fur and NikR regulons**

The role of Fur, the iron-responsive regulator, has been assessed by transcriptional profiling of both a wild-type strain (26695) and a Δfur mutant in iron-restricted and iron-replete conditions (Ernst et al., 2005a). Pair-wise comparisons of these four conditions resulted in identification of complex regulatory patterns, with both genes being positively or negatively regulated by Fur bound to iron or in apo-Fur form, and also in a significant number of Fur-independent iron-regulated genes. Strikingly, out of 97 iron-responsive genes, only 43 were Fur-dependent, pointing to additional regulatory mechanisms responding to iron in *H. pylori*. Fur-independent iron-regulated genes comprised those involved in iron uptake and cofactor metabolism, as well as the amidases. Similar results have been reported with a Δfur mutant in another genetic background (strain G27; Gancz et al., 2006). In that study, the iron-free Fur-repressed genes comprised the oxidative stress response protein SodB and hydrogenase subunits. To clarify these complex iron and Fur regulatory patterns, and to distinguish between direct and indirect effects, it is essential to combine both individual gene expression measurements (as for SodB; Ernst et al., 2005b; or for amiE; van Vliet et al. 2003) and whole-genome DNA-binding assays. One such study used a Fur chromatin immunoprecipitation (ChIP) on chip approach to globally identify the Fur-binding targets (Danielli et al., 2006). Fur-IP-DNA pools were hybridized to *H. pylori* DNA arrays. Two hundred candidate targets were initially identified. Among these regions, only those also identified by transcriptome analysis of wild-type versus Δfur mutant strains were retained. This resulted in a Fur regulon consisting of 59 directly regulated genes, 25 of which were positively regulated (Danielli et al., 2006).

Nickel, like iron, is a double-edged sword since it is both required and potentially toxic, if present at too high an intracellular concentration. In *H. pylori*, nickel is of particular importance as the cofactor of two enzymes essential for colonization, namely urease and hydrogenase. The regulon of the nickel-responsive regulator NikR was investigated under conditions of nickel excess by comparing the response of a wild-type strain to that of a ΔnikR mutant (Contreras et al., 2003). The study revealed that *H. pylori* NikR is a pleiotropic regulator, in contrast to its homologue in *E. coli*, which only represses the NikABCDE nickel uptake system under nickel excess conditions (De Pina et al., 1999). In *H. pylori*, NikR associated with nickel becomes active as an autoregulator controlling nickel uptake (NixA permease), metabolism (structural subunits of urease) and storage (Hpn, Hpn-like), thereby contributing to nickel homeostasis (Contreras et al., 2003). In addition, NikR also controls the expression of stress response and flagellar genes (Contreras et al., 2003). Although NikR was originally thought to be a repressor, its regulon has been shown to comprise both negatively and positively regulated genes. Direct binding of NikR to promoter regions of activated target genes such as ureAB confirmed its role as a nickel-
responsive activator (Delany et al., 2005; Ernst et al., 2005b). Finally, an overlap between the nickel and iron metabolism has been observed in which NikR not only represses the expression of the iron-responsive regulator Fur, but also that of different proteins involved in iron acquisition, including the \textit{exbB/exbD/tonB} operon (\textit{hp1339–1340–1341}) coding for the TonB machinery, a system energizing iron uptake through the outer membrane (Contreras et al., 2003; Delany et al., 2005). The physiological basis for the tight interconnection between the nickel- and iron-responsive gene networks is now better understood as a result of our recent demonstration that the \textit{H. pylori} TonB machinery is, in addition to iron transport, also required for nickel uptake (Schauer et al., 2007). This new finding was substantiated by the identification of the corresponding nickel outer membrane transporter FrpB4, whose expression is exclusively regulated by NikR (Schauer et al., 2007).

Regulons of the heat-shock regulators \textit{HspR} and \textit{HrcA}

The global response of two previously identified heat-shock regulators of \textit{H. pylori}, HrcA and HspR, was analysed using mutant strains deficient in one or both regulators (Roncarati et al., 2007). Expression of 14 genes was found to be negatively controlled by one or by the two regulators, while a set of 29 genes was shown to be positively regulated. DNase I footprinting experiments with the promoter regions of two of the newly identified regulated genes \textit{hp0630} and \textit{flaB} did not show binding of HrcA or HspR, suggesting that these genes are indirect targets. Interestingly, half of the regulated genes were related to flagellar motility and were positively controlled by HrcA or HspR. This points to a possible intersection between stress response and flagellar assembly that was confirmed by the loss of motility of the HrcA and the HspR deletion mutants (Roncarati et al., 2007).

Regulons of the two-component system \textit{ArsRS} and of the orphan regulator \textit{HP1021}

Among the few TCSs of \textit{H. pylori}, HP165–166 has attracted most attention since it was found to play a central role in the \textit{H. pylori} adaptive response to acidity, therefore being named Ars for acid-responsive signalling (Pflock et al., 2005). The importance of this system in \textit{H. pylori} is illustrated by the fact that a mutant of the ArsS histidine kinase \textit{HP165} was unable to colonize mice (Panthel et al., 2003) while the ArsR response regulator \textit{HP166} (of the OmpR family) is essential for \textit{in vitro} growth of \textit{H. pylori} (Beier and Frank, 2000). This suggests unusual distinct functions for ArsR in its phosphorylated and unphosphorylated forms, with the latter regulating the expression of an essential, as yet unknown gene.

Two global studies identified targets of the ArsRS system, using either a DNA magnetocapture assay with bound recombinant ArsR (Dietz et al., 2002) or DNA arrays with a strain deficient in ArsS (Forsyth et al., 2002). The lack of overlap between the results of these studies emphasizes the difficulties and possible side-effects of these approaches. However, recent analysis of ArsRS-mediated acid regulation by these two groups (Loh and Cover, 2006; Pflock et al., 2006a) confirmed some of the data, including the negative autoregulation of ArsR (Dietz et al., 2002) and the regulation of the \textit{Hpn} nickel storage protein (\textit{HP1432})- and arginase (\textit{HP1399})-encoding genes (Forsyth et al., 2002). Little information is available for the response regulator \textit{HP1021}, which apparently misses a cognate sensor. Expression profiling of an \textit{hp1021} deletion mutant only revealed differential expression of ten genes (Pflock et al., 2007a). For the product of five of these genes, the regulation was confirmed by comparative proteomic 2-D GE analysis of the wild-type strain and \textit{hp1021} mutant.

\textit{Role of NikR, Fur, ArsRS, CrdRS and FlgS regulators in the acid response}

Comparison of the numerous transcriptional profiling studies presented above reveals striking overlaps between the subset of genes differentially regulated under several environmental conditions or in mutant strains. Complex regulatory networks have become apparent, in particular for responses to acidity and metal ions. To date, four transcriptional
regulators have been found to be involved in the response of *H. pylori* to acidity. The precursor work of Bijlsma et al. (2002) showed that Fur was required for acid resistance in *H. pylori*, as in other bacteria (e.g. *Salmonella enterica* serovar Typhimurium). The involvement of NikR and ArsR in addition to Fur as effectors of the global response of *H. pylori* to acidity was revealed by comparison of our transcriptome data with that of mutants deficient in these regulators (Contreras et al. 2003; Bury-Moné et al., 2004; Loh and Cover, 2006; Pflock et al., 2006a). For the first time, a nickel-responsive regulator, NikR, was found to be involved in the global acid response (reviewed by van Vliet et al., 2004b).

To assess the role of these effectors in the acid response, several gene expression profiling experiments were performed in mutants exposed to low pH (Table 13.1). In our study, we found that, during growth at pH 5, the number of acid-responding genes dropped from 101 in a wild-type strain to only 36 genes in an isogenic Δfur-ΔnikR double mutant (Bury-Moné et al., 2004). In a more recent study, time-course transcriptome analysis was used to dissect iron and pH regulation in *H. pylori* and compare acid-regulated genes in a wild-type and Δfur mutant strain (Gancz et al., 2006). A list of 95 genes which were aberrantly regulated at acidic pH in the absence of Fur was obtained with a strong overlap with previously identified acid-regulated genes. Fur-dependent acid-regulated genes comprised those involved in ammonia production (amidase, asparaginase), detoxification (SodB and the KatA catalase), pathogenicity (proteins of the cagPAI and NapA) and transcriptional regulation (ArsR). In addition, 89% of the Fur/acid-regulated genes were previously identified as being regulated by Fur, iron or acid. ArsR is an OmpR-like response regulator similar to its orthologue in *S. enterica* which is known to be involved in stationary phase-dependent pH-induced acid tolerance via positive autoregulation (Bang et al., 2002). Low pH has been shown to be a signal triggering the autophosphorylation of the *H. pylori* ArsS histidine kinase and subsequent phosphorylation of its cognate response regulator ArsR (Pflock et al., 2004). Comparison of the transcriptome of a ΔarsS mutant versus a wild-type strain exposed to pH 5 revealed differential expression of as many as 109 genes including several already identified acid-regulated genes such as those encoding urease subunits and the AmiE and AmiF amidases (Pflock et al., 2005, 2006a). For the three corresponding promoter regions, direct binding of phosphorylated ArsR protein was demonstrated. Several genes differentially regulated in the ArsS-deficient mutant were also under the control of NikR and Fur. In another study, global gene regulation in response to acidity of mutants deficient in ArsS, CrdS and FlgS was compared to that of the wild-type strain J99. While no difference was observed with the FlgS mutant of 101 analysed acid-regulated genes, 68 and 63 were no longer responsive to acidity in the ArsS and CrdS mutants, respectively, with about 70% common genes (Loh and Cover, 2006). Surprisingly, no difference in the global genetic response to acidity was observed in crdS mutants of other *H. pylori* strains (Pflock et al., 2007b). Also in contradiction with previous work was the recent study of Wen et al. (2009), who found that FlgS in strain 26695, in addition to its function as a flagellar genes regulator, controls about 100 genes in the response to acidity through a pathway independent of its FlgR cognate regulator. The discrepancies between these different studies might be attributable to different experiments setups and/or to different genetic backgrounds of the *H. pylori* strains analysed. However, it underlines the care required when interpreting transcriptomic results, and the importance of validation of the results by other more direct techniques.

Finally, to make the picture even more complex, one should mention negative autoregulation of each of the three best-characterized regulators, Fur, NikR and ArsR, as well as autorepression being stimulated by acidity for Fur and ArsR. An additional level of complexity is given by cross-regulation whereby NikR negatively controls the expression of fur (Bury-Moné et al., 2004; van Vliet et al., 2004a) and vice versa (Delany et al., 2005). Furthermore, ArsR does not regulate the expression of nikR and fur (Pflock et al., 2006a,b) but Fur represses the expression of arsR in response to iron (Merrell...
et al., 2003b; Gancz et al., 2006), suggesting that the ArsR acid response is part of a complex regulatory network including acid- and metal-dependent regulation.

13.4 Conclusions from Global Gene Expression Studies in H. pylori

Understanding the mechanisms underlying H. pylori’s persistent colonization, and its unique adaptive capacity in the gastric environment, has represented a fascinating challenge for the research community almost since its discovery. A plethora of gene expression profiling studies has been published (see Table 13.1) generating a considerable amount of data, which are often hard to merge. The multiple transcriptomic studies are difficult to compare since they were performed with different reference strains and diverse experimental test conditions, in particular those used to mimic the environment encountered in the host (Table 13.1). While regulators and adaptive mechanisms have started to be identified and transcriptional networks to be unravelled, a closer look at the data produces a rather confusing picture with the expression of a significant number of genes varying in every condition or mutant strain tested.

Despite this, a common theme of these transcriptome analyses is the convincing connection between metal metabolism, acid response and virulence. Accordingly, H. pylori strains deficient in each of the transcriptional regulators involved in these responses (NikR, Fur, ArsRS) results in attenuated or abolished colonization capacities in animal models (Panthel et al. 2003; Bury-Moné et al. 2004; Gancz et al. 2006). Because metal ions become more soluble at low pH, a tempting hypothesis is that higher metal bioavailability is a signal sensed by H. pylori during acid stress (Bury-Moné et al., 2004; van Vliet et al., 2004a,b). It therefore makes biological sense that metal-ion regulators of H. pylori are involved in the response to acidity. We previously proposed a model (Bury-Moné et al., 2004) in which acidity is a ‘spatial–temporal’ signal for H. pylori that indicates its location in the stomach, either in the acidic mucus or close to the neutral epithelial cells, and accordingly regulates several virulence factors. The work of Schreiber et al. (2004) on the spatial orientation of H. pylori in the gastric mucus fits well with such a model.

In addition, a major concern with transcriptomic studies is that they uncover both directly regulated genes and genes differentially expressed as a consequence of indirect effects. Growth phase strongly influences global gene expression in H. pylori (Merrell et al. 2003b; Thompson et al. 2003) including Fur production itself (Danielli et al., 2006), and it might well be that the conditions tested in mutants or upon stress have influenced this parameter. Direct transcriptional regulation analysis has been used to validate the genomic data. Using EMSA (electrophoresis mobility shift assays) or DNase I protection assays, direct binding of Fur, NikR, phosphorylated ArsR or CrdR to the promoter region of a number of genes has been demonstrated (Delany et al., 2002, 2005; Contreras et al., 2003; van Vliet et al., 2003, 2004a,b; Pflock et al., 2004, 2005, 2006a,b; Ernst et al., 2005c; Waidner et al., 2005; Wen et al., 2006). Also, to identify every genomic target of Fur, a ChIP on chip strategy has been used (Danielli et al., 2006). This powerful strategy will be interesting to apply to the study of NikR, phosphorylated ArsR or FlgR in H. pylori cells incubated under different conditions. Second, despite numerous analyses, the DNA-binding site consensus that has been proposed for the Fur, NikR or ArsR regulators is weakly specific (short and AT-rich), and therefore poorly convincing, in an AT-rich organism such as H. pylori. This strongly suggests that additional parameters on the target DNA sequences or other cofactors are still to be identified. Such additional effectors could be small regulatory RNAs, such as identified for the Fur-mediated iron response in E. coli (Massé et al., 2007). In addition, based on currently available data, no hierarchy in the regulatory networks of H. pylori can be established. Nevertheless, the overlapping regulatory pathways (acidity, flagella, metal and heat-shock stresses) are most intriguing and are evocative of the existence of a master regulator such as, for example, ComK, which controls more than 100 genes involved in competence in Bacillus subtilis (Maamar and Dubnau, 2005).
Importantly, such a ‘conductor’ could function through a novel global regulatory mechanism.

13.5 Summary and Outlook

At the end of the 20th century, H. pylori was the first bacterial pathogen for which two complete genome sequences were available. Genomics has rapidly become the approach of choice to study this ‘young’ pathogen. Several complete genome sequences are available today and many more will certainly be published in the coming years. Tools such as massive sequencing, comparative genomics, proteomics and transcriptomics have generated new insights into H. pylori’s spectacular diversity, pathogenicity and gastric adaptation. Thus, Helicobacteromics has provided a better understanding of the genetic drift, genomic instability, host specificity, environmental adaptation and evolutionary biology of this human pathogen. H. pylori has a remarkably variable genome that indicates a high rate of genetic change. It is therefore surprising that recent studies have failed to experimentally capture de novo genetic/genomic changes. The sensitivity of these analyses could be enhanced by novel technologies (i.e. tilling arrays, deep sequencing) to detect single-nucleotide polymorphisms at a genomic level. In addition, ongoing studies aim to track genomic changes occurring during infection by completely sequencing the chromosomes of H. pylori strains isolated from a single patient at different time points during disease progression.

Numerous transcriptome studies have helped to gain a better insight into the regulatory response of H. pylori to environmental and/or stress conditions that this pathogen must deal with in the stomach. Some mechanisms underlying the survival strategies of H. pylori in the gastric environment are now better understood, such as the response to acidity or to metal ions. Transcriptional regulators involved have been identified and some regulatory networks unravelled. However, the regulation of the expression of a number of genes does not depend on any established regulators and many of the networks overlap to a surprisingly high degree. This points to complex regulatory cascades and the involvement of additional regulatory factors, such as unknown transcriptional regulators or small regulatory RNAs. With new high-throughput sequencing technologies such as Illumina SOLEXA or 454-ROCHE, deep sequencing approaches are now available for exploring, for instance, the RNome of a given organism (every RNA expressed in a cell). The groups of J. Vogel (MPIIB Berlin, Germany) and F. Darfeuille (INSERM, Bordeaux, France) recently performed such a global study defining the RNome of H. pylori strain 26695 and identified a number of small non-coding RNAs, some of which could play regulatory roles (Sharma et al., 2010). The future directions will be to follow the changes of the H. pylori RNome under varying conditions or in different mutants and in parallel to test experimentally the role of the candidate regulatory RNAs.

In conclusion, this chapter illustrates the adaptive strategies of H. pylori. A short-term strategy involves coordinated control of gene expression which is an immediate and reversible response affecting the entire bacterial population. A long-term strategy implies genome plasticity, not homogeneous among a population but stabilized under external selective pressure. These characteristics of H. pylori have certainly been determinant in making this bacterium a successful pathogen that has coexisted with humans for hundreds of thousands of years, that once established persistently colonizes the host stomach for decades and that still infects half of all people in the world.

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