Steffen Backert · Yoshio Yamaoka
Editors

Helicobacter pylori Research

From Bench to Bedside

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Foreword

Helicobacter pylori, the Gastric Bacterium Which Still Infects Half the World's Population, Is an Important Part of Gastroenterology and Infectious Disease

It is exciting to participate in this new "state-of-the-art" book about *Helicobacter pylori*, the gastric bacterium which still infects half of the human race and which promises to challenge clinicians and scientists for decades to come. The book is structured so as to place *H. pylori* in perspective as the gastric bacterium attached itself to prehistoric humans and followed their migrations, through the stone-age into the twenty-first century. Thus the first chapter, by Yoshan Moodley, sets us up to ask the questions which can then be answered in depth by the other experts. Where did *H. pylori* come from? How does it survive in the stomach? What unique adaptations have taken place, which allow it to persist throughout life? If it colonised mankind for so long, was there once a useful purpose in having *H. pylori*? Is it still useful in some way? As complete genome sequencing technology becomes widespread, many will use this in-depth initial chapter as a hypothesis generating knowledge-base for our own molecular epidemiology studies. Examples are even given of newer technologies such as RNAseq, for examining gene expression and intergenic control regions. Later, Ichizo Kobayashi predicts that further breakthroughs via OMICS analyses will also provide more insight into the evolution of both the genome and methylome of this highly diverse model organism.

Once the big picture has been described, we want to know how *H. pylori* accomplishes this and "what makes it tick?" So we delve into *H. pylori* biochemistry realising that the metabolism of the bug mirrors the microenvironment of the gastric mucosa (Fischer and deReuse, Praszkier, Sutton and Ferrero; Backert, Zanotti, Lind, Asche and Tegtmeyer; Cover, Holland and Blanke; Arnqvist; Wessler; Pernitzsch, Darfeuille and Sharma). In a hostile acidic milieu, but under the mucus layer, *H. pylori* solve the problems of immediate survival but then must deal with the host's attempts to remove the invader. Certainly, its very plastic

genome is an advantage coupled with the massive numbers of organisms competing for life in that niche. But other mechanisms exist whereby *H. pylori* regulate the immune response, optimising its nutrition without allowing the mucosa to be totally disrupted by too vigorous tissue reaction. Subtle degrees of positive and negative feedback must be at work. A clear explanation of the interrelated virulence factors, primarily CagA and VacA, and then the several newer surface antigens is given in subsequent chapters, perhaps explaining how *H. pylori* disguises itself from the immune response.

As we move from basic to clinical research in *H. pylori*, animal models of the infection become even more important. Chapters on this theme describe the 100-year-long history of various related *Helicobacters* in animals and more recent insights into their genomics and taxonomy (Flahon, Haesebrouck and Smet; Solnick, Eaton and Peek; Müller and Hartung; Oshima, Nakayama and Oshima; McLean; Nicoll, Saw, Hold and El-Omar). How the animal infections provide guidance into the realm of animal models remains important. Chapters on the trials and tribulations of current animal models serve to emphasise that the perfect animal model is yet to be discovered, unless it is still the human. Nevertheless, selection of or direct manipulation of small animal genetics serves to tease out important predictors of epidemiology, colonisation, inflammation and carcinogenesis.

In this context, the clinician will be delighted to study the detailed clinical chapters on human diseases related to *H. pylori* infection (Boltin and Niv; Genta and Lash; Wroblewski and Peek; Sagaert; Koletzko and Megraud). The reasons to treat *H. pylori* and features of the host and the bacterium which drive the various phenotypes of asymptomatic, ulcerated and malignant gastric disorders will give confidence to the clinician seeing patients with gastric disorders. In addition, the spectre of rarer non-gastric disorders is exposed, as these will be seen from time to time and the gastroenterologist or infectious disease specialist will be expected to assist decisions into their management too. Very relevant here is the controversial area of immune modulation from *H. pylori*. It is relevant to many areas, but the tantalising possibility exists that *H. pylori* could still play a useful role, as an immune modulator, like "oil on troubled waters" to decrease the apparent overshoot which commonly occurs in allergy susceptible individuals. Certainly the negative association with asthma in New York children and in adults with various inflammatory gastrointestinal diseases is worthy of study and is authoritatively reviewed in immunology-related and paediatric chapters.

Finally, it is great to see that the common, real-world issues of *H. pylori* treatment are dealt with by authors who have many years of experience successfully curing difficult to treat patients (Molina-Infante and Graham; Malfertheiner and Selgrad; Torres, Correa, Herrero, Piazuelo and Ferrecio; Miftahussurur and Yamaoka; Vale, Vitor and Oleastro; Raghavan and Quiding-Järbrink). While the level of antibiotic resistance has been creeping up, most notably to macrolides and quinolones, logical planning of treatment, with sophisticated microbiological testing, can give us confidence. Several new antibiotics and/or combinations of well-known drugs mean there is a bright, *H. pylori*-free future for patients with the bacterium.

Finally, we are shown glimpses of the twenty-first century future where *H. pylori* infection might be prevented, or eradicated rather simply, in each continent. This is already starting and will become easier as the knowledge in this book is disseminated. Strategies to improve hygiene, vaccinate, treat and even suppress *H. pylori* with foods and probiotic supplements are all relevant here and are now being tested.

In summary then, every science graduate will enjoy the intelligent structure of this book which uses in-depth explanation of the current knowledge to suggest how gastric colonisation with *H. pylori* might be a tool to unlock secrets related to the physiology of the gastrointestinal tract and the gastrointestinal immune system. The clinician also will be delighted with the extensive discussions of important disease associations and then, most importantly, how to render the patient *H. pylori* negative.

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2015

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Part I
Bacteriology and Molecular Biology

Chapter 1
Helicobacter pylori: Genetics, Recombination, Population Structure, and Human Migrations

Yoshan Moodley

Abstract Humans and their stomach bacterium *Helicobacter pylori* share a coevolutionary relationship that spans at least the last 100,000 years and quite possibly even longer. Population and evolutionary genetic research has demonstrated a species-wide phylogeographic structure that faithfully mirrors that of its human host. However, because of its very high genetic diversity and fast generation time, *H. pylori* DNA sequences are often better able to resolve prehistoric human migrations than human DNA markers. The worldwide genetic diversity of *H. pylori* has, thus far, been divided into 7 populations and 14 subpopulations, most of which are now established markers for recent and prehistoric human migrations. Key developments such as the inference of hypothetical ancestral populations and the implementation of coalescent models have provided a clearer understanding of the population historical role of admixture and recombination and helped superimpose a chronology onto the human *H. pylori* association, from which the timing, direction, and magnitude of migration events can be accurately inferred. However, there remain large parts of the world from which *H. pylori* has never been cultured, and this, along with a move to sequence whole genomes rather than just housekeeping genes, will form the basis of future evolutionary genetic research.

Keywords *Helicobacter pylori* • Coevolution • Population • Recombination • Phylogeography • Coalescence • Migrations

1.1 Introduction

In 1984, Barry Marshall and Robin Warren discovered the spiral-shaped stomach bacterium *Helicobacter pylori*, and for demonstrating its role in gastritis and peptide ulcer formation (Marshall and Warren 1984; Marshall et al. 1985), they

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were awarded the Nobel Prize for medicine in 2005. Since its discovery, ongoing research has shown that this bacterium is present in 50 % of human stomachs worldwide, the vast majority of whom are asymptomatic. *H. pylori* is usually contracted early in childhood, and once acquired, bacterial colonization is generally lifelong. The bacterium can be transmitted within families (e.g., from parents to children, Kivi et al. 2003; Tindberg et al. 2001) but also between unrelated people living in close proximity (Delport et al. 2006; Schwarz et al. 2008). Unusually high mutation (Björkholm et al. 2001; Morelli et al. 2010) and homologous recombination rates (Falush et al. 2001; Suerbaum et al. 1998) have resulted in very high DNA sequence diversity that is much greater than that of other bacteria (Achtman et al. 1999) and 50-fold greater than its human host (Li and Sadler 1991). Unlike chromosomal mutations, which can occur at random in any part of the species distribution, recombination necessarily requires mixed colonization (Falush et al. 2001; Raymond et al. 2004; Kersulyte et al. 1999; Taylor et al. 1995) and, by extrapolation, very close human proximity. The end result is a human bacterial species whose range-wide genetic structure mirrors that of its host. In the following review, I will outline the stages of development of evolutionary genetic research that have helped transform our understanding of *H. pylori* – from a newly discovered human pathogen to our species' oldest-known and most faithful commensal.

1.2 The Coevolution of *Helicobacter pylori* and Humans

1.2.1 H. pylori*'s Housekeeping Genes*

Meaningful population and evolutionary analyses of DNA data must dissect out demographic processes (genetic drift and gene flow) from selection processes, since both have the ability to alter allele frequencies. Typically, the selection issue is circumvented in eukaryotic species by only analyzing genes or regions that are, or are at least thought to be, selectively neutral – such as mitochondrial DNA (Cann et al. 1987), nuclear intronic sequences (Matthee et al. 2001), and repeat elements (Bruford and Wayne 1993). The smaller, more conserved genomes of bacteria, however, make such approaches untenable. Instead, the genes chosen for analyses are necessary housekeeping genes, encoding cytoplasmic enzymes. They are distributed across the *H. pylori* genome (Achtman et al. 1999) and are unlinked to genes encoding putative outer membrane or secreted or hypothetical proteins that might be under selection. They are more likely therefore to represent genome-wide selectively neutral variation.

The most incisive evolutionary or population genetic knowledge about *H. pylori* has, until very recently, been inferred from the DNA sequences of a set of seven housekeeping gene fragments. These genes, *atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureA*, and *yphC*, were originally the basis of an attempt at multilocus sequence typing (MLST) of *H. pylori* (Achtman et al. 1999) along the lines of what was then recently and

successfully carried out in *Neisseria meningitidis* (Maiden et al. 1998) and *Streptococcus pneumoniae* (Enright and Spratt 1998). The aim of MLST was to standardize DNA sequence variability in pathogenic microorganisms for efficient comparison of data between different laboratories. Typically, fragments of six to eight housekeeping genes are sequenced, and each unique allele (or haplotype) was given an arbitrary numerical identifier – a sequence type (ST). The resulting allelic profiles then allowed researchers to trace the clonal evolutionary relationships of pathogens, assuming that identical STs were identical by descent – that is, they evolved only once. While this scheme was successful in identifying clonal complexes in several bacterial species, *H. pylori*'s high mutation and recombination rates meant that strains possessing the same ST were the exception rather than the rule (Falush et al. 2003b; Linz et al. 2007). Contrary to its wide utility in other bacterial species, the MLST scheme simply did not provide the necessary variation to adequately quantify population structure in *H. pylori* (Achtman et al. 1999). Instead multilocus nucleotide variation of housekeeping gene fragments was used to deduce evolutionary relationships. This approach proved more fruitful, as researchers were able to make use of a wealth of existing and constantly developing analyses of DNA sequence evolution such as Bayesian inference, phylogenetic reconstruction, and coalescent modeling. However, despite the failure of the MLST approach to bring order to *H. pylori* genetic variation, the seven housekeeping gene sequences are still commonly referred to as the MLST genes.

1.2.2 Geographic Clustering of Housekeeping Gene Sequences

H. pylori's unusually high genetic diversity led to initial suggestions that high recombination and random mating rendered its population structure panmictic (Go et al. 1996; Salaün et al. 1998; Achtman et al. 1999), which means that it had no population or clonal structure. However, with increased sample sizes, geographic patterns of strain relatedness based on sequence similarity slowly became apparent (Kersulyte et al. 2000; Mukhopadhyay et al. 2000) with some early studies suggesting co-migration and association of *H. pylori* and humans for at least 11,000 years ago (Ghose et al. 2002; Yamaoka et al. 2002). However, it was not until model-based Bayesian cluster and assignment analyses were applied to global multilocus DNA sequence data sets of hundreds of strains (Falush et al. 2003b; Linz et al. 2007) could the extent of worldwide population structure in this species start being appreciated. Populations appeared to correlate with their continent of origin which argued strongly against a worldwide panmictic population. Originally, Falush and coworkers (2003b) defined four main modern populations – hpAfrica1, hpAfrica2, hpEastAsia, and hpEurope. Additional regional substructuring within some of these populations led to the designation of subpopulations – hpAfrica1 could be broken down into hspWAfrica and hspSAfrica

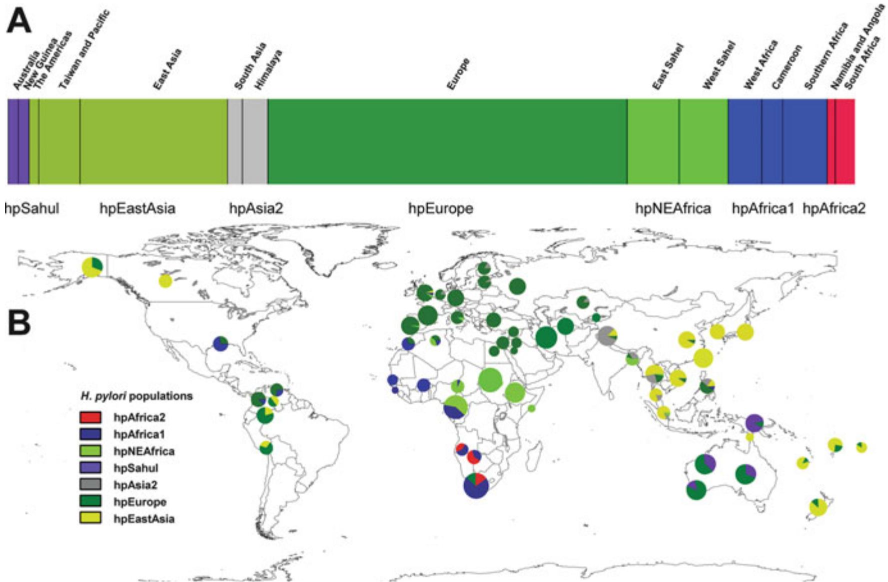

Fig. 1.1 Global overview of genetic structure in *H. pylori*. (**a**) The global population structure of *H. pylori* as determined through Bayesian cluster analysis of the housekeeping gene sequences of 1716 individual strains. Seven populations have been discovered thus far. Locations indicate geographic substructure within each population. (**b**) Worldwide geographic structure of seven *H. pylori* populations. Obvious geographic discontinuities in the distribution of hpEurope and hpAfrica1 reflect recent human movements associated with European colonial expansion and the slave trade, respectively. The structured natural distributions of populations allow them to be used as markers for human migrations

and hpEastAsia into hspEAsia, hspAmerind, and hspMaori. While numbers of populations and subpopulations continue to be discovered or refined, the original method of population definition (e.g., no admixture, using STRUCTURE (Pritchard et al. 2000) software) is now standard. To date, seven modern *H. pylori* populations have been defined globally (Fig. 1.1a). These include the original four plus hpAsia2, hpNEAfrica (Linz et al. 2007), and hpSahul (Moodley et al. 2009). An increase in the number of available strains for analyses has also resulted in an increase in the resolution of subpopulations distributed within almost every modern population (see Table 1.1 for a summary).

1.2.2.1 Recent Human Population Movements

This strong geographic structuring in the global data reflected major recent and long-term events in human settlement history, many of which were then intractable to human DNA. This arose most likely because of the much slower mutation rates of markers typically used to infer human demographic movements such as mitochondrial DNA and Y chromosome (Hudjashov et al. 2007). Therefore, human

Table 1.1 Summary of the modern populations and subpopulations of *Helicobacter pylori*, including their geographic range as far as it is known

Population	Subpopulation	Geographic range
hpAfrica2	hspNorthSan	Namibia, Angola
	hspSouthSan	South Africa
hpAfrica1	hspWAfrica	Senegal, the Gambia, Burkina Faso, Morocco, Algeria, Nigeria, Cameroon, South Africa
	hspCAfrica	Cameroon, Namibia
	hspSAfrica	Namibia, Angola, South Africa
hpNEAfrica	hspENEAfrica	Sudan, Ethiopia, Somalia, Algeria
	hspCNEAfrica	Sudan, Cameroon, Nigeria, Algeria
hpSahul	hspAustralia	Australia
	hspNGuinea	New Guinea
hpAsia2	hspLadakh	India (Himalayas)
	hspIndia	India, Bangladesh, Malaysia, Thailand, the Philippines
hpEurope	Recombinant population	Europe as far east as Southeast Asia
hpEastAsia	hspEAsia	China, India, Malaysia, Singapore, Taiwan, Cambodia, Vietnam, Japan, Korea
	hspAmerind	Canada, the USA, Venezuela, Colombia, Peru
	hspMaori	Taiwan, the Philippines, Japan, Samoa, New Caledonia, Wallis and Futuna, New Zealand

DNA lineages tend to sort more slowly, leading to slower population differentiation and decreased population resolution.

Obvious inconsistencies in the geographic structuring (Fig. 1.1b) were immediate and strong evidence of recent human population movements, many of which are difficult to deduce from human DNA. The presence of hpEurope outside its natural range in the Americas, South Africa, the Philippines, Australia, and the South Pacific Islands reflects the expansion of Europeans during the colonial era beginning about 500 years ago (Falush et al. 2003b; Linz et al. 2007). It shows clearly that colonizing or migrating humans carried with them more than just their own genes and suggests that, in many cases, *H. pylori* lived in intimate association with the people they were colonizing. The particularly high frequency of hpEurope in South America relative to hspAmerind is sometimes taken as evidence of competition and replacement of less diverse indigenous bacterial populations (Domínguez-Bello et al. 2008; Kersulyte et al. 2010). However, this hypothesis does not explain why, even in open modern-day communities, hspAmerind has not been completely replaced by hpEurope. Alternatively, the low frequency of hspAmerind in South America may have resulted in the near extinction of their hosts with the introduction of diseases such as smallpox by old-world colonizers. Other apparent geographic inconsistencies were the presence of the hpAfrica1 in North and South America, where West African people were forcibly brought to the Americas during the colonial-era slave trade, and the distribution of hpEastAsia in

Thailand and Malaysia, carried there by Chinese traders in the last 200 years (Linz et al. 2007; Breurec et al. 2011).

1.2.2.2 Prehistoric Human Migrations

When subpopulation-level structure was analyzed, geographic patterns also suggested large-scale human movements since the end of the Last Glacial Maximum about 18,000 years ago.

Bantu speakers: hpAfrica1

The distribution of hpAfrica1 spans the length of Africa from Morocco and Algeria in the north to South Africa in the south (Fig. 1.2). Geographic substructuring was detected across this considerable distribution, and cluster analyses defined three closely related subpopulations in West and North Africa (hspWAfrica, Falush et al. 2003b), southern Africa (hspSAfrica) (Falush et al. 2003b), and most recently in Central Africa (hspCAfrica, Nell et al. 2013). This differentiation was interpreted as a marker for the expansion of Bantu, a group of languages within the Niger-Congo language family that was spread across sub-Saharan Africa within the last 5000 years from an original homeland in Nigeria/Cameroon. The expansion was enabled through Neolithic technological developments that saw the migration of Iron Age agriculturalists into the summer-rainfall regions of subequatorial Africa that were climatically suitable for their crops, displacing or absorbing resident pastoralists and Stone Age hunter-gatherer communities (Diamond and Bellwood 2003). Debate still continues about the possibility of a Western alternative to the classically accepted eastern migration route into southern Africa via East Africa (Newman 1995; Pakendorf et al. 2011). The discovery of the subpopulation hspCAfrica in Cameroon and Angola but not in South Africa and Namibia (hspSAfrica) supports the existence of a migration route traveling along the west coast of Africa (Fig. 1.2) but which was halted by the Namib Desert prior to reaching South Africa. hspSAfrica is presumed to have evolved during Bantu migrations along the east coast that brought the Nguni speakers to southern Africa. Additionally, hpAfrica1 is also a marker for migrations elsewhere in Africa. The presence of hspWAfrica in North Africa may be taken as evidence of gene flow across the Sahara (Linz et al. 2007), and hspSAfrica in Madagascar suggests migration across the Mozambique Channel by Bantu speakers during or after their migration along the east coast of Africa (Linz et al. 2014).

Nilo-Saharan speakers: hpNEAfrica

In the central Sahel and North Africa, hpAfrica1 shares its distribution with the population hpNEAfrica (Linz et al. 2007), and its frequency decreases eastward to

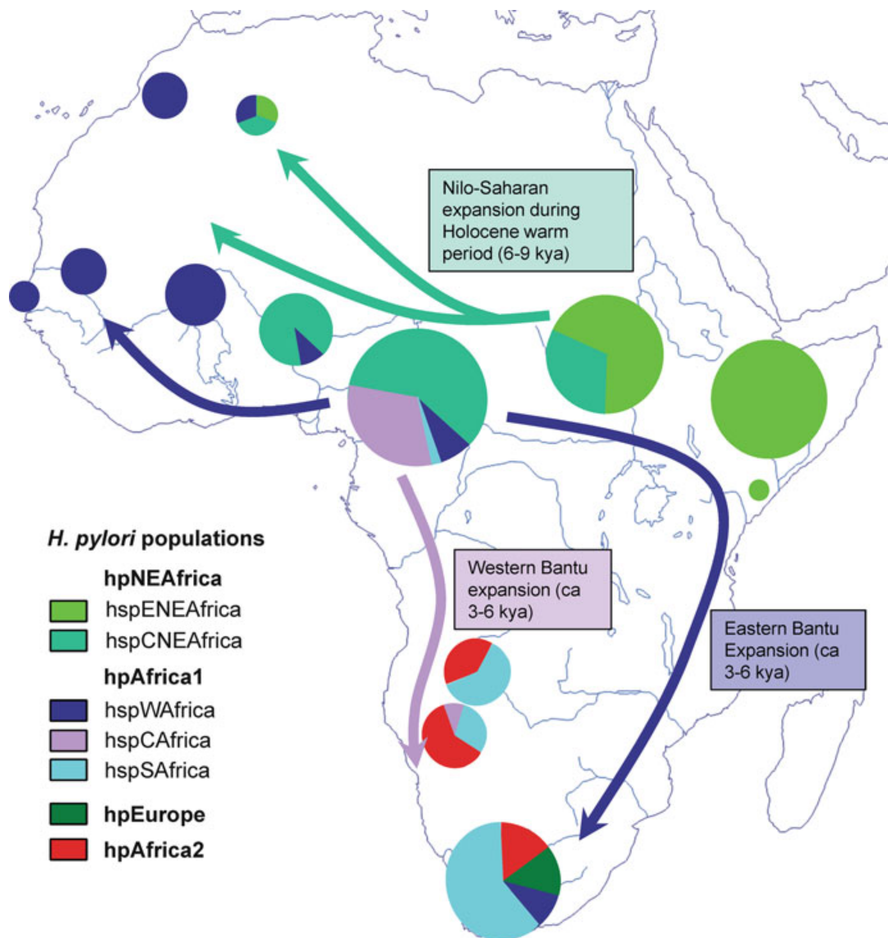

Fig. 1.2 Subpopulation structure among *H. pylori* populations in Africa. The discovery of hspCAfrica in Cameroon and Namibia supports a second wave of Bantu migrations along the west coast of Africa. Among Nilo-Saharan speakers, the evolution of hspENEAfrica coincides with the spread of fishing communities across the Sahara during the Holocene humid period

the Nile and the Horn of Africa while that of hpNEAfrica increases. Therefore the spread of Bantu farmers across the more arid regions of the eastern Sahel appears to have been limited, possibly due to the incompatibility of tropical crops in more arid climates and perhaps the presence of another, older society of Nilo-Saharan speaking pastoralists. Thus, *H. pylori* also provides a convenient marker for the spread of Nilo-Saharan languages in the form of hpNEAfrica (Fig. 1.2). Here again, geographic substructure provides clues as to postglacial movements of among Nilo-Saharans. Nell and coworkers (2013) split hpNEAfrica into hspEastNEA and hspCentralNEA, roughly along the Nile Valley – Sudan, which straddles the Nile, contains both subpopulations at high frequency. The presence of hspCentralNEA in

Cameroon, Nigeria, and Algeria suggests this subpopulation as a marker for human expansions during the Holocene humid period (6000–9000 years ago) that carried Nilo-Saharan languages westward from its home in northeast Africa into the waterlogged Sahara and beyond (Fig. 1.2).

Australians and New Guineans: hpSahul

The peopling of Sahul (the former continent comprising Australia and New Guinea) is thought to have occurred relatively soon after humans left Africa, following the southern coastal route passing southern India, the Andaman Archipelago (then joined to the Asian mainland), and Sundaland (Fig. 1.3). Although human uniparental markers show that indigenous Australians are closely related to New Guineans, they were also indistinguishable from East and southern Asian lineages because of incomplete sorting (Hudjashov et al. 2007). On the other hand, *H. pylori* from these two populations clustered into a single population (named hpSahul, Moodley et al. 2009) and could be further separated into discrete subpopulations, hspAustralia and hspNGuinea (Fig. 1.3), which suggests that they have been isolated from each other since sea levels rose after the Last Glacial Maximum.

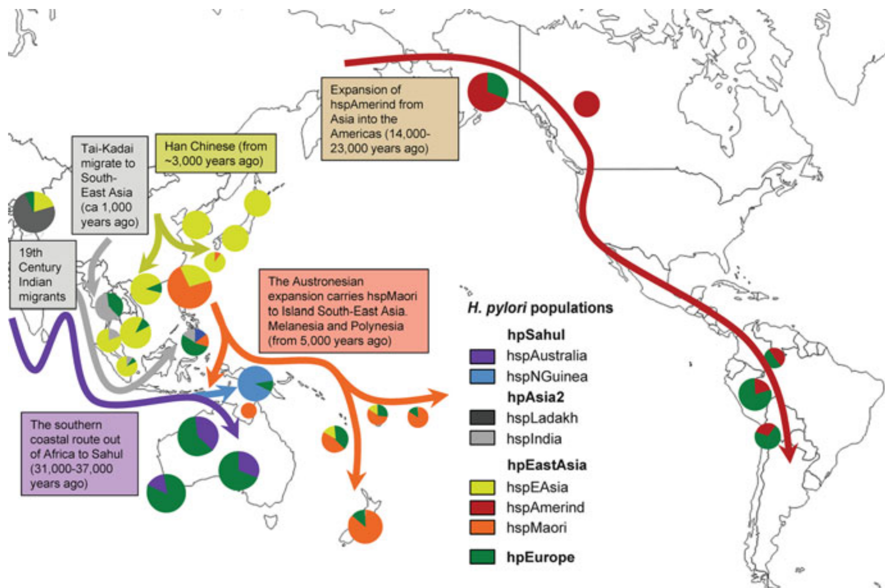

Fig. 1.3 Geographic structuring of subpopulations in the Indo-Pacific is consistent with several human migration events. hpSahul is a marker for post out-of-Africa migration along the southern coastal route, hspAmerind evolved prior to the peopling of the Americas, and hspMaori was carried from Taiwan into the Pacific by Austronesian seafarers

Central and Southeast Asians: hpAsia2

Of all the *H. pylori* populations to have evolved outside Africa, hpAsia2 is perhaps the most intriguing. It evolved among the people who either did not follow a southern coastal migration route, or perhaps those who settled during the early phases of this migration and later began expanding north into the Western and Central Asian hinterland. Its distribution was almost certainly more widespread in the past, prior to the evolution of hpEurope, and hpAsia2 may even have accompanied the first modern humans into Europe 40,000 years ago. The present-day hpAsia2 has largely been replaced by hpEurope in western Eurasia, but it still can be differentiated into subpopulations hspLadakh from the isolated Himalayan region of northern India and hspIndia, which is found among Indians, Malays, and Thais (Tay et al. 2009; Breurec et al. 2011). Its presence in northern Europe among Finnish and Estonians suggests that it may also be a marker for the spread of Uralic languages out of Siberia. Whether other relict hpAsia2 bacteria persist in the mountainous regions of Afghanistan, Pakistan, Tajikistan, Xinjiang, and remote northern Asia/Siberia is yet to be discovered.

Native Americans, Han Chinese, and the Austronesians: hpEastAsia

Among subpopulations of hpEastAsia, hspAmerind was isolated among diverse indigenous American populations in North and South America, and its uniqueness argues against the introduction of the East Asian bacteria into the Americas from recent Chinese or Japanese immigrants. Instead, these differentiated East Asian strains provided the first evidence that *H. pylori* accompanied humans on their migrations across the Bering Strait and into the Americas (Falush et al. 2003b) (Fig. 1.3). Similarly, the homogeneous distribution of the subpopulation hspEAsia across East Asia to Korea and Japan and the fragmentation of the three major language families (Hmong-Mien, Tai-Kadai, and Austroasiatic) originally spoken in South China suggested the expansion of the Chinese language (family, Sino-Tibetan) during the last 3000 years but mainly during the expansion of Zhou Dynasty (1100–221 BC, Diamond 1998). The detection of the subpopulation hspMaori, first isolated in the stomachs of New Zealand Maori but then observed in the Philippines and Samoa (Linz et al. 2007) and again among indigenous in New Caledonians, Wallis and Futuna Islanders, and Taiwanese (Moodley et al. 2009), suggested it as a marker for the expansion of Austronesian language and culture into the Pacific.

1.2.3 Formal Comparisons with Human DNA Data

The strong global and regional geographic structure of population clusters, tantalizingly similar to genetic structure observed in human DNA, suggested an ancient

association between *H. pylori* and its host. Startling formal comparisons between human and *H. pylori* DNA provided the first quantitative evidence for a long-term coevolutionary relationship. In a matrix comparison of pairs of equivalent *H. pylori* and human populations, up to 73 % (Linz et al. 2007) and 62 % (Moodley et al. 2012) of the global diversity in housekeeping gene sequences could be explained by human microsatellite and mitochondrial genomic diversity, respectively.

The genetic structure of a global human sample emphasizes the overarching influence of genetic drift on neutral human DNA markers (Prugnolle et al. 2005; Ramachandran et al. 2005). Under an isolation-by-distance (IBD) model, adjacent populations would be expected to exchange genes relatively regularly compared to populations that are situated further apart from each other. Thus, the geographic distance between a pair of populations is correlated with the neutral genetic distance (or divergence) between populations. IBD was also found to hold true for *H. pylori* populations, further supporting an ancient coevolutionary relationship (Linz et al. 2007).

Perhaps the most compelling evidence for long-term coevolution was provided by the first successful human exodus from Africa, around 60,000 years ago. Upon leaving Africa, migrating human populations were subjected to a series of founder effects in each of which only a proportion of people left an area that was already settled. Combined with the added erosive effect of genetic drift in small populations, these serial founder effects have left an indelible signature of our evolutionary origin in our DNA, as human genetic diversity decreases significantly with distance away from East Africa, the likely cradle of modern humans (Prugnolle et al. 2005; Ramachandran et al. 2005). The same significant relationship between genetic diversity and geographic distance was also demonstrated in *H. pylori*, indicating an African origin for both host and bacterium (Linz et al. 2007). Simulations using a one-dimensional "stepping-stone model" of migration indicated that anatomically modern humans migrated from Africa around 56,000 years ago (Liu et al. 2006) and *H. pylori* likewise approximately 58,000 years ago (Linz et al. 2007). Taken together, these data strongly imply that *H. pylori* arose in Africa and our forefathers carried this pathogen in their stomachs on their migrations out of Africa and to the furthest inhabitable corners of the world.

1.2.4 Recombination and Its Effect on Evolutionary Inference

Strong geographic structuring of population assignment clusters and clear parallels with human DNA seemed counterintuitive in a bacterium with particularly high rates of recombination or admixture (Falush et al. 2001; Suerbaum et al. 1998). However, unlike mutation, recombination requires the physical exchange of

bacteria between hosts, and this can only happen when host individuals live in close proximity to each other. Recombination, therefore, is geographically restricted, and its effect is less pronounced between populations than within populations.

Nevertheless, the intriguing geographic patterns elucidated by Bayesian clustering precluded any formal evolutionary genetic analyses of phylogenetic relatedness between and within populations, which populations were basal or derived, the direction of migrations, population demography, and interpopulation gene flow. That is because of the fundamental assumption of most analytical software that the accumulation of genetic diversity via mutation is lineage specific, that is, it passed on vertically from parent to offspring – identical by descent. Recombination on the other hand allows horizontal gene transfer between unrelated individuals. The effect of this is that mutations that appear identical (identical in state) are not identical by descent. Mutation may also generate homoplasies, but it is generally not of high enough frequency in eukaryotes to seriously bias evolutionary inference. However, in highly recombining species like *H. pylori*, homoplasies generated by horizontal gene transfer could seriously bias standard evolutionary analyses such as reconstruction of clonal phylogenies, dating population splits, and estimating demographic parameters. A standard neighbor-joining phylogenetic analysis of a global data set (Fig. 1.4a) using the Kimura two-parameter model of nucleotide substitution highlights consequences of not explicitly accounting for recombination

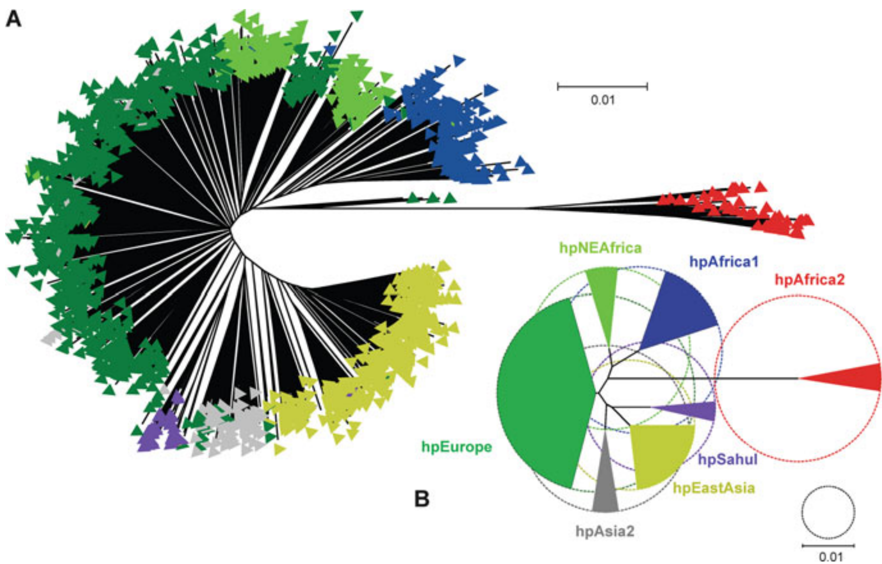

Fig. 1.4 Determining interpopulation relatedness in a highly recombining genome. (**a**) Individual-based neighbor-joining tree showing the influence of homologous recombination. Branches separating individuals are much longer than those separating populations, resulting in a genetic continuum in which only the distantly related hpAfrica is distinguishable. (**b**) Averaging sequence variation within each population reduced the effect of local recombination making interpopulation relationships clearer

in the substitution model. Due to the excess of homoplasies introduced by recombination, branches that lead to the tips of the tree (individual branches) are much longer than those separating populations from each other.

1.2.4.1 Population Trees

Given that recombination is usually geographically restricted, one way of minimizing its effect is to average out individual genetic diversity by a priori defining populations (of e.g., Bayesian clusters, ethnicities, countries, etc.) within each of which recombination may be common but likely to be much less frequent between populations.

Figure 1.4b shows the global population tree for *H. pylori*. Here, relationships between populations are depicted more accurately than in Fig. 1.4a, as individual variation is denoted in circle diameters and not branch lengths. Of the modern populations described, six populations were found to be very closely related to each other, and these included hpAfrica1, hpNEAfrica, hpAsia2, hpEastAsia, hpEurope, and hpSahul. The population hpAfrica2 is not only very divergent to all other *H. pylori* populations but was only isolated in South Africa, among people with both African and European ancestry.

Population trees reconstructed using the fixation index F_{ST}, calculated between pairs of populations, have been useful in retrieving subtle changes in DNA sequence variation, which would otherwise appear to be homogeneously distributed due to admixture. Analyzing a data set comprising ethnicities carrying the highly diverse hpEurope, Latifi-Navid and coworkers (2010) resolved an F_{ST} population tree that largely corroborated known geopolitical events in history, such as the Arab conquest of Iran in the seventh century AD and the Ottoman conquest and the Uzbek-Iranian conflict of the sixteenth century AD. Breurec and colleagues (2011) used the same method to disentangle several human demographic movements in Southeast Asia, where a diversity of three *H. pylori* populations (hpEurope, hpAsia2, and hpEastAsia) are hosted. They extended the prehistoric range of hpEurope into Thailand, Malaysia, and Cambodia, which appears to have been first introduced to Southeast Asia via ancient migration, most likely from the Indian subcontinent, and not exclusively from historically documented recent (nineteenth-century) Indian migration as previously thought (Tay et al. 2009). hpAsia2 (subpopulation hspIndia) was also found to have entered Southeast Asia twice – once most likely with the arrival of Tai-Kadai speakers into what is now Thailand in the early second millennium AD and again with the previously mentioned nineteenth-century Indian migrants (Breurec et al. 2011). Similarly, the subpopulation of hspEAsia was introduced to present-day Vietnam and Cambodia during the Neolithic diaspora out of the Yangtze and Yellow River Valleys around 2000 BC but appears to have arrived in Malaysia, Singapore, and Thailand during recent Chinese migration sometime during the last 200 years. Pairwise F_{ST}, therefore, has been successfully implemented in the inference of past human migrations; however, its disadvantage is that it depends heavily upon the reliability of the assumption of

relatively low recombination rates between, and only minor substructure within, predefined populations or ethnicities.

1.2.4.2 The Linkage Model: The Concept of Ancestral Populations

Falush and coworkers (2003a) attempted to deal with the admixture that results from high recombination by using the Bayesian clustering framework of STRUCTURE to infer a finite number of "ancestral" or precursor populations, to which all nucleotides in a strain's multilocus DNA sequence can then be assigned, thus inferring the level and putative source of admixture at the level of the individual strain. This method, known as the linkage model, relies on the admixture linkage disequilibrium that results when gene flow (migration) occurs between genetically distinct populations. Assignment of each nucleotide to an ancestral population, therefore, is based on linkage to neighboring nucleotides. Although the model depends on the correct inference of the number of ancestral populations, it was nevertheless a seminal leap forward in our understanding of admixture, population history, and evolution in this highly recombining bacterial species and resulted in several high-profile population genetic studies.

Since recombination historically occurred between strains within populations, signal for more ancient between-population events still persists in the *H. pylori* genome, despite very high within-population genetic diversity. The linkage model has thus far identified six ancestral populations (Breurec et al. 2011; Falush et al. 2003b), and one of its major contributions to our present-day understanding of admixture in *H. pylori* is the discovery that hpEurope is a recombinant hybrid population made up of roughly equal African and Central Asian ancestry. The six populations identified were thus ancestral Europe1 (AE1), ancestral Europe2 (AE2), ancestral EastAsia, ancestral Africa1, and ancestral Africa2 (Fig. 1.5). Given the geographic location of populations that contained "pure" AE1 and AE2 isolates, neither population appears to have originated in Europe. Instead, the

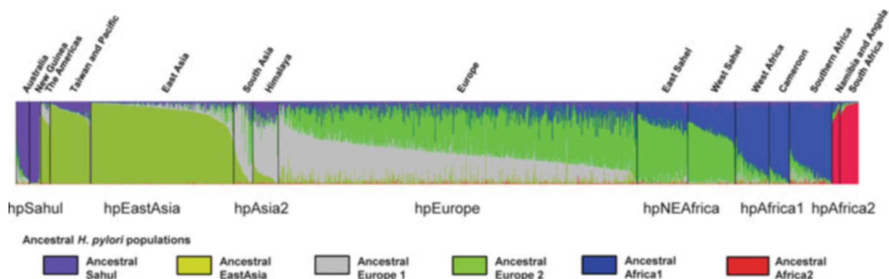

Fig. 1.5 Ancestral populations of *H. pylori* inferred by the linkage model. The multilocus genotype of each strain is divided into "chunks" based on the level of admixture linkage disequilibrium between nucleotides. Modern populations are a produce of admixture between ancient populations. hpEurope is clearly a recombinant population made up of mainly Ancestral Europe1 and Ancestral Europe2 nucleotides

isolates belonging to the modern hpAsia2 population were found to contain the highest levels of AE1 ancestry, locating the source of this ancestral population to somewhere in Central or southern Asia; likewise, the highest AE2 proportions were found among hpNEAfrica isolates, locating its source somewhere in Northeast Africa.

Ancestral EastAsia originated in East Asia, ancestral Africa1 in West Africa, and ancestral Africa2 in South Africa. The proportion of ancestry from each of the five ancestral sources varied among individual isolates and, when grouped by modern population, formed a continuum, suggesting extensive admixture between ancestral populations (Fig. 1.5). This allowed for the detection of more subtle population events occurring in hybrid zones between adjacent ancestral populations. Ladakh in northern India is one such zone inhabited by people of two major human religious groups, Muslims and Buddhists, who have coexisted for almost 1000 years but remained largely isolated due to cultural and religious differences. Human microsatellites and mtDNA were only marginally informative in detecting differences between the two groups. However, a linkage model analysis of *H. pylori* housekeeping gene sequences found that isolates from Buddhists showed a cline of admixture from almost pure ancestral EastAsia to almost pure AE1, clear evidence for the simultaneous introduction of Buddhism and hpEastAsia by human migration from Tibet into a preexisting Ladakhi (hpAsia2) population. Conversely, isolates from Muslims were uniform in AE1 ancestry, indicating that Islam was introduced to Ladakh by a few missionaries rather than by population migration (Wirth et al. 2004).

The pattern of ancestry in modern European *H. pylori* is more complex. Modern humans inhabited Europe between 40,000 and 46,000 years ago (Mellars 2006; Higham et al. 2011; Benazzi et al. 2011) but retreated southward into refugia in the Iberian Peninsula (Torroni et al. 1998, 2001) and Ukraine (Malyarchuk et al. 2008). It is not known whether these original Europeans were infected with *H. pylori* or whether it was first introduced to Europe by the expansion of Neolithic farmers from the Middle East. Although hpEurope comprises roughly 50:50 AE1/AE2 ancestry, the proportion of AE1 is higher in northern Europe, and the proportion of AE2 ancestry is higher in southern Europe (Falush et al. 2003b), suggesting more than one introduction of *H. pylori*. Using principal component analysis (PCA), Linz and colleagues (2007) demonstrated multivariate clines of potential migrations into Europe. These included evidence for the westward spread of domesticated crops from the Fertile Crescent into Europe by Neolithic farmers (AE2), migration of Uralic language-speaking peoples from Siberia into Scandinavia (AE1), and a population expansion from the steppes between the Volga and Don Rivers, associated with the domestication of the horse.

It is extremely unlikely that the hybridization event that resulted in hpEurope actually occurred in Europe. This is because almost all Europeans screened to date contain hpEurope, with very few exceptions. Humans migrating out of Northeast Africa carrying hpNEAfrica would also have to have passed through regions inhabited by hpAsia2 carriers prior to their arrival in Europe. Iran, situated at a crossroad between Europe, Asia, and Africa and containing a large proportion of

the Fertile Crescent in which Neolithic agriculture was developed, was hypothesized as a potential source for one of Europe's ancestral components (Latifi-Navid et al. 2010), but the hpEurope strains carried by Iranians were comprised of equal proportions of AE1 and AE2, similar to other countries in the Middle East (Linz et al. 2007).

hpWAfrica is indigenous to West Africa. However, in an analysis of strains from the Gambia, Senegal, and Burkina Faso, Secka and coworkers (2014) found little to no evidence of AE1 nucleotides in their sample, suggesting that the Nilo-Saharan expansion across the Sahara (6000–9000 years ago), rather than from repeated recent contact with Europeans and North Africans.

1.2.5 Enter Coalescence

Inference of population demographic parameters such as migration rates and effective population sizes and how these change over evolutionary time is made possible by coalescent theory (Kingman 1982). While coalescent models have undergone valuable development (Kuhner et al. 1998; Beerli and Felsenstein 1999; Drummond et al. 2005), their usefulness in analyzing highly recombining *H. pylori* sequences could be severely hampered by high homoplasy. One solution is to implement Hudson's (1985) four-gamete test, to detect recombining regions between pairs of segregating sites, which can then be removed prior to coalescent modeling. The four-gamete test relies on a simple assumption that homoplasies are generated by recombination and not by repeat mutation. Even though *H. pylori* is highly recombining, a small proportion of the recombining regions identified by the four-gamete test would likely be due to repeat mutation. The test is therefore conservative, discarding as much as half the DNA sequence data. Nevertheless, housekeeping gene sequences thus stripped of recombining regions and subjected to coalescent analyses using a two-population "isolation-with-migration" model (Hey and Nielsen 2004) have allowed estimates of bidirectional migration rates between population pairs that are consistent with known human demographic events (Moodley et al. 2009, 2012). It was thus established that gene flow (migration) occurred from East Asia into the Americas, but not the other way around, and notable back migration was detected between Africa and the Middle East and between Central and East Asia, highlighting the interface between these regions as "highways" for human and bacterial gene flow. Newer composite likelihood approaches (e.g., Jaatha software, Naduvilezhath et al. 2011) now provide a more flexible user-defined "isolation-with-migration" coalescent framework in which homologous recombination and changes in effective population size can be directly modeled. This method uses the joint allele frequency spectrum between two populations as a summary statistic to compute the composite likelihood of posterior demographic parameters.

The coalescent has also been used to model recombination directly from DNA sequence data to produce a genealogy that describes the clonal relationships among

individual lineages. The ClonalFrame software (Didelot and Falush 2007) uses a probabilistic Bayesian framework where the number, size, and location of recombination events are model parameters whose posterior distributions are estimated by Markov chain Monte Carlo (MCMC) simulation. The only drawback of this method is that it does not explicitly model the source of imported stretches of DNA sequences but instead assumes that novel mutations are introduced at a uniform rate. This would tend to underestimate the level of recombination relative to mutation. Nevertheless, the method has been used to successfully infer robust near-clonal genealogies at both inter- and intrapopulation scales in a number of studies (Moodley et al. 2009, 2012; Nell et al. 2013), adding greatly to our current knowledge of the evolutionary history of *H. pylori*.

Coalescent models work backward in time, estimating pairwise divergence in population-level models and the height of nodes at which individual lineages coalesce in a genealogy. While these two measures are not strictly the same, the first estimates the divergence between two populations, which would normally predate the time calculated from tree or node coalescence, since any given population may contain lineages that have not fully sorted. This restriction can be circumvented by defining biologically meaningful populations (e.g., those defined by STRUCTURE analysis), rather than more arbitrarily sorting strains into country or ethnic populations. The implementation of coalescent models in analyzing housekeeping gene sequences in *H. pylori* has greatly improved our understanding of the coevolution of this stomach bacterium and its hosts, shedding light on significant human migrations.

1.2.5.1 The Global *H. pylori* Phylogeny

Until the implementation of coalescent models, the phylogenetic relationships among *H. pylori* populations were only vaguely understood, the most striking and obvious observation from both population and individual neighbor-joining phylogenies being the distinctiveness of hpAfrica2 from all other populations (Falush et al. 2003b; Linz et al. 2007). However, genealogical analyses using ClonalFrame produced the first global clonal phylogeny for *H. pylori*, resolving the interrelationships between all known populations (Moodley et al. 2009). This phylogeny was calibrated by attributing certain branches on the tree to known human population splitting events and then smoothing the rates of evolution so that times could be readily compared across branches and the timing of debated human migrations could be estimated. The global phylogeny was refined to include a greater diversity of African strains and an out-group taxon, *H. cetorum*, isolated from cetaceans and *H. pylori*'s closest known relative (Moodley et al. 2012).

The global phylogeny (Fig. 1.6) showed clearly that *H. pylori* is structured into two superlineages: one containing hpAfrica2 and its close relative *H. acinonychis* and the other containing all other populations in a single monophyletic clade. This second superlineage is further split into a clade containing the two sister African populations hpAfrica1 and hpNEAfrica and a clade containing all non-African

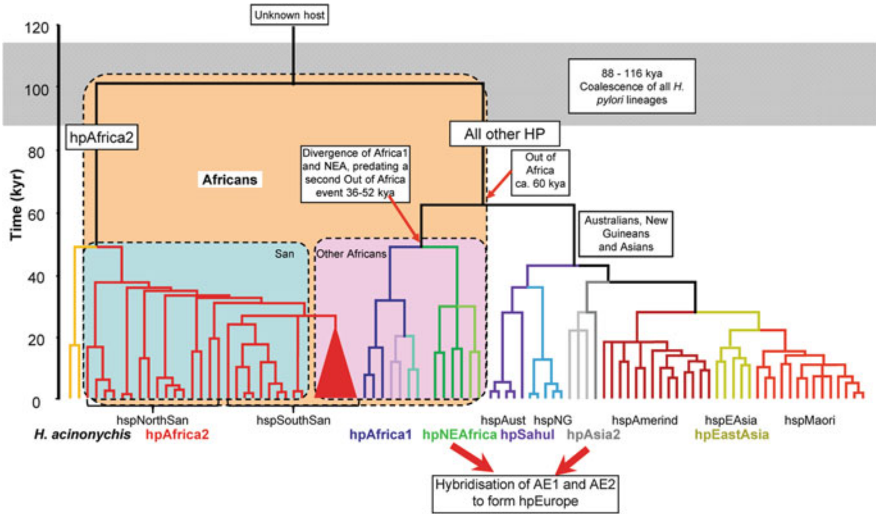

Fig. 1.6 The global *H. pylori* phylogeny obtained through simultaneous coalescent modeling of mutation and recombination. Housekeeping gene sequence diversity is structured into two clonal superlineages that coalesce to approximately 100,000 years. hpSahul is phylogenetically intermediate between African and Asian bacteria, and the San hunter-gatherers of southern Africa are the original hosts of hpAfrica2. Lineages are color-coded according to subpopulations in Figs. 1.3 and 1.4

H. pylori. Within this second clade, hpSahul is basal-most and hpAsia2 and hpEastAsia are sister populations. Subpopulation structure was also resolved within hpEastAsia, showing that hspAmerind is more distantly related to hspEAsia and hspMaori, which form a sister subclade.

1.2.5.2 Age of the *H. pylori* Human Association

If *H. pylori* accompanied humans out of Africa, then the association between the two species must date back prior to that event, seeing as the bacterium would first have to have been acquired by humans and then proliferated to infect the majority of the population by the time the event took place 60,000 years ago. A rooted, fully resolved, and calibrated global clonal phylogeny allowed the estimation of a lower limit for this association. This was the time at which the two *H. pylori* superlineages coalesced to a single common ancestor, and it was found to have occurred approximately 100,000 years ago (range: 88,000–116,000 years, Fig. 1.6, Moodley et al. 2012). However, because of lineage sorting and population bottlenecks, older lineages that might extend this time frame may have gone extinct. It is therefore possible that the human *H. pylori* association could be considerably older than this minimum estimate. That *Helicobacter* species were not successfully identified in gastric biopsies from chimpanzees does not fully reject the hypothesis

of a continuous hominoid-*Helicobacter* association spanning several million years, because of potential technical difficulties in sampling and culturing bacteria and host population structuring. More research in this area, sampling from different populations of bonobos and gorillas, may help push the length of our association further back in time.

1.2.5.3 Sahul Was Colonized Only Once

The dates and numbers of migrations to Sahul are controversial. Using a unique collection of strains from the stomachs of aboriginal Australians and New Guinean Highlanders, Moodley and coworkers (2009) showed that each ethnic group formed a unique subclade (or subpopulation) of its own, with no strain sharing. These subclades formed a single monophyletic group, whose coalescence dated back to 31,000–37,000 years ago (Fig. 1.6). This timing is consistent with the majority of the Pleistocene human archaeological sites but predates the earliest (Pope and Terrell 2007). More importantly, the monophyly of hpSahul evidences a single introduction of *H. pylori* into Sahul, and once it had split into hspAustralia and hspNGuinea, demographic analyses showed little to no migration between the subpopulations. The phylogenetic position of hpSahul, directly intermediate between African and Asian populations, firmly identified it as the marker for the first successful human population migrations outside Africa, along the southern coastal into Southeast Asia and eventually to Sahul. The *H. pylori* in other human population relicts of this migration, such as the Andaman Islanders, the Semang of Malaysia, and the Mani of Thailand, may therefore provide closer genetic links between hpSahul and African populations.

1.2.5.4 The Austronesian Expansion

The expansion of the Austronesian language family was the greatest prehistoric language expansion the world has ever seen, spreading the Malayo-Polynesian subfamily from Madagascar in the Indian Ocean to the Easter Islands in the Pacific. With the exception of Madagascar (Linz et al. 2014), the *H. pylori* subpopulation hspMaori has been observed in the stomachs of Polynesians, Filipinos (Linz et al. 2007), Melanesians, and indigenous Taiwanese (Moodley et al. 2009). A clonal phylogeny for this subpopulation placed Taiwanese strains basal to all others, with sequentially derived branches marking migrations to the Philippines, Melanesia, and Polynesia. Coalescent estimates of bidirectional migration rates between the East Asian mainland and Taiwan, and between Taiwan and the Pacific, show that the overwhelming majority of migration took place in the outward direction. Together with significantly higher genetic diversity in Taiwanese strains, these results established Taiwan as the source for the co-expansion of Austronesian and hspMaori.

1.2.5.5 San Hunter-Gatherers Are the Original Hosts of hpAfrica2

hpAfrica2 is the most distant and curiously distributed of all *H. pylori* populations. Its location at the southern end of Africa led to speculation that it might be associated with the ancient San hunter-gatherers of southern Africa. Its high frequency among existing San ethnicities in southern Africa strengthened this hypothesis. Furthermore, highly similar topologies between rooted global *H. pylori* human mtDNA phylogenies show that hpAfrica2 and the San are basal in the evolutionary histories of their respective species. However, hpAfrica2 is also frequently isolated among Bantu speakers in southern Africa (Falush et al. 2003b; Linz et al. 2007; Moodley et al. 2009; Duncan et al. 2013), and it was not obvious which of the two groups was hpAfrica2's natural host. However, a clonal phylogeny of only hpAfrica2 strains revealed that San isolates were both more diverse and basal to strains isolated from Bantus (Fig. 1.6). Reciprocally, hpAfrica1 strains isolated in San were more derived in an hpAfrica1 phylogeny than Bantu-isolated strains. This placed the San as the unequivocal original hosts of the divergent hpAfrica2 (Moodley et al. 2012). The inclusion of strains from three San ethnicities also resolved the two hitherto unknown subpopulations hspNorthSan and hspSouthSan. The northern ethnicities Khwe and !Xun, speaking Central and Northern Khoisan languages, respectively, were basal to the southern Khomani who spoke southern Khoisan, and migration between the two groups since their split 32,000–47,000 years ago has been predominantly from north to south. Moodley and colleagues (2012) also refined the timing of the split between hpAfrica2 and *H. acinonychis* to 43,000–56,000 years ago (Fig. 1.6), which suggests that the latter species was contracted by large felines from a San host.

1.2.5.6 A Second More Recent Out-of-Africa Migration

hpEurope is a hybrid population comprising nucleotides from two ancestral populations, AE1 and AE2, that evolved in Central or southern Asia and Northeast Africa, respectively. Consequently, and unlike its human hosts, hpEurope is highly diverse, more diverse than most populations in Africa, except southern Africa where the presence of the hpAfrica2 superlineages markedly inflates levels of genetic variation. This high diversity is inconsistent with a single human *H. pylori* migration out of Africa 60,000 years ago. This is because one of hpEurope's ancestral components AE2, exemplified in the modern-day hpNEAfrica, had not yet evolved by then. Coalescent simulations indicate that hpNEAfrica splits from hpAfrica1 sometime between 36,000 and 52,000 years ago, predating the 60,000-year event (Fig. 1.6). AE2 must therefore have left Africa sometime after it evolved in order to come into contact and hybridize with AE2 in western Asia, therefore invoking a second migration of humans out of Africa. Archaeological evidence that Middle Paleolithic technology was predominant in southern Asia, but more recently developed Upper Paleolithic or "Mode 4" stone

tools occurred exclusively in North Africa, the Levant, and Europe (Mellars 2006; Foley and Lahr 2003), corroborates the idea of a second African exodus. However, evidence from uniparentally inherited human DNA markers is still lacking (Macaulay et al. 2005; Oppenheimer 2012).

1.2.5.7 Pygmy Hunter-Gatherers Contracted *H. pylori* Recently from Neolithic Bantus

Nell and coworkers investigated the population demographics of *H. pylori* transmission between hunter-gatherer Baka pygmies and their neighboring Neolithic communities in Cameroon (Nell et al. 2013). They used a composite likelihood approach that included population expansion and **Aboriginal populations and**recombination; they found high gene flow between *H. pylori* populations in Baka and agriculturalists and that the Baka bacterial population was constant rather than expanding. Baka and agriculturalist strains coalesced to a population split between 856 and 3981 years ago, consistent with anthropological evidence that Baka came into recent secondary contact with Neolithic communities 3000–5000 years ago (Schoenbrun 2001). Furthermore, the high number of unrelated strains among the Baka community and their derived positions on a ClonalFrame phylogeny indicated that Baka contracted *H. pylori* from their agriculturalist neighbors. A susceptible-infectious (SI) epidemiological model showed that population-wide infection decreased with population census size, thus explaining the particularly low rate of infection (20.8 %) in the small (~40,000 individuals) Baka population. Given these analyses, it is most likely that Baka were *H. pylori* free prior to contact with Neolithic Bantu agriculturalists, from whom they contracted the bacterium during the last 4000 years.

1.2.6 Outlook: Genomics, Aboriginal Populations, and Ancient DNA

While the population, phylogeographic and evolutionary, research outlined in this review has helped revolutionized our understanding of the long and intricate coevolutionary relationship between humans and *H. pylori*, there are many questions still open to future research.

Thus far, almost all evolutionary and population genetic information on *H. pylori* has been inferred from the original MLST set of seven housekeeping gene sequences. With the costs of genome sequencing technologies decreasing yearly and more *H. pylori* genomes becoming available, whole genome analysis is likely to lead to greater resolution of bacterial and host demography. Already, over 72 *H. pylori* genomes, mostly sequenced with 454 technology, are available on public databases, although some populations such as hpNEAfrica and hpSahul are

not yet represented at the genome level. Nevertheless, future evolutionary analyses could potentially make use of a wealth of genetic information from 1.6 to 1.7 megabase genomes containing approximately 1500 open reading frames. However, genome analysis has its own drawback. The assumption of selective neutrality, vital for evolutionary analyses of housekeeping genes, would no longer apply to whole genomes where several hundred loci are likely to be under varying degrees of selection. Loci under selection or selective sweeps would have to be identified beforehand and excluded from analyses. Alternatively, population demography could be modeled via coalescent simulations that compute a large number of possible scenarios, to which the observed genomic data is compared, identifying regions that do not fit the best neutral model. A benefit of methods is the quantification of regions of the *H. pylori* genome that are under selection. An understanding of these would not only help resolve the history of co-migration but could also identify the loci responsible for medical symptoms and why these have become prevalent in some populations and not others.

Greater sampling coverage among the world's aboriginal people may help elucidate human demographic events in hitherto unstudied ethnicities such as among click-speaking East African Pygmies, the Hadza and Sandawe, among Nilo-Saharans and Bantus along the east coast of Africa, and among the myriad ethnicities in Siberia, islands of Southeast Asia, Oceania, and the Americas.

Ancient DNA may be another avenue of research worth investigating. Increasingly reliable capture methods have already made it possible to retrieve whole human genomes from mummified ancient human remains (Keller et al. 2012; Skoglund et al. 2012; Sánchez-Ouinto et al. 2012). If remains include the gastrointestinal tract, it may also be possible to capture ancient *H. pylori* genomes. Since most recent research in this field has been conducted on remains discovered in Europe, obtaining *H. pylori* from these remains may provide crucial insights as to whether the first hunter-gatherer humans in Europe were infected. Solving this puzzle may also help localize the hybridization event that resulted in the recombinant hpEurope population.

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Chapter 2
Adaptation of *Helicobacter pylori* Metabolism to Persistent Gastric Colonization

Frédéric Fischer and Hilde De Reuse

Abstract *Helicobacter pylori* is an amazingly successful pathogen that persistently colonizes the hostile gastric niche. Persistence of *H. pylori* colonization is an important parameter in the clinical outcome of the infection. *H. pylori* has evolved original mechanisms to colonize and persist within the stomach in spite of the harsh acidic conditions encountered in this environment. Genetic, physiological, and biochemical analyses of *H. pylori* have revealed peculiar properties of its metabolism, some of which are central in the adaptation to the gastric environment. The abundant enzyme urease is essential for *H. pylori* resistance to acidity through urea breakdown and production of buffering ammonia. Acid adaptation and management of the potentially toxic amounts of ammonia are closely associated through original transport and metabolic pathways, some of which involve protein complexes. Several metabolic enzymes have been shown to act in addition as genuine virulence factors in particular as immune modulators. This is illustrated by gamma-glutamyl transpeptidase, asparaginase, and arginase. Finally, given the central role of the nickel-dependent enzyme urease, the uptake and intracellular trafficking pathways of this metal essential for *H. pylori* colonization will be presented. In conclusion, we propose that the constrains of the small *H. pylori* genome and a very specialized niche has resulted in a close association and in overlapping networks between mechanisms of persistence, acid adaptation and metabolic pathways.

Keywords *Helicobacter pylori* • Acid adaptation • Ammonia metabolism • Urease • Hydrogenase • Nickel • Metallochaperone • Gamma-glutamyl transpeptidase • Asparaginase • Arginase • Gastric colonization

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2.1 Introduction

The stomach has long been regarded as a sterile environment because of its extreme acidity. It is now known that the stomach of about half of the human population is infected by *Helicobacter pylori*.

After acquisition during childhood, the infection by *H. pylori* becomes chronic if not treated. Severe pathologies like gastric cancer can occur after decades of *H. pylori* colonization and intense inflammation supporting the view that persistence of the infection is one important parameter in its clinical outcome. It is thus important to define the mechanisms that make this bacterium such a successful and persistent pathogen in its unique and hostile acidic gastric niche. *H. pylori* is a neutrophilic microorganism which means that it grows at pH values between 6 and 8. However, *H. pylori* has evolved original mechanisms to colonize and persist within the stomach in spite of the harsh acidic conditions encountered in this environment. Genetic, physiological, and biochemical analyses of *H. pylori* have revealed peculiar properties of its metabolism, some of which are central in the adaptation to the gastric environment. The nickel-dependent urease enzyme plays a major role in this process through urea breakdown and production of buffering ammonia. The present chapter is focused on the physiological and metabolic adaptations adopted by *H. pylori* to successfully colonize the stomach and to persist in its host. The first part of this chapter will explain how survival and pathogenesis rely on urease enzymatic activity and ammonia metabolism, the second part will concern metabolic enzymes that are in addition virulence factors, while the third part will shed light on the importance of nickel availability to enable successful colonization and persistence.

2.2 *H. pylori* Facing Acidity

Apart from the acidophiles that exclusively grow at low external pH, numerous neutrophilic bacteria are able to survive low-pH exposure. This is, for instance, the case for bacteria such as *Escherichia coli*, *Vibrio cholerae*, and *Salmonella* spp. that need to survive the passage through the stomach but are unable to sustain growth under these conditions. In order to overcome the adverse effects of acidity, most bacteria use acid resistance systems that either involve a reversed F_1F_0-ATPase activity for proton extrusion in response to the membrane potential change or amino acid decarboxylation/protonation pathways coupled to proton efflux (Foster 2004) to maintain cytoplasmic pH homeostasis.

Contrary to the other bacteria passing through the gastric lumen and that cannot divide, *H. pylori* thrives very well within the acidic gastric environment and can even reach densities as high as 10^6 cfu/g in the human stomach (Fig. 2.1). However, *H. pylori* does not possess the "classical" resistance mechanisms described above. Only when urea is provided, *H. pylori* resists low pH exposure and can multiply.

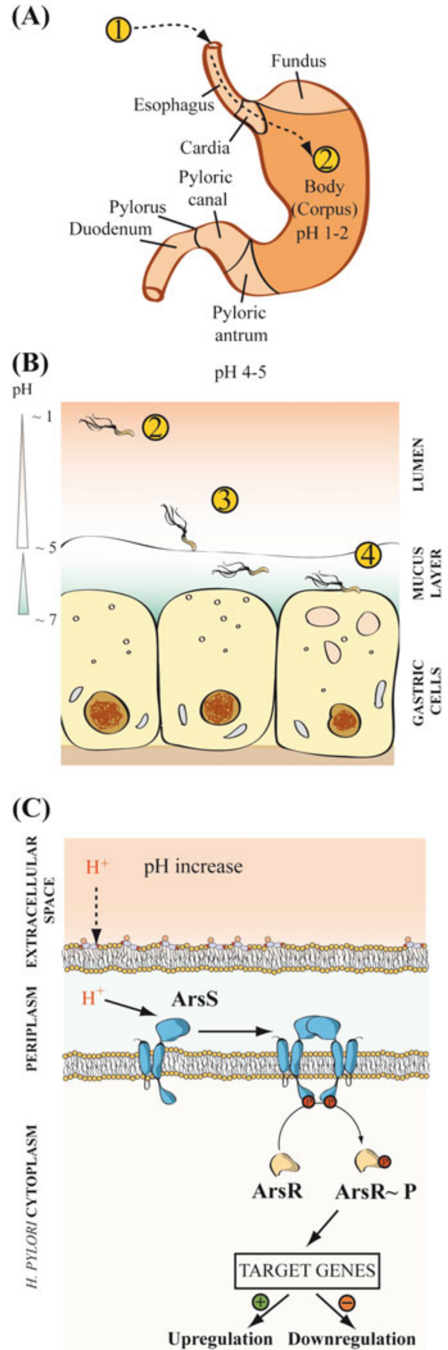

Fig. 2.1 Life cycle of *H. pylori* in the human stomach. (**A**) when *H. pylori* enters the human body through the oral route (*1*), it is exposed to the extremely acidic pH of the stomach lumen (*2*). (**B**) However, its colonization site is the moderately acidic gastric mucus (*3*) where about 80 % of *H. pylori* cells are found (*3*). Around 20 % of the bacteria actually adhere to the gastric epithelial cells where they cause inflammation and tissue injury (*4*). (**C**) to face acidity, *H. pylori* reprograms transcription of numerous genes. The ArsR-S two-component system is central in the acid response of *H. pylori* regulating either positively or negatively the expression of several genes

H. pylori is thus able to survive and multiply under very acidic conditions, but relies on a completely different system that is unique to the gastric *Helicobacter* species and requires massive ammonia production. As we will discuss, acid adaptation involves the nickel-dependent urease enzyme, capable of hydrolyzing urea to produce ammonia and bicarbonate, as well as different enzymes of the ammonia metabolism.

2.2.1 The Dangers of Low pH

Exposure to low pH has dramatic effects on the cells: it triggers protein denaturation, alters enzymatic activity, enhances DNA depurination, and generates membrane damage (Foster 2004). In Gram-negative bacteria, the periplasmic space is the first compartment to experience pH decrease. This drastically perturbs the inward proton gradient and directly decreases the membrane potential, which, in turn, hinders the proton influx toward the periplasm. This affects all the membrane potential-dependent processes, such as ATP synthesis or respiration. Then, accumulation of protons can cause the cytoplasmic pH to decrease, disrupting metabolic pathways, leading to growth arrest, and finally to cell death.

2.2.2 Acid Adaptation and Acclimation of H. pylori to the Gastric Niche

Upon entry through the oral route, *H. pylori* reaches the highly acidic lumen (median pH 1.5) (Fig. 2.1). The bacterium only survives for minutes in this challenging environment and has to migrate rapidly toward the epithelium surface (Schreiber et al. 2005). About ~80 % of the *H. pylori* cells are detected in the moderately acidic gastric mucus (pH ~ 5) (Schreiber et al. 2004). Once there, *H. pylori* may also experience large pH variations under fasting (pH 1) and digestive (pH 4.5–6) conditions. The capacity to deal with this low pH was referred to as "acid acclimation" or "acid adaptation" (Fig. 2.1). In addition, acidity also varies depending on the infection location within the stomach, from the mildly acidic antrum to the highly acidic corpus zones. Many parameters can vary either spatially (lumen, mucus, surface of the epithelial cells, corpus, antrum) and/or overtime within the gastric environment. These parameters not only include the pH value but also the availability (stability, solubility) of nutrients such as amino acids, peptides, and metal ions, etc. It is therefore not surprising that the mechanisms involved in *H. pylori* acid acclimation are not only related to pH managing processes but also to nutrient uptake (Stingl and De Reuse 2005), to chemotaxis, and to motility.

2.2.3 Establishing the Response to Acidity

Once it faces a pH decrease, *H. pylori* triggers a transcriptional reprogramming to express genes involved in acid adaptation and mechanisms of protection against protons.

The global transcriptional response of *H. pylori* to acidity has been analyzed either in vitro using bacteria exposed to acidity (Allan et al. 2001; Ang et al. 2001; Wen et al. 2003; Merrell et al. 2003) or grown under moderately acidic conditions (as in our previous study (Bury-Moné et al. 2004)) or in vivo during Mongolian gerbil colonization (Scott et al. 2007). Gene expression patterns poorly matched when comparing these analyses, both in the nature and the number of regulated genes. This can be explained by the use of different *H. pylori* strains but also because cells were exposed to different culture media and/or acid conditions. Despite those differences, clear common trends could be extracted from these results, some of which were confirmed subsequently.

A major strategy for acid resistance in *H. pylori* is ammonia production. The transcriptomic studies unambiguously showed that the operon containing the genes coding for the urease structural subunits (*ureAB* operon) and the operon encoding the urea channel and urease maturation accessory proteins (*ureIEFGH* operon) were acid-induced in *H. pylori*. In addition, the expression of two other ammonia-producing enzymes, the AmiE amidase and AmiF formamidase, was found to be acid-induced and will be discussed below (Skouloubris et al. 1997, 2001). Acid protection strategy of *H. pylori* is also suggested by the repeated observation of downregulation in the expression of membrane proteins including transporters, permeases, and outer membrane proteins.

Another common observation was 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. 2003) and may be indicative of a strategy to escape acidity or, at least, might suggest a pH-driven response directing the bacteria through the mucus pH gradient toward a suitable site for multiplication and/or adhesion, as suggested by Schreiber et al. (2004). In parallel, major modifications in the expression of genes coding for proteins related to, or regulated by, metal ion availability was evidenced (Bury-Moné et al. 2004). Solubility of nickel and other metals is known to be strongly enhanced at low pH. Urease maturation and activity strictly depend on two nickel ion cofactors within the active site. Therefore, acid resistance is tightly linked to nickel metabolism in *H. pylori*. This aspect will be discussed below.

Most of the studies on transcriptional regulation in *H. pylori* showed the involvement of three major regulators of the acid response network: (i) the two metal regulators Fur and NikR that respond to iron and to nickel, respectively (Van Vliet et al. 2003; Contreras et al. 2003; Bury-Moné et al. 2004), and (ii) the ArsRS (HP0165-HP0166 in strain 26695 (Tomb et al. 1997)) two-component

regulatory system (TCS) that seems to act as a master regulator (Bury-Moné et al. 2004; Pflock et al. 2005; Muller et al. 2009).

When *H. pylori* is exposed to acidic pH, the ArsS sensor histidine kinase, located in the inner membrane, becomes phosphorylated on its His94 residue (Fig. 2.1). This residue of the output domain is crucial for pH sensing. ArsS ~ P then transfers its phosphate to its cognate regulator ArsR that acts as an activator or a repressor on a variety of pH-responsive genes. ArsR is an essential protein for *H. pylori* growth and its phosphorylation is essential for acid acclimation (Muller et al. 2009). The ArsRS system regulates many genes encoding central proteins in the acid response that will be discussed in this chapter (Pflock et al. 2006; Wen et al. 2007). These include genes encoding urease, the AmiE and AmiF aliphatic amidases, the α-carbonic anhydrase, arginase, nickel storage proteins, and several others. The cytoplasmic FlgS histidine kinase which governs the expression of some flagellar genes seems to play an additional minor role in the *H. pylori* transcriptional response to acidity (Marcus et al. 2012).

2.3 Urease, the Major Player in *H. pylori* Resistance to Acidity

H. pylori urease is composed of two subunits, UreA (HP0073) and UreB (HP0072). UreA is a 26.5 kDa protein and the 61.6 kDa UreB subunit carries the active site containing the bi-nickel metallic complex essential for activity. Assembly of this metallocenter is performed by accessory proteins (see below) (Farrugia et al. 2013; De Reuse et al. 2013; Stingl and De Reuse 2005). Each UreA/B dimer associates with two other dimers, forming a $(UreA/B)_3$ complex, which can then cluster with other trimers, to create a giant dodecameric $((UreA/B)_3)_4$ complex whose structure has been solved (Ha et al. 2001). In vivo, urease is present in huge amounts within *H. pylori* cells and can represent up to 10 % of all proteins.

2.3.1 Urease Enzymatic Activity

Urease catalyzes the hydrolysis of urea ($CO(NH_2)_2$) into ammonia (NH_3) and carbamate, the latter being spontaneously degraded into another molecule of ammonia and carbon dioxide. CO_2 mostly exists as a dissolved gas form, and the H_2CO_3 carbonic acid form only represents ~1 % of the species present in solution. At pH 7, it rapidly turns to bicarbonate (HCO_3^-). The *H. pylori* cytoplasmic carbonic anhydrase can further catalyze the interconversion between CO_2 and HCO_3^-. Consequently, the overall reactions lead to molecules capable of proton buffering. The NH_4^+/NH_3 couple represents a strong proton buffering system, since its pK_A is ~9.2, meaning that at neutral or acidic pH, ammonia is almost

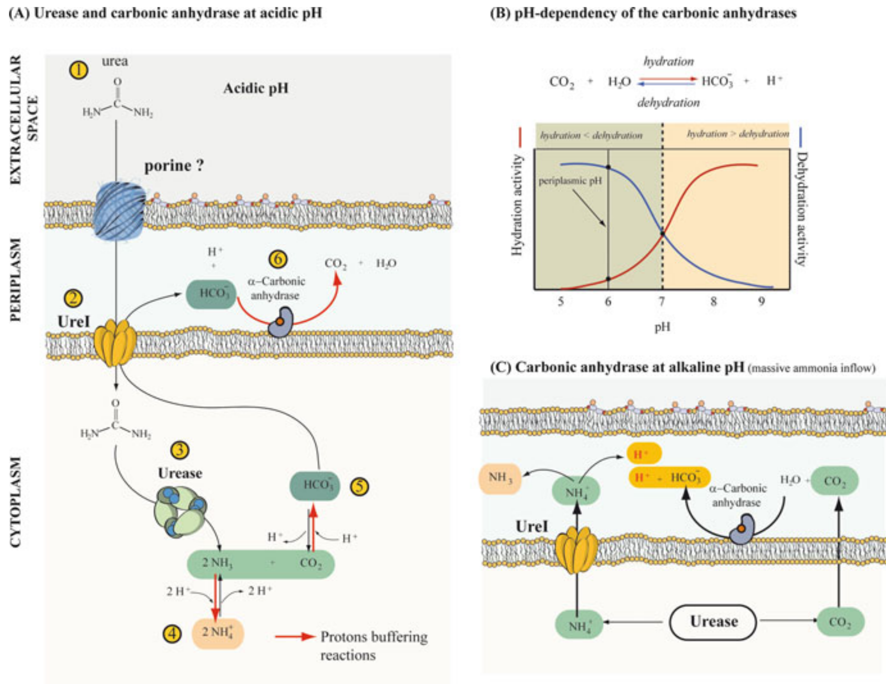

Fig. 2.2 Acid resistance in *H. pylori* and revised model for the role of the α-carbonic anhydrase. *Panel* (**A**) when pH decreases and falls below 6.0, urea (*1*) enters the cell through the UreI channel (*2*). Once in the cytoplasm, it is degraded by urease (*3*) into ammonia and CO_2. Upon protonation, ammonia is transformed into NH_4^+ ions (*4*), thereby buffering the cytoplasm. In solution, CO_2 rapidly turns to bicarbonate HCO_3^-, buffering another H^+ (*5*). HCO_3^- can be exported into the periplasm where the membrane-bound α-carbonic anhydrase catalyzes a dehydration reaction that consumes protons to produce H_2O and CO_2 (6). *Panel* (**B**) carbonic anhydrases catalyze either the hydration of CO_2 or the dehydration of HCO_3^- in a pH-dependent manner. The catalytic efficiency of the hydration reaction increases with pH, with a pK_A value of ~7.0. The opposite is observed for the dehydration reaction. Thus, at the periplasmic pH of 6.0, the dehydration reaction (producing water and CO_2) is strongly favored, while the hydration reaction is not. In case of low-pH exposure, this will avoid periplasmic over-acidification. *Panel* (**C**) if NH_3 production leads to periplasmic alkalinization, the α-CA will rather catalyze the hydration reaction (producing HCO_3^- and H^+). This balancing model should allow *H. pylori* to maintain a periplasmic pH value around 6.0

exclusively converted into its ammonium form NH_4^+. The massive ammonia production by urease activity thus represents the first countermeasure against proton overload under acidic conditions (Mobley and Hausinger 1989; Labigne et al. 1991) (Fig. 2.2).

The urease catalytic constant measured at steady-state is high, ranging from 1,400 to 2,700 s^{-1} (Hu and Mobley 1990), implying a very rapid turnover for urea degradation on one side and nitrogen mineralization on the other side. Based on its kinetic properties, the urease complex should function at a maximal rate at the concentration range of urea found within the human stomach (1–3 mM) (Mobley

and Hausinger 1989; Hu and Mobley 1990). Consequently, urease produces massive amounts of ammonia upon urea entry within the cells, which allow *H. pylori* to rapidly resist pH decrease and proton overload.

2.3.2 Acid-Gated Transport of Urea by the UreI Channel

Urease is a cytoplasmic enzyme that is not required for growth at neutral pH in vitro, but becomes essential under acidic conditions (Fig. 2.2). The *H. pylori* acid acclimation is lost when the urease genes are deleted or when the UreI urea channel gene is disrupted (Skouloubris et al. 1998; Scott et al. 2000; Bury-Moné et al. 2001). Thus, acid acclimation not only depends on urea degradation and ammonia production but also on urea uptake across the inner membrane. Urease and UreI are indeed essential for *H. pylori* to colonize several animal models (Eaton et al. 1991; Skouloubris et al. 1998; Mollenhauer-Rektorschek et al. 2002).

UreI, whose gene lies between the *ureAB* and *ureEFGH* gene clusters, has been identified as a transmembrane channel transporting urea from the periplasmic space into the cytoplasm where it is hydrolyzed by urease to produce ammonia (Skouloubris et al. 1998; Weeks et al. 2000; Weeks and Sachs 2001). UreI is a proton-gated channel responding to the decrease of periplasmic pH, the channel starts to open up when the pH falls below 6.0, and selectively transports urea (Bury-Moné et al. 2001; Gray et al. 2011; Weeks et al. 2000). The UreI structure has been recently solved (Strugatsky et al. 2013): it has a novel channel architecture composed of six protomers assembled in a hexameric ring surrounding a central bilayer plug of ordered lipids. Residues important for the selectivity of the channel have been identified, and the dynamics of urea conduction was characterized providing hints on its capacity to transport urea even at low concentration gradients (McNulty et al. 2013). It has been proposed that UreA-B and the UreE accessory proteins are recruited to UreI at the inner membrane (Voland et al. 2003) and that UreI is in addition permeable to NH_3/NH_4^+ and CO_2 (Scott et al. 2010). Additional data will be necessary to reinforce these views.

When exposed to a low external pH value of 2.5, the *H. pylori* membrane potential, which is −100 mV at neutral pH, starts to considerably fall. When urea is provided, this value stabilizes around −100 mV, indicating that urease is at least partly involved in pH homeostasis of the periplasm that is maintained at about 6.1 (Scott et al. 1998). The control of the periplasm pH upon acid exposure is till now unique to *H. pylori* and implies a complex response dependent on the α-carbonic anhydrase (see below).

2.3.3 Regulation of the Expression of the Urease Genes

Urease genes are clustered into two operons. The *ureA*-*ureB* dicistronic part is followed by an operon composed of *ureI* and the *ureEFGH* genes required for urease maturation and metalation. The urease operon is controlled by the acid-responsive ArsRS two-component system (Marcus et al. 2012), as well as by NikR (Van Vliet et al. 2001, 2002; Muller et al. 2011), the latter responding to intracellular nickel concentration. Urease transcriptional balance thus depends on both acidity and metal content (van Vliet et al. 2004b; Muller et al. 2011). This regulation reflects the need for maximal urease activity under acidic conditions, taking into account its maturation by nickel insertion (De Reuse et al. 2013) (*see* paragraph 2.6).

2.3.4 Role of the Carbonic Anhydrases in H. pylori

Carbonic anhydrases (CA) catalyze the interconversion of carbon dioxide and bicarbonate and are involved in functions such as CO_2 transport or trapping and pH homeostasis. Two genes encoding carbonic anhydrases are found in the *H. pylori* genome (*hp1186* and *hp0004* in strain 26695). The first one encodes a periplasmic α-type CA bound to the inner membrane (Marcus et al. 2005) and homologous to those found in vertebrates. The second one codes for a cytoplasmic ß-CA of a different structural type that is mostly found in prokaryotes and plants (Fig. 2.2). Both enzymes are metalloproteins that require one Zn^{2+} ion to catalyze the reversible hydration of carbon dioxide (CO_2) and dehydration of bicarbonate (HCO_3^-) (reviewed in Frost and McKenna (2014)). CO_2 occupies a central position in the physiology of *H. pylori* owing the large amounts of carbon dioxide produced by urease-mediated urea hydrolysis, the constant bicarbonate supply in the stomach, and its capnophilic nature (strict dependence on CO_2 for growth). In addition, it has been shown that *H. pylori* displays a chemotactic response to bicarbonate through a methyl-accepting chemotaxis receptor protein system, which seems to be coupled with urea and arginine sensing (Cerda et al. 2011).

Both α-CA and β-CA have been shown to be dispensable for in vitro growth under neutral conditions in several *H. pylori* strains (Marcus et al. 2005; Bury-Mone et al. 2008). We only observed a growth defect phenotype with the single and double CA deletion mutants of strain SS1 (Bury-Mone et al. 2008). These mutants were in addition strongly affected in their capacity to colonize the mouse model (Bury-Mone et al. 2008). ^{13}C-NMR measurements demonstrated that most CA activity can be attributed to the α-CA, with the β-CA enzyme playing a minor role (Bury-Mone et al. 2008).

Interestingly, the α-CA plays a role in the urea-dependent response of *H. pylori* to acidic pH (Fig. 2.2b). Indeed, when the α-CA was absent, a delayed production of ammonia from urea was observed (Bury-Mone et al. 2008). The enzyme does not

participate in urease regulation or maturation, indicating that α-CA truly participates in the pH-dependent response. Another study unraveled the involvement of the α-CA in periplasmic pH buffering (Marcus et al. 2005). The authors observed that an α-CA knockout strain (*H. pylori* 26695) could not survive in acidic medium compared to the wild-type strain, a phenotype that was not observed in our study (Bury-Mone et al. 2008). This could be explained by different genetic backgrounds and/or the use of poorly defined complex media for growth (Bury-Mone et al. 2008). Using fluorescent dyes to monitor intracellular and periplasmic pH and measurements of the membrane potential in response to urea uptake by whole *H. pylori* cells, Marcus and coworkers (Marcus et al. 2005) concluded that the α-CA is involved in the buffering of the periplasm and, consequently, in the membrane potential rescue upon acid exposure. The HCO_3^-/CO_2 system displays a $pK_A \sim 6.1$. Thus, a model was proposed in which the CO_2 gas molecule produced by urease passively diffuses across the inner membrane toward the periplasm, where it is hydrated by the α-CA into HCO_3^- and H^+. This proton would then immediately be buffered by NH_3 to form NH_4^+. This would ensure the maintenance of a periplasmic pH ~ 6.1 keeping an –100 mV inner membrane potential (Marcus et al. 2005). The cytoplasmic β-CA would be involved in the system as well, converting the HCO_3^- produced by urea degradation into CO_2. In support of this model is the observation by the same group that UreI is not only involved in urea uptake but also capable of extruding CO_2 and NH_4^+ from the cytoplasm to the periplasm (Scott et al. 2010).

However, our close analysis of the enzymatic properties of the CA enzymes leads us to conclude that this model needs to be reexamined for the following reasons (Fig. 2.2b). Because the activity of these enzymes relies on a zinc ion, their catalytic activity strongly depends on pH. The hydration reaction ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) efficiency increases as a sigmoid when the pH increases, fitting an acid/base titration curve with a pK_A value of ~7.0 (Fig. 2.2b). This property originates from the Zn-hydroxylated center found within the active site (Frost and McKenna 2014). On the contrary, the catalytic efficiency of the dehydration reaction ($HCO_3^- + H^+ \rightarrow CO_2 + H_2O$) decreases as a sigmoid curve when pH increases (Fig. 2.2b). This was clearly demonstrated by Chirica et al. (2001) with purified *H. pylori* α-CA. Consequently, the CA hydration/dehydration reaction ratio directly depends on pH, thereby determining the role of CA as a function of pH variations (Fig. 2.2b).

According to these enzymatic properties, it is unlikely that at the pH ~ 6 found within the periplasm, the α-CA hydrates CO_2 to produce HCO_3^- and H^+, as proposed in the model of Marcus et al. (2005). Under such conditions, one would rather expect the dehydration activity to be increased over hydration.

We propose a more complete model that would work as follows (Fig. 2.2c). At periplasmic pH of approx. 6, the α-CA mostly catalyzes the dehydration reaction, thereby consuming protons and producing CO_2. The same reaction would even be strongly favored at acidic pH (<6.0), meaning that under neutral or acidic conditions, α-CA is involved in proton consumption and buffering, increasing the pH in the periplasm. If NH_4^+ can be exported to the periplasm as stated by Scott et al. (2010), then it would be able to release protons in the alkalinizing periplasm

and counterbalance this pH variation, to bring it back to a more acidic range. When urease catalyzes urea breakdown, however, it triggers a rapid and massive NH_3 accumulation, which increases the cytoplasmic and the periplasmic pH value. Indeed, at pH $\sim$ 6, with a pK_A value of 9.2, ammonia efficiently pumps in protons and turns to its NH_4^+ ionic form, favoring a rapid and strong alkalinization. If the periplasmic pH starts to increase as well, the α-CA would start to catalyze the hydration (HCO_3^- *and* H^+-forming) reaction more efficiently, thereby optimally producing buffering HCO_3^- and H^+ to counterbalance ammonia-driven alkalinization (Fig. 2.2c). In conclusion, we propose that urease and α-CA should rather be regarded as a tangled-up balancing system avoiding over-acidification and over-alkalinization of both the periplasm and the cytoplasm (Fig. 2.2).

2.4 Ammonia Metabolism in *H. pylori*

Considering the large amounts of ammonia that are produced by urease in response to the acid pH exposure, *H. pylori* is expected to have counterbalancing systems to avoid toxic massive ammonia accumulation (De Reuse and Skouloubris 2001). In bacteria, ammonia is assimilated ("stored") into amino acids through amination of α-ketoglutarate into glutamate (Glu) and then by amidation into glutamine (Gln). Not surprisingly, in *H. pylori*, nitrogen from urea has been demonstrated to be incorporated into amino acids (Williams et al. 1996). In addition, urease is required for colonization of gnotobiotic piglets with an artificially neutralized stomach suggesting a metabolic function of urea degradation in addition to its role in acid resistance (Eaton and Krakowka 1994). *H. pylori* was found to have minimalist pathways for nitrogen/ammonia assimilation that are illustrated in Fig. 2.3.

2.4.1 Minimalist Pathways for Nitrogen Assimilation

In *H. pylori* Glu is synthesized from ammonia and α-ketoglutarate by a single glutamate dehydrogenase (GdhA). Indeed, this organism does not possess the classical glutamine oxoglutarate aminotransferase (GOGAT) system found in other bacteria (for a review, see Leigh and Dodsworth (2007)). Gln is then synthesized from Glu and ammonia by the essential glutamine synthetase (GlnA, HP0512). This enzyme cannot be inactivated in *H. pylori* even when Gln is supplied in the medium (Garner et al. 1998; Stingl et al. 2008). In bacteria, Gln plays a central role, since it is involved in almost all the amidation reactions that rely on an amido group donor in the purine/pyrimidine biosynthetic pathway, amino acid biosynthesis, as well as in the bacterial cell wall synthesis (Leigh and Dodsworth 2007). Another way to store ammonia is to perform aspartate (Asp) synthesis from fumarate and ammonia using aspartate ammonia lyase (AspA) and then to amidate it with an asparagine synthetase to produce asparagine (Asn). However, in

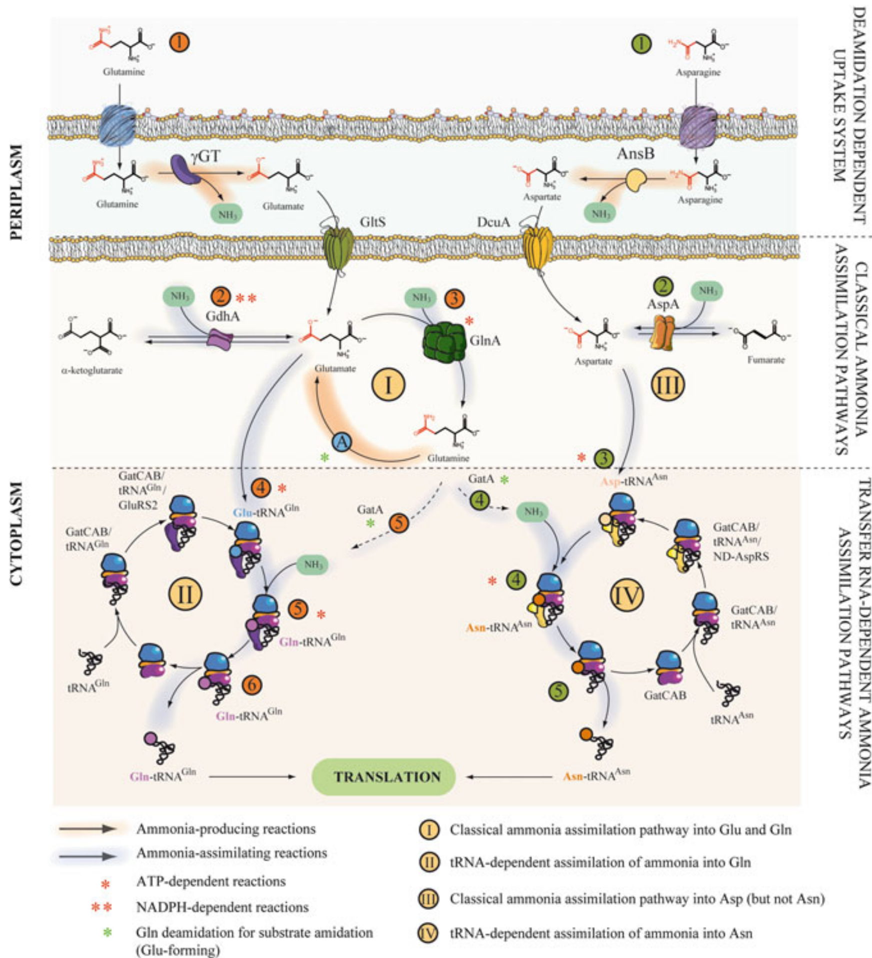

Fig. 2.3 Asparagine and glutamine metabolism in *H. pylori*. Asn (1, *green* label) and Gln (*1*, *orange* label) are both imported in the periplasm, where they are deamidated by the AnsB or GGT enzymes, respectively, providing Asp and Glu and ammonium. Asp and Glu are then transported across the inner membrane through the DcuA and GltS transporters, respectively, toward the cytoplasm. Glu can also be synthesized by the glutamate dehydrogenase (GdhA) (*2*) and Asp by the aspartate ammonia lyase (AspA) (*2*, *III*). Glu serves as a substrate for the Gln synthetase (GlnA) (*3*) that provides Gln (*I*). In *H. pylori* Asn is not synthesized in a free form. Gln can also be synthesized in a tRNA-dependent manner in *H. pylori* (steps *4–6*, *II*). In this pathway, Glu is first misacylated by a glutamyl-tRNA synthetase (GluRS2) onto $tRNA^{Gln}$ (*4*, *orange*); then this intermediate gets amidated by the GatCAB complex (*5*, *orange*), to provide the final $Gln\text{-}tRNA^{Gln}$ molecule suitable for translation (*5*, *orange*). These reactions are performed by the Gln-transamidosome complex (*II*). Asn is specifically synthesized for translation in an analogous tRNA-dependent manner (steps *3–5*, *green*), starting with misacylation of Asp onto $tRNA^{Asn}$ by the nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) (*3*, *green*), followed by amidation by the GatCAB enzyme (*4*, *green*) to provide $Asn\text{-}tRNA^{Asn}$ (*5*, *green*). All reactions occur within the Asn-transamidosome complex (AspRS-ND/$tRNA^{Asn}$/GatCAB) (*IV*)

H. pylori, whole-genome analysis revealed the absence of an Asn biosynthetic pathway (Marais et al. 1999).

2.4.2 Central Role of Glutamine Synthetase in Ammonia Metabolism and Possible Coupling with Urease

Glutamine synthetase (GlnA) that catalyzes the incorporation of ammonia into Glu to form Gln thus plays a central role in the ammonia metabolism of *H. pylori*. This pathway is usually tightly controlled through several levels of regulation including GlnA adenylation. *H. pylori* GlnA does not seem to be subject to this classical retro-inhibition pathway; therefore, Gln synthesis is probably constitutive (Garner et al. 1998).

Some data are supporting the view that there is a coupling between the activities of glutamine synthetase (GlnA) and urease in *H. pylori*. During our analysis of the urease protein interactome by tandem affinity purification (TAP), we found that UreA specifically interacts with GlnA (Stingl et al. 2008). We proposed that this physical interaction of GlnA with UreA allows optimization of the incorporation of urease-derived ammonium into glutamate to produce glutamine through metabolic channeling, a mechanism that has been reported for other protein complexes (see below). In support of this view, a recent study showed that in an *H. pylori* strain artificially overexpressing GlnA, urea-derived ammonia production is enhanced about sixfold (Miller and Maier 2014). Thus, the metabolism of Glu/Gln and probably of amidated amino acids is closely connected to the ammonia production in *H. pylori*. The next paragraphs will describe ammonia-producing and ammonia-assimilating enzymes found in *H. pylori*.

2.4.3 Transport and Metabolism of Amidated Amino Acids

In bacteria, amino acids are mostly used for protein synthesis, but are also taken up as energy, nitrogen, and carbon sources. *H. pylori* preferentially uses amino acids as an energy source and interestingly consumes very large amounts of Asp and Glu as well as their amide counterparts, Asn and Gln (Mendz and Hazell 1995; Stark et al. 1997). In this paragraph, we will present the particularities of the metabolism of these two amidated amino acids Gln and Asn in *H. pylori*.

2.4.3.1 Asp/Asn and Glu/Gln Uptake in *H. pylori*

In *H. pylori*, the important asparaginase and glutaminase activities are catalyzed by two highly active periplasmic enzymes, gamma-glutamyl transpeptidase (GGT,

HP1118) and L-asparaginase (AnsB HP0723) that produce ammonia and Asp or Glu from Asn and Gln, respectively (Cappelletti et al. 2008; Shibayama et al. 2007; Leduc et al. 2010). We tested the role of these enzymes in the uptake of Asp/Asn and Glu/Gln in intact *H. pylori* cells (Leduc et al. 2010). While Asp and Glu uptake were not dependent on these activities, Gln and Asn strictly required GGT and AnsB activities, respectively, to be taken up (Shibayama et al. 2007; Leduc et al. 2010). We identified two inner membrane transporters for Asp (DcuA, HP0724) and for Glu (GltS, HP1506) and demonstrated that they are the sole uptake systems for these two amino acids in *H. pylori* and are unable to take up Gln and Asn (Leduc et al. 2010). We further demonstrated that Asn deamidation by AnsB is a prerequisite to its import by the DcuA transporter. The same is true in the case of Gln, where its deamidation by GGT is required for the GltS transporter to import Glu. This explains why an *H. pylori* GlnA mutant cannot be obtained even when Gln is provided in the growth medium. Consequently, *H. pylori* does not import Asn and Gln under their amidated forms, but uses a coupled deamidation/ transport system that provides Glu and Asp to the cytoplasm, thereby fueling the periplasm with ammonia. Most interestingly, each of the four components of this coupled deamidation/transport systems, namely, GGT/GltS and AnsB/DcuA, is essential for colonization of the animal model (Leduc et al. (2010) and references therein). Their in vivo essentiality can be at least partly attributed to the requirement of amino acids in the gastric environments. However, for GGT and AsnB, important roles linking virulence and amino acid metabolism were demonstrated and will be discussed in paragraph 2.5.

2.4.3.2 Asparagine and Glutamine in Translation

The entire pool of proteinogenic amino acids has to be available in the cells to fuel protein translation. In *H. pylori*, Gln is imported under the form of Glu upon deamidation, and glutamine synthetase (GlnA) enables intracellular Gln biosynthesis. A major issue arises when considering Asn since no predicted Asn synthetase gene was identified in the genome (Marais et al. 1999; Fischer et al. 2012). Moreover, *H. pylori* is also particular in that it is completely deprived of the enzymes ubiquitously implicated in Asn and Gln binding to their respective transfer RNA (tRNA) (Fischer et al. 2012; Chuawong and Hendrickson 2006; Huot et al. 2011; Skouloubris et al. 2003), namely, the asparaginyl-tRNA synthetase (AsnRS) and the glutaminyl-tRNA synthetase (GlnRS). Several studies showed that the metabolism of amidated amino acids (Asn and Gln) is particular in *H. pylori* and involves a specific tRNA-dependent biosynthetic pathway. The system involves an essential enzyme, GatCAB that is one of the *H. pylori* ammonia-assimilating enzymes.

2.4.3.3 How Are Asn-tRNA and Gln-tRNA Generated in *H. pylori?*

In order to be incorporated into proteins, amino acids first have to be aminoacylated onto their cognate tRNA in the form of an aminoacyl-tRNA (aa-tRNA) by aminoacyl-tRNA synthetases (aaRSs). However, both AsnRS and GlnRS are absent in *H. pylori*, making Asn-tRNA^{Asn} and Gln-tRNA^{Gln} synthesis impossible. Like in a large majority of prokaryotes and in organelles, *H. pylori* uses ancestral tRNA-dependent amino acid biosynthesis routes to fuel translation with Asn-tRNA^{Asn} and Gln-tRNA^{Gln} (Sheppard et al. 2008).

These pathways involve the formation of ribonucleoprotein complexes that require the presence of an aaRS, a tRNA, and a tRNA-dependent ammonia-dependent amidotransferase (AdT) composed of three subunits, called GatCAB. The first complex, named the Gln-transamidosome, leads to the formation of Gln-tRNA^{Gln} and involves in *H. pylori* an unconventional glutamyl-tRNA synthetase (GluRS2, HP0643) (Skouloubris et al. 2003), the orphan tRNA^{Gln}, and the GatCAB AdT. GluRS2 is conserved among epsilonproteobacteria. Similarly, the Asn-transamidosome, containing the aspartyl-tRNA synthetase (AspRS, HP0617), the orphan tRNA^{Asn}, and GatCAB, provides Asn-tRNA^{Asn} in a second biosynthetic pathway (Fischer et al. 2012; Silva et al. 2013).

In the Gln-transamidosome (GluRS2/tRNA^{Gln}/GatCAB), the GluRS2 enzyme loads Glu onto the tRNA^{Gln} moiety, thereby producing a misacylated ("wrong") Glu-tRNA^{Gln} species. In *H. pylori*, GluRS2 is specifically devoted to the misacylation of tRNA^{Gln} with Glu for the tRNA-dependent Gln biosynthesis (Skouloubris et al. 2003). This intermediate is then immediately processed by the GatCAB AdT, which amidates the Glu carboxylate into an amide, in an ATP-dependent reaction (Sheppard et al. 2007; Huot et al. 2011). The Asn-transamidosome functions in a similar way, but first involves a nondiscriminating AspRS (ND-AspRS, HP0617) that charges tRNA^{Asn} with Asp, providing the Asp-tRNA^{Asn} intermediate necessary for the next step. The ND-AspRS is also required for normal Asp-tRNA^{Asp} synthesis (Chuawong and Hendrickson 2006). Then, the GatCAB AdT amidates the Asp moiety onto the tRNA^{Asn} molecule and provides Asn-tRNA^{Asn} for protein translation.

GatCAB is an essential enzyme composed of three subunits, with GatA being the amidase, capable of hydrolyzing a free Gln donor, providing ammonium that is then channeled along a molecular tunnel toward the GatB-active site, where the misacylated aa-tRNA lies and where amidation occurs (Nakamura et al. 2006). This enzyme constituted a "classical" example illustrating the existence of ammonia channeling. Gln is by far the best substrate for GatA (Sheppard et al. 2007), but in *H. pylori* GatCAB is also capable of using NH_4^+ *directly*, presumably because massive ammonia concentration may fill up the molecular channel between GatA and GatB (Sheppard et al. 2007). Therefore, it can be considered that in *H. pylori*, GatCAB constitutes an additional system for ammonia assimilation into amino acids.

2.4.3.4 The AmiE and AmiF Aliphatic Amidases

Aliphatic amidases are enzymes catalyzing the hydrolysis of short-chain amides to produce ammonia and the corresponding organic acid. *H. pylori* possesses two such enzymes AmiE (HP0294) (Skouloubris et al. 1997) and AmiF (HP1238) (Skouloubris et al. 2001; Bury-Moné et al. 2003). The expression of both corresponding genes is strongly upregulated in response to acidity and to iron or nickel (Bury-Moné et al. 2004; van Vliet et al. 2004a). AmiE is a "classical" aliphatic amidase; in vitro it is active on propionamide, acrylamide, or acetamide (Skouloubris et al. 1997). We found that the AmiF protein is a novel type of formamidase hydrolyzing formamide to form formic acid and ammonia. AmiF is an AmiE paralogue with a restricted substrate specificity that possibly has evolved to achieve enzymatic specialization after ancestral gene duplication (Skouloubris et al. 2001). The three-dimensional structure of the AmiF enzyme has been solved and indeed defines a new group of formamidases (Hung et al. 2007). These *H. pylori* enzymes are unable to catalyze urea breakdown. The in vivo function and substrates of both enzymes are still unknown, but their catalytic activities liberate ammonia, which clusters them into the ammonia pool-forming proteins of *H. pylori*. Neither of the amidases are important for colonization in the mouse model (Bury-Moné et al. 2003). Interestingly, the amidases are found only in *Helicobacter* species able to colonize the stomach, suggesting that their acquisition might be related to selective pressure in this particular gastric environment (Bury-Moné et al. 2003).

2.5 Metabolic Enzymes Involved in Virulence

A close relation between metabolism and virulence has been established in many pathogens. In *H. pylori*, this is perfectly illustrated by two periplasmic deamidases required for in vivo colonization, gamma-glutamyl transpeptidase (GGT), asparaginase, and also by arginase. The role of GGT will not be discussed here since it is extensively presented in Chap. 7.

2.5.1 Asparaginase

Compared to GGT, there is much less information available on the role of the second periplasmic deamidase of *H. pylori*, the asparaginase AnsB that catalyzes the conversion of Asn to Asp and ammonia (Leduc et al. 2010; Shibayama et al. 2011). Purified AnsB protein was shown to be cytotoxic when applied to cultured AGS and MKN28 gastric epithelial cells (Cappelletti et al. 2008). As for GGT, AnsB was identified as a factor responsible for cell-cycle inhibition of

fibroblasts and gastric cell lines (Scotti et al. 2010). However, in a more recent study, it was shown that the depletion of Asn by asparaginase contributes poorly to induction of the inflammatory response during *H. pylori* infection (Rimbara et al. 2013). Additional studies are needed to understand the precise role of the *H. pylori* asparaginase that might be less determinant for the outcome of the pathologies than GGT. Indeed, no correlation between the severity of the *H. pylori*-associated disease and the level of asparaginase activity was observed (Rimbara et al. 2013).

2.5.2 Arginase

Arginases are enzymes that convert arginine to urea and ornithine. *H. pylori* was found to present a strong membrane-bound arginase activity, and it was proposed that its endogenously produced urea might be used by urease. The corresponding RocF arginase protein is constitutively expressed and was extensively characterized biochemically (Mendz et al. 1998; McGee et al. 2004). It uses cobalt as cofactor, as opposed to mammalian arginases which use manganese and has an acidic pH optimum. Interestingly, arginase activity increases the resistance of *H. pylori* to acid in an arginine-dependent fashion (McGee et al. 1999). However, *H. pylori* arginase is not required for colonization of wild-type mice or of arginase II knockout mice indicating that the major in vivo source for urea is neither bacterial arginase nor host arginase II (Kim et al. 2011). Most interestingly, in *H. pylori* as in some other pathogens, arginase acts as an immune modulator (Das et al. 2010). Indeed, at physiologically relevant arginine concentrations, an *H. pylori* arginase mutant elicits higher amounts of nitric oxide (NO) production in RAW macrophages and accordingly is more affected in its survival. In contrast, peritoneal macrophages from iNOS$^{-/-}$ mice failed to affect the survival of the *rocF* mutant. It was concluded that arginase allows *H. pylori* to evade the immune response by quenching arginine from the inducible nitric oxide synthase (iNOS) thereby downregulating eukaryotic NO production and protecting itself from killing (Gobert et al. 2001). In addition, the *H. pylori* arginase impairs host T-cell function by reducing CD3ζ chain expression (Zabaleta et al. 2004). Thus, arginase might also play a very important role during *H. pylori* infection.

2.6 Metabolism of Nickel, an Essential Metal for the Virulence of *Helicobacter pylori*

Metal acquisition is a critical process for all organisms, because metal ions are often found at very low concentrations in their environments. In spite of their low quantities within the cells, they are essential since many enzymes depend on

metal cofactors for their catalytic activity. To face this scarcity, bacteria have evolved very efficient uptake systems to acquire metals from the environment, as well as systems allowing their intracellular storage, distribution, and maintenance of homeostasis. Indeed, at nonphysiological high intracellular concentrations, metals are toxic. The uptake, storage, distribution, incorporation, and efflux mechanisms are together referred to as "trafficking systems."

2.6.1 *Nickel Is a Virulence Determinant for* H. pylori

Nickel (Ni^{2+}) is a central metal for the acid adaptation and survival of *H. pylori* within the stomach (for reviews, see Stingl and De Reuse (2005), De Reuse et al. (2013), Rowinska-Zyrek et al. (2014)). Indeed, for the urease enzyme to be active, nickel has to be present in sufficient amounts to enable its maturation. Accordingly, the total intracellular nickel concentration is about 50 times that of *E. coli* (Schauer 2007). Since urease is strongly overexpressed and present in very high amounts when the bacterium faces acidic conditions, *H. pylori* must import large amounts of nickel to ensure its optimal activity in a challenging environment. If urease is absent or not maturated, *H. pylori* is unable to colonize the stomach of animal models. In *H. pylori*, [NiFe] hydrogenase is a second enzyme that requires Ni^{2+} as a cofactor (Maier and Bock 1996). Its activity enables *H. pylori* to use H_2 as an energy source, and this enzyme is required for optimal colonization of the mouse model (Olson and Maier 2002). Thus, since Ni^{2+} is critical for both urease and hydrogenase catalytic activities and for proper colonization and survival in the stomach, this metal can be considered as a virulence determinant.

2.6.2 *Nickel Transport and Efflux*

In Gram-negative bacteria, energized transport of metabolites such as iron siderophore complexes through the outer membrane (OM) relies on the TonB machinery and on TonB-dependent transporters (TBDTs). Since nickel is found at very low concentrations within the human stomach, it was expected that *H. pylori* uses efficient uptake systems to obtain appropriate amounts of this metal from its environment. Indeed, we discovered in *H. pylori* the first nickel transport system across a bacterial OM (Schauer et al. 2007, 2008). This uptake machinery requires the FrpB4 TBDT and is energized by the TonB machinery comprising ExbB-ExbD-TonB (Schauer et al. 2007, 2008). The role of ExbD in the control of the nickel activation of urease and thus in pH homeostasis was subsequently confirmed (Marcus et al. 2013). Expression of the *frpB4* gene is repressed by NikR in the presence of nickel, which suggests that this transport system is fully active under nickel-limiting conditions. In addition, FrpB4 is acid-activated allowing *H. pylori* to optimize urease maturation by nickel incorporation under conditions where its

activity needs to be maximal for acid adaptation. Moreover, nickel is more soluble at acidic pH, which makes an acid-induced uptake system appropriate in the gastric environment. Additional mechanisms certainly exist that could allow, for example, nickel entry at neutral pH. FecA3, another *H. pylori* TBDT whose synthesis is under the control of nickel, might be an alternative nickel uptake system (Ernst et al. 2006; Romagnoli et al. 2011).

In *H. pylori*, once nickel has crossed the OM, it can be taken up through the cytoplasmic membrane (CM) by NixA, a high-affinity and low-capacity nickel transporter (Fulkerson and Mobley 2000) of the NiCoT family, whose expression is also repressed by nickel (Wolfram et al. 2006; Muller et al. 2011). Because NixA mutant retains half of urease activity and its capacity to colonize the mouse model is not totally abolished (Nolan et al. 2002), alternative ways of nickel entry across the CM must exist.

Once it has crossed the CM, nickel has to be directed to its proper targets while avoiding potential damages caused by free metal ions. If nickel is in excess with respect to *H. pylori* cellular needs, it has either to be stored or to be exported out of the cell. One nickel export system was described in *H. pylori*. It is a proton-driven RND-type metal efflux-pump encoded by the *cznABC* genes (Stahler et al. 2006). Inactivation of this pump increases *H. pylori* sensitivity to nickel, cadmium, and zinc and impairs colonization of the gerbil stomach (Stahler et al. 2006), underlining the importance of metal homeostasis for *H. pylori* virulence.

2.6.3 Nickel Chaperones and Storage Proteins

Metal overload is toxic to the cells. In *H. pylori*, several storage proteins, also called metallochaperones, have evolved to ensure that nickel remains correctly balanced and gets optimally incorporated into urease and hydrogenase.

2.6.3.1 Role of HspA, the Helicobacter-Specific GroES Homolog

In *H. pylori*, the sole member of the highly conserved and essential GroES co-chaperonin family is HspA (Suerbaum et al. 1994). This protein is original in that it possesses a unique His- and Cys-rich C-terminal extension specifically found within the *Helicobacter* genus. This unusual extension was shown to bind nickel ions (Kansau et al. 1996), and the *hspA* gene is upregulated by NikR in response to nickel (Muller et al. 2011).

HspA is an abundant protein in vivo, and one of its roles in *H. pylori* is to bind free nickel ions to store them in the cytoplasm and prevent the cell from metal overload. Indeed, using an *H. pylori* mutant expressing a C-terminal truncated form of HspA, we observed that this extension is involved in nickel sequestration (Schauer et al. 2010). This mutant strain expressed wild-type urease activity and was strongly affected in its hydrogenase activity. We concluded that HspA

constitutes a nickel storage protein pool specifically devoted to hydrogenase maturation. How nickel is mobilized from HspA and whether HspA provides nickel to other proteins remain to be determined (Schauer et al. 2010).

2.6.3.2 Hpn and Hpn-2: Two Remarkable Histidine-Rich Proteins

H. pylori also possesses two proteins of unusual amino-acid composition that have no orthologs outside the *Helicobacter* species. Hpn and Hpn-2 (also called Hpn-like) are two small proteins (7 and 8 kDa) that are extremely rich in His-residues: 47 and 25 % of the total residues, respectively. Hpn-2 contains additional poly-Glutamine stretches representing 40 % of the total residues. These two proteins form multimers and bind nickel *in vitro*, as expected for His-rich peptides (Gilbert et al. 1995; Ge et al. 2011; Zeng et al. 2008 and 2011; Rowinska-Zyrek et al. 2011).

Like *hspA*, transcription of both genes is upregulated by NikR in response to nickel (Muller et al. 2011). Because *hpn* and *hpn-2* mutants were reported to be more sensitive to high exogenous nickel concentrations than a wild type strain, these proteins were suggested to be involved in nickel storage and detoxification *via* sequestration of excess nickel (Mobley et al. 1999; Seshadri et al. 2007). We recently demonstrated (Vinella et al. 2015) that in *H. pylori* only Hpn is involved in nickel sequestration and that Hpn and Hpn-2 interact with each other. In addition, their combined activities were found to participate in a nickel transfer pathway to urease. Using bioinformatics and top-down proteomics to identify the predicted proteins, we established that Hpn-2 is only expressed by *H. pylori* and its closely related species *Helicobacter acinonychis*. Hpn was detected in every gastric *Helicobacter* species tested and is absent from the enterohepatic *Helicobacter* species. Phylogenomic analysis revealed that Hpn acquisition was concomitant with the specialization of *Helicobacter* to colonization of the gastric environment. Finally, both proteins are essential for colonization of a mouse model by *H. pylori*. Taken together, these results strongly suggest that during evolution, the acquisition of Hpn by gastric *Helicobacter* species was decisive for their capacity to colonize the stomach.

2.6.4 Urease and Hydrogenase Maturation

Urease and hydrogenase are two nickel enzymes necessary for the virulence of *H. pylori*. Complex mechanisms are required for their maturation by nickel incorporation. *H. pylori* is till now the only organism in which a molecular cross talk between the maturation machineries of these two enzymes has been observed. This again underlines the utmost importance of the control of nickel distribution and trafficking in *H. pylori*.

2.6.4.1 Urease Maturation

Urease maturation requires four accessory proteins, UreE, UreF, UreG, and UreH, whose gene cluster lies downstream the *ureAB* operon. The whole process takes place within a multi-protein maturation complex (for a review, see Farrugia et al. (2013)). Interaction between the urease structural and accessory proteins was observed by yeast two-hybrid analysis (Rain et al. 2001; Voland et al. 2003). Using tandem affinity purification, we isolated from *H. pylori* cells a complex composed of the UreA/B structural units, together with the complete UreE/F/G/H activation complex (Stingl et al. 2008). The $((UreA/B)_3)_4$ apo-urease complex is thought to recruit a UreF/G/H complex and the UreE·Ni^{2+} chaperone to catalyze the insertion of two nickel ions. UreE is a metallochaperone that binds one nickel ion with a high affinity (K_D 150 nM) and interacts with UreG (Bellucci et al. 2009). UreH is a scaffold protein necessary for the recruitment of UreF; this heterodimer then binds UreG. This latter protein is an intrinsically unstructured GTPase of weak catalytic activity that dimerizes upon binding of a metal ion (Zambelli et al. 2009). This protein is also capable of binding nickel ($K_D \sim 5$ μM), although less efficiently than UreE. It has been suggested that the binding of the UreF/H complex onto urease induces conformational changes, allowing nickel ion and carbon dioxide to access its active site. UreF was shown to gate the GTPase activity of UreG, which would enhance its GTPase activity and as a result the fidelity of urease metallocenter assembly (Boer and Hausinger 2012). Recently, it was reported that UreF binds two nickel ions per dimer, with a micromolar K_D and site-directed mutagenesis suggested an additional role for a Ni^{2+}-UreF complex in urease maturation (Zambelli et al. 2014).

A recent crystal structure of the *H. pylori* UreF/G/H complex reveals how UreF and UreH facilitate UreG dimerization and how this leads to the correct assembly of its metal-binding site (Fong et al. 2013). The addition of nickel and GTP to the UreF/G/H complex causes the release of the UreG dimer that binds nickel at the dimeric interface. In vitro, the nickel-loaded UreG dimer was shown to activate urease in the presence of UreF/H and in the absence of the UreE metallochaperone. Thus, in the fully assembled UreE/F/G/H, nickel could be channeled from UreE to UreG, before its insertion in the urease-active site. How nickel is transferred from UreE to the binding site of UreG and how the complete activation complex interacts with urease have still to be determined.

2.6.4.2 Hydrogenase Maturation

[NiFe] hydrogenases catalyze the reversible heterolytic cleavage of dihydrogen. The active site of these enzymes is buried within the large subunit (HydB) and contains an $Fe(CO)(CN)_2$ unit and a nickel ion. The small subunit (HydA) carries the Fe-S clusters necessary for dihydrogen activation and electron transfer

(Fontecilla-Camps et al. 2007). *H. pylori* possesses a respiratory-type [NiFe] hydrogenase that catalyzes the oxidation of dihydrogen (Maier et al. 1996).

Hydrogenase maturation requires specialized metallochaperones for nickel delivery and accessory proteins that perform the posttranslational modifications required for the enzyme to be active. HydB contains a complex $NiFe(CN)_2CO$ center, and its maturation requires six accessory proteins for nickel delivery and insertion (HypA and HypB), for Fe delivery (HypC, HypD), and for cyanide (CN) ligand biosynthesis and insertion (HypE, HypF). The whole process has mostly been characterized in *E. coli*. HypA and HypB act together to insert the nickel ion necessary to complete the metallic center within the active site. Upon nickel delivery, a C-terminal peptide gets cleaved by an endopeptidase to obtain the matured large subunit containing the $NiFe(CN)_2CO$ metallic complex. The αβ heterodimer is then translocated to the periplasmic space through the Tat secretion pathway (for a review, see Watanabe et al. (2012)).

2.6.4.3 A Molecular Cross Talk Between Urease and Hydrogenase Maturation Machineries

An interesting particularity of nickel trafficking in *H. pylori* is the interconnectivity between the urease and hydrogenase maturation pathways. This was discovered using *H. pylori* mutants; the data showed that HypA and HypB are both required not only for hydrogenase maturation but also for full urease activation (Olson et al. 2001). In agreement with this, our *H. pylori* interactome analysis evidenced that HypB is actually physically associated to the urease maturation complex (Stingl et al. 2008). Using optical biosensing methods, the *H. pylori* HypA and UreG proteins were shown to compete with each other for UreE recognition (Benoit et al. 2012). Importantly, in vitro translocation of nickel between the two metallochaperones HypA and UreE was demonstrated (Yang et al. 2014). In addition, experiments performed with purified recombinant HypA indicated that this protein is sufficient for the recovery of full urease activity in cell lysates from a *hypA* deletion mutant (Herbst et al. 2010). These results suggest that the function of the hydrogenase accessory proteins HypA in urease activation relies on nickel delivery or exchange rather than on catalytic activity regulation.

2.7 Conclusions and Outlook

In conclusion, this chapter presents how unusual metabolic properties of *H. pylori* contribute to its spectacular capacity to persistently colonize a hostile niche. Its ability to multiply at low pH relies on massive ammonia production whose potential toxicity is prevented by coupling to protein complexes. In *H. pylori* as in other pathogens, some metabolic enzymes are in addition directly involved in virulence acting in particular as immune modulators. Finally, the dependence on the nickel

enzyme urease for acid resistance renders nickel a virulence determinant whose transport and trafficking is tightly controlled.

We propose that the constrains of the small *H. pylori* genome and a very specialized niche have resulted in a close association and in overlapping networks between mechanisms of persistence/acid adaptation and metabolic pathways.

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Chapter 3
Virulence Mechanisms of *Helicobacter pylori*: An Overview

Judyta Praszkier, Philip Sutton, and Richard L. Ferrero

Abstract *Helicobacter pylori* is a highly successful human pathogen, able to establish a chronic infection in the harsh environment of the stomach. These bacteria express a variety of virulence factors that promote their survival under acidic conditions, motility and spatial orientation in gastric mucus and adherence to epithelial cells. Other pathogenicity-associated mechanisms contribute to chronic gastritis by inducing pro-inflammatory responses and by manipulating cellular responses in host cells. Although *H. pylori* elicits a strong inflammatory response, the immune system fails to clear the infection. The pathogen employs a range of evasion strategies to dampen or reduce host immune responses. These strategies enable *H. pylori* to establish an equilibrium with its host, so that the vast majority of the chronically infected individuals do not develop severe disease. However, in a subset of patients, disturbance of this equilibrium in favour of the pathogen may lead to the development of gastroduodenal ulceration, mucosa-associated lymphoid tissue (MALT) lymphoma or adenocarcinoma.

Keywords Virulence factors • Pathogenesis • Urease • Motility • Adhesion • Immunomodulation • Apoptosis • Autophagy

3.1 Introduction

Helicobacter pylori is one of the most successful human pathogens, colonising more than 50 % of the world's population (Suerbaum and Josenhans 2007). The infection is usually acquired in early childhood (Weyermann et al. 2009) and, in the absence of aggressive antibiotic therapy, typically persists for life (Suerbaum and

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Josenhans 2007). Most of the colonised individuals develop asymptomatic chronic gastritis, but in 10–20 % of cases, *H. pylori* infection is associated with the development of severe gastroduodenal disease, including peptic ulcers, gastric MALT lymphoma or adenocarcinoma (Kusters et al. 2006). *H. pylori* infection is the strongest known risk factor for gastric adenocarcinoma, and in 1994 the International Agency for Research on Cancer classified *H. pylori* as a class I carcinogen. The clinical outcome of *H. pylori* infection depends on a complex interplay of many factors, including the virulence determinants expressed by the colonising strain(s), the genetic background of the host and environmental factors.

H. pylori strains show extensive genetic diversity, which is a consequence of the high mutation (Bjorkholm et al. 2001) and recombination frequencies (Suerbaum et al. 1998) in this bacterium. *H. pylori* has undergone a long co-evolution with its human host. Indeed, it is estimated that the association of *H. pylori* with modern humans predates by some 40,000 years the human migration from East Africa that occurred approximately 60,000 years ago (Moodley et al. 2012) (see Chap. 9 for more details). During this long association with humans, *H. pylori* has evolved sophisticated mechanisms to persistently colonise its host and avoid elimination by the immune system. These mechanisms range from colonisation factors that allow the bacterium to survive the harsh acidic environment of the stomach and establish persistent infection in the gastric mucosa, to complex strategies involving virulence factors that enable *H. pylori* to evade and manipulate both innate and adaptive immune responses. The lack of disease progression in the vast majority of persistently colonised individuals points to a delicate balance between the host and the pathogen. This chapter summarises the bacterial factors and biological processes that enable *H. pylori* to establish persistent colonisation and chronic inflammation of the human gastric mucosa. A more in-depth analysis of several of these products and processes will be provided in the chapters to follow.

3.2 *H. pylori* Colonisation and Adherence

3.2.1 Escape from the Stomach Lumen

In order to reach its ideal ecological niche, *H. pylori* must survive the extremely acidic environment of the stomach lumen and penetrate the outer mucous gel layer of the stomach. Once in the mucus, *H. pylori* resides in a very specific niche with an external pH of approximately 5–6. The bacterium is, however, able to increase the pH of its immediate surrounds, as well as of its cytosol and periplasm, by producing urease, which hydrolyses urea to ammonium ions and carbon dioxide (Marshall et al. 1990). Urease is composed of UreA and UreB subunits (Labigne et al. 1991) which are assembled into a catalytically active, nickel-containing dodecamer via the actions of accessory proteins UreE, UreF, UreG and UreH (Mobley et al. 1995). Urease activity is up-regulated under acidic conditions, by a proton-gated urea

channel formed by the inner membrane protein, UreI, allowing rapid entry of urea into the bacteria (Skouloubris et al. 1998; Weeks et al. 2000). *H. pylori* can further tightly control urease activity in response to both an acidic pH and increasing concentrations of nickel ions. This occurs via up-regulation of urease gene expression by the acid-responsive signalling regulon (ArsRS) (Pflock et al. 2006) and the nickel response regulator, NikR (van Vliet et al. 2002), respectively. *H. pylori* mutants lacking either urease activity (through disruption of *ureB*) or a functional UreI were shown to be defective for colonisation in animal models of infection (Eaton et al. 2002; Skouloubris et al. 1998), thus demonstrating the essential role of urease in *H. pylori* pathogenesis (see also Chap. 2).

Having overcome the acidic lumen, *H. pylori* must next confront the viscous mucous gel covering the gastric epithelium. Gastric mucus varies in its viscoelastic properties, from a soft gel at acidic pH to a viscous solution at neutral pH. *H. pylori* is well adapted to this environment and is able to move rapidly, in a corkscrew like manner, through highly viscous solutions that impede the motility of rod-shaped organisms, such as *Escherichia coli* (Hazell et al. 1986). The spiral cell shape of *H. pylori* is believed to enhance its ability to penetrate the mucus, and mutants lacking a helical twist show a colonisation defect. Production and maintenance of the spiral morphology require coordinated activity of multiple enzyme networks that modify the peptidoglycan composition of the cell wall (Bonis et al. 2010; Sycuro et al. 2012).

In addition to its spiral shape, *H. pylori* uses flagella-mediated motility to move through both the gastric lumen and mucus to reach and maintain itself close to the epithelial surface. The importance of motility for *H. pylori* is illustrated by the fact that mutants lacking flagella were unable to colonise the gnotobiotic piglet model of infection (Eaton et al. 1996). *H. pylori* bacteria have two to five sheathed, unipolar flagella which are composed of the basal body, the hook/universal joint and the filament. The flagellar filament is composed of repeating units of two flagellins, FlaA (53 kilodalton, kDa) and FlaB (54 kDa) (Suerbaum et al. 1993). The *flaA* and *flaB* genes are not co-located on the chromosome, nor is their transcription co-regulated (Suerbaum et al. 1993). The flagellar system comprises a network of over 40 mostly unclustered and temporally regulated genes, whose transcription is hierarchical and tightly controlled by the three RNA polymerase sigma factors of *H. pylori*: σ^{28} (FliA), σ^{54} (RpoN) and σ^{80} (Douillard et al. 2009; Josenhans et al. 2002; McGowan et al. 2003; Niehus et al. 2004). *H. pylori* also has an anti-sigma factor, FlgM, which acts as an antagonist to FlaA (Colland et al. 2001; Niehus et al. 2004). Flagellar assembly requires interaction with the peptidoglycan layer through which the flagella has to be extruded. The peptidoglycan-degrading enzymes of the lytic transglycosylase family, Slt and MltD, are required for full motility in *H. pylori* (Roure et al. 2012). Inactivation of *mltD*, but not *slt*, was shown to have a significant impact on *H. pylori* colonisation in vivo (Roure et al. 2012).

Motile bacteria use chemotaxis for spatial orientation, coupling control of flagellar rotation with environmental sensing (Wadhams and Armitage 2004). *H. pylori* uses four methyl-accepting chemoreceptor proteins (TlpA, TlpB, TlpC and TlpD) to sense the external stimuli and repellent ligands. This information is

then relayed via CheW, to a histidine kinase, CheA, which phosphorylates the response regulator, CheY. The phosphorylated CheY interacts with the flagellar motor to alter the rotational direction of the flagellum. *H. pylori* mutants lacking *cheA*, *cheW* or *cheY* are non-chemotactic and show colonisation defects (Terry et al. 2005). In addition, *H. pylori* encodes a novel chemotaxis regulator, ChePep, which preferentially localises to the flagellar pole. *H. pylori chePep* mutants cannot control the rotation of their flagella, but are motile. They are attenuated for colonisation of the stomach and fail to establish bacterial colonies deep in the gastric glands of mice. Interestingly, ChePep homologues are present and functionally conserved in ε-proteobacteria, but not in other bacterial classes (Howitt et al. 2011).

Microarray analyses of *H. pylori* showed that both exposure to low pH in vitro and infection of the gerbil stomach in vivo resulted in increased expression of many of the genes involved in motility and chemotaxis (Merrell et al. 2003; Scott et al. 2007). These findings are consistent with data showing that exposure of *H. pylori* to an acidic environment leads to a large increase in both the proportion and the speed of motile bacteria (Merrell et al. 2003). *H. pylori* exploits the pH gradient of the stomach, which ranges from pH 1.8 in the lumen to a near-neutral pH at the mucus-mucosal interface, to guide it to the epithelial surface. This pH-tactic behaviour is dependent on the chemotaxis receptor, TlpB (Croxen et al. 2006). *H. pylori tlpB* mutants were shown to be motile but could not colonise interleukin-12 p40 (IL-12 p40)-deficient C57BL/6 mice (Croxen et al. 2006). Expression of *tlpB* is regulated at the posttranscriptional level by an abundant small RNA (sRNA), regulator of polymeric G-repeats (RepG), which targets a homopolymeric G-repeat in the leader region of the *tlpB* mRNA. The length of this G-repeat, which varies from 6 to 16 guanine residues in different *H. pylori* strains, influences both the level and type (repression or activation) of regulation. There is also evidence that the length of the *tlpB* G-repeat can change during infection, suggesting that differential expression of *tlpB* may be involved in host adaptation (Pernitzsch et al. 2014).

3.2.2 Adhesion of H. pylori *to Gastric Epithelial Cells*

Although most of the *H. pylori* in the mucosa are free-swimming, some 20 % of the bacteria adhere to the surface of the epithelial cells (Hessey et al. 1990). Binding of *H. pylori* to gastric epithelial cells involves the interactions between specific bacterial adhesins and their cognate receptors on the surfaces of host cells. The known *H. pylori* adhesins all belong to the major outer membrane protein (OMP) family 1 (Alm et al. 2000). This family of proteins is further divided into the *Helicobacter* outer membrane porins (Hop) and Hop-related (Hor) subgroups (Alm et al. 2000; Odenbreit et al. 2009). All but one of the adhesins identified to date are members of the Hop family. The best characterised of these adhesins are the blood group antigen-binding (BabA) and sialic acid-binding (SabA) proteins,

the outer inflammatory protein A (OipA) and the adherence-associated lipoproteins, AlpA and AlpB.

BabA binds to the human fucosylated Lewis^b antigen (Le^b) and related terminal fucose residues on blood group O (H antigen), A and B antigens present on the surface of gastric epithelial cells (Aspholm-Hurtig et al. 2004; Gerhard et al. 1999; Ilver et al. 1998). BabA binding affinity for O, A and B antigens correlates with the blood group expressed by the human host, supporting the notion of adaptation to the host during persistent infection and transmission between hosts (Aspholm-Hurtig et al. 2004). In contrast, SabA binds to sialyl-Lewis x (sLe^x) and Lewis a (sLe^a) antigens whose synthesis is up-regulated during *H. pylori*-induced inflammation (Mahdavi et al. 2002). SabA also binds to erythrocytes (Aspholm et al. 2006) and may play a role in inflammation, by binding and activating neutrophils (Unemo et al. 2005). More details on the effects of BabA and SabA on gastroduodenal disease can be found in Chap. 6.

OipA (formerly called HopH) is involved in the adherence of *H. pylori* to gastric cell lines (Dossumbekova et al. 2006; Yamaoka et al. 2004), but its host receptor has yet to be identified. Expression of functional OipA, which is regulated by slipped-strand mispairing within a CT-rich region present at the 5′ terminus of *oipA*, promoted IL-8 induction in vitro. Conversely, *oipA* inactivation in *cag*PAI$^+$ clinical isolates resulted in approximately 50 % lower IL-8 responses in epithelial cells (Yamaoka et al. 2004). The notion that OipA has a role in IL-8 production in vivo is supported by the observation that OipA expression was significantly associated with high levels of IL-8 in the gastric mucosa of infected patients (Yamaoka et al. 2002). The molecular basis for the effect of OipA on IL-8 production was investigated in a study of the IL-8 promoter in gastric cell cultures (Yamaoka et al. 2004). This study showed that maximal induction of IL-8 transcription required activation of an interferon-stimulated responsive element (ISRE)-like element by the interferon regulatory factor (IRF)-1 (Yamaoka et al. 2004). Moreover, OipA was reported to selectively induce phosphorylation of signal transducer and activator of transcription 1 (STAT1), an upstream mediator of IRF-1 signalling. These results were recapitulated in vivo, where it was found that STAT1 phosphorylation in human gastric biopsy specimens correlated with the presence of functional OipA in the infecting *H. pylori* strain (Yamaoka et al. 2004). It has also been proposed that OipA plays a role in IL-1β, IL-17 and TNF expression and inflammation in the stomach (Sugimoto et al. 2009). Analysis of clinical isolates showed that the presence of functional OipA is associated with high *H. pylori* density, severe neutrophil infiltration, duodenal ulcer disease and gastric cancer (Yamaoka et al. 2002, 2006; Franco et al. 2008). The role of OipA in gastric disease is supported by data showing that inactivation of *oipA* reduces the incidence of cancer in Mongolian gerbils and decreases nuclear translocation of β-catenin (Franco et al. 2008), a cellular protein important for cell adhesion and regulation of genes implicated in carcinogenesis. In vitro studies with murine dendritic cells (DCs) showed that OipA suppresses DC maturation and decreases production of IL-10 (Teymournejad et al. 2014); however, the biological significance of this finding requires further investigation.

AlpA and AlpB, which are encoded by the *alpA/B* operon, are also required for specific adhesion of *H. pylori* to human gastric epithelial cells (Odenbreit et al. 1999). Furthermore, these proteins were shown to be important for colonisation in guinea pig (de Jonge et al. 2004) and murine (Lu et al. 2007) models of infection. Analysis of 200 clinical strains showed that AlpA and AlpB were expressed in all strains, suggesting that these adhesins are likely to have important functions (Odenbreit et al. 2009). The target of both AlpA and AlpB is laminin, a component of the host extracellular matrix (Senkovich et al. 2011).

3.3 Major *H. pylori* Virulence Factors Involved in Pathogenesis

3.3.1 cag *Pathogenicity Island* (cag*PAI*)

The *cag*PAI is a horizontally acquired insertion element of 40-kilobases (kb), consisting of approximately 31 genes, whose presence in a functional form is associated with an increased risk of severe gastroduodenal disease (Covacci et al. 1999; see also Chap. 4). *cag*PAI encodes a bacterial type IV secretion system (T4SS) and its only known effector protein, CagA, which translocates into gastric epithelial cells (Censini et al. 1996; Fischer et al. 2001; Odenbreit et al. 2000). The presence of a *cag*PAI appears to influence the topography of colonisation within the stomach, as *cag*PAI$^-$ *H. pylori* strains were mostly present in the mucous gel layer or near the apical surface of epithelial cells, whereas the *cag*PAI$^+$ strains were found closely adjacent to gastric epithelial cells or in the intercellular epithelial spaces (Camorlinga-Ponce et al. 2004).

The *H. pylori* T4SS is induced by contact with the host cell and forms a large complex spanning the inner and outer membranes of the bacterium, with a pilus-like structure that protrudes from the bacterial surface (Rohde et al. 2003). It is currently unclear how the *H. pylori* T4SS is able to deliver not only CagA but also *H. pylori* cell wall peptidoglycan, into the host cell. The internalised peptidoglycan is recognised by the cytoplasmic pathogen-recognition molecule, nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) (Viala et al. 2004). NOD1 sensing of *H. pylori* peptidoglycan triggers in epithelial cells a pro-inflammatory signalling cascade, characterised by the translocation of nuclear factor-κB (NF-κB) to the nucleus (Viala et al. 2004) and activation of the mitogen-activated protein kinases (MAPKs), p38 and extracellular signal-regulated kinase (ERK), leading to induction of CXC chemokine responses (Allison et al. 2009). *H. pylori* can also activate this pro-inflammatory signalling cascade via the actions of outer membrane vesicles (OMVs) which deliver peptidoglycan to cytosolic NOD1 (Hutton et al. 2010; Kaparakis et al. 2010). Both T4SS- and OMV-dependent activations of the NOD1 signalling pathway involve cholesterol-rich microdomains, or lipid rafts, in host-cell membranes (Hutton et al. 2010; Kaparakis

et al. 2010). Interestingly, CagA translocation into host cells also requires the presence of functional lipid raft domains (Jimenez-Soto et al. 2009; Lai et al. 2008). *H. pylori* T4SS delivery of CagA (Jimenez-Soto et al. 2009; Kwok et al. 2007) and peptidoglycan (Hutton et al. 2010; Kaparakis et al. 2010) into cells was shown to be dependent upon binding of the *cag*PAI-encoded protein, CagL, to its cognate host-cell receptor, $\alpha_5\beta_1$ integrin. CagA itself was shown to interact with the host factors, β_1 integrin (Jimenez-Soto et al. 2009; Kwok et al. 2007) and phosphatidylserine (Murata-Kamiya et al. 2010).

Once CagA has translocated into epithelial cells, it localises to the plasma membrane and undergoes tyrosine phosphorylation within the EPIYA motif that is found in tandemly arranged segments located in the C-terminal half of the protein. The number and organisation of these segments differ between *H. pylori* strains and are thought to contribute to differences in strain pathogenicity (Argent et al. 2004; Higashi et al. 2002). There are four distinct EPIYA segments (A to D), each of which contains a single EPIYA motif, with the EPIYA A, B and C segments predominating in *H. pylori* isolates from Western countries and EPIYA A, B and D segments predominating in the generally more virulent East Asian isolates (Higashi et al. 2002). The cellular kinases responsible for phosphorylating the EPIYA motifs within CagA are oncoproteins belonging to the Src and Abl family kinases (Poppe et al. 2007; Selbach et al. 2002; Tammer et al. 2007).

CagA translocation and tyrosine phosphorylation lead to a perturbation of mammalian signal transduction cascades, morphological effects such as cell cytoskeletal rearrangement, elongation and scattering that has been designated the "hummingbird" phenotype, as well as modification of cellular functions (Selbach et al. 2003; Tammer et al. 2007; Tsutsumi et al. 2003). These in vitro observations are recapitulated in vivo, with the finding that CagA is actively translocated to gastric epithelial cells and tyrosine-phosphorylated and binds Src homology region 2 (SH2) domain-containing phosphatase-2 (SHP-2) in inflamed human gastric mucosa (Tsutsumi et al. 2003). The ability of CagA to perturb host-cell functions is dependent on its SHP-2 binding activity, which is determined by the number and sequences of tyrosine phosphorylation sites (Higashi et al. 2002). It should be noted that non-phosphorylated CagA also contributes to pathogenesis, through interactions that lead to induction of pro-inflammatory and mitogenic responses, suppression of apoptosis, loss of cell polarity and disruption of gastric barrier function (see Chap. 4 for a detailed discussion).

3.3.2 Vacuolating Cytotoxin VacA

VacA is a pore-forming toxin secreted by a classical autotransporter pathway (see Chap. 5 for more details). The protein is synthesised as a 140 kDa precursor, which is processed to produce a 33-amino acid signal peptide, the mature 88 kDa secreted toxin, an approximately 12 kDa secreted peptide and a C-terminal domain that remains associated with the bacteria (Cover and Blaser 1992; Schmitt and Haas

1994; Telford et al. 1994). The secreted VacA can undergo spontaneous proteolytic cleavage into the N-terminal p33 and C-terminal p55 fragments that are thought to represent the functional domains of VacA (Torres et al. 2005) and that remain non-covalently associated (Cover et al. 1997; Lupetti et al. 1996; Telford et al. 1994). The p33 domain is important for membrane channel formation (McClain et al. 2001; Ye et al. 1999), whereas the p55 domain is required for binding to host cells (Garner and Cover 1996). Both domains are required for toxin oligomerisation (Gangwer et al. 2007; Genisset et al. 2006). Active VacA induces structural and functional changes in epithelial cells in vitro, the most noticeable being formation of large intracellular vacuoles, the phenotype that gave the toxin its name (Leunk et al. 1988).

Most of the secreted VacA was shown to bind to cultured epithelial cells and to use lipid rafts as entry sites so as to be internalised by clathrin-independent endocytosis (Gauthier et al. 2005). A number of studies have indicated that VacA may also exert an antagonistic effect on CagA functions (see Chap. 5 for a detailed discussion). Once intracellular, VacA causes a wide range of alterations to the host cell. The large membrane-bound vacuoles induced by VacA in the cytoplasm of gastric cells originate from the late endosomal pathway and are a consequence of disruption of the late endosomal/lysosomal compartments (Papini et al. 1994). However, the role of these cytoplasmic vacuoles in *H. pylori* pathogenesis is unclear. As discussed in Sect. 6 below, VacA induces apoptosis of gastric epithelial cells, independently of its vacuolating activity and also promotes autophagy in these cells.

All *H. pylori* strains encode *vacA*, yet display considerable heterogeneity in their production of the vacuolating cell phenotype (Atherton et al. 1995). This diversity is largely due to polymorphisms in the *vacA* gene. The highest level of sequence diversity is found in the signal (s), intermediate (i) and middle (m) regions of *vacA* and forms the basis of a classification system. The signal sequences of the s1 and s2 alleles of VacA are processed at different sites, such that the mature s2 toxin contains a 12-amino acid hydrophilic extension at its N-terminus, which abolishes its cytotoxic activity and reduces its ability to form membrane channels, without abrogating toxin secretion (Letley et al. 2003; McClain et al. 2001). The i region, defined as either i1 or i2, is important for toxicity (Rhead et al. 2007; Winter et al. 2014); however, its role in VacA functions is not yet known. The m region of VacA contains the cell-binding site, with m1-type toxins having higher binding affinities for host cells than m2-type toxins and also showing different cell-type specificities (Pagliaccia et al. 1998; Wang et al. 2001).

H. pylori strains with the s1/m1 *vacA* alleles have higher levels of vacuolating activity in vitro than those carrying s1/m2 alleles (Atherton et al. 1995). Epidemiological studies are consistent with these in vitro observations, as *H. pylori* strains that encode s1 and m1 *vacA* alleles are associated with a higher risk of gastric carcinoma than strains with s2 and m2 alleles. Furthermore, s1/m1 *vacA* genotypes are strongly associated with peptic ulcers (Atherton et al. 1995, 1997; Strobel et al. 1998). The i1 allele of *vacA* shows a strong association with gastric adenocarcinoma (Rhead et al. 2007; Winter et al. 2014). Interestingly, in the murine

model of infection, *H. pylori* bacteria producing the s2/i2 form of VacA colonised mice more efficiently than those producing the s1/i1 form of VacA or those lacking VacA, potentially suggesting a different biological role for the weakly active s2/i2 toxin. Strains producing more active VacA induced more severe and extensive metaplasia and inflammation in the mouse stomach than strains producing s2/i2 toxin (Winter et al. 2014). Thus, specific *vacA* alleles may contribute to the pathogenicity and clinical outcomes of *H. pylori* infection.

3.3.3 Other Putative Autotransporter Proteins of H. pylori

H. pylori genomes contain three *vacA*-like genes encoding proteins of 260–348 kDa (Tomb et al. 1997). The C-terminal regions of these proteins show homology to the C-terminal region of VacA, which is a β-barrel domain that is required for secretion of VacA through an autotransporter (type V) pathway. On the basis of this similarity, three proteins are predicted to be autotransporters: immunomodulating autotransporter (ImaA), flagella-associated autotransporter A (FaaA) and VacA-like protein C (VlpC) (Radin et al. 2013; Sause et al. 2012). These proteins are all present on the surface of *H. pylori* (Radin et al. 2013; Sause et al. 2012). However, whereas ImaA and VlpC localise to a bacterial pole, FaaA localises to a sheath overlying the flagellar filament and bulb and is important for flagellar morphology and function. The *faaA* mutant strain shows decreased motility, reduced flagellar stability and an increased proportion of flagella in nonpolar sites (Radin et al. 2013).

Expression levels of *imaA*, *faaA* and *vlpC* were up-regulated during colonisation of the mouse stomach (Radin et al. 2013; Sause et al. 2012). *imaA* was identified as belonging to the ArsRS regulon and thus its increased expression in vivo is likely a response to gastric acid (Sause et al. 2012). The mechanism(s) by which *faaA* and *vlpC* expression levels are regulated remain(s) unknown. Consistent with the idea that ImaA, FaaA and VlpC may have important roles in colonisation, competition experiments in mice showed that mutants for each of these autotransporters were outcompeted by wild-type bacteria in vivo (Radin et al. 2013; Sause et al. 2012). Indeed, a single challenge study confirmed that an *H. pylori faaA* mutant was attenuated in its ability to colonise, when compared with the wild-type strain; however, this was apparent during the early (4 days post-infection) but not late (1 month post-infection) stages of infection (Radin et al. 2013; Sause et al. 2012). It was suggested that FaaA may be important early in the infection process, when fully formed and functional flagella are required for *H. pylori* entry into the mucous layer (Radin et al. 2013; Sause et al. 2012). Similar to the findings above, mice challenged with *H. pylori imaA* mutant bacteria alone or in competition with wild-type bacteria demonstrated that this mutant also had a colonisation defect in vivo (Sause et al. 2012). In this case, however, ImaA reduced expression of inflammatory chemokines and cytokines in infected stomachs and cultured epithelial cells, suggesting that this autrotransporter may be important for dampening host immune responses (Sause et al. 2012). The immunomodulatory activity of ImaA was

observed in *H. pylori* strains that harbour a *cag*PAI, suggesting that ImaA downregulates the inflammatory responses triggered by the T4SS (Sause et al. 2012). Interestingly, ImaA exhibits some similarity to the bacterial integrin-binding protein, invasin (Sause et al. 2012). As the T4SS pilus is known to mediate its pro-inflammatory effects through binding to $\alpha_5\beta_1$ integrin (Hutton et al. 2010; Kwok et al. 2007), it was suggested that ImaA and the T4SS may compete for integrin binding (Sause et al. 2012). Thus, in the absence of ImaA, the T4SS is better able to deliver the pro-inflammatory effectors, CagA and peptidoglycan (Sause et al. 2012).

3.3.4 γ-Glutamyl Transpeptidase

γ-Glutamyl transpeptidase (GGT) is produced by all strains of *H. pylori* (Chevalier et al. 1999). It plays an important role in the amino acid metabolism of *H. pylori*, by synthesising glutamate from both glutamine and glutathione, neither of which can be assimilated from the environment by this bacterium (Shibayama et al. 2007). *H. pylori* GGT hydrolyses glutamine and glutathione outside the cell, with the resulting products of this reaction being glutamate and ammonia and glutamate and cysteinylglycine, respectively. The glutamate produced is transported by a Na^+-dependent reaction into *H. pylori* cells (Shibayama et al. 2007). The enzyme is catalytically active even at the low pH of the gastric mucosa and its expression appears to be constitutive (Chevalier et al. 1999). GGT is synthesised as a 60 kDa inactive proenzyme that undergoes autocatalytic processing to form an enzymatically active heterodimer of 40 and 20 kDa subunits (Chevalier et al. 1999; Shibayama et al. 2003).

Several lines of evidence indicate that GGT is a virulence factor of *H. pylori*. Although deletion of *ggt* did not impair the in vitro growth of *H. pylori* (Chevalier et al. 1999), *ggt* mutants were attenuated for colonisation of mice and gnotobiotic piglets (Chevalier et al. 1999; McGovern et al. 2001; Oertli et al. 2013). The degree of attenuation appears to depend on the *H. pylori* strain and/or the experimental animals used. It has been suggested that GGT may be associated with *H. pylori*-induced peptic ulcer disease (PUD) in that *H. pylori* isolates from patients with PUD showed significantly higher levels of GGT activity than those from patients with non-ulcer dyspepsia (Gong et al. 2010). Definitive evidence for this suggestion is, however, currently lacking.

GGT is thought to contribute to the *H. pylori*-induced damage to the gastric epithelial cells by promoting apoptosis and by modulating the immune response. Purified, enzymatically active GGT induced apoptosis and reduced viability in AGS gastric epithelial cells (Shibayama et al. 2003). Furthermore, GGT was shown to induce production of H_2O_2, leading to DNA damage, apoptosis and activation of inflammatory pathways (Gong et al. 2010). It has been suggested that GGT might contribute to the damaging effect of *H. pylori* on gastric cells by depleting glutamine and glutathione, which are important nutrients for maintenance of healthy

gastrointestinal tissue (Shibayama et al. 2007). As discussed in Sect. 4.3 and in more detail in Chap. 5, GGT also exerts immunomodulatory effects on T-cells.

3.3.5 High Temperature Requirement A (HtrA) Serine Protease

H. pylori HtrA belongs to a family of serine proteases that is widely conserved in both single and multicellular organisms. This family of proteins can be distinguished from other serine proteases by their sequence homology and oligomeric structure, as well as by the presence of a protease domain and one or two carboxy terminal PDZ (**p**ost synaptic density protein, **D**rosophila disc large tumour suppressor, Dlg1, and **z**onula occludens-1 protein) domains (Clausen et al. 2011). HtrA serine proteases are involved in important cellular processes, including bacterial virulence (Clausen et al. 2011). The HtrA secreted by *H. pylori* (Bumann et al. 2002) cleaves the extracellular domain of the cell adhesion protein E-cadherin, present on the surface of host cells, resulting in the loss of cell-cell contact and enabling the bacterial entry into the intracellular space of epithelial cells (Hoy et al. 2010). *H. pylori* HtrA cleavage of E-cadherin was highly efficient at physiological and high temperatures and at pH 5–8, with highest activity observed at pH 6–7 (Hoy et al. 2013). Expression of HtrA was up-regulated by oxidative stress (Huang and Chiou 2011) and environmental acidification (Merrell et al. 2003). These characteristics of HtrA might aid *H. pylori* in colonising the gastric environment. Interestingly, HtrA also appears to be essential for *H. pylori* survival in vitro (Hoy et al. 2010; Salama et al. 2004), suggesting that the protein may have functions other than the cleavage of E-cadherin. Indeed, many HtrAs play an important role in protein quality control, with some also acting as chaperones to stabilise specific proteins (Clausen et al. 2011).

3.3.6 Other Pro-inflammatory Virulence Factors of H. pylori

Various new bacterial virulence factors are emerging as putative contributors to *H. pylori* pathogenesis in human gastric mucosa. For example, it has been suggested that the TNFα-inducing protein (Tipα, HP0596) contributes to *H. pylori* oncogenicity. Tipα is a homodimeric protein that has been shown to be important for colonisation of mouse gastric mucosa (Godlewska et al. 2008) and is secreted independently of the T4SS (Suganuma et al. 2005). This protein binds specifically to nucleolin, a cell surface receptor on gastric epithelial cells (Watanabe et al. 2010), whereupon it is internalised into the cytosol and then nucleus (Suganuma et al. 2008). In a mouse gastric epithelial cell line (MGT-40), Tipα was shown to induce expression of chemokine genes, such as *Ccl2*, *Ccl7*, *Ccl20*,

Cxcl1, *Cxcl2*, *Cxcl5* and *Cxcl10* (Kuzuhara et al. 2007). Tipα was also shown to induce epithelial-mesenchymal transition in human gastric cancer cell lines (Watanabe et al. 2014).

Duodenal ulcer-promoting gene A (*dupA*) was originally found to be associated with an increased risk for duodenal ulcers and a reduced risk for gastric atrophy and cancer (Lu et al. 2005). Subsequent studies, however, indicated that this association held in some geographical regions, but not in others (Abadi et al. 2012; Alam et al. 2012; Arachchi et al. 2007; Argent et al. 2007; Gomes et al. 2008; Imagawa et al. 2010; Nguyen et al. 2010; Shiota et al. 2010). *dupA* is associated with increased IL-8 production from gastric mucosa in vivo (Hussein et al. 2010; Lu et al. 2005). This gene is located in a plasticity region and encodes a protein that is functionally homologous to the T4SS ATPase protein, VirB4. In some *H. pylori* genomes, *dupA* is located adjacent to homologues of other *vir* genes, and a complete *dupA* cluster was predicted to form a third T4SS, *tfs3a* (Kersulyte et al. 2009). The presence of a complete *dupA* cluster was found to significantly increase the risk of duodenal ulcer compared to *H. pylori* infection with an incomplete *dupA* cluster or without the *dupA* gene (Jung et al. 2012). These data suggest that the epidemiological studies into the role of *dupA* in pathogenicity should be revisited, with the focus on the presence of an intact *dupA* cluster. Indeed, the extensive genetic diversity of *H. pylori*, which contributes to its success as a pathogen, also increases the difficulty of delineating the molecular basis of the pathogenesis of *H. pylori*-induced diseases.

3.4 Avoidance and Modulation of the Host Immune Response

Although *H. pylori* is an extracellular pathogen, this bacterium is able to disrupt epithelial integrity (Amieva et al. 2003). Thus, *H. pylori* products are likely to enter the lamina propria and come into contact with immune cells (Necchi et al. 2007). Although controversial, there is also some evidence to suggest that the bacterium can invade and replicate within intracellular compartments of epithelial cells, macrophages and DCs (Ricci et al. 2011). *H. pylori* induces vigorous inflammatory host responses, with a large influx of neutrophils, macrophages, DCs and lymphocytes, but the immune system fails to clear the infection, attesting to the success of the various sophisticated strategies used by the pathogen to evade and subvert this system. These strategies include: evasion of the innate immune system, modulation of phagocytosis and neutrophil functions, inhibition of lymphocyte proliferation, and skewing of T-cell-mediated adaptive immune responses toward tolerogenicity (Baldari et al. 2005).

3.4.1 Evasion of Detection by the Innate Immune System

Host cells are able to detect conserved components of microorganisms, known as microbe-associated molecular patterns (MAMPs), via pattern recognition receptors (PRRs). The best characterised PRRs are the Toll-like receptors (TLRs), which recognise specific classes of MAMPs and respond by activating intracellular signalling pathways that lead to activation of the master transcriptional regulator, NF-κB, and pro-inflammatory gene expression. *H. pylori* has developed several strategies that largely allow it to avoid detection by TLRs (Takeda and Akira 2005). The best understood of these strategies involve lipopolysaccharide (LPS) and flagellin.

LPS consists of an O side chain, a core oligosaccharide and lipid A, which is anchored to the bacterial membrane. The lipid A of *H. pylori* LPS is predominantly tetraacylated, whereas the LPS of *E. coli*, which is 1,000-fold more biologically active than that of *H. pylori*, is hexaacylated (Moran et al. 1997). Pathogenic bacteria can evade the host innate immune system by concealing or removing the negatively charged phosphate groups present on the lipid A disaccharide backbone. The resultant net loss of negative surface charges makes the bacterial membrane more resistant to cathelicidin antimicrobial peptides (CAMPs). CAMPs, which are found in macrophages and neutrophils and at the mucosal surface, are an important component of the host innate immune response and a link between the innate and adaptive immune systems (Diamond et al. 2009). The low biological activity of *H. pylori* LPS has been shown to be due to the removal of phosphate groups from the 1′- and 4′-positions of lipid A by two lipid phosphatases, LpxE and LpxF, respectively (Cullen et al. 2011). Dephosphorylated LPS is attenuated for TLR4 activation and highly resistant to CAMPs. Importantly, dephosphorylation of lipid A by LpxE and LpxF is required for effective colonisation and survival of *H. pylori* in mice (Cullen et al. 2011). Studies indicated that *H. pylori* LPS initiates inflammatory signalling in human epithelial cells via TLR2, rather than the more classical sensor of Gram-negative LPS, TLR4 (Smith et al. 2011; Yokota et al. 2007). TLR2 recognises a variety of microbial components, including lipoproteins, lipoteichoic acid and atypical LPS molecules whose structures differ from those recognised by TLR4 in the number of acyl chains in the lipid A moiety (Takeda and Akira 2005).

Recognition of many but not all bacterial flagellins by TLR5, which is present on the membrane of various cell types, including epithelial cells, leads to activation of the innate immune system (Takeda and Akira 2005). However, the major flagellin of *H. pylori*, FlaA, is much less well recognised by TLR5 than the flagellins of other enteric mucosal pathogens, such as *Salmonella typhimurium* (Gewirtz et al. 2004). Moreover, unlike the flagellins of *Escherichia* and *Salmonella*, FlaA is not released from the bacteria. The evolutionarily conserved recognition sequence for TLR5 is located in the N-terminal D1 domain of bacterial flagellin, within a region that is required for flagellar filament assembly and motility. Substitution of amino acids 89–96 of the flagellin (FliC) from *S. typhimurium*, with the corresponding amino acids from *H. pylori* FlaA, abolishes its recognition by TLR5 but also renders the bacteria non-motile (Andersen-Nissen et al. 2005). *H. pylori* has preserved its

motility by selecting for compensatory changes in other regions of FlaA, suggesting that avoidance of detection by TLR5 is important for the persistence of *H. pylori* at mucosal sites (Andersen-Nissen et al. 2005).

3.4.2 Modulation of Phagocytosis and Neutrophil Function

The engulfment and killing of microorganisms by the process known as phagocytosis are an important part of host innate defence against many pathogens. The role of macrophages in *H. pylori* pathogenesis, however, remains very controversial. Indeed, professional phagocytes appear to be ineffective in killing *H. pylori*. Reduced levels of *H. pylori* opsonisation by phagocytes have been attributed to its urease activity (Rokita et al. 1998) and the environmental conditions in the stomach (Berstad et al. 1997). Furthermore, *cag*PAI$^+$ *H. pylori* strains are able to retard phagocytosis in a *cag*PAI-dependent but CagA-, VacA- and urease-independent manner (Allen et al. 2000; Ramarao et al. 2000). Following their engulfment, these more virulent *H. pylori* strains stimulate rapid and extensive homotypic phagosome fusion, leading to formation of megasomes containing large numbers of viable *H. pylori*. Formation of these megasomes, which were shown to be stable for 24 h, requires live, metabolically active *H. pylori* (Allen et al. 2000). *cag*PAI$^+$ *H. pylori* strains induce the recruitment and retention of coronin 1 protein on phagosomes and prevent phagosome fusion with lysosomes (Zheng and Jones 2003). This inhibition of phagosome maturation is dependent on VacA and urease (Schwartz and Allen 2006; Zheng and Jones 2003). Although *H. pylori* strains that do not encode *cag*PAI, and which express a non-toxigenic form of VacA, are capable of subverting bacterial killing by macrophages for up to 24 h, their survival is inferior to that shown by *cag*PAI$^+$ bacteria (Zheng and Jones 2003). The ability of toxigenic alleles of VacA to modulate autophagy may also contribute to the survival of *H. pylori* in macrophages, by allowing the surviving phagocytosed bacteria to escape killing (Raju et al. 2012). Despite the in vitro evidence for *H. pylori* survival in macrophages, further investigations are required in in vivo models to confirm the biological relevance of these observations.

One mechanism by which *H. pylori* has been shown to be capable of interfering with its phagocytosis by antigen-presenting cells is via the actions of a cholesterol-**α**-glucosyltransferase (HP0421). This enzyme, also known as type 1 capsular polysaccharide biosynthesis protein J (CapJ), catalyses the conversion of cholesterol to cholesteryl α-glucosides (Lebrun et al. 2006; Wunder et al. 2006). Although *H. pylori* is auxotrophic for cholesterol, its envelope contains high concentrations of cholesteryl glucosides (Tannaes and Bukholm 2005). The pathogen extracts cholesterol from the plasma membranes of epithelial cells, but excessive cholesterol promotes phagocytosis of the bacteria by antigen-presenting cells, thereby enhancing T-cell activation. Conversely, α-glucosylation of cholesterol by cholesterol-α-glucosyltransferase abrogates phagocytosis of *H. pylori* and T-cell activation (Wunder et al. 2006). In addition to these effects, cholesterol

glucosylation by CapJ is important for tight binding of *H pylori* to gastric epithelial cells and for the assembly of a functional T4SS, as a *capJ* mutant was impaired in its ability to translocate CagA into the cytosol of host cells (Wang et al. 2012).

3.4.3 Inhibition of Lymphocyte Proliferation

H pylori uses the secreted proteins VacA (Gebert et al. 2003) and GGT (Shibayama et al. 2007), to inhibit lymphocyte activation and proliferation. VacA is able to inhibit proliferation of primary human B lymphocytes, as well as $CD4^{+}$ and $CD8^{+}$ T-cells (Torres et al. 2007). The VacA receptor on human immune cells is the β2 (CD18) integrin subunit (Sewald et al. 2008). In transformed Jurkat T-cells, VacA was shown to down-regulate transcription of IL-2, required for efficient lymphocyte activation and proliferation (Gebert et al. 2003). VacA does this by blocking nuclear translocation of the global regulator of immune response genes, nuclear factor of activated T-cells (NFAT). In activated primary human T-cells, VacA has also been shown to inhibit IL-2-driven cell-cycle progression independently of IL-2 secretion, by blocking the activation of regulatory proteins important for G1 cell-cycle transition (Oswald-Richter et al. 2006; Sundrud et al. 2004). Interestingly, murine T-cells, splenocytes and CD4+ T-cells do not significantly respond to VacA and this resistance is, at least in part, due to the impaired binding of VacA to murine cells (Algood et al. 2007; Sewald et al. 2008).

In common with *H. pylori* VacA, GGT inhibits proliferation of stimulated primary T-cells and peripheral blood mononuclear cells (PBMCs), but without affecting secretion of IL-2 (or IFN-γ) and without induction of apoptosis (Oertli et al. 2013; Schmees et al. 2007). The inhibition of lymphocyte proliferation involves induction of cell-cycle arrest in G1 phase through disruption of Ras-dependent signalling and requires the structural integrity of the catalytic domain of GGT and the presence of glutamine (Oertli et al. 2013; Schmees et al. 2007). It has been suggested that the inhibitory effect of GGT on T-cells is mediated indirectly by the formation of metabolites during transpeptidation (Oertli et al. 2013; Schmees et al. 2007).

3.4.4 Skewing of Adaptive Immune Responses Toward Tolerogenicity

H. pylori bacteria can manipulate adaptive immune responses to promote their persistence. One mechanism by which this may occur is via the preferential induction of regulatory T-cell (Treg) responses. This is evident in heavily colonised but asymptomatic carriers, who show Treg-predominant responses (Robinson et al. 2008). Similar findings were observed in a mouse model, in which depletion

of Tregs led to spontaneous clearance of the infection (Arnold et al. 2011). *H. pylori*-induced disease in humans was associated with low Treg responses and significantly higher levels of gastric T-helper 1 (Th1) and Th2 cells, whereas in disease-free infected subjects the balance was shifted toward elevated Tregs and a reduced T-helper response (Robinson et al. 2008). Thus, it was suggested that disease is a consequence of an inadequate regulatory response to *H. pylori* infection (Robinson et al. 2008).

DCs play a crucial role as a link between innate and adaptive immunity, being exquisitely adept at acquiring, processing and presenting antigens to T-cells. DCs present antigens in a way that promotes tolerance, at least in part, via regulation of Treg responses (Maldonado and von Andrian 2010). Priming by tolerogenic DCs converts naive T-cells into FoxP3$^+$ Tregs through antigen presentation in the absence of co-stimulatory signals or cytokines (Maldonado and von Andrian 2010). *H. pylori* is able to reprogram DCs toward a tolerogenic phenotype in vitro and in vivo. Indeed, DCs that have been exposed to *H. pylori* appear to preferentially prime Tregs over pro-inflammatory Th1 and Th17 responses and also fail to produce pro-inflammatory cytokines (Kao et al. 2010; Oertli et al. 2012; Wang et al. 2010). The importance of DCs in the development of *H. pylori*-specific immune tolerance is highlighted by the finding that systemic depletion of DCs breaks tolerance and facilitates clearance of the bacteria (Oertli et al. 2012). *H. pylori* VacA and GGT proteins play critical, non-redundant and non-synergistic roles in the tolerising effects of this pathogen on murine DCs in vitro and in vivo, by mechanisms that are independent of their suppressive activities on T-cells. Isogenic *H. pylori* mutants lacking either GGT or VacA are incapable of preventing LPS-induced DC maturation, fail to drive DC tolerisation and are attenuated for mouse colonisation (Oertli et al. 2013). Furthermore, *vacA* mutants induce stronger Th1 and Th17 responses and more severe gastric pathology (Oertli et al. 2013). VacA and GGT were reported to induce the expression of miR-155 and Foxp3 in human lymphocytes via a cAMP-dependent pathway (Fassi Fehri et al. 2010). Both VacA and GGT promote efficient induction of Tregs in vivo, while VacA is required to prevent allergen-induced asthma. The immunomodulatory effects of GGT are dependent on its enzymatic activity, whereas those of VacA are not linked to its vacuolating cytotoxicity, as strains expressing the toxigenic (s1/m1) or non-toxigenic (s2/m2) forms of VacA are equally tolerogenic in vitro (Oertli et al. 2013).

3.5 Mitigation of Inflammatory Responses

H. pylori infection leads to chronic gastritis, which generates reactive oxygen species (ROS) and nitric oxide (NO). The pathogen limits the bactericidal effects of these pro-inflammatory mediators, enabling it to chronically colonise its host. The inflammatory response induced by *H. pylori* generates large amounts of ROS, which encompass superoxide anions, hydroxyl radicals and hydrogen peroxide.

H. pylori survive these oxidative stress conditions using a variety of stress resistance proteins. These include catalase (KatA), superoxide dismutase (SodB) and three peroxidases, an alkylhydroperoxide reductase (AhpC) and two thiolperoxidases (Tpx and bacterioferritin comigratory protein, Bcp), which catalyse the breakdown of hydrogen peroxide, superoxide and organic peroxides, respectively. *H. pylori* also encodes the neutrophil activating protein (NapA), which sequesters toxic levels of iron, and NADPH quinone reductase, MdaB (Stent et al. 2012). Furthermore, *H. pylori* bacteria respond to inactivation of important oxidative stress resistance proteins by increasing the expression of their oxidative stress resistance proteins, including KatA, SodB and NapA (Olczak et al. 2005). Disruption of *katA*, *sodB*, *ahpC*, *tpx*, *bcp* or *mdaB* in *H. pylori* results in an oxidative stress-sensitive phenotype that severely affects the ability of mutants to colonise the stomach (Harris et al. 2003; Olczak et al. 2002, 2003; Seyler et al. 2001; Wang and Maier 2004; Wang et al. 2005).

Infection by *H. pylori* leads to up-regulation of inducible nitric oxide synthase (iNOS) in the gastric mucosa, leading to mucosal damage. Data obtained using cultured macrophages indicate that this induction of iNOS is dependent on the urease released from *H. pylori* (Gobert et al. 2002). *H. pylori* bacteria mitigate the bactericidal effects of NO, which is generated during the conversion of L-arginine to L-citrulline by iNOS, via the actions of an arginase, RocF (Gobert et al. 2001). The constitutively produced RocF inhibits production of NO by competing with the host for L-arginine, which is hydrolysed to L-ornithine and urea; the latter is then used as a substrate by urease. Loss of RocF activity leads to significant NO-dependent killing of *H. pylori* in vitro (Gobert et al. 2001). However, *rocF* is not essential for *H. pylori* colonisation of wild-type (McGee et al. 1999) or arginase II knockout mice (Kim et al. 2011). RocF activity is stimulated by Trx1 (HP0824), one of the two thioredoxins of *H. pylori*. Trx1 acts as a chaperone, converting denatured or suboptimally folded RocF into its catalytically active structure and reversing the damage caused by reactive oxygen and nitrogen intermediates (McGee et al. 2006).

3.6 Modulation of Apoptosis and Autophagy by *H. pylori*

Apoptosis and autophagy are intricately connected but opposing processes that can be induced in response to cellular stress and must be finely balanced to regulate cell death and survival. Perturbation of this balance can lead to pathologies such as cancer. *H. pylori* is capable of inducing and inhibiting both apoptosis and autophagy. The major known *H. pylori* virulence factors involved in these processes are VacA, CagA and GGT.

3.6.1 Apoptosis

VacA induces apoptosis of gastric epithelial cells by targeting the mitochondria, where it accumulates in the inner membrane and causes depolarisation, outer membrane permeability, cytochrome C release and mitochondrial fragmentation (Cover et al. 2003; Galmiche et al. 2000; Willhite et al. 2003; Yamasaki et al. 2006). The ability of VacA to form anion-selective membrane channels is required for cytochrome C release, mitochondrial outer membrane permeabilisation (MOMP) and cell death (Willhite and Blanke 2004). VacA induces the activation of the pro-apoptotic proteins, Bax and Bcl-2 homologous antagonist/killer (Bak), thus leading to apoptosis (Yamasaki et al. 2006). VacA-mediated MOMP and activation of Bak require the mitochondrial recruitment and hyperactivation of dynamin-related protein 1 (Drp1), a critical regulator of mitochondrial fission within cells (Jain et al. 2011). GGT induces apoptosis in gastric epithelial cells via a mitochondria-mediated pathway (Kim et al. 2007) and by inducing the loss of survivin, an inhibitor of apoptosis (Valenzuela et al. 2013). Recently, another putative *H. pylori* virulence factor, HP0986 (TNFR1 interacting endonuclease A, TieA), was reported to actively induce apoptosis in cultured human and murine macrophages via a Fas-mediated pathway (Alvi et al. 2011).

There is also evidence that *H. pylori* can prevent or block apoptosis. In a transgenic mouse model, *H. pylori* CagA was shown to interact with the apoptosis-stimulating protein of p53 (ASPP2) and thereby inhibit apoptosis by promoting proteasomal degradation of the p53 tumour suppressor (Buti et al. 2011). Host cells that had been co-cultured with *H. pylori* and then treated with the p53-activating drug doxorubicin were more resistant to apoptosis than cells not exposed to the bacterium (Buti et al. 2011). In a separate study, it was reported that CagA is also able to mediate activation of the pro-survival factor ERK and the anti-apoptotic protein, myeloid leukaemia cell differentiation protein 1 (Mcl-1) (Mimuro et al. 2007). Using the Mongolian gerbil model, the authors showed that *H. pylori* is able to activate the ERK-Mcl-1 pathway in vivo so as to suppress apoptosis in gastric pit cells, thereby leading to gland hyperplasia and persistent bacterial colonisation (Mimuro et al. 2007). In agreement with these findings, another group demonstrated *H. pylori* CagA-dependent induction of Mcl-1 expression via up-regulation of a known negative regulator of Mcl-1, the tumour suppressor microRNA (miRNA) *miR-320* (Noto et al. 2013). Consistent with the Mongolian gerbil data, *H. pylori* was shown to induce Mcl-1 expression in a CagA-dependent manner in the murine gastric mucosa, as well as in tissues from a human population at high risk for gastric cancer (Noto et al. 2013). Moreover, Mcl-1 epithelial expression levels increased at each stage of neoplastic progression in gastric tissues from human subjects infected with *cagA*$^+$ strains of *H. pylori* (Noto et al. 2013). Thus, down-regulation of *miR-320* and subsequent induction of Mcl-1 by *cagA*$^+$ *H. pylori* strains suppresses apoptosis, potentially promoting *H. pylori* persistence within the gastric mucosa but also possibly gastric carcinogenesis.

3.6.2 Autophagy

Autophagy is a tightly controlled major catabolic pathway in eukaryotes, which is required for the lysosomal/vacuolar degradation of cytoplasmic proteins and organelles. *H. pylori* induces autophagy in gastric epithelial cells (Tang et al. 2012; Terebiznik et al. 2009), as well as in professional phagocytes (Wang et al. 2010). VacA is necessary and sufficient to induce autophagy in gastric epithelial cells, with the induction of autophagy responsible for a decrease in the levels of intracellular VacA and vacuole biogenesis within intoxicated cells. Thus, autophagy may represent a mechanism by which host cells limit VacA-mediated damage (Terebiznik et al. 2009). In the AZ-521 human gastric epithelial cell line, VacA-induced autophagy was shown to be mediated by VacA binding to and internalisation via the low-density-lipoprotein receptor-related protein 1 (LRP1) (Yahiro et al. 2012). Knockdown of LRP1 abrogated VacA internalisation and significantly down-regulated autophagy in vitro (Yahiro et al. 2012). LRP1 is also required for the induction of autophagy-mediated degradation of CagA in response to m1 forms of VacA in AGS gastric epithelial cells. Signalling through p53 degradation is involved in m1VacA-induced autophagy in these cells (Tsugawa et al. 2012).

In addition to the effects of VacA in promoting autophagy, this cytotoxin can also block autophagy. Indeed, prolonged exposure of human gastric epithelial cells to VacA was found to disrupt toxin-induced autophagy, by blocking the maturation of autolysosomes (Raju et al. 2012). VacA alters the degradative properties of the endocytic pathway by subverting the sorting and activation of cathepsin enzymes (Satin et al. 1997). VacA-induced autolysosomes lack the key lysosomal hydrolase, cathepsin D, and so cannot complete the process of degrading their cargo, leading to accumulation of ROS and the signalling adaptor, p62 (Raju et al. 2012). Interestingly, impairment of autophagy and the accumulation of p62 lead to enhanced tumourigenicity (Moscat and Diaz-Meco 2009). Further work is required to determine whether VacA subversion of autophagy may promote gastric cancer development.

Finally, *H. pylori* can modulate autophagy through a mechanism involving the microRNA, *MIR30B* (Tang et al. 2012). Expression of *MIR30B* was elevated in gastric mucosal tissues from infected patients, as well as during infection of gastric epithelial cell lines, and this effect was a specific response to *H. pylori*. Moreover, elevated *MIR30B* expression in human gastric tissues was inversely correlated with the mRNA levels of the genes encoding two of the proteins that are important in regulating autophagy, autophagy-related protein 12 (ATG12) and BCL2-interacting coiled-coil protein (BECN1). Inhibition of autophagy by *MIR30B* was demonstrated to increase the number of VacA-dependent large vacuoles and enhanced the intracellular survival of the pathogen, demonstrating that autophagy is involved in regulating the levels of intracellular VacA (Tang et al. 2012). Taken together, these data show that *H. pylori* VacA is able to use different strategies to interfere with autophagy in gastric epithelial cells.

3.7 Conclusions and Outlook

H. pylori is arguably one of the most successful human pathogens, persistently colonising the gastric mucosa of more than 50 % of the world's population. This pathogen induces vigorous inflammatory host responses. However, due to the many effective strategies employed by the bacterium to subvert host immune responses, the infection cannot be easily cleared. *H. pylori* uses its numerous virulence factors to establish chronic infection, alter cellular signalling cascades, cause damage to the mucosa and modulate host immune responses. The multitude of virulence factors, together with their complex interactions and allelic variations, render it difficult to dissect the individual contributions of these factors to the chronicity of *H. pylori* infection and its long-term consequences. Moreover *H. pylori* is not only highly heterogeneous but also genetically unstable, adding to the difficulty in studying the virulence mechanisms of this human pathogen. As discussed here, *H. pylori* can alter cell proliferation, apoptosis and autophagy processes, as well as down-regulate cellular tumour suppressor genes. All together, these changes contribute to oncogenesis and the development of more severe gastric disease. Although the last decade has seen great advances in our understanding of the virulence mechanisms of *H. pylori*, much of this knowledge has been gained from experiments conducted in vitro or in animal models and awaits confirmation from clinical and epidemiological studies. Such studies must encompass populations from diverse geographic locations, as both bacterial and host polymorphisms are likely to contribute to the pathogenesis of *H. pylori* infection in humans.

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Chapter 4
Roles of the *cag*PAI and CagA on Gastroduodenal Diseases

Steffen Backert, Giuseppe Zanotti, Judith Lind, Carmen Isabell Asche, and Nicole Tegtmeyer

Abstract *Helicobacter pylori* is a highly successful human-specific Gram-negative bacterium. Infections with this pathogen in the stomach can induce pathologies ranging from chronic gastritis, peptic ulcers, to gastric cancer. Highly virulent *H. pylori* isolates harbor the cytotoxin-associated genes (*cag*) pathogenicity island, which encodes a typical type IV secretion system (T4SS). This T4SS constitutes a syringe-like pilus structure for the translocation of virulence factors such as the CagA effector protein into gastric epithelial and immune cells. This is achieved by a number of T4SS proteins such as CagL, CagI, CagY, and CagA, which can interact with the host cell integrin member $\alpha_5\beta_1$ followed by transport of CagA across the host cell membrane. After delivery, CagA undergoes phosphorylation by oncogenic tyrosine kinases and mimics a host factor for the activation or inactivation of multiple intracellular signaling cascades. Here we review the current status in the characterization of phosphorylation-dependent and phosphorylation-independent signaling events by CagA and the CagA-independent T4SS activities in vivo and in vitro, which include the induction of membrane dynamics, actin-cytoskeletal rearrangements, disruption of cell-to-cell junctions, as well as pro-inflammatory, proliferative and anti-apoptotic nuclear responses. The contribution of these signaling pathways to pathogenesis during *H. pylori* infection is discussed.

Keywords *Helicobacter pylori* • Type IV secretion • VirB1–VirB11 • VirD4 • Signaling • Gastric cancer • Infection • Tyrosine kinase • SH2 domain • Inflammasome

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4.1 Introduction

Helicobacter pylori is one of the best adapted human pathogens that colonizes the surface region in the gastric mucosa of the stomach. Approximately half of the world's population carries this microbe, often causing asymptomatic gastritis in infected people, but also more severe gastric diseases such as mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer may arise. Colonization commonly occurs early in childhood and *H. pylori* can persist lifelong, if not treated by antimicrobial therapy. Although *H. pylori* infections are commonly associated with elevated inflammation parameters that are generated by the host innate and adaptive immune systems, the bacteria are not eliminated. Various mechanisms of immune evasion were documented and *H. pylori* became a prime example of chronic bacterial infections. Host-pathogen interactions are highly complex and determine the clinical outcome of infections. The development of gastric diseases is controlled by the bacterial genotype, genetic predisposition of the host, and environmental factors. For example, specific polymorphisms in host genes with important roles in pro-inflammatory and immune-regulatory signal transduction such as interleukin-1β (IL-1β), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), or Nod-1 (nucleotide oligomerization domain-1) have been linked to a higher risk of developing *H. pylori*-triggered gastric diseases (for more details, see Chaps. 3, 13, and 16 of this book). *H. pylori* strains are highly heterogeneous both in their DNA sequences and virulence properties. Dozens of bacterial genes have been described to control the pathogenesis of *H. pylori*. There are two classical virulence factors expressed by *H. pylori*, the CagA protein encoded in the *cag* (cytotoxin-associated genes) pathogenicity island (*cag*PAI) and the vacuolating cytotoxin A (VacA). VacA interacts with multiple host molecules and can trigger various downstream signaling cascades as discussed in Chap. 5. Here we summarize our current knowledge on the multiple *cag*PAI and CagA functions as well as the multitude of affected host signaling cascades with focus on their importance in *H. pylori* pathogenesis.

4.2 The *cag*PAI Encodes a Type IV Secretion System

In the *H. pylori* research field, worldwide interest is focused on the effector protein CagA. CagA is encoded by highly virulent type I isolates, but is absent in less virulent type II strains. Thus, the protein has been recognized as a molecular marker for the *cag*PAI locus (Hatakeyama 2003; Backert et al. 2015). The *cag*PAI encodes functional components of a type IV secretion system (T4SS). This T4SS represents a pilus-like structure (called the T4SS pilus), induced upon host cell contact and protruding from the bacterial membrane (Fig. 4.1a). T4SS machineries are evolutionary related to DNA conjugation systems (Backert and Meyer 2006). The class

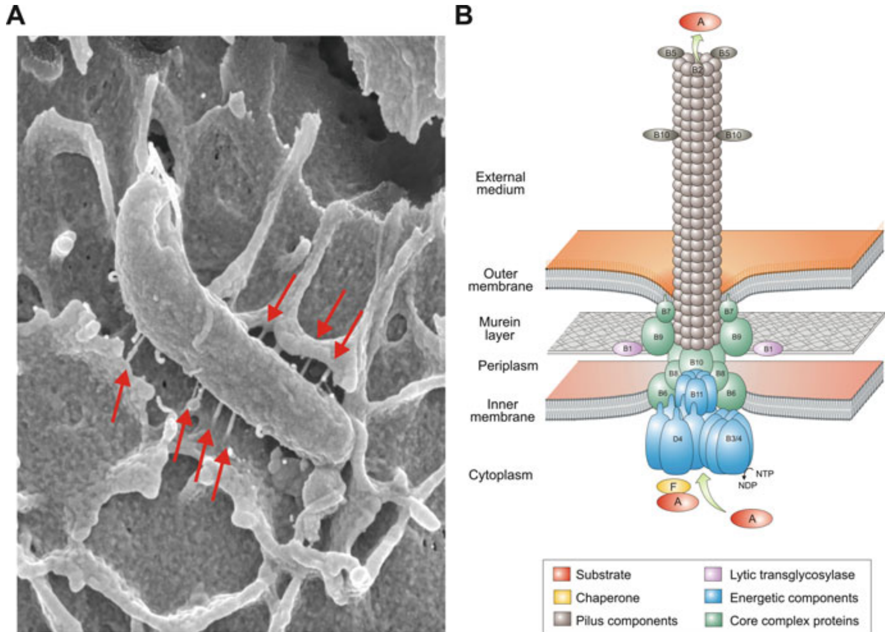

Fig. 4.1 Model for the assembled type IV secretion (T4SS) complex in *Helicobacter pylori* (*Hp*). (**a**) The T4SS encoded by the *cag* pathogenicity island is a multicomponent protein complex spanning the inner and outer membranes. Typical T4SS pili are shown by scanning electron microscopy, connecting the bacterium with the membrane of AGS gastric epithelial cells (*arrows*). (**b**) The *Hp* T4SS exhibits homology to the VirB/VirD4 T4SS machinery of *Agrobacterium tumefaciens*. T4SS assembly and subcellular localization of the proteins are shown in a simplified manner. Pilus components, core complex proteins, energetic components, and others factors are highlighted with different colors as indicated. The reported substrates for the *Hp* T4SS are CagA and peptidoglycan. (For more details, see text. Panel **a** was from Hauck (2007) with kind permission from Nature Publishing and panel **b** was adapted from Tegtmeyer and coworkers (2011) with kind permission from Wiley.)

of T4SSs is highly diverse both with regard to the delivered factors (DNA, proteins, DNA-protein complexes, or peptidoglycan) and recipient organisms. The latter can be either a bacterium of the same or other species or even species from different kingdoms (e.g., mammalian, fungal, or plant cells). In addition to *H. pylori*, pathogenicity-linked T4SSs have also been found in *Agrobacterium*, *Legionella*, *Bordetella*, *Bartonella*, and other species. T4SSs commonly comprise 11 VirB proteins (encoded by the *virB1–virB11* genes) and the NTPase coupling protein (VirD4). The prototypic and by far best characterized T4SS is the T-DNA transfer apparatus of *Agrobacterium tumefaciens* (Waksman and Orlova 2014). The agrobacterial VirB proteins can be classified in three groups: (1) the core or putative channel components (VirB6–VirB10), (2) the pilus-associated proteins (VirB2, and possibly VirB3 and VirB5), and (3) the energetic factors (the NTPases: VirB4 and VirB11). In addition, VirB1 is an enzyme having muraminidase activity, allowing

localized lysis of murein layer in the membrane to achieve T4SS assembly at a specific site of the cell (Backert et al. 2015). A model for the individual steps in assembling of the agrobacterial T4SS has been described in numerous review articles (Backert and Meyer 2006; Waksman and Orlova 2014). When having a look at the *H. pylori* T4SS, all 11 VirB and VirD4 orthologs are encoded in the *cag*PAI as well as some accessory proteins (Fischer 2011), resulting in a T4SS model (Fig. 4.1b). Immunogold labeling and electron microscopy (EM) have shown that the tips of the T4SS pilus are covered with CagL (Kwok et al. 2007). In addition, EM revealed that CagL, CagI, and CagH proteins were involved in T4SS pilus formation and deletion of a conserved C-terminal hexapeptide motif, which is shared among these proteins, abolished CagA delivery (Shaffer et al. 2011). The T4SS pilus is also decorated locally or completely by CagY (VirB10) (Rohde et al. 2003). CagY is a very large protein (about 250-kDa) that contains two transmembrane domains with the mid region (also called the repeat domain) exposed to the extracellular environment (Rohde et al. 2003). It carries an unusual sequence structure owing to an extraordinary number of direct DNA repeats that are predicted to result in in-frame intersections or deletions (Barrozo et al. 2013). Interestingly, CagY rearrangements are driven by the host and are sufficient to result in gain or loss of function in the T4SS. This molecular switch allowed modification of the immune response to benefit persistent infection of *H. pylori* (Barrozo et al. 2013). Major other components of the T4SS structure, also visualized by EM, are the VirB7 and VirB9 proteins (Tanaka et al. 2003). Interestingly, T4SS pilus formation requires a host cell receptor (Kwok et al. 2007). This is achieved by a number of T4SS proteins, including CagI, CagL, CagY, and CagA, interacting with the host cell integrin member $\alpha_5\beta_1$, followed by translocation of CagA into the host (Kwok et al. 2007; Jiménez-Soto et al. 2009). Binding of CagA to phosphatidylserine has also been shown to play a role in the translocation process (Murata-Kamiya et al. 2010).

4.3 Crystal Structures of *cag*PAI Proteins

In the last few years, several pieces of information have been gathered about the architecture of bacterial T4SSs, both from EM and single-crystal X-ray diffraction studies. The most complete picture of the apparatus comes from the 18Å–23Å EM structure of the T4SS of *Escherichia coli* conjugative plasmid R388 (Low et al. 2014). This complex includes 8 proteins (from VirB3 to VirB10) that form a supramolecular assembly spanning the entire cell envelope. The complex can be divided in two parts, the core complex, characterized by C_{14} symmetry, and the inner membrane complex, with a lower C_2 symmetry. The two complexes are connected by a small stalk. In the overall, the entire membrane-spanning machinery is about 340Å high and 255Å wide. A more detailed description at the atomic level

comes from the crystal structure of the O-layer (Chandran et al. 2009; Terradot and Waksman 2011) and from EM studies of the outer membrane T4SS core complex from plasmid pKM101 (Fronzes et al. 2009). The O-layer includes 14 copies of VirB10, VirB7, and VirB9, with the VirB10 components forming a tetradecameric channel and the 14 VirB7/VirB9 complexes surrounding and stabilizing it. The T4SS pilus is composed of the major subunit VirB2 and VirB5 (CagL) (Backert et al. 2008). The structure of the latter, which decorates the pilus surface, has been determined (Barden et al. 2013). CagL consists of a four-helix bundle, containing the Arg-Gly-Asp (RGD) motif that represents a recognition site for integrin binding (Kwok et al. 2007). Finally, the structure of VirB11 ATPase revealed a hexameric ring and functions as a gating molecule at the inner membrane, which is proposed to cycle through closed and open forms by ATP binding/hydrolysis (Yeo et al. 2000; Hare et al. 2007).

The crystal structures of some other members of the *cag*PAI, which are important for CagA secretion or IL-8 induction, have also been determined: CagZ, a 23-kDa protein essential for CagA translocation, consists of a single compact L-shaped domain containing seven α-helices (Cendron et al. 2004); CagS is a 23-kDa single-domain protein characterized by an all-α structure and an unusually high methionine content (Cendron et al. 2007); CagD 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).

The translocated effector protein CagA in strain 26695 consists of 1,186 amino acids and is structurally organized in several domains. Various crystal structures of the large N-terminal portion, residues 1–876 and 261–829 (Hayashi et al. 2012) and residues 1–884 (Kaplan-Türköz et al. 2012), consist of three domains, one of which is made by two sub-domains (or four domains in total, according to a different interpretation). Domain I (24–221) is composed of 10 α-helices. It makes very few contacts with the other domains, such that its orientation is mobile with respect to the rest of the molecule. Domain II (residues 303–644) constitutes the molecular core and contains a large antiparallel β-sheet flanked by an all α-helical subdomain (residues 370–446) and two long α-helices. A long, partially flexible α-helix connects domain II to domain III (residues 645–824), whose structure is that of a classical four-helix bundle. The C-terminal region (residues 825–1186, about 30% of the entire protein) of CagA seems to be intrinsically disordered, a fact that could possibly facilitate the interaction with other proteins inside the host cell. In fact, the structure of microtubule affinity-regulating kinase 2 (MARK2) in complex with 120 amino acids of the C-terminal region (residues 885–1005) has been determined, but only a 14-residue peptide of CagA is ordered in the crystal (Nesić et al. 2010). Recently, the complex between domain I of CagA (residues 25–220) with the apoptosis stimulating protein of p53-2 (ASPP2) has been also solved (Nešić et al. 2014), confirming that different parts of CagA interact with different target proteins to hijack host cell-signaling cascades associated with disease outcome.

4.4 Pathological Function of the *cag*PAI Type IV Secretion System

Clinical, epidemiological, and functional studies have shown that the presence of *cag*PAI and CagA is associated with the development of gastric disease. Various animal infection models have been developed and provided comprehensive evidence for the significance of *cag*PAI and CagA in *H. pylori* pathogenesis (Ogura et al. 2000; Rieder et al. 2005; Franco et al. 2005; Noto et al. 2013). For example, Mongolian gerbils infected with highly virulent *cag*PAI-positive *H. pylori* have been found to develop similar pathology as compared to infected humans. Gerbils developed gastric dysplasia in almost all animals by 4-week infection, which was accompanied by adenocarcinomas in ~25% of gerbils (Franco et al. 2005). After 8 weeks, ~75% of infected animals exhibited gastric adenocarcinomas. Importantly, infection with isogenic mutants indicated that CagA and the T4SS were necessary for gastric cancer development in the gerbil model (Franco et al. 2005; Noto et al. 2013). For more details and other models, please refer to Chap. 10 of this book. In addition to the *H. pylori* infection model systems, a first direct causal link between CagA and oncogenesis in vivo was reported by the production of transgenic C57BL/6J mice expressing CagA (Ohnishi et al. 2008). After 72 weeks, these transgenic mice exhibited gastric epithelial hyperplasia and some mice developed polyps and adenocarcinomas in the stomach and small intestine. Systemic CagA expression in these mice further induced leukocytosis with IL-3/GM-CSF hypersensitivity, and some animals exhibited myeloid leukemias and B-cell lymphomas (Ohnishi et al. 2008). These findings were supported using two other model organisms, zebrafish and Drosophila (Neal et al. 2013; Reid et al. 2012; Muyskens and Guillemin 2011). Transgenic expression of CagA in these systems exhibited significantly increased rates of intestinal epithelial cell proliferation, upregulation of c-Jun N-terminal kinase (JNK) signaling, and Wnt target genes associated with small cell carcinoma and adenocarcinoma (Neal et al. 2013; Wandler and Guillemin 2012). These experiments demonstrate that *H. pylori* can trigger the development of gastric adenocarcinoma in gerbils and other model systems in a manner dependent on a functional T4SS and that sole expression of CagA is sufficient to produce severe malignant lesions in transgenic mice. Thus, CagA and the *cag*PAI T4SS play central roles during *H. pylori* pathogenesis in vivo.

4.5 Phosphorylation-Dependent Host Cell Signaling of Translocated CagA

CagA represents a prime example of tyrosine phosphorylatable effector proteins (CagA^{PY}) of bacteria. Site-directed mutagenesis and mass spectrometry revealed numerous phosphorylation sites in CagA known as the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs A, B, C, and/or D (Hatakeyama 2003; Yamaoka 2010; Backert

et al. 2010). The host tyrosine kinases active on these EPIYA motifs were identified as members of the c-Src and c-Abl families (Wessler and Backert 2008). The resulting phosphotyrosines together with some flanking residues commonly act as recognition motifs for eukaryotic signaling factors. They recruit in particular cellular binding partners that contain SH2 (Src homology 2) domains, but not PTB (phosphotyrosine binding) domains and thereby target and subvert eukaryotic signal transduction pathways in ways that benefit the pathogen (Selbach et al. 2009). This indicates that CagA was specifically designed during evolution to target SH2 domain containing host cell factors (Fig. 4.2a). Altogether, 12 phospho-dependent interaction partners have been identified over the years (Table 4.1). The first reported interaction partner of $CagA^{PY}$ was the tyrosine phosphatase SHP-2 (Higashi et al. 2002). Since then, nine other host cell factors were also found to interact with CagA in a phosphorylation-dependent fashion: the tyrosine phosphatases SHP-1; phosphoinositide-3-kinase (PI3K); the signaling adaptor proteins Crk, Grb2, and Grb7; the tyrosine kinases Csk, c-Src, and c-Abl; as well as the Ras GTPase-activating protein Ras-GAP (Tsutsumi et al. 2003; Suzuki et al. 2005; Tammer et al. 2007; Selbach et al. 2009; Zhang et al. 2015). Thus, $CagA^{PY}$ seems to mimic a tyrosine-phosphorylated host cell protein and

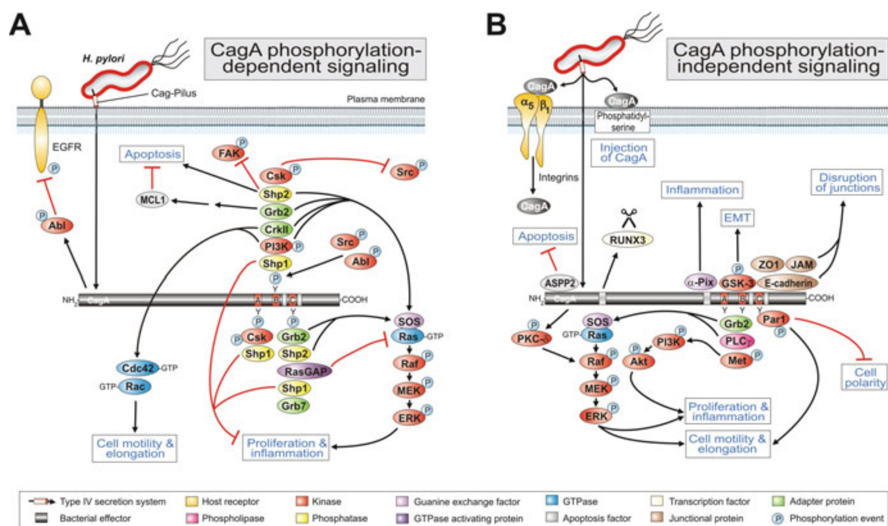

Fig. 4.2 Model for the role of *H. pylori* CagA in host cell-signaling processes which may affect pathogenesis. CagA phosphorylation-dependent (**a**) and phosphorylation-independent (**b**) signal transduction events are shown. CagA is translocated across the host cell membrane of infected gastric epithelial cells which requires integrin β_1 and phosphatidylserine. The tyrosine kinases c-Src and c-Abl phosphorylate delivered CagA. CagA can then modulate various signaling cascades associated with cell polarity, cell proliferation, actin-cytoskeletal rearrangements, cell elongation, disruption of tight and adherens junctions, pro-inflammatory responses, and suppression of apoptosis, as depicted. *Black arrows* indicate activated signaling pathways and *red arrows* correspond to inactivated cascades. (For more details, see text. Panels **a** and **b** were updated from Backert and coworkers (2010) with kind permission from Wiley.)

Table 4.1 Host cell proteins described to interact with phosphorylated CagA and proposed roles in *H. pylori* infections

Interaction partner	Proposed function	Experimental evidence	Applied methods[a]	References
Abl	Phosphorylation of CagA and CrkII adapter proteins	Infection, transfection of CagA	coIP, IF	Tammer et al. (2007)
				Poppe et al. (2007)
				Brandt et al. (2007)
CrkI, CrkII, CrkL[b]	Cell scattering, loss of AJs, MAPK signaling	Infection, transfection of CagA	coIP, IF, PD, immunoblot	Suzuki et al. (2005)
				Tammer et al. (2007)
				Brandt et al. (2007)
Csk	c-Src inactivation, loss of CagA-Shp-2 interaction	Transfection of CagA	coIP	Tsutsumi et al. (2003)
	Dephosphorylation of cortactin, ezrin, and vinculin	Proteomics	SILAC/MS	Selbach et al. (2009)
Grb2[b]	Cell scattering, activation of Ras-MAPK signaling	Proteomics	SILAC/MS	Selbach et al. (2009)
Grb7	Yet unknown	Proteomics, infection	SILAC/MS, coIP	Selbach et al. (2009)
PI3-kinase[b]	ATK signaling	Proteomics, infection	SILAC/MS, coIP	Selbach et al. (2009)
				Zhang et al. (2015)
Ras-GAP	Yet unknown	Proteomics	SILAC/MS	Selbach et al. (2009)
Shp-1	Yet unknown	Proteomics, infection	SILAC/MS, coIP	Selbach et al. (2009)
Shp-2	Cell scattering, activation of ERK	Transfection of CagA	coIP	Higashi et al. (2002)
	Tyrosine dephosphorylation of FAK	Transfection of CagA	coIP	Higashi et al. (2004)
	Downregulation of hBD3	Infection	IF, RT-PCR, immunoblot	Tsutsumi et al. (2006)
c-Src[b]	Phosphorylation of CagA	Infection	coIP	Selbach et al. (2003)

[a]Abbreviations: AJs adherens junctions, *IF* co-localization by immunofluorescence, *coIP* co-immunoprecipitation, *PD* pull-down experiments, *SILAC/MS* stable isotope labeling with amino acids in cell culture combined with mass spectrometry of bound proteins

[b]Using the coexpression system in COS-7 cells, Tsutsumi et al. (2003) overexpressed CagA together with the p85 subunit of PI3-kinase, c-Src, Grb2, or Crk-II, but none of the above reported interactions during infection between CagA and these proteins were found during these transfection studies

therefore appears to function as a master key or picklock to hijack specific cascades of the host. The various CagA^{PY}-SH2 domain interactions appear to have complex roles in *H. pylori*-triggered cytoskeletal rearrangements, cell scattering, and elongation (summarized in Fig. 4.2a).

Infection of AGS gastric epithelial cells with *H. pylori* in vitro results in migration of the cells and an elongated morphology, known as the "hummingbird phenotype" (Segal et al. 1999). This phenotype requires host cell motility by a yet unknown T4SS factor (Churin et al. 2003), while cell elongation is clearly induced by CagA^{PY} (Backert et al. 2001). Transfection experiments have shown that CagA^{PY}-SHP-2 interaction activates the phosphatase activity of SHP-2, which contributes to AGS cell elongation by stimulating the Rap1 → B-Raf → Erk signaling pathway (Higashi et al. 2004). It was also shown that the CagA^{PY}-SHP-2 complex suppresses the activation of epidermal growth factor receptor (EGFR) and downstream signaling, which is associated with enhanced *H. pylori* survival (Bauer et al. 2012). The authors found that an antimicrobial peptide, human β-defensin 3 (hBD3), which is highly active against *H. pylori*, is downregulated by EGFR suppression. These findings revealed a novel mechanism how T4SS-positive strains make use of CagA to evade a key innate mucosal defense pathway to support persistent *H. pylori* infection (Bauer et al. 2012).

Additional reports demonstrated that the elongation phenotype also requires tyrosine dephosphorylation of three well-known actin-binding proteins—vinculin, ezrin, and cortactin (Selbach et al. 2003, 2004; Moese et al. 2007). The phosphatase involved in this scenario is not SHP-2 and still remains to be identified. Instead it was shown that CagA^{PY} can hamper c-Src activity in two ways, by direct binding of both proteins to each other and by interaction of CagA^{PY} with Csk, a negative regulator of c-Src (Tsutsumi et al. 2003; Selbach et al. 2003). Since c-Src is the first kinase phosphorylating CagA, inactivation of c-Src by CagA^{PY} forms a typical negative feedback-loop mechanism, controlling the amount of intracellular CagA^{PY}. Interestingly, vinculin, ezrin, and cortactin are also targets of c-Src, and c-Src inactivation by CagA^{PY} causes the tyrosine dephosphorylation of these factors (Tegtmeyer and Backert 2011). Moreover, binding of CagA^{PY} to PI3K and/or CrkII is involved in activating the small Rho family GTPase members Rac1 and Cdc42 (Suzuki et al. 2005; Selbach et al. 2009), while binding of CagA^{PY} to SHP-2 or Grb2 can stimulate pro-inflammatory and proliferative responses through the mitogen-activated protein (MAP) kinase cascades (Fig. 4.2a). Finally, CagA^{PY} can also interact with SHP-1, Grb7, and Ras-GAP with yet unknown consequences for the host cell (Fig. 4.2a). Taken together, CagA^{PY} can bind to a remarkably high number of host cell factors to activate signaling mediating cell scattering, elongation, and probably other phenotypes.

Translocation and phosphorylation of CagA appear also important for the interplay of *H. pylori* with macrophages. Heme oxygenase (HO-1), an anti-inflammatory enzyme, is released by macrophages in response to CagA phosphorylation in *H. pylori*-infected human patients and mice, while blocking of phagocytosis prevented CagA^{PY} and HO-1 induction (Gobert et al. 2014). Genetic ablation

of the *hmox-1* gene in mice resulted in increased gastritis, which was associated with enhanced M1/Th1/Th17 responses, reduced regulatory macrophage response, as well as lower *H. pylori* colonization. These findings provide a mechanism by which *H. pylori* manipulate immune responses, supporting its own survival by induction of macrophage HO-1 (Gobert et al. 2014).

4.6 Phosphorylation-Independent Signaling of CagA

Early microarray studies of *H. pylori*-infected T84 cells showed that expression of 670 host genes changed and 479 of these genes occurred independent of the phosphorylation state of the CagA protein (El-Etr et al. 2004). Thus, not all the interactions of intracellular CagA require its tyrosine phosphorylation. Subsequently, 12 cellular binding partners of non-phosphorylated CagA have been reported (Table 4.2). Non-phospho CagA interactions have been found to induce loss of cell polarity, mitogenic responses, and pro-inflammatory signaling (Fig. 4.2b). The first interaction partner of non-phosphorylated CagA described has been the adapter protein Grb2 (Mimuro et al. 2002). Interestingly, Grb2 is the only binding factor that has been described to interact with both non-phosphorylated and phosphorylated CagA (Mimuro et al. 2002; Selbach et al. 2009). In particular, non-phosphorylated CagA was shown to utilize Grb2 for recruiting Grb2-associated Sos (son of sevenless), a guanine-exchange factor (GEF) of the small GTPase Ras, to the plasma membrane (Fig. 4.2b). This CagA/Grb2/Sos complex triggers Ras-GTP production, which in turn activates the Raf → MEK → ERK signaling pathway contributing to cell scattering (Mimuro et al. 2002) and stimulation of nuclear responses mediating cell proliferation and transcription of the anti-apoptotic myeloid cell leukemia sequence-1 (MCL-1) protein (Mimuro et al. 2007). CagA was also described to function as a mimetic of the eukaryotic Grb2-associated binder (Gab) adaptor protein in transgenic Drosophila which feeds into the same MAP kinase signaling pathway (Hatakeyama 2003; Botham et al. 2008). CagA-triggered MAP kinase activation can stimulate the transcription factor NF-κB mediating the onset of multiple target genes such as IL-8 (Brandt et al. 2005). In addition, it was shown that CagA targets various tumor suppressors such as p53 and RUNX3. CagA-positive *H. pylori* strains more strongly suppressed p53 as compared with low-risk strains during infection in vivo and in vitro. Degradation of p53 protein was shown to be induced by CagA-mediated signaling via host-specific E3 ubiquitin ligases, thus contributing in tumorigenicity (Wei et al. 2015). In addition, non-phosphorylated CagA can bind to RUNX3, which is often inactivated in gastric cancer. Interaction with RUNX3 proceeds by a WW domain located at the amino-terminus of CagA (Tsang et al. 2010). Importantly, CagA induces the ubiquitination and subsequent degradation of RUNX3, in this way turning off the transcriptional activity of RUNX3 (Fig. 4.2b). These data

Table 4.2 Host cell proteins described to interact with non-phosphorylated CagA and proposed roles in *H. pylori* infections

Interaction partner	Proposed function	Experimental evidence	Applied methods[a]	References
α-Pix [b]	Inflammation	Infection	coIP, MS	Baek et al. (2007)
ASPP2	Activation of ASPP2 and p53 degradation, apoptosis inhibition	Proteomics	LC-MS/MS, coIP, IF, Y2H	Buti et al. (2011), Nešić et al. (2014)
β1-integrin	Internalization of CagA	Infection	Y2H, coIP, Biacore studies	Jiménez-Soto et al. (2009)
CagA	Multimerization	Transfection of CagA	coIP, mutagenesis	Ren et al. (2006)
Calcineurin	Dephosphorylation of NFAT, nuclear translocation	Transfection of CagA	Coexpression, IF	Yokoyama et al. (2005)
c-Met [c]	Cell scattering, activation of PI3-kinase, Erk, β-catenin, and NF-κB [d]	Infection, transfection of CagA	coIP, IF	Churin et al. (2003), Suzuki et al. (2009)
E-cadherin	Destabilization of AJs, β-catenin signaling	Transfection of CagA	coIP	Murata-Kamiya et al. (2007)
GSK-3	Snail-mediated EMT trough GSK-3 depletion	Infection, transfection of cagA	IF, coIP, immunoblot	Lee et al. (2014)
p120-catenin	E-cadherin/c-Met/ p120 complex, cell scattering, invasion	Infection	IF, coIP, cell invasion assay, immunoblot	Oliveira et al. (2006, 2009)
Grb2 [e]	Cell scattering, activation of Ras-MAPK signaling	Transfection of CagA	PD, coIP, IF	Mimuro et al. (2002)
Par1b/ MARK2	Inhibition of Par1 activity, disruption of apical junctions	Transfection of CagA	coIP, IF, MF	Saadat et al. (2007), Zeaiter et al. (2008)
Par1b	Inhibition of mitosis	Transfection of CagA	IF, FACS	Umeda et al. (2009)
Par1a, Par1c, Par1d	Enhancement of cell elongation	Transfection of CagA	coIP, IF	Lu et al. (2009)
Par1b	Inhibition of Par1	3D structure	Crystallization, binding, and kinase assays	Nesić et al. (2010)
PLC-γ [f]	Cell scattering	Infection	coIP	Churin et al. (2003)

(continued)

Table 4.2 (continued)

Interaction partner	Proposed function	Experimental evidence	Applied methods[a]	References
RUNX3	Oncogene by blocking tumor suppressor RUNX3	Infection, transfection of CagA	Mutagenesis, coIP	Tsang et al. (2010), Liu et al. (2012)
TAK1[g]	Activation of NF-κB	Transfection of CagA, infection	EMSA, qPCR, coIP, PD, IF	Lamb et al. (2009)
ZO-1, JAM	Disruption of lateral junctions and cell motility	Infection, transfection of CagA	IF, MF	Amieva et al. (2003),
				Bagnoli et al. (2005)

[a]Abbreviations: *AJs* adherens junctions, *EMSA* electrophoretic mobility-shift assay, *FACS* fluorescence-activated cell sorting, *IF* co-localization by immunofluorescence, *coIP* co-immunoprecipitation, *EMT* epithelial-mesenchymal transition, *MF* membrane fractionation by Iodixanol Gradients, Opti-Prep™, *MS* mass spectrometry of bound proteins, *PD* pull-down experiments, *qPCR* quantitative real-time polymerase chain reaction, *Y2H* yeast two-hybrid screen
[b]Dependency of $CagA^{PY}$ was not investigated but α-PIX does not contain an SH2 domain and is therefore unlikely to interact with CagA in a phospho-specific manner
[c]Activation of c-Met was not observed in another study and attributed to cross-reactivity of the phospho-c-Met antibody with $CagA^{PY}$ (Snider and Cardelli 2009)
[d]Activation of CagA-induced NF-κB activation and IL-8 secretion was not observed when CagA was co-transfected with dominant-negative c-Met in AGS cells (Brandt et al. 2005)
[e]An interaction of Grb2 with non-phosphorylated CagA (Mimuro et al. 2002) or $CagA^{PY}$ (Selbach et al. 2009) was reported during *Hp* infection; but none of the latter interactions were found in studies using transfected CagA (Tsutsumi et al. 2003; Churin et al. 2003)
[f]PLC-γ coIP revealed a strong background signal for non-injected CagA in a translocation-defective Δ*virb11* mutant
[g]An interaction of CagA with TAK1 was excluded in another study showing that anti-TAK1 antibodies artificially precipitate CagA from a T4SS-defective strain (Sokolova et al. 2014)

together suggest the presence of distinct EPIYA-independent domains within CagA, which have crucial functions in protein targeting and modification of host cell transcription.

Another remarkable outcome of phosphorylation-independent CagA activities in polarized epithelial cells is the disturbance of the cell-to-cell integrity (Fig. 4.2b). In particular, the proper architecture of the gastric epithelium is controlled by highly organized tight and adherent junctions (Wessler and Backert 2008). Infection and transfection experiments showed that CagA interferes with these intercellular junctions in multiple ways. For example, CagA associates with ZO-1 (zona occludens-1), a tight-junction scaffolding protein and the transmembrane protein JAM (junctional adhesion molecule), causing an ectopic assembly of tight-junction components at sites of bacterial attachment (Amieva et al. 2003). The non-

phosphorylated form of CagA has also been reported to bind to the cell-cell junctional transmembrane protein E-cadherin (Murata-Kamiya et al. 2007). Later on, immunoprecipitation studies showed that CagA forms a complex with c-Met recruiting E-cadherin and the armadillo-domain protein p120 catenin, suggesting that binding of CagA to E-cadherin is probably not direct (Oliveira et al. 2009). However, there is much debate going on whether or not the 135-kDa c-Met receptor is phosphorylated and activated upon infection with *H. pylori* (Snider and Cardelli 2009). Thus, the function of c-Met signaling during *H. pylori* infection is not fully clarified and should be investigated more thoroughly in future. Some controversy also exists whether CagA can disrupt the E-cadherin complex associated with the release of β-catenin, which has been proposed for transfected CagA or *H. pylori*-infected AGS cells (Franco et al. 2005; Murata-Kamiya et al. 2007). It has been noted by some authors that AGS cells do not express E-cadherin and exhibit abnormal β-catenin allocation, making them not suitable for the study of related signaling (Oliveira et al. 2009). Considering this fact, it was shown, using MDCK cells expressing wild-type E-cadherin and β-catenin without any mutations, that *H. pylori*-induced β-catenin signal transduction proceeds independently of CagA during infection (Sokolova et al. 2008). Similarly, some controversy also exists with regard to the proposed interaction of CagA with transforming growth factor beta-activated kinase 1 (TAK1) (Lamb et al. 2009; Sokolova et al. 2014) (Table 4.2).

However, the importance of CagA in inducing the loss of cell polarity is much clearer. The host kinase Par1b (partitioning-defective 1), also called MARK2 (microtubule affinity-regulating kinase), is a central regulator of cell polarity and was reported to have a crucial impact on *H. pylori*-induced signal transduction. Non-phosphorylated CagA can directly bind Par1b that results in the inhibition of its kinase activity, triggering the loss of cell polarity (Saadat et al. 2007; Nesić et al. 2010). Furthermore, more recent studies showed that CagA not only binds to Par1b but also to other members of this kinase family (Par1a, Par1c, and Par1d) and that these interactions contribute to the *H. pylori*-triggered AGS cell elongation phenotype (Lu et al. 2009). Recent data also suggest that CagA can also interact with glycogen synthase kinase 3 (GSK-3) and acts as a pathogenic scaffold protein that induces a Snail-mediated epithelial-mesenchymal transition via the depletion of GSK-3 activity (Lee et al. 2014). Taken together, these results suggest that transfected CagA can interfere with Par1 members, c-Met, GSK-3, and E-cadherin signaling and may also activate NF-κB, thereby contributing to *H. pylori*-induced pro-inflammatory responses. Finally, there are two more reported binding partners of CagA, α-Pix (Baek et al. 2007), and integrin β_1 (Jiménez-Soto et al. 2009; Kaplan-Türköz et al. 2012). While the interaction with α-Pix has a proposed role in inflammation, CagA-integrin β_1 interaction may be involved in delivery of CagA into the host cell (Fig. 4.2b). Importantly, the downstream pathways of CagA emerged to be highly diverse and possible cross talk among them and other bacterial factors need to be dissected in more detail.

4.7 T4SS-Dependent but CagA-Independent Cellular Signaling Induced by *H. pylori*

In this section, various T4SS-dependent but CagA-independent signaling events induced by *H. pylori* will be discussed (Fig. 4.3). Early studies indicated that *H. pylori* can actively inhibit its own uptake and killing by professional phagocytes (Ramarao et al. 2000). This anti-phagocytic effect was dependent on vital bacteria expressing the T4SS, because various isogenic *virB* mutants blocked this phenotype (Ramarao et al. 2000). Interestingly, the actual factor involved was not CagA, because isogenic Δ*cag*A mutants also abrogated phagocytosis. These experiments indicated that *H. pylori* express a yet unknown T4SS effector with anti-phagocytic capability that may play a crucial function in the immune escape of this persistent pathogen (Fig. 4.3). Infection of bone-marrow-derived macrophages by *H. pylori*-induced pathology via microRNAs, such as miR-155, as important regulators of inflammatory and innate immune responses. Increase of miR155 expression was T4SS-dependent but CagA-independent and resulted in reduced macrophage apoptosis (Koch et al. 2012). However, most of the studies were performed to

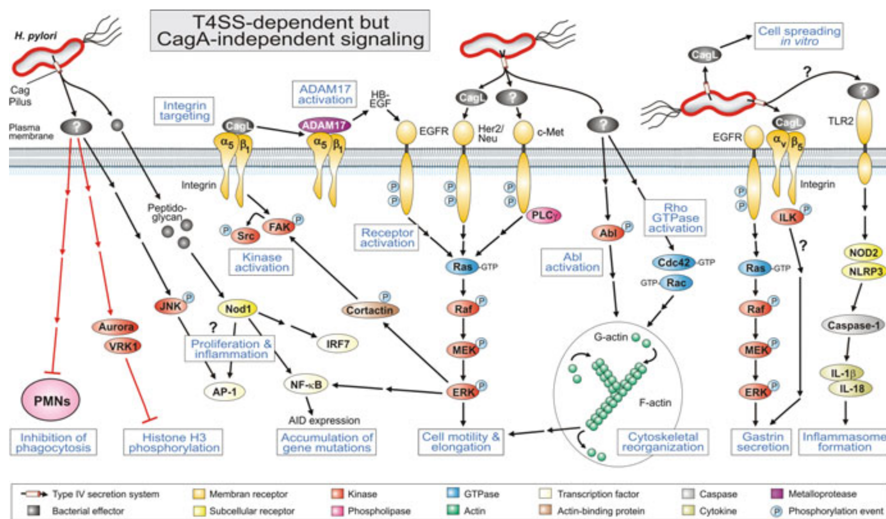

Fig. 4.3 Model for the role of *H. pylori* T4SS-dependent but CagA-independent host cell-signaling processes which may affect pathogenesis. The multitude of known T4SS-dependent but CagA-independent pathways involve in the activation of receptor and non-receptor tyrosine kinases, pro-inflammatory signaling, Rho GTPase activation, scattering and motility of gastric epithelial cells, as well as suppression of histone phosphorylation and *H. pylori* phagocytosis by immune cells. Two particular T4SS factors have been reported to be involved in some but not all of these responses. The known signaling functions for injected peptidoglycan as well as pilus-exposed or recombinant CagL are shown. For numerous other pathways, the actual T4SS factor is yet unknown as also indicated. (For more details see text. This figure was updated from Backert and coworkers (2010) with kind permission from Wiley.)

investigate the interaction of *H. pylori* with gastric epithelial cells. Phosphoproteomics of epithelial cells infected with *H. pylori* revealed the induction of multiple tyrosine-phosphorylated proteins. The majority of enriched phosphopeptides were from kinases of the MAPK family and the use of isogenic mutants showed that both CagA and the T4SS are key regulators of tyrosine phosphorylation events (Glowinski et al. 2014). In addition, histone H3 phosphorylation was found to be altered by a T4SS-dependent but CagA-independent pathway (Fig. 4.3). Infection with *cag*PAI-positive *H. pylori* strains decreased H3 phosphorylation levels at two phosphorylation sites, serine residue 10 and threonine residue 3 (Fehri et al. 2009; Ding et al. 2010). It appeared that mitotic histone H3 kinases such as Aurora B and vaccinia-related kinase 1 (VRK1) were not fully activated in *H. pylori*-infected cells, leading to a transient pre-mitotic cell cycle arrest (Fehri et al. 2009). Together, these data demonstrate that *H. pylori* subvert cellular key processes, including cell cycle progression, by a not yet identified T4SS effector. Furthermore, the results of numerous reports revealed that other components of the T4SS, but not CagA itself, were necessary for the induction of pro-inflammatory signaling, including the activation of transcription factors NF-κB and AP-1 (Fig. 4.3). This implicated that the T4SS might deliver effectors in addition to CagA or that the T4SS itself triggers the effect. Despite systematic mutagenesis of all *cag*PAI genes and other efforts, the hypothetical additional effector remained unknown for many years. One proposed candidate was bacterial peptidoglycan, because it can be identified by Nod1, an intracellular pathogen-recognition molecule (Viala et al. 2004). These results implicated that T4SS-dependent delivery of peptidoglycan is responsible for activation of Nod1 → NF-κB-dependent pro-inflammatory responses such as secretion of IL-8 (Viala et al. 2004). However, the actual bacterial T4SS factor(s) and pathways that activate both transcription factors, NF-κB and AP-1, are highly controversial in the literature and still not fully defined (Backert and Naumann 2010). Remarkably, T4SS-positive *H. pylori* can activate the NF-κB-dependent induction of a DNA-editing enzyme (AID) in gastric epithelial cells, that leads to the accumulation of mutations in tumor suppressor p53 (Matsumoto et al. 2007). Thus, induction of AID by *H. pylori* infection might be a mechanism whereby gastric carcinogenesis-related gene mutations accumulate.

Infection of gastric epithelial cells with *H. pylori* was also reported to profoundly activate various receptor tyrosine kinases (RTKs) in a T4SS-dependent manner including EGFR (Keates et al. 2001; Churin et al. 2003), hepatocyte growth factor receptor c-Met, and Her2/Neu (Churin et al. 2003). Studies on the downstream signaling indicated that each of these RTKs can activate the MAP kinase members MEK and ERK1/2 (Fig. 4.3). However, while induction of EGFR has been shown to induce pro-inflammatory responses leading to the secretion of IL-8 (Keates et al. 2001), activation of c-Met (but not EGFR or Her2/Neu) was involved in cell scattering and motogenic responses of infected gastric epithelial cells (Churin et al. 2003). At later time points of infection, EGFR can be inactivated by CagA as mentioned above (Bauer et al. 2012). Interestingly, the small Rho GTPases Rac1

and Cdc42 and the non-receptor tyrosine kinase c-Abl are also activated by a T4SS-dependent but CagA-independent process and play a role in stimulating scattering and motility of infected gastric epithelial cells (Fig. 4.3). However, the actual T4SS factor involved for many of the above events is still unknown.

In vitro studies showed a profound role of recombinant CagL in activating the host tyrosine kinases EGFR, ErbB3/Her3, FAK, and c-Src (Tegtmeyer et al. 2010). Investigation on the molecular mechanism of EGFR activation by CagL has demonstrated the involvement of ADAM17, a metalloprotease implicated in catalyzing ectodomain shedding of receptor tyrosine kinase ligands. In non-stimulated cells, the inactive form of ADAM17 forms a complex with integrin $\alpha_5\beta_1$ (Fig. 4.3). During acute *H. pylori* infection, however, it was demonstrated that CagL binding to integrin $\alpha_5\beta_1$ activates ADAM17 by dissociating ADAM17 from the complex (Saha et al. 2010). In addition, CagL immobilized on petri dishes binds host cells and, thus, mimics human fibronectin (Tegtmeyer et al. 2010). Fibronectin is a 250-kDa protein containing an RGD motif that plays crucial roles in promoting cell adhesion, migration, and intracellular signaling. It was shown that purified CagL alone can directly trigger intracellular signaling pathways upon contact with mammalian cells and can even complement the spreading defect of fibronectin$^{-/-}$ knockout cells in vitro (Tegtmeyer et al. 2010). Treatment of AGS cells with purified CagL was also demonstrated to be sufficient to result in IL-8 induction, which required the RGD motif in CagL and activation of integrin $\alpha_5\beta_1$ (Gorrell et al. 2012). Using different wild-type or Δ*cagL* mutant strains showed that IL-8 induction occurred independently of CagA translocation. Further studies revealed another surface-exposed Phe-Glu-Ala-Asn-Glu (FEANE) interaction motif located close to the RGD site. This site enhanced the interaction of CagL with integrin $\alpha_5\beta_1$ supporting CagA translocation and was referred to as RGD helper sequence, RHS (Conradi et al. 2012). CagL was also shown to be related to increased gastrin expression resulting in hypergastrinemia, a major risk factor for gastric adenocarcinoma formation. Gastric epithelial cells stably transfected with a human gastrin promoter luciferase construct increased the promoter activation of gastrin via integrin-linked kinase (ILK) and integrin $\alpha_V\beta_5$ (Wiedemann et al. 2012). Another role of CagL was seen in bone-marrow derived DCs, where it resulted in induction of pro-IL-1β and formation of mature IL-1β and NLRP3 (NOD-like receptor pyrin domain-containing 3) induction in response to *H. pylori* infection. This activity was mediated via different host innate immune receptors including Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain 2 (NOD2) (Kim et al. 2013). CagL and the activity of MAP kinases were also important for *H. pylori*-mediated regulation of eosinophil migration (Nagy et al. 2011). The results indicate that *H. pylori* increases production of the chemokines CCL2, CCL5, and granulocyte-macrophage colony-stimulating factor (GMC-SF) by gastric epithelial cells and that these molecules induce eosinophil migration. Interestingly, CagL sequence analyses revealed that isolates from the gastric cancer patients had a higher rate of amino acid sequence polymorphisms—Y58 and

E59—than those of the non-gastric cancer patients (Yeh et al. 2011). The CagL Y58E59 polymorphism increased risk of gastric cancer up to 4.6-fold and infected patients had higher integrin $\alpha_5\beta_1$ expression than noninfected patients. Furthermore, CagL-Y58E59 *H. pylori* infection predisposed an upward shift in integrin $\alpha_5\beta_1$ in the corpus, leading to more severe corpus chronic inflammation (Yeh et al. 2011). However, expression of isogenic CagL Y58/E59 variants in *H. pylori* strain 26695 significantly blocked translocation and phosphorylation of CagA as compared to complemented wild-type CagL (Tegtmeyer et al. 2014). The involved signaling should be studied in detail in future studies.

4.8 Conclusions

H. pylori represents a highly successful human pathogen, which can trigger severe clinical symptoms in a small subset of patients. The investigation of bacteria-host interactions and virulence factors such as CagA and the T4SS has provided us with crucial insights in mechanisms leading to *H. pylori* pathogenesis. A list of more than 20 known cellular interaction partners of CagA is quite amazing for a bacterial effector protein. The current model suggests that CagA mimics a eukaryotic signaling factor either located in a large multiprotein complex or simultaneously in various subcellular areas of infected host cells. The large variety of binding partners also reflects the integrated network of complex signal transduction pathways in target cells, which may have important impact on the multi-step pathogenesis of *H. pylori*. In the future, it will be important to search for additional translocated effector molecules of the *cag*PAI T4SS and various other T4SSs present in the *H. pylori* chromosome (Backert et al. 2015). Finally, the importance of CagA for *H. pylori* itself is also not yet clear. Using a polarized epithelium model system, Δ*cagA* mutants were shown to be defective in cell surface colonization, but exogenous addition of iron to the apical medium partially rescues this defect, suggesting that one of CagA's effects on host cells is to facilitate iron acquisition from the host via a mechanism involving the transferrin receptor (Tan et al. 2011). To test whether CagA is important in promoting iron acquisition in vivo, the colonization of *H. pylori* in iron-replete *vs*. iron-deficient Mongolian gerbils was carried out. While wild-type *H. pylori* and Δ*cagA* mutants colonized iron-replete gerbils at similar levels, Δ*cagA* mutants are markedly impaired in colonizing iron-deficient gerbils (Tan et al. 2011). Iron depletion accelerated the development of *H. pylori*-induced premalignant and malignant lesions in a CagA-dependent manner (Noto et al. 2013). *H. pylori* strains harvested from iron-depleted gerbils or grown under iron-limiting conditions exhibited enhanced virulence and induction of inflammatory factors. Future studies should investigate the molecular basis of this important disease-associated phenomenon.

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Chapter 5
Helicobacter pylori Vacuolating Toxin

Timothy L. Cover, Robin L. Holland, and Steven R. Blanke

Abstract The vacuolating cytotoxin (VacA) was one of the first *H. pylori* virulence factors identified. All *H. pylori* strains contain a *vacA* gene, but there is variation among *H. pylori* strains in the levels of VacA secretion and activity of VacA proteins. Strains containing allelic types of *vacA* that produce more active forms of the toxin are associated with human gastroduodenal disease. Experiments in animal models suggest that VacA may contribute to *H. pylori* colonization of the stomach, and the toxin has been linked to gastric epithelial damage. VacA induces a variety of effects in cultured epithelial cells, including alterations in membrane trafficking within the endolysosomal system, mitochondrial dysfunction, and cell death. Most VacA effects on cells are a consequence of intracellular toxin activities. Unlike most intracellular-acting bacterial toxins that enzymatically modify target molecules within eukaryotic cells, VacA causes cellular alterations primarily through the formation of intracellular ion-conducting channels. In this chapter, we review the structure and function of VacA, along with the roles of VacA in the pathogenesis of *H. pylori*-associated diseases.

5.1 Introduction

Helicobacter pylori colonizes the human stomach, and persistent infection is a risk factor for peptic ulcer disease and gastric cancer. The development of these diseases is dependent on the actions of specific *H. pylori* virulence factors and is also

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influenced by host and environmental factors. One of the important *H. pylori* virulence factors is the vacuolating cytotoxin (VacA). This toxin was originally identified based on its ability to cause vacuolation of cultured host cells (Leunk et al. 1988; Cover and Blaser 1992), but is now known to have many additional activities. In this chapter, we discuss progress in our understanding of the structure-function relationships that underlie the actions of VacA, the unusual manner by which VacA is taken up into and trafficked within host cells, and the ability of VacA to form ion-conducting channels in the membranes of intracellular organelles. In addition, we discuss the role of VacA in the pathogenesis of *H. pylori*-associated gastric disease.

5.2 The *vacA* Gene *vacA* Transcription

A single chromosomal copy of *vacA* is present in all sequenced *H. pylori* strains (Cover et al. 1994; Telford et al. 1994; Schmitt and Haas 1994). *VacA* orthologues are present in *H. cetorum* (a gastric Helicobacter found in dolphins and whales) (Kersulyte et al. 2013), and a degenerated *vacA* gene has been detected in *H. acinonychis* (found in large cats) (Dailidiene et al. 2004). The *vacA* transcript is monocistronic, with a transcriptional start point about 120 nucleotides upstream from the ATG start site (Schmitt and Haas 1994; Forsyth et al. 1998). There is considerable variation in *vacA* transcription levels among *H. pylori* strains (Forsyth et al. 1998).

5.3 Secretion and Proteolytic Processing of VacA

In *H. pylori* strain 60190, *vacA* encodes a 1287 amino acid (140 kDa) protoxin (Cover et al. 1994), which undergoes distinct proteolytic processing steps (Fig. 5.1). First, the 33 amino acid amino-terminal signal sequence is cleaved, presumably as the toxin is transported across the cytoplasmic membrane into the periplasmic space. Further processing results in an 88 kDa mature secreted form of the toxin, a 12 kDa secreted peptide, and a ~33 kDa carboxyl-terminal domain that remains associated with the bacterial cell (Cover and Blaser 1992; Nguyen et al. 2001; Bumann et al. 2002; Telford et al. 1994). The process of VacA secretion is thought to occur by a type V (or autotransporter) secretion pathway, and the secreted 88 kDa toxin is considered to be the "passenger domain" (Fischer et al. 2001). Analogous to other autotransporter proteins, the VacA carboxyl-terminal domain is predicted to have a β-barrel structure and is required for secretion of the VacA passenger domain (Schmitt and Haas 1994).

The 88 kDa VacA toxin is released from the bacteria into the extracellular space as a soluble protein (Cover and Blaser 1992) or as a component of membrane blebs (Fiocca et al. 1999). VacA can also remain on the surface of *H. pylori*, spatially

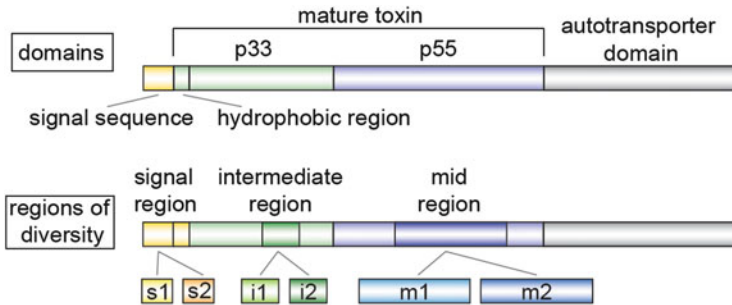

Fig. 5.1 VacA structure. Proteolytic cleavage of the VacA protoxin yields a signal peptide, an 88 kDa secreted protein, and the autotransporter domain. The secreted 88 kDa protein is composed of two domains (p33 and p55), and the autotransporter domain is processed to yield a 12 kDa secreted peptide and a 33 kDa cell-associated protein. Three main regions of *vacA* diversity are currently recognized: the signal region (s1 and s2 alleles), intermediate region (i1 and i2), and mid-region (m1 and m2 alleles)

organized into distinct toxin-rich domains (Ilver et al. 2004), and found predominantly in a unipolar location (nonflagellar pole) (Radin et al. 2013). The mechanisms that govern release of VacA as a soluble protein or retention on the bacterial surface have not been elucidated. Surface-bound VacA may promote *H. pylori* adherence to gastric epithelial cells, and upon bacterial contact with host cells, VacA can be transferred directly to host cells (Ilver et al. 2004).

5.4 Properties of the 88 kDa Secreted VacA Protein

The secreted 88 kDa toxin can undergo limited proteolysis to yield two fragments, designated as p33 and p55 (Telford et al. 1994; Willhite et al. 2002; Ye et al. 1999; Nguyen et al. 2001; Torres et al. 2005) (Fig. 5.1). The p55 domain has a predominantly β-helical structure (Gangwer et al. 2007), which is a feature shared by many bacterial autotransporter proteins. The structure of the p33 domain has not been determined, but it contains a strongly hydrophobic region near the amino-terminus that is required for membrane channel formation and many of the toxin's cell-modulating effects (see Sect. 6) (de Bernard et al. 1998; Ye and Blanke 2000; Vinion-Dubiel et al. 1999; McClain et al. 2003).

Neither p33 nor p55 alone has detectable effects on eukaryotic cells. In contrast, a mixture of p33 and p55, when added exogenously to host cells or co-expressed intracellularly within cells, can reconstitute VacA cellular activity (Ye et al. 1999; Gonzalez-Rivera et al. 2010). Both the p55 domain and the p33 domain have important roles in binding of VacA to host cells (see Sect. 7) (Garner and Cover 1996; Wang et al. 2001; Reyrat et al. 1999; Pagliaccia et al. 1998; Torres et al. 2005). About 422 amino acids at the amino-terminal end of VacA (which includes the p33 domain and about 100 amino acids of the p55 domain) are

sufficient to induce cell vacuolation if VacA is expressed intracellularly in transiently transfected cells (Ye et al. 1999; de Bernard et al. 1998).

VacA monomers can assemble into large water-soluble oligomeric structures (Cover and Blaser 1992). Multiple types of oligomeric VacA structures have been visualized, including single layered forms (containing 6–9 subunits), bilayered forms (containing 12 or 14 subunits), and two-dimensional crystals (Lupetti et al. 1996; Cover et al. 1997; Adrian et al. 2002; Czajkowsky et al. 1999; Chambers et al. 2013). When exposed to either acidic or alkaline pH, VacA oligomers dissociate into monomeric components, which can reassemble into oligomeric structures following neutralization (Cover et al. 1997; Yahiro et al. 1999). VacA oligomers have relatively little effect on cultured human cells in vitro, whereas the monomers generated by acidification or alkalinization of oligomers are highly active (de Bernard et al. 1995). As discussed further in Sect. 6, the water-soluble oligomeric structures are likely to be structurally similar to membrane pores formed by VacA.

5.5 *VacA* Allelic Diversity and Association of *vacA* Genotypes with Disease

The activity of VacA in culture filtrates from different *H. pylori* strains is highly variable (Leunk et al. 1988; Cover and Blaser 1992), due to nonsense mutations, internal duplications, deletions, or 1-bp insertions within the *vacA* gene (Ito et al. 1998), as well as amino acid sequence variation (Atherton et al. 1995). Variation among strains in the levels of vacuolating activity in culture filtrates has also been linked to differences in *vacA* transcription or differences in VacA secretion efficiency (Forsyth et al. 1998). Several allelic families of *vacA* have been described, based on nucleotide sequence variation in specific regions of *vacA* (Fig. 5.1). The emergence of sequence variation may be due to a strong selective pressure on *vacA* as *H. pylori* colonizes and adapts to new hosts and different host environments (Gangwer et al. 2010).

The "m (for middle) region" of *vacA* is about 800 nucleotides in length and encodes part of the p55 domain (Atherton et al. 1995). Two main families of *vacA* alleles, designated as m1 and m2, can be differentiated based on diversity within the m-region (Atherton et al. 1995). Type m1 and m2 forms of VacA differ in ability to cause alterations in specific cell types, due at least in part to distinct cell-binding properties (Pagliaccia et al. 1998; Wang et al. 2001). The VacA determinants that influence the cell-type specificities of m1 and m2 forms of VacA have been mapped to a 148-residue region within p55 (Skibinski et al. 2006).

Sequence diversity also exists within the "s-region," which encodes the signal sequence and the amino-terminus of the processed mature toxin. Two major allelic clusters in this region are designated as s1 and s2 (Atherton et al. 1995). In contrast to s1 forms of VacA, s2 forms of VacA fail to induce cellular vacuolation (Atherton

et al. 1995). The signal sequences of s1 and s2 VacA proteins are processed at different sites, resulting in a mature form of s2 VacA with a 12 amino acid extension that inactivates the toxin (Letley et al. 2003; McClain et al. 2001).

A third approach for classifying *vacA* alleles is based on diversity within a region between the s- and m-regions (encoding part of the p33 domain), known as the "i (for intermediate) region." Two main families of alleles, designated as i1 and i2, are recognized (Rhead et al. 2007; Basso et al. 2008). Type i1 VacA proteins typically exhibit increased activity in gastric epithelial or T-cell culture assays compared to type i2 forms of VacA (Rhead et al. 2007; Gonzalez-Rivera et al. 2012).

Epidemiologic studies have revealed strong associations between specific *vacA* i-, m-, and s-region allelic types and the occurrence of disease in *H. pylori*-infected humans. There is a higher incidence of peptic ulcer disease and gastric cancer in individuals infected with *H. pylori* strains possessing s1 *vacA* alleles than in persons infected with strains harboring s2 *vacA* alleles (Fig. 5.2) (Atherton et al. 1995, 1997; van Doorn et al. 1998; Figueiredo et al. 2002). The demonstration that s2 forms of VacA lack vacuolating toxin activity in cell culture assays (Atherton et al. 1995; McClain et al. 2001; Letley et al. 2003) provides a possible functional explanation for these epidemiologic observations. Several studies have reported that, in comparison to strains harboring m2 *vacA* alleles, strains harboring m1 *vacA* alleles are associated with a higher risk of gastric carcinoma (as well as gastric

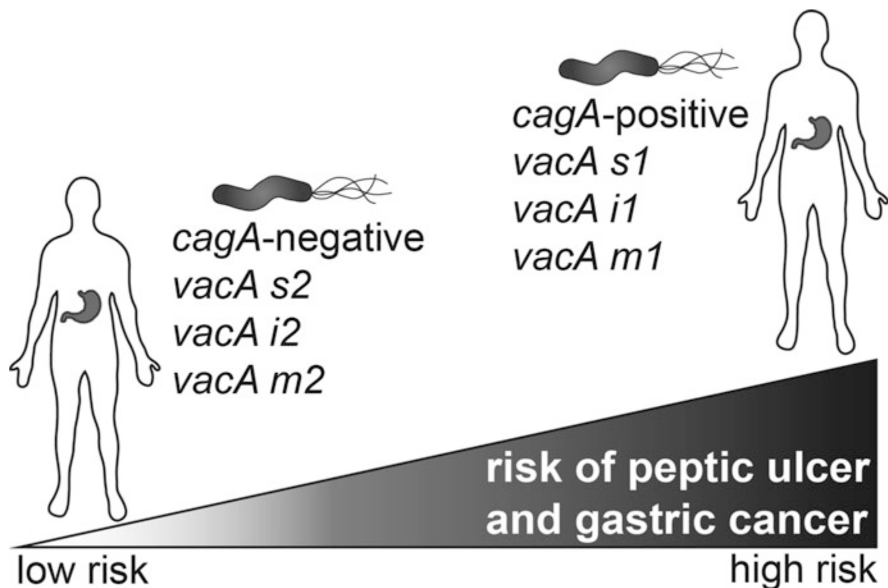

Fig. 5.2 Role of VacA in *H. pylori*-associated gastric disease. Humans harboring *H. pylori* strains that contain *cagA* and s1, i1, or m1 *vacA* alleles are at a greater risk for gastric disease, compared to humans harboring *H. pylori* strains that are *cagA* negative and contain s2, i2, or m2 *vacA* alleles

histologic precursors of gastric cancer, such as epithelial damage, atrophic gastritis, and intestinal metaplasia) (Figueiredo et al. 2002; Atherton et al. 1997). Strains carrying s1/m1 *vacA* alleles are associated with increased bacterial load and neutrophil infiltration within the human gastric mucosa (van Doorn et al. 1998; Atherton et al. 1997; Figueiredo et al. 2002). The i-region has been reported to be a more reliable predictor of severe gastric disease than the s- or m-regions of *vacA* (Rhead et al. 2007; Basso et al. 2008; Winter et al. 2014).

Interpreting the results of epidemiologic studies can be complicated by confounding factors, including the effects of acid-suppressive agents, aspirin or other nonsteroidal anti-inflammatory agents, and the gender or age of patients. Further challenges arise due to difficulty in culturing *H. pylori* strains from individuals with gastric cancer. Evaluating an association between *vacA* alleles and disease risk can also be complicated by the existence of other strain-specific bacterial factors that contribute to disease. One of the best understood examples is a chromosomal region known as the *cag* pathogenicity island (*cag* PAI), which encodes the CagA effector protein and a type IV secretion system (T4SS). The *cag* PAI can be either present or absent in individual *H. pylori* strains. *H. pylori* isolates associated with high disease risk frequently harbor a functional *cag* PAI and produce the most active forms of VacA (Atherton et al. 1995; van Doorn et al. 1998), making it difficult to assess the independent contributions of VacA and the *cag* PAI. In addition to the *cag* PAI, several other genetic markers of virulence, including an adhesin known as BabA, in-frame *oipA* (*hopH*) alleles, and type I *hopQ* alleles, are present in *H. pylori* strains containing type s1 *vacA* alleles more frequently than in *H. pylori* strains containing type s2 *vacA* alleles (Atherton et al. 1995; van Doorn et al. 1998). For more details on the latter factors, please refer to Chaps. 3, 4 and 6.

5.6 Membrane Channel Formation by VacA

VacA can insert into the plasma membrane of human cells or planar lipid bilayer systems to form anion-selective membrane channels (Czajkowsky et al. 1999; Iwamoto et al. 1999; Tombola et al. 1999a, b). Low-resolution images of membrane-associated VacA indicate that the toxin forms hexagonal ring-shaped structures (Czajkowsky et al. 1999; Adrian et al. 2002), similar in appearance to water-soluble VacA oligomers (Czajkowsky et al. 1999). VacA channel formation probably results from interaction of VacA monomers with the membrane, followed by subsequent oligomerization and membrane insertion (Czajkowsky et al. 1999; Iwamoto et al. 1999).

The mature 88 kDa VacA toxin is predicted to contain only one strongly hydrophobic region, located near the amino-terminus of p33 (Vinion-Dubiel et al. 1999). This region contains three tandem GXXXG motifs (defined by glycines at positions 14, 18, 22, and 26) (McClain et al. 2003), which are characteristic of

transmembrane dimerization sequences. Mutagenesis of several residues within this region (including G14 and G18) abolishes the capacity of VacA to form membrane channels in planar lipid bilayers (McClain et al. 2003) and abolishes vacuolating cytotoxin activity (McClain et al. 2003; Ye and Blanke 2000; de Bernard et al. 1998), suggesting that the amino-terminal hydrophobic region of VacA inserts into the membrane. Additional regions of VacA also may insert into the membrane (Wang et al. 2000).

VacA can permeabilize the plasma membrane of epithelial cells, resulting in leakage of ions and other small molecules (Tombola et al. 2001; Szabo et al. 1999), presumably as a consequence of VacA insertion into the plasma membrane and membrane channel formation. VacA-induced cell vacuolation has been attributed to VacA channel formation in the membranes of late endocytic compartments (Montecucco and Rappuoli 2001). VacA-induced alterations in mitochondrial membrane permeability may be due to formation of VacA channels in mitochondrial membranes (Willhite et al. 2003; Willhite and Blanke 2004). Although many cellular effects of VacA are attributable to membrane channel formation, several VacA effects are probably the consequences of channel-independent actions (see Sect. 8).

5.7 VacA Interactions with Host Cells: Binding, Uptake, and Trafficking

5.7.1 Intracellular Actions of VacA

VacA is internalized into cultured cells (Garner and Cover 1996; Gauthier et al. 2004, 2005; McClain et al. 2000), and inhibition of VacA uptake into cells results in attenuated cellular activity of the toxin (Patel et al. 2002; Schraw et al. 2002). Ectopic expression of functional VacA directly within the cytosol of several mammalian cell lines results in both vacuole biogenesis and modulation of mitochondrial function (de Bernard et al. 1997; Willhite and Blanke 2004; Willhite et al. 2003), which provides evidence that VacA functions within the cytosol. Despite nearly 20 years of study, the mechanism by which VacA is trafficked to intracellular sites of toxin action (late endosomes and mitochondria) remains one the most poorly understood aspects of VacA biology. Most studies of VacA internalization and intracellular trafficking have been carried out using immortalized cells of epithelial origin. These studies are relevant for understanding VacA interactions with gastric epithelial cells, but it remains to be seen whether VacA binds and is taken up into immune cells by similar mechanisms.

5.7.2 Interactions of VacA with the Epithelial Cell Surface

Both the p33 and p55 fragments of VacA bind to liposomes, suggesting that both may contribute to VacA interactions with the plasma membrane of target cells (Moll et al. 1995; Pagliaccia et al. 2000; Wang et al. 2000; Czajkowsky et al. 1999). Correspondingly, a mixture of p33 and p55 recombinant fragments binds to target cells to a much greater extent than either fragment individually (Torres et al. 2005).

Early studies using radiolabeled VacA suggested that the toxin binds to the plasma membrane of sensitive cells nonspecifically or, alternatively, binds to an abundant, low-affinity receptor (McClain et al. 2000; Ricci et al. 2000). In contrast, indirect immunofluorescence and flow cytometry-based studies indicated that VacA binding to HeLa cells is saturable (Massari et al. 1998), and competitive binding studies (Wang et al. 2001) suggested that the toxin might bind specifically to a component on the surface of host cells. Cross-linking studies revealed three interacting proteins on the surface of human-derived AZ-521 gastric cells: receptor protein tyrosine phosphatase β (RPTP-β, also known as Ptprz or PTP-zeta), receptor protein tyrosine phosphatase α (RPTP-α), and low-density lipoprotein receptor-related protein (LRP1) (Yahiro et al. 1999, 2003, 2012) (Fig. 5.3). Each of these proteins influences the susceptibility of specific cell types to VacA (Padilla et al. 2000; Fujikawa et al. 2003; Yahiro et al. 2004), and RPTP-β is required for VacA-induced epithelial damage in a mouse model (Fujikawa et al. 2003).

More recently, the abundant plasma membrane sphingolipid, sphingomyelin (SM), was demonstrated to confer sensitivity to VacA across a number of different cell lines (Gupta et al. 2008, 2010). SM is important for binding VacA to the cell surface and interacts with VacA, indicating that SM functions as a VacA receptor. Moreover, SM was demonstrated to be important for VacA binding to the cell surface, uptake, and intracellular trafficking in a manner relevant for toxin activity (Gupta et al. 2010).

The relative contributions of RPTP-β, RPTP-α, LRP1, and SM to VacA cellular binding, uptake, and trafficking are not fully clear. Membrane lipid rafts are important for VacA cellular activity (Patel et al. 2002; Schraw et al. 2002; Gauthier et al. 2004; Ricci et al. 2000), and SM has an important role in VacA association with membrane rafts (Gupta et al. 2008, 2010). Based on the idea that membrane rafts may function as specialized signaling platforms on the cell surface, one plausible idea is that VacA binding to SM-enriched rafts provides a nucleating center for other cell-associated factors, including RPTP-β, RPTP-α, or LRP1, to assemble into a functional complex required for toxin uptake into cells.

5.7.3 Uptake of VacA into an Intracellular Compartment

VacA is taken up by cells and trafficked by an unusual pinocytic mechanism, not previously described for other intracellular-acting bacterial exotoxins (Fig. 5.3)

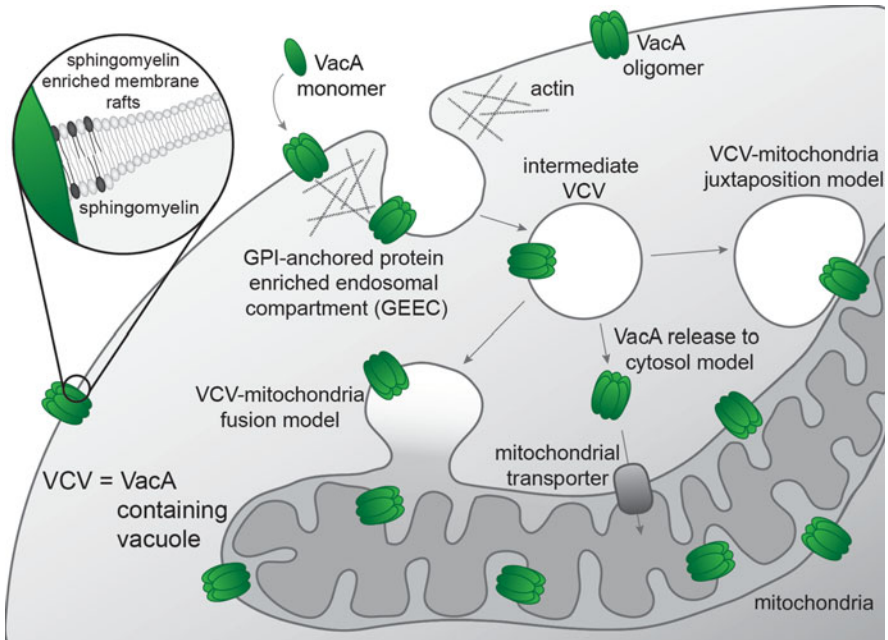

Fig. 5.3 Intracellular trafficking of VacA. Upon interacting with the cell surface, VacA monomers oligomerize into pore structures that are associated with lipid-enriched microdomains on the plasma membrane. After binding to sphingomyelin in the microdomains, VacA is internalized by an actin-dependent mechanism into GPI-anchored protein-enriched endosomal compartments (GEECs). After internalization, VacA traffics to the mitochondria by a currently unknown mechanism. There are three proposed models: (1) the VacA-containing vesicles (VCVs) associate with mitochondria, allowing for direct transfer, (2) VacA is released into the cytosol and is translocated through mitochondrial transporters, or (3) the VCVs fuse with the mitochondrial membrane

(Ricci et al. 2000; Gauthier et al. 2005, 2006). Sites of VacA binding to the plasma membrane are localized above F-actin structures, which are regulated by the small GTPase Rac1 (Gauthier et al. 2005). Following uptake from the cell surface, VacA enters VacA-containing vesicles (VCVs) that are similar to previously described glycosylphosphatidylinositol-anchored protein-enriched endosomal compartments (GEECs) (Gauthier et al. 2005, 2007). It is hypothesized that these noncanonical early endosomal compartments promote VacA trafficking to intracellular sites of toxin action (late endosomes and mitochondria).

GPI-anchored proteins within GEECs are typically recycled back to the plasma membrane, but VacA within GEECs transitions to compartments enriched in markers characteristic of early endosomal compartments and then ultimately to late endosomes. The trafficking of VacA from GEECs requires polymerized actin structures, which contact early VCVs (Gauthier et al. 2007). Although little is

known about this form of actin-dependent intracellular toxin trafficking, the capacity of VacA to exploit such a pathway suggests unusual requirements for intracellular VacA transport.

5.7.4 VacA Trafficking to Mitochondria

Ectopic intracellular expression of VacA results in localization of the p33 domain of VacA to mitochondria (Galmiche et al. 2000), suggesting that VacA might translocate from within the cytosol of intoxicated cells across mitochondrial outer membranes (Willhite and Blanke 2004; Domanska et al. 2010). Conceptually, the translocation of VacA to mitochondria from the cytosol would require that VacA exit from the endolysosomal system, which is consistent with the mode of action of many other intracellular-acting bacterial exotoxins.

Nonetheless, there is a striking dearth of direct evidence to indicate that VacA translocates to the cytosol prior to mitochondrial localization. Recently, VacA-containing vesicles (VCVs) possessing several canonical endolysosomal markers were demonstrated to align in close juxtaposition with the mitochondria (Calore et al. 2010), suggesting the possibility that VacA, rather than escaping to the cytosol prior to targeting mitochondria, might instead be transferred directly from endosomal compartments to the mitochondria (Fig. 5.4). The amino-terminal p33 domain of VacA promotes toxin import across the mitochondrial outer membrane and insertion into the inner membrane by a mechanism requiring the import channel of the mitochondrial TOM20 complex (Domanska et al. 2010). These findings suggest that VacA is transferred from intracellular trafficking vesicles directly to mitochondria by a mechanism requiring p33-mediated toxin interactions with existing mitochondrial protein import machinery and that VacA reaches the mitochondria via VCV-mediated transfer of the toxin from the endolysosomal system. The functional and molecular properties of VCVs required for targeting to mitochondria have not been identified.

5.7.5 VacA Uptake and Trafficking in Immune Cells

Relatively little is known about the binding, uptake, and trafficking of VacA in immune cells. Studies of VacA interactions with primary human T cells indicate that VacA binds to the CD18 receptor (β2 integrin) and is then internalized through a clathrin-independent process that requires Ser/Thr kinases of the protein kinase C (PKC) family (Sewald et al. 2008, 2011).

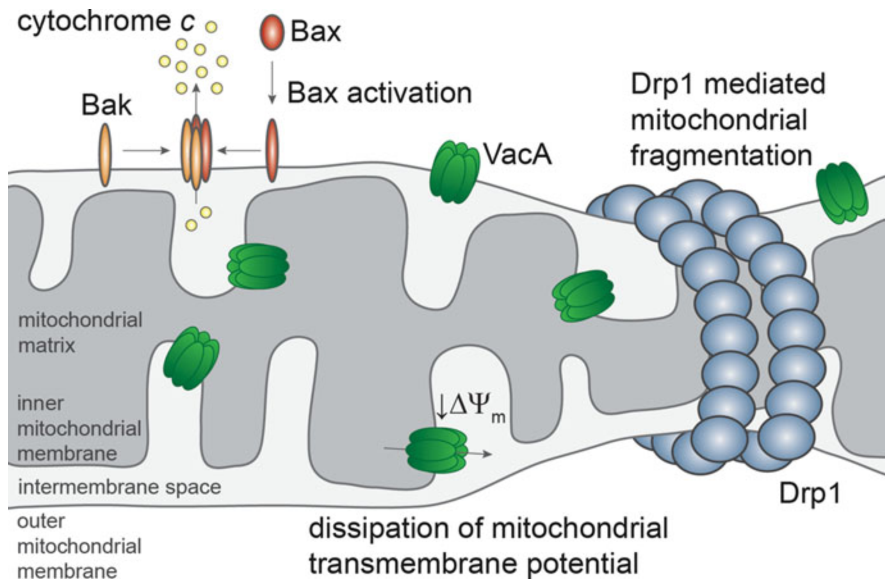

Fig. 5.4 VacA effects on mitochondria. Upon reaching the mitochondria, VacA exerts three prominent effects. (1) VacA activates the proapoptotic protein Bax to translocate to the mitochondria, where it forms a pore with another proapoptotic protein Bak, releasing cytochrome c into the cytosol, (2) VacA induces mitochondrial fragmentation by recruiting the mitochondrial fission protein Drp1 to the mitochondria, and (3) VacA dissipates the transmembrane potential of the inner mitochondrial membrane

5.8 Effects of VacA on Host Cells In Vitro

VacA effects on host cells in vitro have been studied using viable intact *H. pylori*, *H. pylori* culture supernatants containing VacA, VacA purified from *H. pylori* culture supernatants, or recombinant VacA produced by *E. coli*. The oligomeric form of VacA has relatively little activity and therefore is typically activated by exposure to low pH or high pH prior to contact with cells (de Bernard et al. 1995). A wide range of cell types, including gastric epithelial cells and several types of immune cells, are susceptible to the effects of VacA. VacA can cause an array of different effects within an individual cell type, ranging from subtle morphologic or functional alterations to cell death (Fig. 5.5).

5.8.1 VacA as a Modulator of Epithelial Cell Function

5.8.1.1 Alterations in Endosomal Compartments

Transformed cell lines derived from multiple tissue types and from multiple mammalian species undergo vacuolation in response to VacA (Leunk et al. 1988;

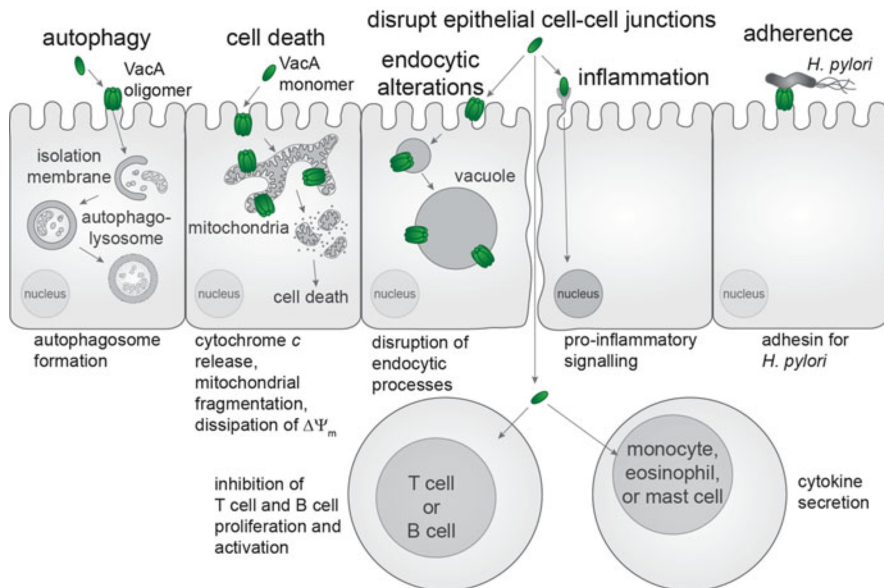

Fig. 5.5 Cellular alterations caused by VacA. Exposure of cultured cells to VacA results in endocytic alterations, the induction of autophagy, and in some cases cell death due to VacA-induced mitochondrial alterations (cytochrome c release, dissipation of the mitochondrial transmembrane potential, and mitochondrial fragmentation). Additionally, VacA disrupts epithelial cell-cell junctions, allowing the toxin to interact with immune cells in the lamina propria. VacA has both proinflammatory and anti-inflammatory effects and may facilitate adherence of *H. pylori* to gastric epithelial cells

Pagliaccia et al. 1998). Primary human gastric epithelial cells are also susceptible (Smoot et al. 1996). The intraluminal pH of VacA-induced vacuoles is acidic (Cover et al. 1992; Papini et al. 1994), and the membranes of these vacuoles are enriched in Rab7 and other markers for late endocytic compartments (Papini et al. 1994, 1997; Li et al. 2004; Molinari et al. 1997). VacA-dependent vacuole formation is enhanced by the presence of weak bases such as ammonium chloride (Cover and Blaser 1992; Li et al. 2004).

Intracellular expression of VacA results in cell vacuolation (de Bernard et al. 1997; Ye et al. 1999), and when added externally to cultured cells, VacA localizes to membranes of VacA-induced vacuoles (Fiocca et al. 1999; Ricci et al. 1997). A current model posits that VacA is internalized into cells and forms anion-selective channels in the membranes of late endocytic compartments, and in the presence of permeant weak bases, vacuoles arise due to swelling of late endosomal compartments (Fig. 5.5) (Montecucco and Rappuoli 2001). VacA mutant toxins that are deficient in membrane channel formation lack the capacity to cause cell vacuolation, regardless of whether they are added to the surface of cells or expressed intracellularly (Vinion-Dubiel et al. 1999; Ye and Blanke 2000; McClain et al. 2003), and chemical inhibitors of anion channel function block

VacA-induced cellular effects (Tombola et al. 1999b). Correspondingly, wild-type VacA causes swelling of isolated endosomes and enhances the v-ATPase proton pump activity of endosomes in a chloride-dependent manner, whereas mutant toxins lacking channel activity do not induce swelling of isolated endosomes (Genisset et al. 2007). Multiple cellular factors, including vacuolar ATPase, Rab7, Rac1, syntaxin 7, dynamin, and GPI-anchored proteins and connexin 43 (Papini et al. 1997; Hotchin et al. 2000; Li et al. 2004; Suzuki et al. 2001, 2003; Radin et al. 2014), are required for VacA-induced vacuolation and may have roles in VacA internalization or vesicle swelling.

Underlying the process of VacA-mediated cell vacuolation, it remains unclear whether VacA inserts directly into endosomal membranes to form channels, whether the VacA channels form in the plasma membrane prior to endocytosis, or whether both of these processes occur. Another area of uncertainty involves the source (or sources) of membrane required for the formation of intracellular vacuole compartments. One model proposes that VacA stimulates progressive enlargement of pre-existing vesicular compartments, and another proposes that VacA stimulates fusion of multiple smaller endocytic compartments. It has been suggested that vacuoles could arise from late endosomes without a requirement for fusion of different compartments, via a process involving fusion of late endosomal internal membranes with the late endosomal limiting membrane (de Bernard et al. 2002; Genisset et al. 2007; Suzuki et al. 2003).

VacA can cause detectable alterations in late endocytic compartments even in the absence of visible vacuolation. For example, VacA can inhibit the intracellular degradation of epidermal growth factor (Satin et al. 1997) and can inhibit procathepsin D maturation (associated with mis-targeting of pro-cathepsin D outside the cell) (Satin et al. 1997). In addition, VacA can cause apical mislocalization of the transferrin receptor, resulting in perturbation of transferrin recycling (Tan et al. 2011).

5.8.1.2 Autophagy

In addition to inducing intracellular vacuoles, VacA stimulates the formation of autophagosomes (Terebiznik et al. 2006, 2009). Low-density lipoprotein receptor-related protein-1 (LRP1) is reported to be a cell surface receptor that mediates VacA-induced autophagy (Yahiro et al. 2012). VacA-induced autophagy is associated with decreased intracellular glutathione levels, accumulation of reactive species, and activation of AKT kinase (Kimura et al. 2001; Tsugawa et al. 2012). It has been proposed that autophagy is a host mechanism to limit toxin-induced damage (Terebiznik et al. 2009) and may also promote intracellular survival of *H. pylori* in gastric epithelial cells (Terebiznik et al. 2006). Although acute exposure of cells to VacA triggers autophagy, chronic exposure to VacA may lead to a disruption of autophagy (Raju et al. 2012).

5.8.1.3 Cell Death

Vacuolated cells exclude trypan blue (Leunk et al. 1988), and if VacA is removed from the medium overlying vacuolated cells, the cells continue to proliferate. Conversely, VacA can inhibit cell proliferation and cause cell death following exposure of cells to high doses of the toxin for prolonged time intervals (Kuck et al. 2001; Cover et al. 2003). VacA-induced cell death was initially thought to be an apoptotic phenomenon, but more recent studies have shown that cell death may also occur through programmed necrosis (Radin et al. 2011), which, unlike apoptosis, stimulates an inflammatory response. VacA-induced cell death potentially occurs through multiple mechanisms involving autophagy, mitochondrial alterations, activation of signal transduction pathways, and endoplasmic reticulum stress, as discussed further below.

5.8.1.4 Alterations in Mitochondria

VacA induces reduction of the mitochondrial transmembrane potential and release of cytochrome *c*, both of which reflect alterations in mitochondrial membrane permeability (Kimura et al. 1999; Galmiche et al. 2000; Willhite et al. 2003; Willhite and Blanke 2004). These changes result in a reduction in cellular ATP levels and impaired cell cycle progression (Kimura et al. 1999), impairment of glutathione metabolism, impaired resistance of cells against oxidative stress (Kimura et al. 2001), activation of caspase 3, and cleavage of PARP (Galmiche et al. 2000). In addition, VacA causes mitochondrial network fragmentation through a Drp1-dependent process (Jain et al. 2011). Since release of cytochrome *c* from mitochondria and activation of caspase 3 are proapoptotic phenomena, VacA-induced mitochondrial alterations are thought to have an important role in VacA-dependent cell death (Kuck et al. 2001; Cover et al. 2003; Galmiche et al. 2000).

Both p33 and p55 expressed within mammalian cells and full-length VacA applied to the surface of cells localize to the mitochondria (Galmiche et al. 2000; Willhite and Blanke 2004; Calore et al. 2010; Foo et al. 2010). Similarly, when VacA is added to isolated mitochondria in cell-free systems, the toxin is translocated into mitochondria and associates with the mitochondrial inner membrane (Galmiche et al. 2000; Domanska et al. 2010; Calore et al. 2010; Foo et al. 2010). Mutant forms of VacA defective in the capacity to form membrane channels fail to cause cytochrome *c* release, and chemical inhibitors of VacA channel formation inhibit VacA-induced cytochrome *c* release (Willhite et al. 2003; Willhite and Blanke 2004), suggesting that VacA can act to form channels in mitochondrial membranes. Alternatively, the observed changes in mitochondrial membrane permeability may result from indirect processes, including the activation of endogenous channels found in mitochondria, through activation of the proapoptotic proteins Bax and Bak (Yamasaki et al. 2006; Calore

et al. 2010; Jain et al. 2011), through reduced expression of pro-survival Bcl2 proteins, or through endoplasmic reticulum stress (Akazawa et al. 2013).

5.8.1.5 Effects of VacA on Cellular Signal Transduction Pathways

Two classes of mitogen-activated protein (MAP) kinases (p38 and ERK1/2) and the activating transcription factor 2 (ATF-2) signaling pathway are activated in gastric epithelial cells in response to VacA (Nakayama et al. 2004; Hisatsune et al. 2007). VacA-induced activation of the p38/ATF-2 signal pathway is likely to be independent of VacA effects on late endocytic compartments and mitochondria (Nakayama et al. 2004). One potential consequence of VacA-induced p38 activation is upregulation of cyclooxygenase-2 (COX-2) expression, leading to increased prostaglandin E2 (PGE2) production (Hisatsune et al. 2007). Other cellular effects attributed to activation of signal transduction pathways include activation of G protein-coupled receptor kinase interactor (Git1) (Fujikawa et al. 2003), upregulated expression of vascular endothelial growth factor through an epidermal growth factor receptor-dependent pathway (Caputo et al. 2003), and activation of a PI3K-dependent signaling pathway that leads to phosphorylation of protein kinase B (AKT) and glycogen synthase kinase-3β (GSK3β) and subsequent translocation of β-catenin to the nucleus (Nakayama et al. 2009). These effects occur relatively rapidly, suggesting that they are the consequences of VacA interactions with specific cell surface components, without a requirement for internalization of the toxin (Fujikawa et al. 2003; Caputo et al. 2003).

5.8.1.6 Effects of VacA on Epithelial Cell Permeability and the Cytoskeleton

VacA lowers the transepithelial electric resistance (TER) of polarized epithelial cells due to increased paracellular epithelial permeability of the monolayers (Papini et al. 1998). The mechanisms by which VacA alters paracellular permeability are not understood, but VacA-induced alterations in actin filaments and the microtubule network within intoxicated cells (Pai et al. 1999; Hennig et al. 2005; Tabel et al. 2003) may be contributory (Wang et al. 2005). In addition to changes in paracellular permeability, VacA increases the transepithelial flux of certain molecules, including urea and bicarbonate (Szabo et al. 1999; Tombola et al. 2001; Debellis et al. 2001; Guarino et al. 1998), by a mechanism attributed to the formation of VacA channels in the plasma membrane (Szabo et al. 1999). Release of small molecules may have a favorable effect on survival of *H. pylori* within the acidic gastric mucus layer (Debellis et al. 2001), and release of factors such as Fe^{3+}, Ni^{2+}, sugars, and amino acids may support the growth of *H. pylori* (Papini et al. 1998).

5.8.2 VacA as a Modulator of Immune Cell Function

VacA can cause alterations in many types of immune cells in vitro. VacA can potentially gain access to immune cells in the lamina propria in vivo through disruptions in the gastric epithelial layer. In addition, *H. pylori* residing within the gastric mucus layer may have direct access to intraepithelial T lymphocytes and dendritic cells (DCs).

5.8.2.1 Effects of VacA on T and B Lymphocytes

VacA causes alterations in both CD4+ T cells and CD8+ T cells (Gebert et al. 2003; Boncristiano et al. 2003; Sundrud et al. 2004; Oswald-Richter et al. 2006; Torres et al. 2007). When added to Jurkat T cells, VacA inhibits the production of interleukin 2 (IL-2) (a factor required for T-cell viability and proliferation) and downregulates surface expression of the IL-2 receptor (Gebert et al. 2003; Boncristiano et al. 2003; Sundrud et al. 2004). VacA treatment of primary human CD4+ T cells results in inhibition of activation-induced proliferation, mitochondrial depolarization, ATP depletion, and cell cycle arrest (Sundrud et al. 2004; Oswald-Richter et al. 2006). The inhibitory effects of VacA on IL-2 secretion are much more prominent in Jurkat cells than in primary cells, whereas the inhibitory effects of VacA on T-cell proliferation are more prominent in primary human CD4+ T cells (Sundrud et al. 2004; Oswald-Richter et al. 2006; Sewald et al. 2008).

VacA can inhibit activation of nuclear factor of T cells (NFAT) (Gebert et al. 2003; Boncristiano et al. 2003), a transcription factor that globally regulates of immune response genes required for optimal T-cell activation. Within Jurkat T cells, VacA alters the expression of numerous genes, including the Ca^{2+}-calmodulin-dependent phosphatase calcineurin (an enzyme that dephosphorylates NFAT) (Gebert et al. 2003). It has been proposed that VacA blocks calcium influx into cells from the extracellular milieu, thereby inhibiting the activity of calcineurin (Gebert et al. 2003; Boncristiano et al. 2003). VacA can also activate MAP kinases (p38 and MKK3/6) and the Rac-specific nucleotide exchange factor Vav in T cells (Boncristiano et al. 2003; Oswald-Richter et al. 2006). All of these effects can occur without a substantial increase in apoptosis or cell death.

Some effects on T cells are dependent on the formation of VacA channels in cell membranes (Boncristiano et al. 2003; Sundrud et al. 2004; Oswald-Richter et al. 2006), and other effects are the result of activation of altered signaling in T cells via a channel-independent mechanism (Boncristiano et al. 2003). Most of the known effects of VacA on T cells are expected to result in localized immunosuppression, but VacA also stimulates expression of COX-2 in T cells, which is expected to have a proinflammatory effect (Boncristiano et al. 2003).

VacA interferes with antigen presentation by B lymphocytes. In one model system, VacA interfered with proteolytic processing of tetanus toxoid and inhibited

the invariant chain (Ii)-dependent pathway of antigen presentation mediated by newly synthesized major histocompatibility complex (MHC) class II molecules (Molinari et al. 1998). This effect is likely due to VacA-induced alterations on endocytic compartments, resulting in alterations in endocytic trafficking. VacA also inhibits activation-induced proliferation of B cells (Torres et al. 2007).

5.8.2.2 Effects of VacA on Other Types of Immune Cells

Within macrophages, VacA disrupts proper vesicular maturation by inducing the formation of large vesicular compartments (termed megasomes) (Allen et al. 2000; Zheng and Jones 2003) and the recruitment and retention of the tryptophan aspartate-containing coat protein (TACO or coronin 1) to phagosomes (Zheng and Jones 2003). VacA is reported to stimulate activation of p38 MAP kinase, cause increased expression of COX-2 (Boncristiano et al. 2003), and block cytokine production in macrophages (Weiss et al. 2013). Finally, it has been reported that VacA can activate various signal transduction pathways in macrophages (Hisatsune et al. 2008) and can stimulate macrophages to undergo apoptosis (Menaker et al. 2004).

VacA stimulates both mast cell chemotaxis and production of proinflammatory cytokines by mast cells (Supajatura et al. 2002; de Bernard et al. 2005). Binding of VacA to a mast cell line induces an oscillation in levels of cytosolic calcium and exocytosis of secretory granules (de Bernard et al. 2005). VacA can cause upregulated expression of chemokines in eosinophils (Kim et al. 2007) and apoptosis of eosinophils as a later event (Kim et al. 2010). The former effect is reported to occur via a pathway involving calcium influx, mitochondrial generation of reactive oxygen intermediates, and NF-κB activation (Kim et al. 2007).

Neutrophils and DCs are also susceptible to VacA. VacA induces the activation of p38 MAP kinase and increased expression of COX-2 in neutrophils (Boncristiano et al. 2003; Brest et al. 2006) and inhibits DC maturation (Kim et al. 2011). Isogenic *H. pylori* mutants lacking VacA are unable to prevent LPS-induced DC maturation and fail to drive DC tolerization as assessed by induction of Treg properties in cocultured naïve T cells (Oertli et al. 2013). Thus, VacA contributes to a tolerizing effect of *H. pylori* on DCs.

5.8.3 Effects of VacA on Parietal Cells and Acid/Base Balance

When added to gastric glands or cultured parietal cells, VacA inhibits acid secretion by blocking the recruitment of H,K-ATPase-containing tubulovesicles to the apical membrane through a mechanism linked to an influx of extracellular calcium,

activation of calpain, and proteolysis of ezrin (Kobayashi et al. 1996; Wang et al. 2008). VacA-induced alterations of parietal cells may contribute to the hypochlorhydria sometimes observed in *H. pylori*-infected persons. VacA also increases bicarbonate efflux from gastric epithelial cells (Debellis et al. 2001) and inhibits duodenal bicarbonate secretion, through a histamine-dependent process (Tuo et al. 2009).

5.9 Synergistic and Antagonistic Associations Between VacA and CagA

VacA and CagA are strain-specific *H. pylori* virulence factors that contribute to the pathogenesis of gastric disease associated with *H. pylori* infection. Like VacA, CagA modulates host cell function in several ways, primarily by disrupting signal transduction within intoxicated cells. There are several fundamental differences in the processes by which VacA and CagA modulate host epithelial cells. CagA, as a T4SS effector delivered to the eukaryotic cytosol, modulates the functional properties of cells with which *H. pylori* has direct physical contact. In contrast, secreted VacA can act on cells to which *H. pylori* is directly attached as well as epithelial cells at distal sites (see Chap. 4 for more details). Thus, gastric epithelial cells are predicted to be subject to the modulating effects of both VacA and CagA.

The cell modulatory effects of VacA and CagA are in some cases synergistic. For example, studies of *H. pylori* colonizing the apical surface of polarized epithelial monolayers indicate that both VacA and CagA facilitate iron acquisition (Tan et al. 2011). However, most studies to date indicate that the cellular activities of VacA and CagA are primarily antagonistic. VacA and CagA inhibit each other's effects on epithelial cells, with CagA downregulating cellular vacuolation and VacA downregulating CagA-induced cell alterations (Argent et al. 2008; Tegtmeyer et al. 2009). CagA activates the NFAT pathway via activation of calcineurin, whereas VacA blocks calcineurin activation through decreased calcium influx, thereby downregulating the NFAT pathway (Yokoyama et al. 2005). Additionally, CagA is degraded through an autophagic process; therefore, VacA-induced autophagy leads to degradation of CagA (Tsugawa et al. 2012). Strikingly, the capacity of VacA to induce the death of epithelial cells is blocked by CagA (Oldani et al. 2009), and CagA further inhibits VacA-dependent apoptosis by blocking the cellular uptake of VacA from the cell surface (Akada et al. 2010). Overall, these findings are consistent with the idea that VacA and CagA promote *H. pylori* persistence by functioning together to remodel the gastric niche occupied by the bacterium and at the same time limit the degree to which the gastric mucosa is damaged.

5.10 Role of VacA In Vivo

5.10.1 Role of VacA in H. pylori *Colonization of the Stomach*

Mice, gerbils, and gnotobiotic piglets can be colonized by *H. pylori vacA* knockout mutant strains (Eaton et al. 1997; Ogura et al. 2000; Salama et al. 2001; Wirth et al. 1998), which indicates that, in these animal models, VacA production is not an absolute requirement for gastric colonization. However, wild-type *H. pylori* strains colonize better than *vacA* mutant strains, based on both competition experiments and experiments using individual strains (Salama et al. 2001; Oertli et al. 2013). In addition, VacA-immunized mice are protected against challenges with *H. pylori* (Marchetti et al. 1998; Ghiara et al. 1997). *H. pylori* strains producing less active s2/i2 forms of VacA colonize mice more efficiently than *vacA* knockout mutant strains or strains that produce more active forms of VacA (Winter et al. 2014). These studies provide evidence that VacA may contribute to an improved ability of *H. pylori* to colonize the stomach.

Since murine T cells are resistant to VacA (Algood et al. 2007), there are limitations in the use of mouse experiments for evaluating the role of VacA in long-term *H. pylori* infection. Nevertheless, one study reported that a *vacA* mutant strain induced stronger Th1 and Th17 responses and triggered more severe gastric pathology in mice than did a wild-type strain; this was attributed to an ability of VacA to promote the induction of Tregs (Oertli et al. 2013). Therefore, it seems plausible that VacA might have a role in enabling *H. pylori* to persistently colonize human hosts.

5.10.2 Role of VacA in Gastroduodenal Disease

As discussed in Sect. 5, strains of *H. pylori* that contain certain allelic forms of *vacA* are associated with an increased risk of symptomatic gastroduodenal disease (Fig. 5.2), but drawing conclusions from these epidemiologic studies is limited by the possibility of confounding variables. Caution must be used when evaluating the role of VacA in animal models, since some of the cellular components with which VacA interacts are found exclusively in human cells (Algood et al. 2007). Nevertheless, direct administration of VacA protein into the stomachs of mice results in gastric mucosal injury and gastric inflammation (Fujikawa et al. 2003; Supajatura et al. 2002; Telford et al. 1994). A gastric mucosal inflammatory response may occur as a consequence of either VacA-mediated damage to the gastric epithelium or direct proinflammatory effects of VacA on various types of intoxicated cells (Boncristiano et al. 2003; Hisatsune et al. 2007; Supajatura et al. 2002; Kim et al. 2007).

Comparing the histologic alterations in animals infected with isogenic wild-type or *vacA*-null mutant strains revealed that VacA may have a small but detectable effect on the development of gastric ulceration in gerbils (Ogura et al. 2000). Strains producing more active forms of VacA induced more severe and extensive metaplasia and inflammation in mice than did strains producing less active s2/i2 forms of VacA (Winter et al. 2014). Other studies have not detected an effect of VacA on gastric histology in animal models (Eaton et al. 1997; Ogura et al. 2000; Salama et al. 2001) or reported that VacA has an anti-inflammatory effect (Oertli et al. 2013).

5.11 Conclusions and Outlook

While the vast majority of intracellular-acting bacterial toxins function as enzymes to covalently modify and alter the properties of host intracellular targets, VacA forms intracellular membrane channels, leading to alterations in membrane trafficking within the endolysosomal system and altered dynamics and function of the intracellular mitochondrial network. Exciting areas for future research will be to ascertain how VacA forms and regulates toxin channels at discrete sites within the cell and how intracellular channel formation contributes to host cell dysfunction.

One of the most difficult experimental challenges is to understand how VacA contributes to *H. pylori* colonization, persistence, and gastroduodenal disease. For bacteria that are largely human specific, such as *H. pylori*, animal models have inherent limitations (see Chap. 10 for more details). In future studies, it will be important to elucidate the contributions of the toxin to the long-term remodeling of the gastric microenvironment, which may ultimately contribute to the development of disease.

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Chapter 6
Roles of the BabA and the SabA Adhesins in Gastroduodenal Diseases

Anna Arnqvist

Abstract Adhesion is an important prerequisite for colonization and it is the initial step in infections with pathogenic bacteria. Adherence to host epithelial surfaces is the result of bacterial surface proteins, called adhesins, and their specific interaction with cognate protein- or glycoconjugate receptors on the host cells. Often, the bacteria have a set of complementary adhesins that are specific for different host receptors. Alternative mechanism has been suggested to mediate *H. pylori* adhesion, and this chapter will focus on the two well-characterized adhesins BabA and SabA. In the healthy gastric mucosa, the Lewis b antigen (Leb) is present in the gastric epithelial lining of blood group O (H-antigen), B, and A individuals. *H. pylori* binding to ABO/Leb is mediated by the blood group antigen-binding BabA adhesin. As the inflammation develops, Leb is downregulated and the levels of sialylated antigens increase. Sialyl-Lewis x/a antigens (sLex/a) are specifically recognized by the *H. pylori* sialic acid-binding adhesin SabA. Even though bacterial adherence per se cannot cause disease, adherence is considered as a crucial step in pathogenesis since it is needed for bacterial delivery of effector molecules into the host cell. The presence of receptors and host-immune responses are two factors that differently affect adhesion. To achieve long-term colonization, *H. pylori* must regulate the expression of a cognate adhesin to fit the available receptors. Adhesion to the gastric epithelial cells promotes gain of nutrients, but too tight adhesion may be intimidating because of the risk of clearance by the bacteria for life-threatening immune responses. Thus, expression levels of the adhesins must be fine-tuned in accord to host receptor expression levels. This chapter will also discuss *H. pylori* adhesion in relation to severe gastric diseases.

Keywords *Helicobacter pylori* • Adhesion • Blood group antigen-binding adhesin BabA • Sialic acid binding adhesin SabA • ABO blood group antigen/Lewis b antigen (ABO/Leb) • Sialyl-Lewis x antigen (sLex) • Homologous recombination • Slipped-strand mispairing

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6.1 Introduction

Bacterial colonization of the human host is the initial step to establish and maintain an infection. Bacterial adhesion to host cells is mediated via ligand-receptor interactions. Bacteria recognize and bind to certain receptors, including proteins, glycoproteins, or glycolipids on the host cell mucosal surface. Oligosaccharides present on the highly glycosylated secreted and membrane-bound mucins and to glycoproteins and glycosphingolipids on the host cell surfaces act as receptors for *H. pylori* attachment. Proteins on the bacterial cell surface that facilitated binding to the host receptor are called adhesins. A given bacterium often carries multiple adhesins. Adhesins interact with their cognate receptors with high specificity, which contributes to host and tissue tropism. Some adhesins are polymorphic and recognize several receptor moieties. The affinities of adhesin-receptor interactions are variable, from very high-affinity interactions to those of low affinity. Clustering of adhesins and/or receptors can cause multivalency effects and increase the binding. Expression of the host cell receptor repertoire exhibits a wide individual variation due to genetic predisposition, as well as variation in relation to healthy or inflamed mucosa.

In gastric biopsy material, the majority of *H. pylori* are found in the mucus layer but a subset is firmly attached to the epithelial cell surface (Hessey et al. 1990). *H. pylori* has also been identified intracellularly (Aspholm et al. 2006b; Semino-Mora et al. 2003). For *H. pylori*, it is a delicate balance to on the one hand stay close to the host cell to gain nutrients and on the other hand have the capacity to loosen the grip when the host cellular response becomes too vigorous or upon host cell shedding. Adhesion of *H. pylori* to the gastric mucosa also aids the bacteria to resist the shear force during the peristaltic movements. To persistently colonize the gastric mucosa for the lifetime is challenging, but obviously *H. pylori* have the ability to adapt to the continuous changes that occur in the stomach environment. The capacity to attach to gastric epithelial cells contributes to bacterial delivery of effector molecules to the host and subsequent host responses, events that sometimes lead to the development of disease. Therefore, adhesion is a significant step in the pathogenesis of *H. pylori*-related peptic ulcer disease and gastric cancer. This chapter discusses the two best-characterized *H. pylori* adhesins, the BabA adhesin that mediate binding to fucosylated blood group antigens in healthy gastric mucosa and the SabA adhesin that recognizes sialic acid sialyl-Lewis x antigen in inflamed mucosa, how their respective expression is regulated, and their role in gastric diseases.

6.2 The Blood Group Antigen-Binding Adhesin BabA

6.2.1 Identification of the Blood Group Antigen-Binding Adhesin BabA

Fucosylated H antigens are complex carbohydrates with a α1.2 fucose residue linked to a terminal Galβ (Fig. 6.1). The H-antigen forms the base for the ABO blood group antigens. Addition of a fucose residue to the H-antigen forms the difucosylated Lewis b antigen (Leb) (Fig. 6.1). In blood group A individuals, the H and Leb antigens are extended with terminal N-acetylgalactosamine (GalNAc), whereas in the blood group B individuals, H or Leb have instead been extended with galactose (Gal) (Fig. 6.1). Individuals with a so-called positive secretor status also expresses the secretor (fucosyl) transferase, in saliva, tears, milk and gastro-intestinal mucus secretions, and epithelium, thus adding the α1.2 fucose residue to form the H-antigen that then can be extended into the ABO blood group antigens (reviewed by (Clausen and Hakomori 1989). Individuals of nonsecretor phenotype do not express the fucosyl transferase in gastric mucus and epithelium. The first functional host receptor for *H. pylori* to be identified was the fucosylated H and Lewis b antigens (Leb) (Borén et al. 1993). Since cell lines are transformed cells, the ABO/Lewis antigens are usually not expressed at all or only in limited amount. Therefore, the finding of Leb as a functional receptor for *H. pylori* was dependent on the usage of a newly developed in vitro adherence assay (Falk et al. 1993). Fluorescently labeled *H. pylori* were overlaid on to human gastric histo-tissue sections, which showed that *H. pylori* is bound to gastric surface mucous cells. Colostrum samples reflect the individual blood group phenotype. Later the same year, Borén and co-workers (1993) used the same in vitro adherence assay in combination with colostrum samples from individuals with different Lewis blood group antigens to show that the *H. pylori* receptor on human gastric surface mucous cells contains Leb (Borén et al. 1993). *H. pylori* is a human- and primate-specific pathogen and the Leb antigen is not expressed in mice. However, *H. pylori* mediates specific adhesion to the gastric and intestinal tract of transgenic mice that specifically expressed the human α-1,3/4 fucosyl transferase (Falk et al. 1995). When these transgenic mice were infected with *H. pylori,* it was demonstrated that Leb binding was associated with the development of chronic gastritis (Guruge et al. 1998).

Fig. 6.1 Composition of fucosylated H1 and Leb ABO blood group antigens. Addition of a fucose residue to the H1 antigen forms the Leb antigen. In blood group A and B individuals, the H1 or corresponding Leb antigens are either extended with an N-acetylgalactosamine (GalNAc) or a galactose (Gal)

The first study to analyze the prevalence of Leb-receptor binding activity in a collection of strains showed that 66 % of them exhibited Leb binding (Ilver et al. 1998). Receptor-binding activity was measured using ^{125}I-labeled Leb semi-synthetic HSA glycoconjugate (Aspholm et al. 2006a; Ilver et al. 1998). Affinity analysis using Scatchard assays, in which the binding affinity, the association constant *Ka*, is calculated, showed that Leb binding in strain CCUG17875 was of high affinity (the $K_a = 1 \times 10^{-10}$) (method is described in (Aspholm et al. 2006a). Based on using electron microscopy and Leb-receptor conjugate labeled with gold particles, the number of Leb-binding adhesins was estimated to be approximately 500 per bacterial cell surface. Far-western blot analysis, i.e., receptor overlay analysis with soluble Leb-receptor conjugate, was used to identify a Leb-binding protein. To isolate the Leb-binding protein, an affinity purification method called receptor activity-directed affinity tagging (retagging) was established (Ilver et al. 1998). An N-terminal amino acid sequence of the Leb-binding adhesin was determined and used to design degenerated PCR primers for PCR amplification of a DNA fragment, which then was used as a probe for the screening of a plasmid library. Initially, two sets of alleles were identified that encoded for proteins that displayed identical N- and C-terminal domains but contained different central domains. An additional, extended N-terminal sequence made it possible to distinguish the clones and determine which that encoded for the Leb-binding protein. The corresponding protein was named the blood group antigen-binding adhesin BabA, and the gene was consequently named *babA* (Ilver et al. 1998). The gene corresponding to the second set of clones was named *babB*. The function of the BabB protein is yet elusive.

At the time it was not known that the *H. pylori* strain that was used to identify the Leb receptor expressed a BabA adhesin with a specialist phenotype, meaning that its BabA adhesin specifically recognized H1 and Leb of blood group O individuals but not A-Leb or B-Leb. This strain, P466, originates from South America, where a majority of the population is of blood group O phenotype (Aspholm-Hurtig et al. 2004). Binding of strain CCUG17875 to gastric mucosa could be blocked with Leb, A-Leb, or B-Leb, while binding of strain P466 only was blocked with Leb. Close to 400 *H. pylori* strains from different geographic regions (Sweden, Germany, Spain, Japan, and Alaska) were analyzed for their receptor-binding phenotypes, and it then became evident that 95 % of them bound to A-Leb, B-Leb, and Leb. In contrast, strains from South America (Peru, Venezuela Amazons, and Colombian mestizo) displayed different binding patterns since 60 % of them only recognized Leb and not A-Leb or B-Leb (Aspholm-Hurtig et al. 2004). Strains that only bound Leb were named "specialists," and strains that exhibited the ABO/Leb phenotype were called "generalists." Receptor-overlay analysis (far-western probed with receptor glycoconjugate) and a DNA transformation approach to shuffle specialist and generalist *babA* alleles both showed that the BabA protein alone determines the "specialist"- or the "generalist"-binding modes. Moreover, phylogenetic analysis of *babA* sequences from specialist and generalist strains suggested that long-term adaptation of the *babA* alleles occurs in relation to

the types of receptors that are available in the local population (Aspholm-Hurtig et al. 2004).

Besides binding to the host epithelial cells, the BabA adhesin mediates binding to Lewis b antigen expressed on MUC5AC, MUC5B, and MUC1 mucins (Lindén et al. 2002, 2004). BabA also exhibits binding to MUC5B in saliva and the glycoprotein gp-340 (Walz et al. 2005, 2009). An update of BabA carbohydrate-binding specificities to further explore the structural requirements for carbohydrate recognitions was recently published (Benktander et al. 2012).

6.2.2 Location of the **babA** *Gene*

The BabA adhesin was first described for strain CCUG17875 (Ilver et al. 1998). To identify its genomic location, screening of an ordered cosmid library constructed from the NCTC11638 strain (Bukanov and Berg 1994) identified two *babA* alleles and one *babB* gene (Ilver et al. 1998). DNA sequence analysis showed that the *babA1* allele in comparison with the *babA2* allele carried a 10 bp frameshifting deletion in its very 5′ end that causes a premature stop. Knockout deletion analysis confirmed that the *babA2* allele encoded for the BabA adhesin and that the *babA1* allele was silent. The results of DNA sequence analyses showed that the *babA* and the *babB* genes encode for proteins with a high level of amino acid identity in their N-terminal domains, display unique central domains, and share the ~300 most C-terminal amino acids. Both the BabA adhesin and the BabB protein belong to the Hop (*H. pylori* outer membrane porins) family that is characterized by a conserved N-terminal signal peptide, a variable middle domain, and a conserved C-terminal membrane-spanning β-sheet motif, of which the middle domain determines the specificity of the protein (Alm et al. 2000). Thus, the middle domain of BabA is likely to contain the Leb-binding domain.

Today, when the *babA* gene has been identified and sequenced in a large number of strains, it is evident that most strains carry only one *babA* allele. Comparison of the two first genome sequences of the 26695 and J99 strains showed that the *babA* and the *babB* alleles are located in reciprocal loci (Alm et al. 1999; Tomb et al. 1997). Then, the *babB* gene was found to be located in a third, alternative locus. The gene encoding for this third *bab* paralog has been named *babC* (Colbeck et al. 2006; Oh et al. 2006). The three loci where the *bab* genes are found are now called locus A, B, and C, respectively (Hennig et al. 2006). The *babA* gene is most often found in the A locus (Hennig et al. 2006; Oh et al. 2006). Some occasional strains have multiple copies of the *babA* allele, while some strains lack the gene. Noteworthy is that the majority of *H. pylori* strains seem to carry a *babB* gene.

6.2.3 *Mechanisms That Switches BabA Expression On and Off*

When the first genome sequences were published, high levels of homologous sequences in the 5′ and 3′ ends of the *hop* genes were found, indicating the possibility for homologous recombination events (Alm et al. 1999, 2000). In particular, Pride and Blaser reported about chimeric *babA/B* genes in 2 out of 42 strains (Pride and Blaser 2002). The same study suggested that *H. pylori* homologous recombination events were RecA dependent and DNase insensitive, which suggested that they probably were of intragenomic origin, i.e., gene conversion (Pride and Blaser 2002). Intragenomic changes of the *babA* gene were later found when rhesus macaques were experimentally infected with the BabA-expressing J166 *H. pylori* strain (Solnick et al. 2004). Output clones recovered 17 weeks postinfection had lost BabA expression, either by homologous recombination with *babB* creating a *babA/B* chimeric gene or by insertion or deletion of nucleotides in the dinucleotide CT repeat tract, 5′ of *babA*. One or two nucleotide changes in the CT repeat tract via slipped-strand mispairing cause frameshifts and thus a truncated protein. The opposite event, recombination of *babA* into the *babB* gene that results in a Lewis-binding *babB/A* chimera, was found in another study (Bäckström et al. 2004). Here, homologous recombination between the *bab* genes was demonstrated when Leb-binding clones were identified and isolated from an *H. pylori* strain carrying a silent *babA* allele. Clones of the Leb-binding phenotype were enriched by using biotinylated Leb conjugate and streptavidin-magnetic beads, and they were then identified using colony screening (Bäckström et al. 2004). The acquired Leb-binding clones were the result of homologous recombination of the silent *babA* allele into the *babB* gene (located in B locus). This chimeric *babB/A* gene carried a dinucleotide CT repeat tract in the 5′ end and was thus subjected to frequent reversible on/off phase shift variations. Phase shift variation via slipped-strand mispairing is a faster process than phase shift via homologous recombination. Homologous recombination of *babA* into the B locus in the 17875 strain was determined to occur with a frequency of 1×10^{-5} (Bäckström et al. 2004). The frequency for homologous recombination in the opposite direction, recombination of the *babB* gene into the A locus, occurs with similar frequency in strain J166, e.g., 3×10^{-5} or 3×10^{-6} per cell division (Amundsen et al. 2008). The same study showed that *bab* recombinations are promoted by RecA and the double break repair enzyme AddA. Phase shift via slipped strand occurs with a higher frequency. The *babA* gene situated in the B locus turns expression on and off with a frequency of 5×10^{-3}. Additional studies have also described homologous recombination event and slipped-strand mispairing between the *bab* genes (Colbeck et al. 2006; Hennig et al. 2006).

Besides gene conversion and slipped-strand mispairing, BabA expression can be switched off via mutations. Experimental infection of rhesus monkeys with strain J99 showed that BabA expression is turned off via single base-pair mutations (Styer et al. 2010). Another study where Mongolian gerbils were long-term infected with

H. pylori also showed that BabA expression was switched off (Ohno et al. 2011). Here output clones had switched off BabA expression due to either one base-pair insertions or deletions that introduced a premature stop codon. In addition, clones with larger deletions (31, 70, 84 bp, respectively) were also identified (Ohno et al. 2011).

Other recombination events can also switch off Leb binding. Output clones from Mongolian gerbils infected with strain 7.13 displayed BabA proteins with Leb-non-binding phenotype (Styer et al. 2010). Genetic analysis showed that the 7.13 strain carry two *babA* alleles, similar to strain CCUG17875, where a *babA2* allele encodes for a Leb-binding adhesin and the *babA1* allele is silent. In the Leb-non-binding 7.13 output clones, a DNA fragment encoding for six amino acids was replaced resulting in the expression of Leb-non-binding BabA proteins.

6.2.4 Regulation of BabA Expression Levels

The first *H. pylori* genome analyses showed that there are a few RNA polymerase (RNAP) sigma (σ) factors and a few genes that encode for transcriptional regulatory proteins (Alm et al. 1999; Tomb et al. 1997). It was also shown that the *H. pylori* genomes contain many simple sequence repeats, which are typical hot spots for slipped-strand mispairing and thus to act as contingency loci that are known to contribute to generation of heterologous populations. BabA expression levels and Leb-binding activity vary between strains, but a few studies concerning regulatory mechanisms have been conducted. BabA expression in strain CCUG17875 was found to be higher when BabA was expressed from the A locus than when expressed from the B locus (Bäckström et al. 2004). The transcriptional start sites for the *babA* gene (located in the A locus) and the *babB* gene (located in the B locus) were determined with primer extension analysis. The −10 promoter region of *babA* (5′-**TATAAT**) had a perfect match to the −10 consensus sequence of *E. coli* σ^{70} housekeeping promoters (5′-**TATAAT**) compared to the −10 promoter region of the *babB* (5′-G**A**T**AA**G) (Bäckström et al. 2004). Even though a consensus sequence for *H. pylori* −35 promoter element has not been determined, the −35 region of the A locus (5′-A**TGACA**) in CCUG17875 have an almost perfect match to the *E. coli* −35 promoter region (5′-**TTGACA**). Besides the differences in nucleotide composition of the binding sequences for the RNAP σ-factor (i.e., the −35 and the −10 regions), the distance between these motifs are known to affect transcription initiation. The second *babA* allele of strain CCUG17875, *babA1*, has five extra As between the −10 and the −35 promoter regions. It seems possible that the length of this A tract affects promoter activity, but it has not been confirmed experimentally. Hennig and co-workers (2006) analyzed BabA expression levels relative the spacing between the ribosomal binding site and the *babA* ATG translational start codon in 35 strains. Although there was a variation, it did not correlate to the BabA expression levels (Hennig et al. 2006). They also did not find any sequence variations in the promoter regions that were associated with the BabA

expression levels. The recent mapping of the *H. pylori* transcriptome showed the presence of many small RNAs (sRNAs) and a massive antisense transcription, which suggested that *H. pylori* uses riboregulation to regulate gene expression (Sharma et al. 2010). However, no role of riboregulation on BabA expression has so far been demonstrated.

6.2.5 BabA Expression and Gastric Disease

There is a continuous interest in the BabA adhesin and its role in disease outcome. Over the years, a series of papers have reported about BabA and its association to severe mucosal inflammation and increased risk of peptic ulcer disease and gastric cancer (Aspholm-Hurtig et al. 2004; Colbeck et al. 2006; Fujimoto et al. 2007; Gerhard et al. 1999; Hennig et al. 2004; Ilver et al. 1998; Lehours et al. 2004; Odenbreit et al. 2009; Olfat et al. 2005; Oliveira et al. 2003; Sheu et al. 2006; Song et al. 2014; Yamaoka et al. 2002; Yu et al. 2002). Different approaches have been used to evaluate the association between BabA and disease. A series of studies applied PCR to detect the presence of the *babA* gene. Often, primers that amplify the *babA2* allele are used. Using such approach, *babA* located in other locus than the A locus will not be found unless additional primer pairs are used. Other studies have analyzed BabA expression by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), immunoblots, or assays that detect for BabA protein expression with Leb-binding activity such when RIA assay with ^{125}I-labeled Leb-receptor conjugate is used (Aspholm et al. 2006a; Ilver et al. 1998) or when enzyme-linked immunosorbent assay (ELISA) is used (Solnick et al. 2004). The collection of clinical isolates that has been assayed for BabA expression and binding to Leb has demonstrated differences in BabA expression levels as well as differences in binding affinity to the Leb receptor among strains. These differences probably mirror the ability of *H. pylori* to adapt local environmental conditions during the lifelong persistent infection (Aspholm-Hurtig et al. 2004; Fujimoto et al. 2007; Ilver et al. 1998; Yamaoka et al. 2002).

Already in 1999, Gerhard and co-workers reported that there was an epidemiologic association between *cagA*, *vacAs1*, and *babA2* genotypes and higher incidence of ulcer disease and gastric cancer (Gerhard et al. 1999). Later, BabA expression in combination with CagA- and VacAs1-expressing strains has been confirmed to be associated with severe gastric disease (Azevedo et al. 2008; Ishijima et al. 2011). Moreover, BabA-mediated binding to Leb plays an important role for the initiation of contact-dependent signaling mediated by the type IV secretion system (T4SS). When Leb-transfected cells were infected with a wild-type *H. pylori* strain and isogenic BabA and T4SS mutants, it was shown that BabA-Leb-binding induced T4SS-dependent host cell signaling, which increased mRNA levels of genes coding for pro-inflammatory cytokines as well as precancerous-related factors. These data were also supported by in vivo data from experimental *H. pylori* infection of Mongolian gerbils (Ishijima et al. 2011).

It was recently suggested that *H. pylori* has the capacity to jeopardize the host genome integrity via direct bacterial-host cell contact. *H. pylori* adhesion, via BabA-Leb binding, induced higher levels of double-strand breaks in the chromosomal DNA of infected host cells than a *babA* deletion mutant (Toller et al. 2011). Similarly, host cells, pre-incubated with soluble Leb prior to infection, exhibited reduced double-strand break induction. Other virulence-associated factors such as the VacA cytotoxin, the γ-glutamyl transpeptidase (GGT), and the *cag* pathogenicity island (*cag*PAI) were tested but did not promote double-strand breaks in host target cells (Toller et al. 2011). DNA damages that are not precisely repaired may increase carcinogenesis.

For experimental infection, the rhesus macaque is a highly relevant animal model since they are naturally infected by *H. pylori* and because it mimics the clinical outcome observed in humans. Several studies have focused on BabA expression and Leb binding (Mahdavi et al. 2002; Styer et al. 2010). Although not naturally infected by *H. pylori*, the Mongolian gerbil is an attractive alternative experimental model for studying *H. pylori* infection, and it has been used for studies of BabA (Ohno et al. 2011; Styer et al. 2010). Mongolian gerbils were infected with the BabA-expressing strain TN2GF4 for 18 months (Ohno et al. 2011). Besides BabA expression, the degree of inflammation was followed by histological examination and by scoring mononuclear cell (MNC) and polymorphonuclear cell (PMN) infiltration during the same time span. Cellular infiltration increased after 1 month and gradually increased to reach a peak after 6 months. Gastric ulcers developed to varying degree. Output clones were examined for BabA expression and Leb binding. Similar to what has been reported for rhesus macaques and gerbils previously, BabA expression was lost during the course of infection. After 1 month, 80 % of the output clones displayed BabA expression, but the levels declined to 33 % after 3 months. No output clones expressed BabA after 6 and 18 months postinfection. Among the 1-month output clones, BabA expression levels had increased significantly. The changes in expression levels could not be explained by any changes in DNA sequences in the promoter regions nor in the *babA* open reading frame (ORF). There was also a correlation between output clones isolated from gerbils with gastric ulcer, which displayed no BabA expression. In the same study, it was suggested that the BabA expression levels directly or indirectly contribute to cellular inflammation (Ohno et al. 2011). Gerbils were infected with isolated output clones with high or low expression levels. No differences in cellular infiltration occurred after 1 month, but after 3 months, the level of cellular infiltration of gerbils infected with output clones with low BabA expression levels was lower compared to those infected with output clones with high BabA expression levels (Ohno et al. 2011). Thus, the differences in BabA expression levels as well as in Leb-binding affinity among strains are likely to affect disease outcome.

6.3 The Sialic Acid-Binding Adhesin SabA

6.3.1 H. pylori *Binding to the Inflammation-Associated Sialyl-Lewis x/a Antigen Receptor*

A *babA* knockout deletion mutant was found to adhere to gastric histo-tissue sections from an *H. pylori*-infected patient, and the binding could not be fully blocked with soluble Leb-receptor conjugate (Mahdavi et al. 2002). In contrast, no binding to histo-tissue sections from healthy individuals was detected. Together these results suggested that *H. pylori* exhibit an additional receptor-adhesin interaction for binding to the gastric mucosa. Instead of the ABO/Leb as receptors, it was shown that *H. pylori* recognizes sialyl-Lewis x and a antigens (sLex/a) in inflamed gastric mucosa (Mahdavi et al. 2002). The sLex receptor was identified using thin-layer chromatography (TLC), mass spectrometry (MS), and ^{1}H nuclear magnetic resonance (^{1}H-NMR). First, glycosphingolipids were separated using TLC, which thereafter were overlaid with *H. pylori*. Binding to the sialyl-Lewis x (sLex) and sialyl-diLewis x (sdiLex) was confirmed with monoclonal antibodies. Stronger binding was obtained to sdiLex. The glycosphingolipid bound to *H. pylori* was confirmed to be sdiLex using MS and ^{1}H-NMR (Mahdavi et al. 2002). Only minute levels of sLex are found in healthy gastric mucosa, and there is a reciprocal regulation of the fucosylated ABO/Leb and sialylated sLex expression during *H. pylori* infection (Lindén et al. 2008; Mahdavi et al. 2002). An *H. pylori* infection alters expression of several genes involved in glycan biosynthesis and in particular upregulation of the gene encoding the *N*-Acetylglucosamine (GlcNAc) transferase β3GlcNacT5 (Marcos et al. 2008). Biosynthesis of Lewis antigens is dependent on β3GlcNacT5, and when β3GlcNacT5 was overexpressed, the expression levels of sLex increased. It has also been shown that *H. pylori* colonization density increases in patients with high expression levels of sLex (Sheu et al. 2006).

6.3.2 *Identification of the Sialic Acid-Binding Adhesin SabA*

The retagging technique was once again used for identification and purification of an *H. pylori* adhesin. The sLex-receptor conjugate was used in affinity purification of the sialic acid-binding adhesin, SabA (Mahdavi et al. 2002). Using mass spectrometry, four peptides were found to match one gene (JHP662 in strain J99), and two of these peptides also matched a related gene (JHP659 in strain J99). Deletion mutagenesis verified that JHP662 encoded the SabA adhesin since binding to sLex was abolished. The JHP659 gene was called *sabB*. Similar to the BabA and the BabB proteins, the SabA adhesin and the SabB protein share a high degree of homologies in their N- and C-terminal domains. The SabA and SabB proteins both belong to the Hop family of proteins. Mapping of the receptor epitope revealed that the NeuAcα2-3Gal-disaccharide is the minimal sialylated binding epitope that is

required for SabA-mediated binding (Aspholm et al. 2006b). Interestingly, when clinical SabA-expressing isolates were tested for binding to a series of sialylated glycans such as sialyl-di-Lex, sLea, and sialyl-lactosamine, it was found that the majority exhibited polymorphism in sialyl-binding properties and that it is an inherent property of the SabA adhesin itself (Aspholm et al. 2006b).

6.3.3 The SabA Adhesin Is the **H. pylori** *Hemagglutinin*

H. pylori was early described to hemagglutinate human erythrocytes (Emody et al. 1988). It was for many years a debate about the *H. pylori* hemagglutinin, i.e., the protein that is responsible for sialic acid-dependent hemagglutination of erythrocytes. Initially, the HpaA protein was described as the *H. pylori* hemagglutinin (Evans et al. 1993), but construction of a deletion mutant did not abolish the hemagglutination activity (O'Toole et al. 1995). However, when a *sabA* deletion mutant strain was tested for its function in sialic acid-dependent hemagglutination of erythrocytes, no agglutination was observed, which suggested that the SabA adhesin is the *H. pylori* hemagglutinin (Aspholm et al. 2006b). An additional role of SabA is its role in neutrophil activation via binding to sialic acid-containing receptors and thus to mediate nonopsonic activation of human neutrophils and oxidative burst (Unemo et al. 2005). Besides being a key player for oxidative metabolism, SabA has also been suggested to be important for the induction of phagocytosis (Petersson et al. 2006).

6.3.4 Mechanisms for Regulation of SabA Expression

Screening of single clones for sLex-binding activity showed that approximately 1 % switched off their binding (Mahdavi et al. 2002). The analysis of the *sabA* DNA sequence identified a simple dinucleotide CT sequence repeat, a typical hot spot for slipped-strand mispairing and thus phase variation. sLex-binding versus sLex-non-binding clones exhibited differences in the number of CTs which generated frameshifts that either put the *sabA* ORF in or out of frame (Mahdavi et al. 2002). Variation in the number of CT repeats and frameshifts were later confirmed with immunoblot analysis and α-SabA antibodies (Lehours et al. 2004; Yamaoka et al. 2006). Similar to the *babA* gene, *sabA* can also be located in alternative genomic loci without the CT repeats in the 5′ end of the *sabA* ORF (Sheu et al. 2006). Recently, the exact location of the *sabA* and the *sabB* genes was mapped in a thorough analysis of some fifty North American clinical strains (Talarico et al. 2012). Strain J99, in which the *sabA* gene corresponds to JHP0622 (*sabA* locus), the *sabB* gene corresponds to JHP0659 (*sabB* locus), and a third gene *omp27* (*hopQ*) corresponds to JHP1103, was used as reference. In this set of strains, 84 % of the *sabA* gene was located in the *sabA* locus and the

remaining was found in the *hopQ* locus. A significant number of strains lacked the *sabB* gene, while there seemed to be a selection to maintain the *sabA* gene. The same study showed that gene conversion events occur at the *sabA*, *sabB*, and *hopQ* loci. Similar as for *babA* and *babB* gene conversions, the *sabA* gene conversion events were affected by RecA, the nuclease-helicase AddA that is involved in double-strand break repair and RecG (Amundsen et al. 2008; Talarico et al. 2012). Thus, expression of the SabA adhesin can be switch on and off via phase variation, often by slipped-strand mispairing during replication of the CT nucleotide repeat tract but also via gene conversions.

There is a variation in SabA expression levels among strains (Aspholm et al. 2006b; Sheu et al. 2006; Yamaoka et al. 2006). Differences in SabA expression levels can often be explained by differences in the promoter strength (Åberg et al. 2014). In addition, a simple thymine (T) nucleotide repeat tract is located in close proximity to the −35 region of the *sabA* promoter and was suggested to affect promoter strength (Goodwin et al. 2008; Kao et al. 2012). There are wide differences in the length of the T-tract, from T_5 to T_{28} where T_{13} to T_{19} are to the most common length (Kao et al. 2012; Åberg et al. 2014). Variation of T-tract length changes along the course of infection. Output pools from mice as well as from human antrum, and corpus stomach regions displayed variation in sLex-binding phenotype as well as T-tract length (Åberg et al. 2014).

The actual effect of changes in the T-tract length was elucidated using site-directed in vitro mutagenesis to create a series of isogenic mutants with varying numbers of Ts (Åberg et al. 2014). The analysis of promoter strength, mRNA levels, SabA protein expression, as well as sLex-binding, both to receptor conjugate and human gastric histo-tissue sections, showed a multiphasic expression pattern. The maximum and minimum mRNA and protein levels were observed with a T-tract length interval of approximately ten base pairs, which corresponds to one turn of the DNA helix (Åberg et al. 2014). The effect of T-tract length variations and *sabA* mRNA levels has been confirmed by Harvey et al. (2014).

Simple sequence repeats between the −10 and −35 promoter elements can affect the docking of the RNAP σ-factor. The *sabA* T-tract has a promoter proximal location, and the effect of the T-tract length was likely to operate by an alternative mechanism. Exchange of specific lengths of the T-tract with adenine and guanine showed that the T-tract does not only work as a spacer to adjust the size and hence expression, but it probably affects DNA topology (Harvey et al. 2014; Åberg et al. 2014). In silico and functional biochemical analysis showed the T-tracts affect the local DNA structure and thus binding of the RNAP. A model where changes in the T-tract length affect the axial alignment between the core promoter and UP-like elements has been suggested (Åberg et al. 2014). Thus, without input from known trans-acting regulators, changes in the T-tract length act as a promoter rheostat to fine-tune SabA expression. Clones with optimal SabA expression levels, which best fit the host prerequisites, survive and continue colonization. In addition to *sabA*, other genes with simple sequence repeat motifs located at similar positions in other

genes were also shown to be affect promoter output in a similar way (Åberg et al. 2014).

6.3.5 SabA Expression and Regulation by Acidic Conditions

The majority of *H. pylori* reside in the mucus layer, and a minor population (about 20 %) is situated close to the gastric epithelium (Hessey et al. 1990). There is a pH gradient from very acidic conditions in the lumen through the mucus layer to be close to neutral at the epithelium. Thus, pH is an important environmental factor that *H. pylori* have to sense and respond to. Using DNA arrays and RT-qPCR, it was shown that *sabA* expression decreases as a response to acidic conditions and that the repression is regulated via the acid responsive regulon ArsRS (Bury-Moné et al. 2004; Merrell et al. 2003; Pflock et al. 2006; Yamaoka et al. 2006). The ArsR response regulatory protein probably acts to regulated SabA expression via its direct binding to the *sabA* promoter region located −20 to +38 relative the transcriptional start site (Harvey et al. 2014). Considering the neutral pH close to the epithelium where the bacteria make use of adhesion properties, it seems logical to downregulate adhesion properties in order to loosen the grip from the shedding host gastric epithelial cell when it reaches the lumen. Alternatively, when ArsRS senses acidic pH, downregulation of binding aids the bacteria to avoid attachment to receptor moieties present on mucins where the pH is acidic and instead promotes motility and thus the ability to swim toward the environment of neutral pH.

6.3.6 SabA and Gastroduodenal Diseases

The first study to assay for sLex binding among clinical *H. pylori* isolates showed that 37 % among 95 European isolates bound to sLex. sLex-positive strains often exhibited binding to Leb as well. Among the sLex-binding strains, almost half of them showed binding to sLea (Mahdavi et al. 2002). The analysis of SabA expression in a collection of 200 clinical isolates from the USA and Colombia diagnosed with either gastritis, duodenal ulcer, or gastric cancer showed that 66 % of the gastritis isolates, 88 % of the duodenal ulcer isolates, and 89 % of the strains isolated from patients with gastric cancer exhibited SabA expression (Yamaoka et al. 2006). A study based on 145 Taiwanese clinical isolates showed that all isolates expressed BabA and 31 % expressed SabA. Here, no differences in SabA expression and outcome of disease were found (Sheu et al. 2006).

Upon *H. pylori*-induced gastritis, the gastric mucosa is infiltrated with neutrophils. The SabA adhesin seems to have an essential function in adherence of *H. pylori* to sialylated neutrophils. SabA-mediated binding to neutrophils was a

prerequisite for nonopsonic activation of neutrophils and is thus likely to have a role in phagocytosis of *H. pylori* (Unemo et al. 2005).

6.4 BabA- and SabA-Mediated Binding to Mucins

The majority of individuals are of positive secretor phenotype, i.e., express ABO/Leb antigens in the gastric mucus. *H. pylori* that resides in the stomach mucus layer binds to secreted highly glycosylated mucins (Lindén et al. 2002). The secreted MUC5AC and the MUC6 mucins are the two major mucins expressed in the stomach mucus layer (Lindén et al. 2002; Van den Brink et al. 2000). The BabA adhesin mediates binding of *H. pylori* to MUC5AC mucin in individuals of positive secretor phenotype that expresses Leb glycans (Lindén et al. 2002). In addition, *H. pylori* mediate binding to mucins carrying sLex by the SabA adhesin (Lindén et al. 2008). Similar as in humans, the glycosylation and spatial distribution of mucins change in rhesus macaques as a consequence of the *H. pylori* infection (Cooke et al. 2009; Lindén et al. 2008). Experimental infection of rhesus monkeys showed that individuals that display a weak-secretor phenotype had more stable levels of fucosylation, lower degree of inflammation, and lower bacterial infection load, whereas individuals with a positive secretor phenotype displayed increased levels of inflammation-associated (sialylated) glycans and a transient decrease in fucosylated (Leb) glycans. This suggested that the secretor phenotype determines the dynamics of mucosal glycosylation in response to *H. pylori* infection and that *H. pylori* infection is associated with an increase in sialylated (sLex/a) mucosal antigens and a reciprocal decrease in fucosylated (Leb) mucosal antigens (Lindén et al. 2008). At acidic conditions, the BabA-mediated binding to Leb-containing glycans on the mucins is abolished (Lindén et al. 2004). Moreover, the bacterial-mucin interplay seems to co-regulate both *babA* and *sabA* expression as well as bacterial growth. The effects were host specific because mucins from different individuals with different disease state caused different responses (Skoog et al. 2012). The MUC1 mucin is present in the gastric glands. Adherence to the MUC1 mucin may limit the *H. pylori* infection and protect the gastric glands from *H. pylori* colonization (Lindén et al. 2009). Thus, MUC1 act as a decoy due to the release of the MUC1 extracellular domain upon *H. pylori* binding (Lindén et al. 2009). Mice that carried a deletion in MUC1 exhibited increased *H. pylori* colonization and increased inflammation relative wild-type mice (McGuckin et al. 2007).

6.5 BabA- and SabA-Mediated Adhesion of *H. pylori* Outer Membrane Vesicles to the Gastric Mucosa

Gram-negative bacteria shed outer membrane vesicles (OMVs) (Kulp and Kuehn 2010). The exact role of OMVs is yet elusive but it is clear that they function as vehicles to deliver bacterial components to host cells. Since the composition of the OMVs represents the outer membrane of the bacteria and also contain additional components from mainly the periplasmic space, they are carrier of host-effector molecules and thus disease-promoting factors (Kulp and Kuehn 2010). *H. pylori* OMVs carrying the VacA cytotoxin are present in human gastric biopsy specimens (Fiocca et al. 1999). Two-dimensional $^{31}P^{1}H$ NMR correlation spectra have been used to determine the phospholipid composition of *H. pylori* OMVs, and comprehensive mass spectrometry analyses have been used to identify their protein composition (Olofsson et al. 2010). Phosphatidylethanolamine and cardiolipin were the dominating phospholipids (Olofsson et al. 2010), and the majority of outer membrane proteins were found in the OMVs and among them the BabA and the SabA adhesins (Mullaney et al. 2009; Olofsson et al. 2010). Adhesion is a key step for the delivery of toxins and effector molecules to target tissues. Using electron microscopy in combination with soluble Leb- and sLex-receptor conjugates and gold particles confirmed that *H. pylori* OMVs carry the BabA and the SabA adhesins and that both bound their respective receptor. Receptor displacement assay showed that BabA on intact *H. pylori* bacterial cells and OMV-BabA are bound to the Leb receptor with the same affinity, which suggested that the BabA adhesins exhibit similar folding in the outer membrane as in the OMVs (Olofsson et al. 2010). Moreover, the same study showed that both the BabA and the SabA adhesins on OMVs mediate receptor-specific adhesion to human gastric mucosa.

6.6 Conclusion and Outlook

For *H. pylori* the life in the human stomach is similar to a roller coaster ride. Close attachment to the epithelium offers a nutrient-rich, replicative niche but with a high risk of eradication by host-immune responses or clearance by shear forces caused by the peristaltic movement. Life further out in the mucus layer offers a famine lifestyle and a high risk of acid exposure. The glycans on mucins and on the gastric epithelial cells that function as receptors for *H. pylori* vary from person to person. The glycans that are expressed depend on the individual expression of the transferase enzymes involved in glycan biosynthesis. In addition to this, the *H. pylori* infection per se alters regulation of the glycan transferases, which results in variable expression levels in different locations in the stomach. Therefore, it is absolutely essential for *H. pylori* to continuously adapt its adhesion properties to fit the local gastric environment in order to stay colonized (Fig. 6.2). Detailed knowledge about the molecular terms that operate to fine-tune the expression of the BabA and SabA

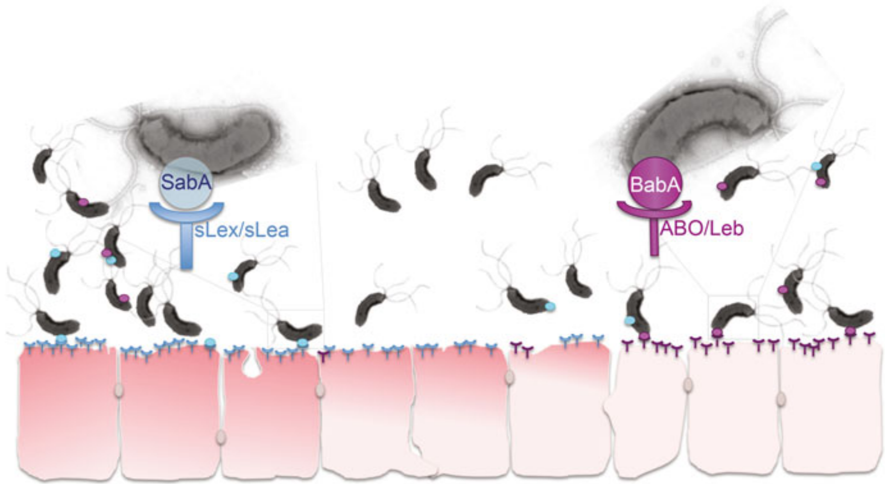

Fig. 6.2 Role of *H. pylori* adhesion in sickness and in health. The so far best-characterized *H. pylori* adhesins are the blood group antigen-binding BabA adhesin and the sialic acid-binding adhesin SabA. In the healthy gastric mucosa, the BabA adhesin binds to the fucosylated ABO/Leb antigens, while adhesion to inflamed gastric mucosa mainly is mediated by the binding of the SabA adhesin to the sLex and sLea antigens. The glycosylation of the gastric mucosa varies during the persistent *H. pylori* infection, and, therefore, the bacterium needs to adapt its adhesion properties accordingly. Expression of the BabA and the SabA adhesins can be switch on and off via homologous recombination or SSM. SSM also plays an important role in fine-tuning the expression levels of the SabA adhesin. A thymine (*T*) nucleotide repeat tract located close to the *sabA* −35 promoter element is a target for SSM, and changes in the T-tract length affect promoter strength and thus transcription initiation. Altogether, homologous recombination and SSM generate heterogeneous populations that include best-fit clones ready to adapt to any host changes such as changes in the glycosylation pattern and available receptor structures

adhesins in relation to continuously changes in the local environment during the persistent *H. pylori* infection remains to be solved.

Successful colonization is the first step for the development of chronic gastritis, peptic ulcer disease, and gastric cancer. Blocking attachment is an attractive target for future design of new drugs. Even though a large number of strains have been assayed for their Leb-binding activity and the nucleotide composition of the *babA* gene has been determined, no consensus for Leb binding in BabA has yet been determined (Aspholm-Hurtig et al. 2004). Further research to gain deep knowledge about the molecular details for ligand-receptor binding is crucial for design of drugs that can inhibit adhesion to the gastric mucosa. Therefore, structural determination will be essential to identify the Leb-receptor-binding domain. Recently, a crystal structure of a recombinantly expressed extracellular domain of the SabA protein (1–460 amino acids) was described to have a "club shape." A conserved cavity in the SabA head domain that may function as binding site was identified. The authors made attempts to co-crystallize the protein together with sLex but did not succeed.

However, upon testing the expressed SabA protein in surface resonance experiments, they could detect binding to sLex but not to Leb, Lea, or Ley. Some binding of low affinity was also detected in Lex. This difference in binding specificity of the recombinantly expressed SabA and native SabA may be explained by differences in steric blocking, hydrophobicity, or charges in the local environment of the SabA protein (Pang et al. 2014). Based on the analysis of the SabA primary and tertiary structure of the suggested binding pocket, four highly conserved amino acid residues were mutated. A Q159A substitution reduced biding to sLex, while the Y148A and Q162A substitutions showed reduced binding to Lex, and the K152A substitution did not result affect binding to neither of sLex or Lex (Pang et al. 2014). The SabA carbohydrate-binding domain contains amino acid residues that are conserved both between SabA orthologs but also to the BabA protein (Pang et al. 2014). Further research is needed to fully determine the relation between structure and function.

Besides the BabA and the SabA adhesins, *H. pylori* have other adhesion properties that aid in attachments and probably pathogenesis, but their function is beyond the scope of this chapter. These additional attachment mechanisms have to be taken into account to fully describe the adhesion process as well as in design of future drugs.

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Chapter 7
Emerging Novel Virulence Factors of *Helicobacter pylori*

Silja Wessler

Abstract Through the expression of a wide range of differently functioning virulence factors, *Helicobacter pylori* possesses multiple possibilities to interact with host target cells that in combination determine the development and progress of diseases. Prominent *H. pylori* factors such as the type IV secretion system (T4SS), cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), and blood-group antigen-binding adhesin (BabA)/sialic acid-binding adhesin (SabA) are discussed in other chapters. Besides those well-established pathogenic and virulence factors, a series of other bacterial factors exist, including neutrophil-activating protein (NapA), γ-glutamyl transpeptidase (GGT), tumor necrosis factor-alpha-inducing protein alpha (Tipα), the cell-translocating serine/threonine kinase (CtkA), and bacterial proteases, such as the serine protease high-temperature requirement A (HtrA) or the collagenase Hp0169. These factors are described as secreted *H. pylori* proteins that might be implicated in pathogenesis as well. As additional novel disease-associated factors, the *Helicobacter* outer membrane proteins (Hop) HopQ and HopZ and the duodenal ulcer-promoting gene A (DupA) are also currently under intensive investigation. Since these *H. pylori* factors are either secreted or surface exposed, they can directly interfere with host cell functions to modulate cellular responses. Research on these bacterial structures is still at the beginning; however, they represent novel target molecules for supporting therapeutic intervention strategies.

Keywords CtkA • DupA • GGT • HopQ • HopZ • *H. pylori* • HtrA • NapA • Tipα

7.1 Introduction

As a highly dynamic and complex bacterial organism, *H. pylori* encodes multiple different factors and structures, which have the capability to modulate host cell functions. The molecular mechanisms of the type IV secretion system (T4SS) that translocates the bacterial effect protein CagA, the secreted pore-forming protein

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VacA, or different adhesins, such as BabA or SabA, are subject of many studies, and most of them are well characterized of how they interact with host cell proteins. The cellular and molecular mechanisms of these proteins are discussed in other chapters (see Chaps. 3, 4, 5, and 6). Indeed, additional factors might contribute to bacterial pathogenesis of *H. pylori*. Obviously, *H. pylori*-induced pathogenesis is a multistep process reflecting many different modes of interference with host cell functions (Backert and Clyne 2011; Posselt et al. 2013; Wessler and Backert 2008). In particular, GGT, NapA, Tipα, CtkA, HopQ, HopZ, and HtrA attracted much attention as bacterial compounds in the last years that are implicated in many different mechanisms to induce cellular responses (Table 7.1), hence acting as emerging novel factors of *H. pylori*. These factors share their extracellular localization allowing direct contact with molecules of host cells to interfere with their unique functions (Fig. 7.1). However, it is still of debate whether those *H. pylori*

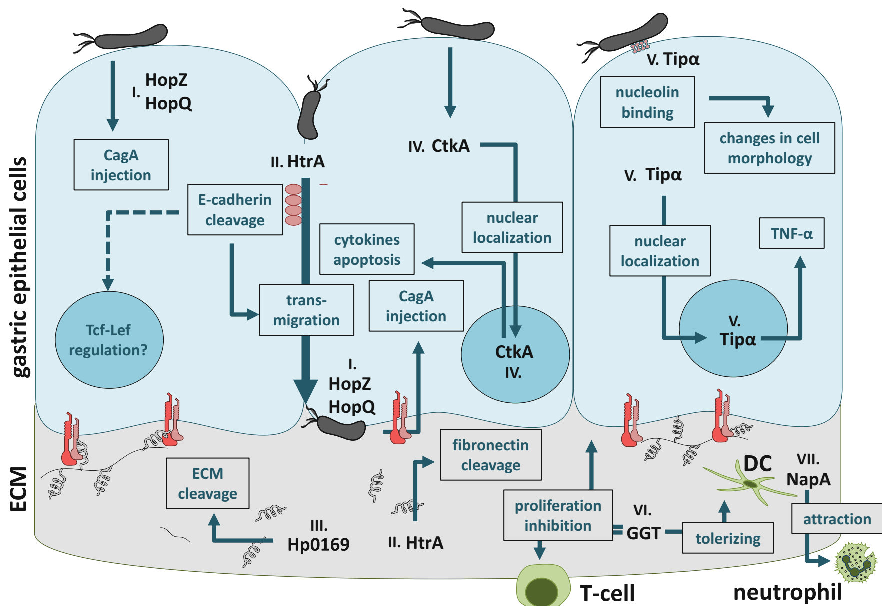

Fig. 7.1 Emerging novel factors with possible functions in *H. pylori* pathogenesis. The adhesins HopQ and HopZ (*I*) contribute to *H. pylori* adherence on gastric epithelial cells. HopQ expression promotes CagA translocation via an unknown host receptor. HtrA (*II*) is secreted by *H. pylori* and cleaves the cell adhesion molecule and tumor suppressor E-cadherin allowing transmigration of *H. pylori*. If E-cadherin cleavage affects Tcf-Lef signaling is unknown. HtrA also targets fibronectin, a component of the extracellular matrix (ECM). Hp0169 (*III*) functions as a collagenase with possible substrates in the ECM. CtkA (*IV*) is a dual-specific bacterial serine/threonine and tyrosine kinase, which enters the cell and translocates into the nucleus, where it is involved in gene regulation leading to cytokine release and apoptosis. Tipα (*V*) binds to nucleolin on the cell surface leading to changes of the gastric cell morphology and/or adhesion. Tipα also translocates into the nucleus to induce TNF-α expression. The enzyme GGT (*VI*) has multiple effects by inhibition of T cell and epithelial cell proliferation. Dendritic cells (DCs) are tolerized in response to GGT. Neutrophils are attracted by NapA (*VII*)

structures represent pathogenic or virulence factors or if they can facilitate the molecular interaction of other bacterial factors with host cells, which are discussed in this chapter.

7.2 The Effects of GGT on Epithelial Cells and Immune Cells

H. pylori commonly encode the gamma-glutamyl transpeptidase (GGT) as a constitutively expressed enzyme (Chevalier et al. 1999) that converts glutamine into glutamate and ammonia and glutathione into glutamate and cysteinylglycine. GGT is expressed as a 61 kDa protein that contains an N-terminally located signal peptide composed of 26 amino acids. After cleavage of the signal peptide, pro-GGT is further processed into a 40 kDa N-terminal polypeptide and a 21 kDa C-terminal domain (Chevalier et al. 1999). The 20 kDa variant of GGT has been identified in the soluble fraction of *H. pylori* culture supernatants, and the 38 kDa subunit was found in the structure bound and soluble fractions of *H. pylori* (Backert et al. 2005), indicating that GGT is actively secreted by *H. pylori*. GGT expression and activity have been correlated with the development of peptic ulcer disease (Gong et al. 2010). Initially, GGT was suggested as an essential factor for *H. pylori* colonization (Chevalier et al. 1999), but this statement has been softened a few years later in a study describing that GGT-negative *H. pylori* were able to colonize piglets and mice to a significant lower extent compared to isogenic wild-type strains (McGovern et al. 2001). The fact that *H. pylori* GGT knockout mutants can colonize animals was then repeatedly published (Chevalier et al. 1999; McGovern et al. 2001; Oertli et al. 2013) implying that GGT is not essential per se but obviously an important factor in the establishment of *H. pylori* infection. The reason for the reduced colonization of GGT-negative bacteria is still not clear as GGT obviously does not function as an adhesin or virtually contributes to bacterial survival. However, if GGT activity alters, bacterial growth is contradictory in the literature. Gong and co-workers reported that *H. pylori* growth correlated with the activity of GGT and that higher GGT activity favors bacterial growth in vitro (Gong and Ho 2004). Other publications indicated that GGT-negative *H. pylori* strains grew equally well as compared to the corresponding wild-type strain (Chevalier et al. 1999; McGovern et al. 2001; Rossi et al. 2012).

GGT from *H. pylori* and *H. bilis* inhibited AGS cell proliferation (Fig. 7.1), even though higher concentrations were required and involved an apoptosis-independent mechanism (Rossi et al. 2012). However, apoptosis as another cellular function for *H. pylori* GGT was also described (Shibayama et al. 2003). Similar effects on host cell death have also been observed for *H. suis* GGT (Flahou et al. 2011). Increasing concentrations of GGT, which was enriched from *H. pylori* membrane fractions, induced apoptosis in AGS cells, while an inhibitor targeting GGT activity decreased apoptosis significantly (Shibayama et al. 2003). This observation has

been confirmed by other studies. Recombinant GGT purified from *Escherichia coli* triggered apoptosis in AGS cells. Activation of caspase-3 and caspase-9 was induced by GGT stimulation, and a possible regulation of the apoptosis-related factors Bax and Bcl-2 was suggested by expression analyses (Boonyanugomol et al. 2012; Kim et al. 2007). GGT-dependent regulation of the inhibitor of apoptosis protein (IAP) survivin was recently also detected. The protein level of survivin is decreased in the mucosa of *H. pylori*-infected patients with gastritis (Valenzuela et al. 2010) and in *H. pylori*-colonized MKN-45 and AGS cells in vitro (Valenzuela et al. 2013). Further analysis revealed that the protein level of survivin is downregulated via a proteasomal pathway (Valenzuela et al. 2013). Many studies describing apoptosis-inducing properties of GGT have been performed using recombinant GGT. However, a GGT knockout mutant still induced T-cell apoptosis to a similar extent (Beigier-Bompadre et al. 2011), indicating that GGT might not play a major role in *H. pylori*-mediated induction of apoptosis in T cells.

Along with its apoptosis-inducing properties, enzymatically active GGT acts as a novel immunosuppressive factor by inhibiting T-cell proliferation (Fig. 7.1), which could contribute to the persistence and/or immune evasion of *H. pylori* (Schmees et al. 2007). T-cell inhibition resulted from depriving the extracellular space of glutamine (Wustner et al. 2015). Interestingly, GGT-mediated apoptosis was not observed in T cells (Schmees et al. 2007). Mechanistically, *H. pylori* GGT can induce a cell cycle arrest at the G1/S phase transition in T cells (Schmees et al. 2007) and in gastric epithelial cells (Kim et al. 2010). In epithelial cells, downregulation of the cell cycle-associated proteins cyclin E, cyclin A, cyclin-dependent kinase (Cdk) Cdk 4, and Cdk 6 and upregulated Cdk inhibitors p27 and p21 led to cell cycle arrest and apoptosis (Kim et al. 2010). In cultured AGS and MKN-28 cells, GGT-mediated upregulation of COX-2 and HB-EGF was observed (Busiello et al. 2004). Even though the functional consequences of enhanced COX-2 and HB-EGF expression were not associated to a cellular phenotype, the authors speculated that GGT influences the cell cycle via this pathway (Busiello et al. 2004). If those effects also occur in T cells leading to the inhibition of proliferation has not been analyzed in detail yet (Schmees et al. 2007). T-cell inhibition was initially reported as a VacA-mediated effect (Gebert et al. 2003); hence, GGT is an additional important factor in T-cell inhibition underlining the importance of secreted factors in *H. pylori* pathogenesis. Furthermore, in a following publication, it was shown that both GGT and VacA contributed to *H. pylori's* tolerizing effects on murine dendritic cells (DCs) in vitro and in vivo (Fig. 7.1) (Oertli et al. 2013), which can also contribute to the prevention of asthma in vivo (Engler et al. 2014). This effect was independent of T cells indicating important functions of GGT in manipulating the immune system and establishing persistent infection. These kinds of combined effects of VacA and GGT were also described for the induction of miRNA-155 (Fassi Fehri et al. 2010). GGT/VacA-induced miRNA-155 directly targets the protein kinase A inhibitor α (PKIα) mRNA in a cAMP-Foxp3-dependent manner in T cells (Fassi Fehri et al. 2010). However, whether this pathway contributes to the T-cell inhibition or other responses is yet unknown.

The research on GGT is still at the beginning as reflected by the small but accumulating number of publications. However, these studies on the relatively diverse pathways and cellular responses are steadily increasing and will more and more complete the picture of GGT as a novel and important factor in *H. pylori* pathogenesis.

7.3 NapA Affects the Host Immune System

The effects of neutrophil-activating factor A (NapA) on *H. pylori*-infected host cells appear to be restricted on immune cells, while functions on gastric epithelial cells were not yet described. Initially, *H. pylori* NapA was identified as an extracellular 150 kDa multimeric protein complex in water extracts of *H. pylori* (Evans et al. 1995). It was suggested that NapA is released from the cytosol by autolysis, but the exact pathway of NapA delivery into the supernatant is yet unknown.

It was consistently described that NapA can attract neutrophils (Fig. 7.1) via chemotaxis and promotes the adherence of neutrophils to endothelial cells. In principle, recombinantly expressed NapA from *Bacillus subtilis* and *E. coli* was used for these studies (Table 7.1), but also NapA enriched from *H. pylori* water extracts has been associated with increased adherence of neutrophils to endothelial cells, which could be decreased by adding a polyclonal anti-NapA antibody (Evans et al. 1995). LPS-free NapA produced in *B. subtilis* significantly induced chemotaxis of neutrophils and monocytes (Satin et al. 2000). Furthermore, recombinant NapA resulted in an increased transendothelial migration of neutrophils, which was slightly reduced using a NapA-deletion mutant of *H. pylori* without exerting a direct effect on endothelial cells (Brisslert et al. 2005). When rat mesenteric venules were directly exposed to NapA, leukocytes were stimulated to adhere to the endothelium. The authors then indicated that NapA has the capability to cross the endothelial layer to promote leukocyte adherence directly through the activation of β2-integrins. They excluded a paracellular route of NapA delivery but suggested a mechanism that involves a NapA transport within the endothelial cells (Polenghi et al. 2007). In transwell filter assays using Caco-2 cells as a polarized cell culture model, it was repeatedly shown that NapA itself could translocate from the apical to the basolateral compartment. The mechanism of NapA transmigration in this model remained unknown (Montemurro et al. 2002). However, these data consistently indicate that NapA plays a prominent role in the attraction of neutrophils to the site of infection.

In expression analyses of mononuclear cells (MNC), NapA induced the expression of tissue factor (TF) and PAI-1 (plasminogen activator inhibitor-2) (Montemurro et al. 2001). In peritoneal mast cells (PMC) from rats indicated that NapA induced the release of β-hexosaminidase and interleukin-6 (IL-6). Both could be blocked using pertussis toxin suggesting the involvement of heterotrimeric G proteins (Montemurro et al. 2002). It was further implied that recombinant NapA induced the production of reactive oxygen intermediates (ROI) in the host

(Evans et al. 1995), which involved activation of the plasma membrane NADPH oxidase via phosphatidylinositol 3-kinase (PI3-K) and Src family tyrosine kinases (Satin et al. 2000). This signaling pathway might be upstream of the activated MAPK (mitogen-activated protein kinase) ERK1/2 and p38 in NapA-treated neutrophils, which was shown by Nishioka and colleagues a few years later (Nishioka et al. 2003). Inhibition of ERK1/2 and p38 significantly inhibited chemotaxis in these cells (Nishioka et al. 2003). These studies were mainly performed using recombinant NapA protein. In contrast, using a deletion mutant of *H. pylori*, a NapA-dependent increase of oxidative burst was observed (Petersson et al. 2006) making more experiments necessary to investigate oxidative burst in response to NapA.

NapA forms dodecameric multimers that bind iron in a ferroxidase site (Tonello et al. 1999; Yokoyama et al. 2012; Zanotti et al. 2002). A functional ferroxidase site appears to be important for the formation of dodecamers to protect DNA from oxidative damage without direct binding (Kottakis et al. 2008). As potential host cell binding partners for NapA, sulfated oligosaccharide structures (Namavar et al. 1998) and acid glycosphingolipid fraction on neutrophils (Teneberg et al. 1997) were identified. Later it was speculated that NapA represents a toll-like receptor-2 (TLR2) agonist to induce NF-κB-mediated IL-12 and IL-23 release in neutrophils and monocytes (Amedei et al. 2006). Among the induced cytokines, NapA-mediated IL-1β release from monocytes appears to trigger an increase in cell survival through preventing apoptosis (Cappon et al. 2010). The possible NapA function as a TLR2 ligand was investigated in tumor growth in a bladder cancer mouse model. Interestingly, local administration of NapA decreased tumor growth by triggering tumor necrosis (Codolo et al. 2012) suggesting that recombinant NapA could be applied in anticancer strategies. The assumption that NapA functions as a therapeutic agent has been confirmed and expanded by further reports. In vivo recombinant NapA induced Th1 polarization and decreased T-cell-specific allergic responses, which might be a novel strategy of prevention and treatment of allergic diseases or asthma (Amedei et al. 2006; Codolo et al. 2008). A similar beneficial effect of NapA on *Trichinella spiralis*-mediated pathogenesis was also detected (Del Prete et al. 2008). NapA is highly immunogenic as many *H. pylori*-infected patients have antibodies against NapA and produced a significant immunoprotective response in mice (Satin et al. 2000). Using attenuated measles virus (MV) vectors, animals immunized with MV strains expressing the secretory NapA antigen developed strong humoral immunity against NapA (Iankov et al. 2011). This model was further developed for the therapeutic efficacy of oncolytic MV strains expressing NapA as a therapeutic transgene for the treatment of metastatic breast cancer pleural effusion (Iankov et al. 2012). According to the measles virus model, oncolytic adenovirus harboring *napA* as an immunomodulatory gene exhibited therapeutic effects on neuroendocrine tumors (Ramachandran et al. 2013). It was further demonstrated that MV-encoded NapA-tagged chimeric antigen can induce significantly stronger immune response (Iankov et al. 2013) indicating that NapA can enhance immunogenicity of poor immunogens as a novel approach in vaccine development and immunotherapy. In summary, these results

identify NapA as a virulence factor relevant for the pathogenic effects of *H. pylori* at the sites of infection and point to NapA as a therapeutic agent in immunotherapy and cancer-related disorders.

7.4 Tipα: A Multifunctional Factor?

The induction of cytokines in epithelial cells in response to *H. pylori* infection is dependent on a functional T4SS (Backert et al. 2010; Fischer et al. 2001). In addition to these well-established data, the tumor necrosis factor-alpha-inducing protein alpha (Tipα) was suggested as a novel bacterial factor that activated nuclear factor kappa B (NF-κB) and TNF-α in Bhas 42 (BALB/3T3 cells transfected with *v-H-ras* gene) and MGT-40 cells (mouse gastric epithelial cell line) exhibiting transforming activities (Suganuma et al. 2005). This unexpected observation was confirmed in macrophages (Godlewska et al. 2008), a study indicating that Tipα also has an influence on mice colonization (Godlewska et al. 2008), which might account for the NF-κB activation. Because TNF-α acts as a promoter of tumor progression (Bauer et al. 2012; Vendramini-Costa and Carvalho 2012; Walczak 2011), Tipα has consequently been suggested as a carcinogenic factor that might trigger inflammation and/or carcinogenesis in patients infected with *cag*PAI-negative *H. pylori* strains. In this context, future studies are required to investigate the pathophysiological role of Tipα in vivo.

Structurally, Tipα contains an N-terminal signal peptide and forms a homodimer via one or two interchain disulfide bonds mediated by cysteine residues C25 and C27 at low pH values and hydrophobic interactions (Jang et al. 2009; Tosi et al. 2009; Tsuge et al. 2009). It might also share some homologies with penicillin-binding proteins of Gram-positive bacteria (Kuzuhara et al. 2005) implying that the *tipα* gene was acquired by horizontal gene transfer. Tipα was also described as a highly immunogenic factor. In vivo, recombinant Tipα can stimulate the production of specific antibodies (Voland et al. 2002), which could induce a partially protective response against *H. pylori* colonization (Inoue et al. 2009). In DNA microarray and ELISA analyses, several chemokines and cytokines (e.g., Ccl2, Ccl7, Cxcl1, etc.) were upregulated in Tipα-treated cells (Kuzuhara et al. 2007a; Tang et al. 2013). Mechanistically, it was further suggested that Tipα can be internalized by epithelial cells to translocate into the nucleus (Fig. 7.1), where it binds DNA (Suganuma et al. 2008). Supporting this assumption, in DNA-binding experiments, recombinant Tipα bound different elements of the TNF-α promoter (Kuzuhara et al. 2007b) leading to the hypothesis that Tipα could directly transactivate gene promoters to trigger cytokine/chemokine synthesis and carcinogenesis. Hence, it is unclear to which extent Tipα-induced NF-κB or Tipα itself activates cytokine synthesis. Recently, nucleolin was described as a cell surface receptor for Tipα (Watanabe et al. 2010a, b) proposed as the origin of signal transduction leading to TNF-α transactivation. The main function of nucleolin as chromatin-associated protein containing histone chaperone activities

involves the regulation of many different aspects of gene transcription and DNA metabolism (Storck et al. 2007). On cancer cells, nucleolin was also detected on the cell surface, where it is implicated in signal transduction events and also in viral and bacterial infections (Abdelmohsen and Gorospe 2012). Tipα-nucleolin interaction was described to change the cell morphology and adhesion (Fig. 7.1) which was interpreted as "epithelial-mesenchymal transition" (EMT) (Watanabe et al. 2014). Generally, EMT is considered as a morphogenetic reprogramming of epithelial cells leading to the loss of typical epithelial properties and the increase of a highly motile, mesenchymal morphology (Lamouille et al. 2014; Nieto 2013). *H. pylori*-infected AGS cells strongly develop an EMT-like phenotype as the consequence of CagA injection and tyrosine phosphorylation (Backert et al. 2001; Moese et al. 2004). Indeed, the authors observed an upregulation of vimentin in Tip-α-treated cells representing a protein marker for mesenchymal cells and an increase in cell adhesion and spreading (Watanabe et al. 2014). Although a Tipα-knockout *H. pylori* mutant exists (Godlewska et al. 2008), a large number of publications describe effects of recombinant Tipα (Table 7.1). In summary, function of Tipα in *H. pylori* pathogenesis needs to be validated since an in vivo proof with a Tip-α-deletion mutant of *H. pylori* is still missing in most reports.

7.5 JHP0940 Encodes the Bacterial Kinase CtkA

Comparing the genomes of the *H. pylori* strain Hp26695 with J99 revealed various strain-specific open reading frames (ORFs). One of these ORFs, JHP940, is located within a plasticity region of J99 but is absent in Hp26695. Based on the observation that JHP940 was more frequently expressed in gastric cancer strains, JHP940 was suggested as a potential pathogenicity marker (Occhialini et al. 2000; Yakoob et al. 2010). This correlation between genetic *jhp0940* variation and *H. pylori*-associated disease was further expanded on patients with chronic gastritis and duodenal and gastric ulceration (Armitano et al. 2013; Yakoob et al. 2010). In contrast to these studies, *jhp0940*-positive *H. pylori* isolates were not significantly associated with gastric ulcer, duodenal ulcer, or gastric carcinoma in other reports (Romo-Gonzalez et al. 2009; Santos et al. 2003; Sugimoto et al. 2012). The reasons for these contradictory studies are not fully clear but might reflect geographic differences in *H. pylori* isolates.

Although JHP940 is not expressed in all isolates, it could exert cellular responses during infection with *H. pylori*. JHP940 is expressed as a 36 kDa protein, which stimulated NF-κB-mediated TNF-α and IL-8 secretion in human macrophages (Rizwan et al. 2008). Functionally, JHP940 acts as an active, autophosphorylating serine/threonine kinase, which enters, as a recombinant protein, the nucleus of human cells to induce NF-κB activity and cytokine secretion (Fig. 7.1). Therefore, JHP940 was renamed cell-translocating kinase A (CtkA) (Kim do et al. 2010). Recently, CtkA secretion was demonstrated to be involved in the induction of host

Table 7.1 Proposed functions of emerging *H. pylori* pathogenicity factors

Name[a]	Function	Physiological role	Cellular phenotype	Method[a]	References
CtkA	Kinase	Apoptosis		Rec. CtkA	Tenguria et al. (2014)
		Inflammation	Induction of cytokines	Rec. CtkA	Kim do et al. (2010), Rizwan et al. (2008), and Tenguria et al. (2014)
			Nuclear localization	Rec. CtkA	(Kim do et al. 2010)
GGT	Hydrolase	Supports colonization/ persistence	Supports adherence	Knockout	Chevalier et al. (1999), McGovern et al. (2001), and Oertli et al. (2013)
			Inhibits T cells	Knockout	Beigier-Bompadre et al. (2011) and Schmees et al. (2007)
				Rec. GGT	Boonyanugomol et al. (2012) and Schmees et al. (2007)
				GGT inhibitor	Schmees et al. (2007)
			DC tolerization	Knockout	Oertli et al. (2013)
				GGT inhibitor	Oertli et al. (2013)
		Epithelial cell survival	Induces apoptosis	Knockout	Boonyanugomol et al. (2012), and Kim et al. (2007), Shibayama et al. (2003), and Valenzuela et al. (2013)
				Enriched GGT	Shibayama et al. (2003)
				GGT inhibitor	Shibayama et al. (2003)
				Rec. GGT	Flahou et al. (2011) and Kim et al. (2007, 2010)
			Inhibits proliferation	Rec. GGT	Rossi et al. (2012)
		Prevents asthma		Knockout	Engler et al. (2014)
				Rec. GGT	Engler et al. (2014)

(continued)

Table 7.1 (continued)

Name[a]	Function	Physiological role	Cellular phenotype	Method[a]	References
HopQ	Adhesin		CagA translocation	Knockout	Belogolova et al. (2013) and Jimenez-Soto et al. (2013)
HopZ	Adhesin		Binding to epithelial cells	Knockout	Peck et al. (1999) and Yamaoka et al. (2002)
Hp0169	Protease	Supports colonization of *H. pylori*	Degrades components of the ECM	Knockout	Kavermann et al. (2003)
				Rec. Hp0169	Kavermann et al. (2003)
HtrA	Protease	Disruption of adherence junctions	Cleaves E-cadherin	Rec. HtrA	Hoy et al. (2010, 2012)
				Inhibitor of HtrA	Hoy et al. (2010, 2012)
			Cleaves fibronectin	Rec. HtrA	Boehm et al. (2012) and Hoy et al. (2010)
NapA	–	Effects on neutrophils	Chemotaxis	Rec. NapA	Nishioka et al. (2003) and Satin et al. (2000)
			Adhesion	Rec. NapA	Evans et al. (1995), Nishioka et al. (2003), and Polenghi et al. (2007)
				Blocking antibody	Evans et al. (1995)
				Water extract	Evans et al. (1995)
			Transendothelial migration	Knockout	Brisslert et al. (2005)
				Rec. NapA	Brisslert et al. (2005)
				Cell culture supernatant	Brisslert et al. (2005)
			Induction of oxidative burst	Rec. NapA	Nishioka et al. (2003) and Satin et al. (2000)
				Enriched NapA	Evans et al. (1995) and Satin et al. (2000)
			Decrease of oxidative burst	Knockout	Petersson et al. (2006)

(continued)

Table 7.1 (continued)

Name[a]	Function	Physiological role	Cellular phenotype	Method[a]	References
		Effects on monocytes and DCs	Induction of cytokines	Rec. NapA	Amedei et al. (2006) and Cappon et al. (2010)
				Knockout	Amedei et al. (2006)
			Anti-apoptosis	Rec. NapA	Cappon et al. (2010)
				IP NapA	Cappon et al. (2010)
			TLR2 ligand	Rec. NapA	Amedei et al. (2006)
		Further applications	Prevention of allergic disorders/asthma	Rec. NapA	Amedei et al. (2006) and Codolo et al. (2008)
			Antitumor (immune) response	Rec. NapA	Codolo et al. (2012)
				Rec. viruses	Iankov et al. (2012) and Ramachandran et al. (2013)
			Enhances vaccination against viruses	Rec. measles virus	Iankov et al. (2011, 2013)
Tipα	–	Involved in colonization		Knockout	Godlewska et al. (2008)
		Modulation of the immune system	DNA binding	Rec. Tipα	Jang et al. (2009) and Kuzuhara et al. (2007b)
			Induction of cytokines and chemokines	Rec. Tipα	Kuzuhara et al. (2007a), Suganuma et al. (2005, 2008), and Tang et al. (2013)
		Induction of “EMT”	Induction of cell migration/spreading	Rec. Tipα	Watanabe et al. (2014)

[a]Abbreviations: *CtkA* cell-translocating serine/threonine kinase A, *EMT* epithelial-mesenchymal transition, *GGT* γ-glutamyl transpeptidase, *Hop* outer membrane protein, *HtrA* high-temperature requirement A, *IP* immunoprecipitation, *NapA* neutrophil-activating protein, *rec.* recombinant, *Tipα* tumor necrosis factor-alpha-inducing protein alpha, *TLR2* toll-like receptor 2

cell apoptosis (Fig. 7.1) (Tenguria et al. 2014). Furthermore, it could be shown that CtkA is also an autophosphorylating tyrosine kinase (Tenguria et al. 2014) with unknown substrates. Although the implication of bacterial tyrosine kinases in bacterial physiology became more accepted in the last decade, CtkA is the first identified tyrosine kinase in *H. pylori*. Together with its translocating capacity and its threonine/serine and tyrosine kinase activity, CtkA is an attractive novel factor in the research of *H. pylori* pathogenesis.

7.6 *Helicobacter pylori* Secretes Proteases That Target Host Cell Proteins with Important Functions in Pathogenesis

Comparable to secreted NapA, GGT, or Tipα, various proteases represent interesting virulence factors if they are cell surface presented or secreted. The first identified active protease secreted or exposed by *H. pylori* with a putative function in *H. pylori* pathogenesis was Hp0169, which acts as a calcium-dependent collagenase that degrades components of the extracellular matrix of host cells (Kavermann et al. 2003). In vitro, type I collagen was used as a substrate to demonstrate proteolytic activity of Hp0169 (Fig. 7.1). Together with the observation that genomic deletion of the *hp0169* gene led to a drastic defect in stomach colonization of mice indicated that Hp0169-targeted ECM components are crucially necessary for colonization (Kavermann et al. 2003).

In fact, *H. pylori* express a wide range of different (hypothetical) proteases with unidentified functions (Lower et al. 2008). For instance, *H. pylori* sheds a protease that degrades PDGF (platelet-derived growth factor) and TGF-β (transforming growth factor beta) (Piotrowski et al. 1997; Slomiany et al. 1996). The identity of this protease is of high interest since this protease might directly influence distinct signal transduction pathways, which are under direct control of *H. pylori*. Generally, pharmacologically interesting proteases have an extracellular localization for accessible druggability. In a comprehensive study, the whole genome of *H. pylori* was analyzed to predict hypothetical proteases exhibiting an extracellular localization. Among 14 proteins with hypothesized proteolytic activities (e.g., Hp1012, Hp0506, etc.), HtrA was identified as a secreted active serine protease (Lower et al. 2008). HtrA has previously been localized in supernatants of *H. pylori* cultures, but activity and biological significant substrates were unknown (Bumann et al. 2002). In organisms, HtrA proteases are widely expressed, and its functions as a serine protease and chaperone in bacterial physiology have been well described in *Escherichia coli* and some other bacteria (Clausen et al. 2011; Hansen and Hilgenfeld 2013). In *E. coli*, three different types of HtrAs are synthesized (DegP, DegQ, and DegS). The closest homologue to *H. pylori* HtrA in *E. coli* is DegP, which is active as multiple trimers (Clausen et al. 2011). The structure of HtrA is highly conserved. Its N-terminal signal peptide is necessary for periplasmic localization. After a stretch of approximately 200 amino acids, there is a serine protease domain containing a classical catalytic triad composing an asparagine, histidine, and serine. The protease domain is followed by two PDZ domains which are important for protein-protein interaction and promote the formation of trimer, hexamer, dodecamers, etc. (Clausen et al. 2011). The proteolytic activity of *H. pylori* HtrA has initially been demonstrated in 2008 by the detection of HtrA in casein zymography analysis. In this study, HtrA was identified as an active monomer of ~50 kDa and as a multimer of >170 kDa (Lower et al. 2008). These data were consistent with an earlier report describing a metalloproteinase-like protease secreted with a native molecular size of approximately 200 kDa, which was detected by casein zymography (Windle and Kelleher 1997). Although the

identity of this protease remained unknown in this report, it appears likely that this is the first description of active *H. pylori* HtrA. The authors suggested that this surface-exposed metalloprotease activity might be involved in proteolysis of a variety of host proteins in vivo and thereby contribute to gastric pathology (Windle and Kelleher 1997).

If *H. pylori* HtrA exhibits a role for pathogenesis remained speculative for a long time. From other pathogens, it has been proposed that HtrA proteases increase the viability of bacteria under stress conditions (Ingmer and Brondsted 2009). This role was mainly attributed to the observation that HtrA acts a chaperone in the protein quality control of microbes and degrades misfolded proteins. Eventually, *H. pylori* HtrA increases viability under stress conditions since it tolerated extreme temperatures, pH, and ion concentrations (Hoy et al. 2013). However, data are accumulating that there is also a more direct role of HtrA in bacterial pathogenesis.

7.7 HtrA Can Affect *H. pylori* Pathogenesis via Direct Cleavage of E-Cadherin and Fibronectin

Besides its function as a chaperone to degrade misfolded proteins in the periplasm, HtrA can directly interfere with proteins exposed on the surface of host cells. E-cadherin and fibronectin were identified as the first substrates with biological functions for *H. pylori* HtrA (Fig. 7.1) (Hoy et al. 2010). The role of HtrA-mediated fibronectin cleavage is not well understood. Importantly, *H. pylori* CagL targets β1-integrins to inject CagA (Kwok et al. 2007). This interaction occurs via the RGD motif, a well-characterized integrin-binding motif in fibronectin. Hence, it might be interesting to investigate if HtrA-mediated fibronectin cleavage facilitated CagL binding to β1-integrin to promote CagA delivery in host cells.

The molecular and functional mechanism of HtrA-mediated E-cadherin cleavage is better understood. The gastric epithelium forms an effective first protection barrier against intruding bacteria, which requires a highly structured architecture establishing and maintaining an apical-basolateral domain structure. As important intercellular structures, gastric epithelial cells express different junctions mediating cell-to-cell adhesion and intercellular communication. Among them, the functional integrity of adherence junctions plays an important role. The transmembrane protein E-cadherin is the key molecule in the establishment and maintenance of adherence junctions (van Roy 2014). The extracellular E-cadherin domain forms homophilic interactions between the extracellular E-cadherin domains of two adjacent cells. The intracellular domain of E-cadherin recruits signaling proteins into a multiprotein complex. Among them, $p120^{ctn}$ binds to the juxtamembrane, while another catenin, β-catenin, binds to the intracellular E-cadherin domain and bridges E-cadherin to the actin cytoskeleton via binding to α-catenin (van Roy 2014). HtrA cleaves off the extracellular E-cadherin domain resulting in the disruption of adherence junctions allowing *H. pylori* to enter the intercellular

space of gastric epithelial cells (Fig. 7.1). This mode of transmigration can be blocked by a specific HtrA inhibitor that prevents E-cadherin shedding (Hoy et al. 2010). HtrA-triggered E-cadherin cleavage and consequent disruption of adherence junctions are not unique for *H. pylori*. A similar mechanism has been observed for *Campylobacter jejuni* and has been suggested for other Gram-negative pathogens of the gastrointestinal tracts indicating that the interference of HtrA with host cells functions is rather a prevalent mechanism in bacterial pathogenesis (Boehm et al. 2012; Hoy et al. 2012).

In contrast to other pathogens, functional investigation of HtrA in *H. pylori* infections is rather challenging because it was not possible to create a genomic deletion or mutations in the *htrA* gene of *H. pylori* (Hoy et al. 2010; Salama et al. 2004). Similar to the reports on Tipα or NapA, most data were obtained from studies using recombinant HtrA protein (Table 7.1). Therefore, small molecule inhibitors have been designed via structure-based virtual screening based on a comprehensive prediction of ligand-binding sites on a computational protein model (Hoy et al. 2010; Lower et al. 2011). A preliminary X-ray structure of *H. pylori* HtrA confirmed the computation model (Perna et al. 2014). In vitro and in cell culture studies, pharmacological inhibition of HtrA activity using defined lead structures decreased E-cadherin ectodomain shedding and bacterial transmigration through the intercellular space (Hoy et al. 2010, 2012).

The functional integrity of adherence junctions attracted much attention to the research community since it has been discovered that $p120^{ctn}$ and β-catenin have an additional role in tumor development (van Roy 2014). Besides contributing to the integrity and stability of the E-cadherin-mediated AJ, β-catenin and $p120^{ctn}$ can be released form E-cadherin and can translocate into the nucleus where they act as important cofactors for TCF/Lef transcription factors of cancer-related target genes. Therefore, functional E-cadherin-based AJs are also considered as important tumor suppressors (van Roy 2014). Hence, it might be interesting if HtrA-mediated E-cadherin cleavage might also promote *H. pylori*-dependent gastric carcinogenesis through the disruption of intercellular adhesions and the induction of E-cadherin-originated signal transduction pathways.

7.8 The *Helicobacter* Outer Membrane Proteins HopQ and HopZ Contribute to Bacterial Adherence

H. pylori express a large family of *hop* genes encoding outer membrane proteins (Alm et al. 2000). Among this large family, HopZ and HopQ acquired increased attention as reports are accumulating indicating that these factors play important roles in *H. pylori*-host interactions.

HopZ is encoded by two alleles, which were differentially distributed in *H. pylori* strains. The intact HopZ protein functions as an adhesion in vitro and in vivo (Peck et al. 1999; Yamaoka et al. 2002) but had no influence on CagA

translocation (Odenbreit et al. 2002). The on/off expression status of the phase-variable *hopZ* gene could not consistently be associated with *H. pylori* diseases (de Jonge et al. 2004a, b; Lehours et al. 2004). These data might indicate that HopZ function is redundant and can be compensated by other adhesins.

Corresponding to HopZ, HopQ is also expressed from two *hopQ* alleles that share 75–80 % identical nucleotide sequences and could be classified as type I and type II *hopQ* alleles according to their relatedness in different *H. pylori* strains (Cao and Cover 2002). Type I *hopQ* alleles were stronger associated with *cag*$^+$/type s1-*vacA* strains from patients with peptic ulcer disease (Cao and Cover 2002). While type I *hopQ* alleles were found in Western and Asian *H. pylori* strains, type II *hopQ* alleles were mainly identified in Western *H. pylori* (Cao et al. 2005).

HopQ is localized on the surface of *H. pylori* (Sabarth et al. 2005) implying that it could represent a functional adhesin. *H. pylori hopQ* mutants showed different phenotypes in adherence dependent on the *H. pylori* strain. In Hp26695 and J178, *hopQ* mutants were hyperadherent, but no alteration were observed in J99 and 87-29. Correspondingly, CagA injection and tyrosine phosphorylation were enhanced in AGS cells infected with hyperadherent *hopQ* mutants, but IL-8 secretion was not affected (Loh et al. 2008). In coinfection experiments of two different *H. pylori* isolates, HopQ expression in one competing strain was involved in the restriction of CagA translocation by the other strain. Although CagA translocation of the *hopQ*-negative *H. pylori* was not investigated in this study, these data suggest that HopQ expression may play a role in CagA translocation by a yet unknown mechanism (Jimenez-Soto et al. 2013). However, HopQ was recently identified as an important factor promoting CagA translocation in a study employing *H. pylori hopQ* knockout and complemented mutants (Fig. 7.1). Here, deletion of *hopQ* also decreased NF-κB and MAPK activity and exhibited negative effects on IL-8 secretion. The *hopQ* mutant did not show a defect in bacterial adhesion to host cells (Belogolova et al. 2013). In summary, HopQ is an interesting novel candidate with signal transduction-inducing capacities, which needs to be investigated in more detail.

7.9 DupA as a Marker for *H. pylori*-Associated Disorders

The first description of *dupA* as a duodenal ulcer-promoting gene revealed that it was the only gene in 500 *H. pylori* strains from East Asia and South America that was significantly associated with the induction of duodenal ulceration with neutrophil infiltration, while DupA exhibited protective effects on atrophy, intestinal metaplasia, and gastric cancer (Lu et al. 2005; Zhang et al. 2008). Many different reports followed and described an association between the presence of *dupA* and *H. pylori*-dependent diseases in different geographic populations. The correlation between DupA and duodenal ulcer was not confirmed in other reports (Argent et al. 2007; Douraghi et al. 2008; Gomes et al. 2008). These inconsistent data could be explained by the finding that *dupA* genes are polymorphic and most *H. pylori*

strains contain a longer *dupA* allele (Hussein et al. 2010) suggesting that *dupA* has two genotypes, which are responsible for the conflicting data. Underlining this consideration, *H. pylori* containing the long *dupA* variant induced a significant higher IL-12p40 level in PBMCs but not in epithelial cells (Hussein et al. 2010). This could also point to a cell-type-specific response. Aforementioned studies mainly base on the comparison of *dupA*-positive versus *dupA*-negative *H. pylori* strains but not on the genes sequence. A more extensive analysis indicated that not *dupA* alone but a *dupA* gene cluster determines the risk of developing duodenal ulcers (Jung et al. 2012). Since DupA shares high homologies to the VirB4 ATPase, it was speculated that DupA participates in T4SS-dependent processes. The existence of additional *vir* genes around the *dupA* gene could indicate an addition T4SS system resembling the *cag*PAI and the ComB system. However, as long as neither DupA protein expression nor a biochemical function in *H. pylori* could be demonstrated experimentally, it remains a theoretical debate.

7.10 Conclusions and Outlook

The interaction of VacA and the T4SS-delivered CagA with host cells has been intensively investigated and, hence, is relatively well understood how they manipulate host cell signaling contributing to *H. pylori*-associated disorders. Investigation of GGT, NapA, CtkA, HopQ, HopZ, Tipα, or HtrA in *H. pylori* pathogenesis is a comparatively young field leading to the accumulation of novel interesting data, which might add important pieces into puzzle of the multiple mechanisms of *H. pylori* factors interfering with host cell functions (Table 7.1). More efforts are necessary to identify receptors or target molecules in host cells to increase our understanding of the complex network of *H. pylori*-host interactions. However, most of these factors share their extracellular localization making them attractive candidates for pharmacological inhibition or vaccination strategies.

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Chapter 8
The Primary Transcriptome and Noncoding RNA Repertoire of *Helicobacter pylori*

Sandy R. Pernitzsch, Fabien Darfeuille, and Cynthia M. Sharma

Abstract The intense study of *Helicobacter pylori*, one of the most prevalent human pathogens, has contributed much to understanding of bacterial virulence mechanisms. While genome sequencing revealed a high genetic diversity among *Helicobacter* strains, its transcriptional organization has been less understood. The *H. pylori* genome encodes for only a small number of transcriptional regulators, and little is known about the role of posttranscriptional gene regulation and the mechanisms and functions of small regulatory RNAs (sRNAs) in Epsilonproteobacteria. Until recently, *Helicobacter* was even regarded as a bacterium without RNA-based regulation (riboregulation). However, the development of deep sequencing technology and its application to massively parallel high-throughput cDNA analysis (RNA-seq) has revolutionized transcriptome analysis of pro- and eukaryotes. A differential RNA-seq (dRNA-seq) study of *H. pylori* strain 26695 selective for primary transcriptome analysis allowed for genome-wide mapping of transcriptional start sites. This study also revealed an unexpectedly complex and compact transcriptional output from the small *H. pylori* genome. Besides an extensive antisense transcription, more than 60 sRNA candidates were identified, including potential regulators of *cis*- and *trans*-encoded target mRNAs. This indicates that posttranscriptional regulation represents an extensive layer of gene expression control in *Helicobacter*. In this chapter we review how RNA-seq has facilitated transcriptome annotation and identification of novel regulatory features in *H. pylori* and what is currently known about sRNAs in this widespread gastric pathogen.

Keywords *Helicobacter pylori* • RNA sequencing (RNA-seq) • Deep sequencing • Terminator exonuclease • Differential RNA-seq (dRNA-seq) • Small regulatory RNAs • Posttranscriptional regulation • Transcriptional start sites • Primary transcriptome

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8.1 Introduction

The human stomach is a dynamic and hostile environment, in which the gastric pathogen *Helicobacter pylori* encounters a variety of environmental stressors. These include pH fluctuations, nutrient limitations, ever changing availabilities of metal ions, and oxidative stress caused by the host's immune system (Sachs et al. 2003). Despite being very well adapted to the human gastric niche (Salama et al. 2013; Suerbaum and Josenhans 2007), *H. pylori* requires regulatory systems for rapidly changing its gene expression to adapt to different colonization conditions. Gene expression changes induced by environmental stimuli or mutation of transcriptional regulators have been previously analyzed in *H. pylori* mainly using microarrays in combination with qRT-PCR, primer extension, and in vitro promoter-binding assays (reviewed in Danielli et al. 2010; Josenhans et al. 2007). These studies have provided important insight into gene expression control at the transcriptional level. However, until recently, little was known about the transcriptional organization at single-nucleotide resolution and posttranscriptional gene regulation in *H. pylori*. Small regulatory RNAs (sRNAs) are an emerging class of posttranscriptional gene expression regulators that act during bacterial stress response or virulence control in pathogens (Papenfort and Vogel 2010; Storz et al. 2011). While *H. pylori* was previously regarded as an organism without riboregulation (Mitarai et al. 2007), deep sequencing-based transcriptome analysis has revealed a wealth of sRNAs in this pathogen (Sharma et al. 2010).

The development of high-throughput DNA sequencing technology and its application to sequencing of cDNA libraries, so-called RNA sequencing (RNA-seq), has provided a powerful method for both annotating and quantifying the transcriptional output from an organism's genome. RNA-seq has revolutionized our view of the extent and complexity of both eukaryotic and prokaryotic transcriptomes (Wang et al. 2009; Croucher and Thomson 2010; Barquist and Vogel 2015). It has been successfully used to improve genome annotations and has revealed a wealth of novel noncoding transcripts (ncRNAs). In this chapter, we summarize the analysis of the primary transcriptome of *H. pylori* using a novel differential RNA-seq (dRNA-seq) approach (Sharma et al. 2010). This approach allows for the specific mapping of primary transcripts and has defined a genome-wide map of transcriptional start sites (TSS) in *H. pylori*. While *H. pylori* was the first organism to be studied by dRNA-seq, this approach has been subsequently applied to many other pro- and eukaryotes (Sharma and Vogel 2014). In addition to a very complex and tightly packed transcriptional output, an extensive antisense transcription from the opposite strand of protein-coding genes as well as more than 60 potential sRNAs were uncovered in the small *H. pylori* genome. We describe how these *H. pylori* transcriptome characteristics uncovered by dRNA-seq have revealed new regulatory features in this important human pathogen. This approach has opened a new perspective on the extent of RNA-mediated regulation not only in *H. pylori* but also in several other bacterial pathogens (Sharma and Vogel 2014), including the related food-borne pathogen *Campylobacter jejuni*.

8.2 Primary Transcriptome Analysis of *Helicobacter pylori*

8.2.1 Bacterial Transcriptome Analysis Using RNA-Seq

The transcriptome describes the identity and abundance of all cellular transcripts that are expressed under a specific condition. In contrast to genomes, transcriptomes can be highly dynamic, and transcript abundances can rapidly change in response to changing cell states, environments, or stress conditions. Throughout their life cycle, RNAs can be modified or cleaved for maturation or function, and processing by diverse RNA degradation enzymes mediates transcript stabilization or degradation. In the last two decades, hybridization-based methods such as microarrays and tiling arrays that rely on PCR probes or oligonucleotide probes were commonly used for transcriptome analyses in diverse organisms, including bacteria such as *H. pylori* (Josenhans et al. 2007). These include studies on transcriptome changes in response to important stress conditions for *H. pylori* such as pH fluctuations (Merrell et al. 2003; Wen et al. 2003; Bury-Mone et al. 2004) or metal ion availabilities (Danielli et al. 2010). Regardless of the power of array-based approaches for transcriptome analyses and determining gene expression profiles, they have major limitations. For example, probe design of microarrays is mostly restricted to regions of known or predicted genes, excluding not only ncRNAs such as sRNAs but also 5′ and 3′ untranslated regions (UTRs) of open reading frames (ORFs). Even though tiling arrays cover intergenic regions (IGRs) and other regulatory sequences (e.g., UTRs), disadvantages such as cross-hybridizations and a limited detection of low abundant transcripts still exist for array-based approaches. The development of RNA-seq technology has greatly facilitated transcriptome analyses by allowing for transcript identification and quantification at single-nucleotide resolution over a large dynamic range (Wang et al. 2009; Croucher and Thomson 2010; Sorek and Cossart 2010). Compared to microarrays, RNA-seq is more sensitive, provides absolute quantity levels, is not affected by on-chip sequence biases, and gives additional information on variations in transcript processing. Furthermore, RNA-seq does not rely on specific probe design and allows for the detection of known and novel transcripts.

In a typical bacterial RNA-seq workflow, either total RNA or selectively fractionated RNA, e.g., size-selected RNA or RNA from a co-immunoprecipitation experiment, is first converted into a cDNA library. Upon PCR amplification, the cDNA library is analyzed by one of the currently availiable next-generation sequencing platforms, such as Solexa (Illumina), 454 (454 Life Sciences) or Solid (ABI), resulting in millions of short cDNA sequences ("reads"). These reads are then aligned ("mapped") to the respective reference genome and gene expression can be visualized as cDNA coverage plots at single-nucleotide resolution in a genome browser. The cDNA read patterns and counts can then be used to annotate transcripts or to determine gene expression changes. Different protocols have been developed to construct cDNA libraries for specific and general applications. To perform strand-specific RNA-seq, which is important to distinguish between sense

and antisense transcription, cDNA library preparation protocols have been designed that are based on ligation of a 5′ RNA linker combined with either 3′ poly(A)-tailing of RNAs and oligo(dT)-priming for reverse transcription or cDNA synthesis from a ligated 3′ linker (see references in Borries et al. 2010; Passalacqua et al. 2012; Heidrich et al. 2015). The first bacterial RNA-seq studies included depletion steps for ribosomal RNA (rRNA) and transfer RNA (tRNA), which comprise 95 % of the cellular RNA pool, to enrich for sequencing of mRNAs and sRNAs (reviewed in Sorek and Cossart 2010). However, since the sequencing depth and affordability of the next-generation sequencing platforms is constantly improving, depletion steps are no longer required. This reduces library preparation biases associated with such depletion steps.

Besides the annotation of transcriptome features, including 5′ and 3′ transcript boundaries or identification of novel transcripts such as sRNAs or small ORFs, RNA-seq is nowadays also widely used for gene expression profiling and is replacing microarray approaches (Croucher and Thomson 2010; Sorek and Cossart 2010). Several recent RNA-seq-based transcriptome studies in bacteria, including Epsilonproteobacteria (Chaudhuri et al. 2011; Butcher and Stintzi 2013; Taveirne et al. 2013), indicate that this method can be used to perform comprehensive as well as quantitative expression profiling of the transcriptome under various stress conditions or between mutant and wild-type strains.

8.2.2 Differential RNA-Seq for Primary Transcriptome Analysis

Several RNA-seq approaches have been developed for annotation of transcript 5′ ends on a genome-wide scale (Wurtzel et al. 2010; Mendoza-Vargas et al. 2009; Cho et al. 2009; Singh and Wade 2014; Lin et al. 2013; Salgado et al. 2013). Many of these approaches cannot distinguish between primary and processed transcripts. In contrast, the differential RNA-seq approach allows for a selective sequencing of primary transcripts and, in turn, global identification of transcriptional start sites (TSS) and associated promoter sequences (Sharma et al. 2010; Sharma and Vogel 2014; Bischler et al. 2015). Primary transcripts carry a 5′ triphosphate end (5′PPP), whereas processed transcripts, such as the abundant ribosomal RNAs or tRNAs, carry a 5′ monophosphate (5′P) or, less frequently, a 5′ hydroxyl (5′OH) group (Fig. 8.1a). The dRNA-seq approach is based on a differential treatment of RNA with 5′ P-dependent terminator exonuclease (TEX) that specifically degrades 5′P RNAs, whereas primary transcripts are not affected (Fig. 8.1b). Thus, TEX treatment results in a relative enrichment of primary transcripts and depletes processed RNAs. To distinguish between primary and processed RNAs by dRNA-seq, two differential cDNA libraries are sequenced and compared: one library (TEX-) is generated from untreated, total RNA and the other (TEX+) from RNA enriched for primary transcripts by TEX treatment (see Borries et al. 2010; Heidrich et al. 2015;

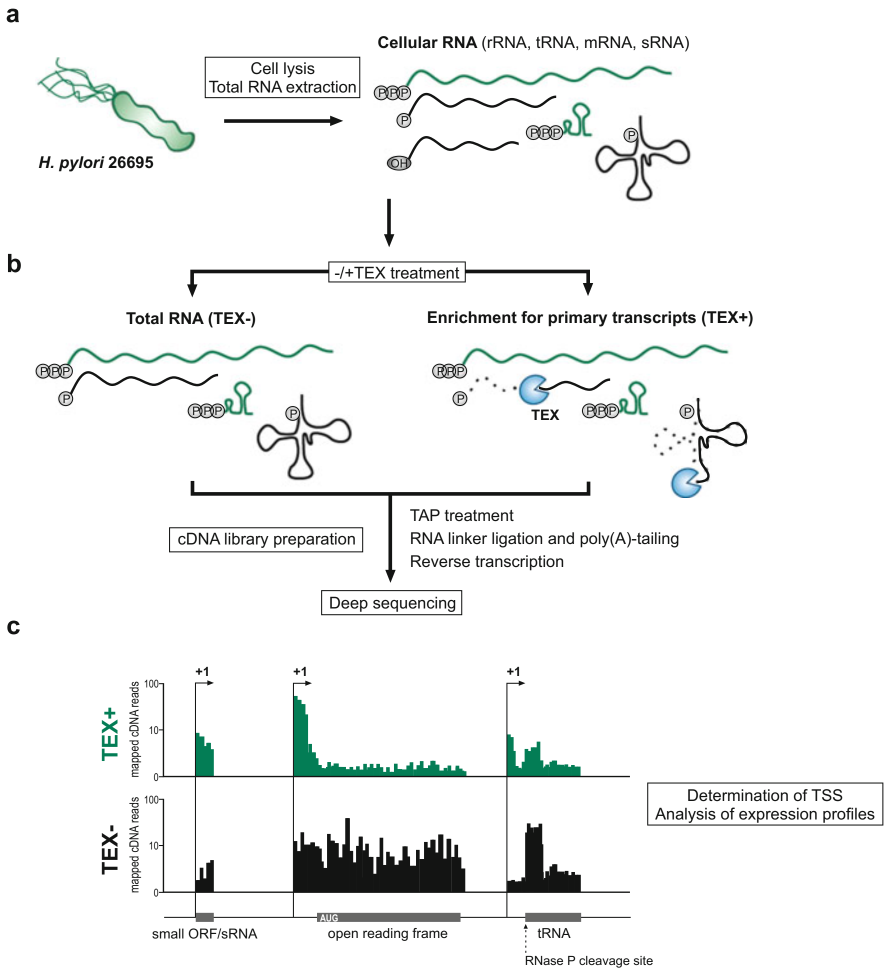

Fig. 8.1 Workflow of the RNA-seq approach exemplified for *H. pylori* strain 26695. (**a**) Total cellular RNA consists of primary (*green*) transcripts with a 5′ triphosphate (PPP) and processed transcripts (*black*) with a 5′ monophosphate (P) or, less frequently, a 5′ hydroxyl group OH. (**b**) For generation of dRNA-seq cDNA libraries, RNA samples are split into two halves. One is left untreated (TEX-), whereas the other one is subjected to 5′-dependent terminator exonuclease (TEX) treatment (TEX+). TEX (*blue*) specifically degrades RNAs with a 5′P. Following differential TEX treatment, 5′ PPP ends will be trimmed to 5′P by tobacco acid pyrophosphatase (TAP), allowing RNA linker ligation. RNAs with a 5′ OH are not accessible for linker ligation and, thus, are not represented in the final cDNA library. Upon poly(A)-tailing of the 3′ end using poly (A) polymerase and reverse transcription, cDNA libraries are analyzed by deep sequencing. (**c**) Sequenced cDNA reads of the TEX −/+libraries are mapped to the reference genome and are visualized as cDNA coverage plots representing the number of reads (*left scale*) per nucleotide in a genome browser. TEX treatment leads to a characteristic enrichment pattern at the 5′ ends of primary transcripts, thereby allowing for mapping of transcriptional start sites (TSS) (*black arrows*). In contrast, 5′ ends of processing sites are not enriched (*dotted arrow*)

Bischler et al. 2015 for a detailed protocol). Since TEX also removes abundant processed RNAs including 16S and 23S rRNAs, no additional rRNA depletion steps are required, and in contrast to many other bacterial RNA-seq studies, dRNA-seq is typically performed on total RNA.

After the differential TEX treatment, the RNA 5′PPP ends are converted into 5′P ends by tobacco acid pyrophosphatase (TAP) treatment to allow for RNA linker ligation. Upon poly(A)-tailing of the RNA and conversion into cDNA by reverse transcription using oligo(dt)-priming, the resulting cDNA libraries are amplified and sequenced on one of the next-generation sequencing platforms. While the first dRNA-seq study to reveal the primary transcriptome of *H. pylori* strain 26695 employed 454 pyrosequencing (Sharma et al. 2010), nowadays dRNA-seq mainly employs Illumina sequencing, allowing for higher sequencing depth (Sharma and Vogel 2014). Sequencing of the dRNA-seq libraries results in a characteristic, sawtooth-like enrichment pattern: in the TEX+ sample cDNA reads cluster toward the 5′ ends of primary transcripts, whereas those from the TEX- sample are randomly distributed along transcripts (Fig. 8.1c). These enrichment patterns allow for globally annotating TSS for all expressed genes under the condition (s) examined. While TSS were manually annotated in the dRNA-seq studies of *H. pylori* and diverse other organisms, several algorithms have been developed that now allow for an automated TSS annotation (Bischler et al. 2015 and reviewed in Sharma and Vogel 2014).

8.3 *Helicobacter* Transcriptome Features Identified by dRNA-Seq

8.3.1 Global Transcriptional Start Site Maps

Previously, the 5′ ends of transcripts and respective promoters were mapped on a gene-by-gene basis using laborious RNase S1 protection experiments (Berk and Sharp 1977), primer extension assays (Thompson et al. 1979), or RACE (rapid amplification of cDNA ends) approaches (Argaman et al. 2001; Vogel et al. 2003; Bensing et al. 1996). For example, the TSS of several abundantly transcribed *H. pylori* genes, such as *ureA* or *cagA*, were mapped using these methods (Spohn et al. 1997; Spohn and Scarlato 1999; Shirai et al. 1999). The dRNA-seq-based primary transcriptome analysis of *H. pylori* strain 26695 allowed, for the first time, mapping of TSS in a bacterium on a genome-wide scale (Sharma et al. 2010). dRNA-seq analysis of *H. pylori* 26695 grown under five different growth conditions, combined with 454 pyrosequencing at a depth of 200,000–500,000 cDNA reads per library, revealed about 1,900 unique TSS. This dense TSS map exceeded the number of ~1,700 annotated genes and uncovered an unexpectedly complex transcriptional output from the small *H. pylori* genome. Identified TSS were classified according to their location relative to flanking genes into five different

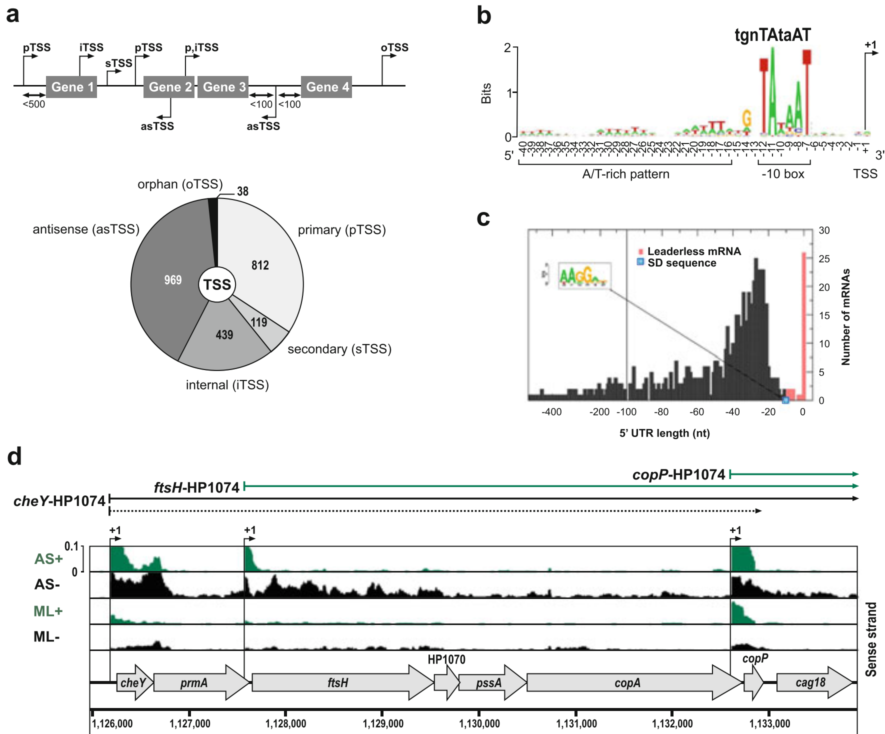

Fig. 8.2 Annotation of transcriptional start sites and 5′ UTRs in *H. pylori* strain 26695. (**a**) (*Top*) Representation of TSS categories based on gene expression and location according to flanking genes: primary (p), secondary (s), internal (i), antisense (as) and orphan (o) TSS. (*Bottom*) Pie chart showing the distribution of TSS to the different TSS categories for *H. pylori* strain 26695. (**b**) Bioinformatics-based motif searches upstream of the 1,906 identified *H. pylori* TSS revealed an extended −10 promoter element (tgnTAtaAT) and a periodic A/T-rich pattern as the consensus promoter motif for the housekeeping sigma factor, σ^{80}. (**c**) Frequency of 5′ UTR lengths based on annotated primary and secondary TSS. More than 30 leaderless mRNAs with a 5′ UTR length <10 nt (*read bars*) were identified. A commonly identified ribosome binding site motif (SD sequence, AAGGag) is shown in the inset. (**d**) cDNA reads of mid-log growth (ML−/+) and acid stress (AS−/+) libraries were mapped to the *cheY*-HP1074 operon (*black arrow*). In addition to the pTSS upstream of *cheY*, two iTSS, upstream of *ftsH* (cell division) and *copP* (copper transport) were detected using dRNA-seq. This suggests transcription of suboperons (*green arrows*) that uncouple expression of these downstream genes. *Gray arrows* represent the annotated ORFs and +1 denotes identified TSS in *H. pylori* 26695 (Sharma et al. 2010). The figure was adapted from Sharma et al. 2010

categories: (I) primary (pTSS) and (II) secondary TSS (sTSS) located <500 bp upstream of ORFs that correspond to main and alternative promoters of mRNA and rRNA/tRNA genes, respectively; (III) internal TSS (iTSS) that are transcribed sense within genes but initiate after the annotated start codon; (IV) antisense TSS (asTSS) that are located antisense either within or ±100 nt up- or downstream of annotated genes; and (V) orphan TSS (oTSS) that have no annotation in close

proximity and might correspond to novel sRNAs or mRNAs (Fig. 8.2a). Besides more than 800 pTSS and 110 sTSS, a wealth of novel noncoding transcripts was detected. The discovery of more than 960 asTSS, including at least one asTSS for more than half of all *H. pylori* ORFs, indicated that antisense RNA-mediated regulation might be a major level of gene expression control in *H. pylori*.

The *H. pylori* genome encodes three sigma factors: the housekeeping sigma factor RpoD (σ^{80}) and the two alternative sigma factors RpoN (σ^{54}) and FliA (σ^{28}), which control the expression of motility genes (Lertsethtakarn et al. 2011). In *Escherichia coli*, the RNA polymerase recognizes, upon assembly with one of the sigma factors, specific promoter sequences such as the −10 (5′-TATAAT consensus) and −35 (5′-TTGACA consensus) boxes of the housekeeping RpoD-driven promoters (Burgess and Anthony 2001). Analysis of the upstream regions of the 1,900 TSS mapped in *H. pylori* provided the first global evidence that promoters of RpoD in Epsilonproteobacteria consist of an extended −10 box (5′-tgnTAtaAT) preceded by a periodic A/T-rich pattern but lack a −35 box (Fig. 8.2b). Such a promoter motif was previously suggested based on the analysis of a small number of *H. pylori* genes (Forsyth and Cover 1999) and bioinformatics-based promoter predictions in *C. jejuni* (Petersen et al. 2003; Wosten et al. 1998). Different dRNA-seq analyses of *C. jejuni* have further confirmed this global promoter consensus for the pathogenic Epsilonproteobacteria (Dugar et al. 2013; Porcelli et al. 2013).

Genomes are mainly annotated in an automated fashion, which can be error prone through selection for the longest possible open reading frames. The genome-wide TSS mapping can improve genome annotations by providing global information about 5′ transcript boundaries. Furthermore, transcripts that may contain smaller ORFs missed by genome annotations are also identified. The dRNA-seq analysis of *H. pylori* revealed several genes where transcription started downstream of the annotated start codon. Further examination of these genes and incorporation of information about start codon conservation in different *H. pylori* strains allowed for re-annotation of the start codons for at least 18 genes. In general, a combined transcriptome and genome sequencing will increase the accuracy of genome annotations of novel strains and species, especially those that utilize different promoter consensus sequences from those of the model enterobacteria.

8.3.2 5′ UTR Lengths

Until recently, the full repertoire of 5′ UTRs for a bacterium was largely unknown. The annotation of primary and secondary TSS upstream of open reading frames allowed for the exact mapping of 825 5′ UTRs in *H. pylori* (Fig. 8.2c, Sharma et al. 2010). Analysis of the 5′ UTR length distribution showed that at least 50 % of them are 20–40 nt in length (Fig. 8.2c). A decrease in frequency for shorter 5′ UTRs was observed, since these leaders are too short to harbor sufficient sequence information required for ribosome binding and translation initiation (Ramakrishnan 2002). A classical consensus AAGGag sequence was identified as a Shine–Dalgarno (SD) motif for most of the *H. pylori* mRNAs. However, a surprising

number of genes (2.2 %) have a 5′ UTR length <10 nt and correspond to leaderless mRNAs, lacking an SD sequence. Leaderless mRNAs are typically translated by specialized ribosomes without SD interaction (Moll et al. 2002, 2004) and transcriptional initiation occurs at the start codon. Besides these putative leaderless transcripts, 337 long 5′ UTRs (>60 nt) were detected that could contain posttranscriptional control elements such as *cis*-encoded transcription attenuators or riboswitches (Serganov and Nudler 2013). For example, a search for conserved structural RNA motifs in the 5′ UTRs revealed a potential thiamine pyrophosphate (TPP) riboswitch upstream of the *pnuC* gene encoding a nicotinamide mononucleotide transporter in *H. pylori*, which is highly conserved in bacteria, archaea, and plants and has been suggested to lead to transcription attenuation in the presence of TPP (Rodionov et al. 2002). Future studies will show the function and mechanism of this potential *H. pylori* riboswitch, and examination of other long 5′ UTRs might reveal novel regulatory RNA elements and/or RNA thermometers (Kortmann and Narberhaus 2012).

8.3.3 Operon and Suboperon Structure

In bacteria, many genes, especially those encoding for functionally related proteins or components of the same enzymatic pathway or structural complex, are encoded in operons and are transcribed as polycistronic transcripts. It was previously assumed that *H. pylori* lacks an extensive operon organization (Tomb et al. 1997; Thompson et al. 2003). However, dRNA-seq analysis indicated that there are multiple iTSS within open reading frames, including genes in predicted operons (Mao et al. 2009). Combination of the dRNA-seq-derived TSS map with conventional RNA-seq that covers full-length transcripts provided a genome-wide operon map of *H. pylori* strain 26695 and revealed that 87.5 % of all genes are located within 337 primary operons. In addition, 126 alternative operons and 66 single genes overlapping the 3′ part of polycistronic transcripts were detected. For example, in the eight-gene operon composed of *che*Y-HP1074 (Fig. 8.2d), two iTSS were identified, one within *prmA* (HP1068) and another within *copA* (HP1072), which lead to uncoupling of the *ftsH*-HP1074 and *copP*-HP1074 suboperons from the primary operon *che*Y-HP1074. Bacteria might use such suboperons to uncouple and differentially regulate certain genes from the rest of the primary operon under certain stress or growth conditions and thereby increase their transcriptome complexity. Global transcriptome studies have also reported transcription of suboperons in other bacterial species such as *Mycoplasma* (Guell et al. 2009).

8.3.4 Noncoding RNAs

Posttranscriptional regulation constitutes an important layer of gene expression control in the cell. In bacteria, the 50–400-nt-long small regulatory RNAs are

posttranscriptional regulators that control gene expression during stress responses and virulence control (Papenfort and Vogel 2010; Storz et al. 2011). While some sRNAs can directly bind proteins and modulate their activity, most of the functionally characterized sRNAs act as antisense RNAs by base-pairing interactions on either *cis*- or *trans*-encoded mRNAs (Waters and Storz 2009). Whereas *cis*-encoded sRNAs originate from the opposite DNA strand and share full complementarity with their target transcripts, *trans*-encoded sRNAs are encoded at distinct genomic locations and regulate target mRNAs by short and imperfect base-pairing interactions. Most of the functionally characterized *trans*-encoded sRNAs repress translation of their target mRNAs by base-pairing near to, or directly at, the ribosome binding site (RBS) and start codon. This translational repression is often coupled to transcript degradation, but several mechanisms of activation of gene expression have also been reported (Frohlich and Vogel 2009). It is now also clear that sRNAs can regulate multiple genes of functionally related pathways by either direct binding to multiple target mRNAs or by regulation of transcription factors and thereby act as global regulators in response to environmental stress conditions (Storz et al. 2011). The RNA chaperone Hfq is a key player in sRNA-mediated regulation in enterobacteria such as *E. coli* and *Salmonella* and is required for sRNA stabilization and/or facilitation of sRNA–mRNA interactions (Vogel and Luisi 2011). Despite its crucial roles in enterobacteria, an obvious Hfq homolog is absent in about 50 % of all bacteria, and it seems to be dispensable for sRNA-mediated gene regulation in Gram-positive bacteria (Chao and Vogel 2010), indicating that additional RNA-binding proteins might be involved in posttranscriptional regulation.

Even though the genome of *H. pylori* was sequenced nearly 20 years ago, little is known about its posttranscriptional gene regulation including sRNAs and RNA-binding proteins. None of the enterobacterial sRNAs, except for the housekeeping RNAs, transfer-messenger RNA (tmRNA), signal recognition particle RNA (SRP/4.5S RNA), and M1 RNA (RNase P), are conserved at the sequence level in *H. pylori* (Sharma et al. 2010). Bioinformatics-based prediction and a small-scale cDNA cloning approach identified only a few candidates for natural antisense transcripts and sRNAs in *Helicobacter* (Livny et al. 2006; Xiao et al. 2009a, b). The limited knowledge of riboregulation in *H. pylori* might be also due to the traditional probe design of microarrays used for transcriptome studies, which were so far mainly restricted to known or predicted mRNAs.

Although sRNAs have been intensively investigated during the last years, knowledge of their regulatory potential and mechanisms is mainly based on work in the model organisms *E. coli*, *Salmonella*, and other Gammaproteobacteria. Systematic searches for sRNAs using experimental methods or biocomputational predictions have greatly facilitated the identification of sRNA genes on a genome-wide scale in various bacteria (Backofen and Hess 2010; Sharma and Vogel 2009). In particular, RNA-seq-based approaches have revealed a wealth of potential sRNAs in diverse bacterial species (Croucher and Thomson 2010; Sorek and Cossart 2010; Barquist and Vogel 2015). The abovementioned dRNA-seq analysis of *H. pylori* strain 26695 not only helped to define a global map of TSS but also revealed many noncoding transcripts. Diverse candidates were identified that are

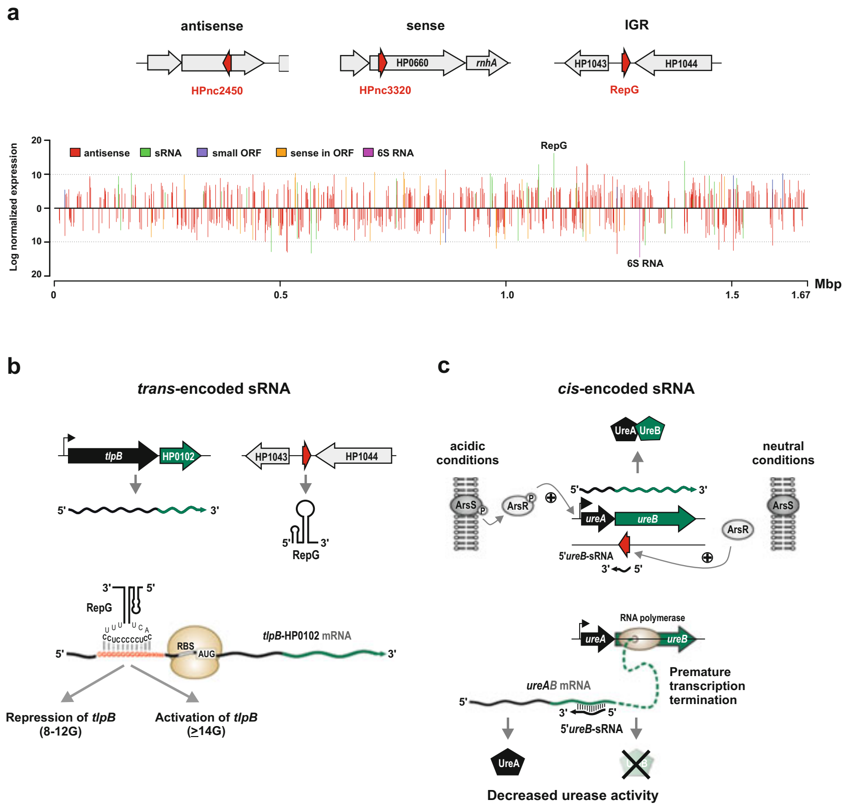

Fig. 8.3 Examples of *cis*- and *trans*-acting sRNAs in *H. pylori*. (**a**) (*Upper panel*) Examples for sRNA candidates that were discovered in intergenic regions or that are located sense or antisense to open reading frames. (*Lower panel*) Relative expression levels for newly detected transcripts (noncoding RNAs and novel small mRNAs) along the *H. pylori* 26695 chromosome. The figure was adapted from Sharma et al. 2010. (**b**) Regulation of expression of the chemotaxis receptor TlpB by the *trans*-encoded RepG sRNA (Pernitzsch et al. 2014). The 87-nt-long RepG sRNA is encoded in the intergenic region between the orphan response regulator HP1043 and the hypothetical protein HP1044. The C/U-rich terminator loop of RepG directly binds to a G-repeat in the 5′ UTR of the *tlpB*-HP0102 mRNA. Dependent on the G-repeat length, RepG mediates repression (8–12 G) or activation (≥14 G) of TlpB. (**c**) Regulation of urease expression by the *cis*-encoded antisense 5′*ureB*-sRNA (Wen et al. 2011, 2013). (*Left*) Expression of the *ureAB* operon, which encodes for the urease apoenzyme, is induced under acidic conditions by the phosphorylated ArsR response regulator of the acid-responsive ArsSR two-component system. (*Right*) Expression of the *cis*-encoded antisense transcript, 5′*ureB*-sRNA, is induced by unphosphorylated ArsR under neutral conditions. An interaction between 5′*ureB*-sRNA and the 5′ region of *ureB* leads to premature transcription termination of the *ureAB* mRNA. Truncation of *ureB* mRNA represses its translation, resulting in a reduced amount of UreB and decreased urease activity

either expressed as single genes from intergenic regions or antisense/sense to coding genes (Fig. 8.3a), suggesting that *H. pylori* has the potential for extensive riboregulation.

8.3.4.1 Housekeeping RNAs

The housekeeping RNAs of *H. pylori*, tmRNA, M1 RNA, and SRP/4.5S RNA were found to be highly expressed in the dRNA-seq study. These conserved RNAs are essential for a variety of cellular processes such as mRNA translation, tRNA maturation, as well as protein translocation. The housekeeping tmRNA, which is involved in *trans*-translation (i.e., rescue of stalled ribosomes and degradation of truncated, potentially toxic proteins), was shown to be essential and required for stress response as well as natural competence in *H. pylori* (Thibonnier et al. 2008). While tmRNA and its protein cofactor, SmpB, have similar functions in *H. pylori* and *E. coli*, tmRNA possesses certain sequence constrains that are required to achieve ribosome rescue in a given organism (Thibonnier et al. 2010). Thus, while such housekeeping RNAs and their functions are ubiquitous in eubacteria, they have coevolved with the translation machinery of their host organisms and, thus, possess specialized characteristics.

One of the most abundant transcripts in the dRNA-seq study of *H. pylori* was found to be a homolog of the housekeeping RNA, 6S RNA, which was missed in previous bioinformatics predictions (Barrick et al. 2005; Sharma et al. 2010). 6S RNA is an abundant and ubiquitous riboregulator that mimics an open promoter complex and thereby sequesters RNA polymerase (RNAP) bound to the housekeeping sigma factor (σ^{70}), resulting in transcriptional activation of σ^{S}-dependent genes (Cavanagh and Wassarman 2014). Despite only little sequence conservation to the *E. coli* 6S RNA, the 180-nt-long 6S RNA from *H. pylori* folds into the same characteristic long hairpin structure which was shown to be essential for its binding to RNA polymerase in *E. coli* and *Bacillius subtilis*. The dRNA-seq data also detected 14–20-nt-long “product RNAs” (pRNAs), which are transcribed using 6S RNA as a template and are important for the recycling of RNAP during outgrowth or supply of new nutrients such as NTPs (Wassarman and Saecker 2006), and thus provides further evidence that this RNA is a functional 6S RNA homolog. However, detection of pRNAs arising from both strands of 6S RNA, as well as the absence of RpoS in *H. pylori*, suggests that, like tmRNA, the *H. pylori* system has slightly diverged from the *E. coli* paradigm. Whether 6S RNA has a role during stress response or stationary phase growth in *H. pylori* or, like in *Legionella*, impacts on its virulence (Faucher et al. 2010) still needs to be investigated.

8.3.4.2 Mechanisms and Functions of Antisense/Base-Pairing RNAs in *H. pylori*

Trans-Encoded Antisense RNAs

First insight into sRNA-mediated posttranscriptional regulation of gene expression in *Helicobacter* was gained based on the expression of an artificial antisense RNA (asRNA) with full complementarity to the 5′ UTR and start codon of the essential

alkyl hydroperoxide reductase gene *ahpC* (Croxen et al. 2007). This antisense RNA has been shown to repress expression of this abundant enzyme, indicating that sequestration of the ribosome binding site and thus abrogation of target mRNA translation might be a feature of sRNA-mediated regulation in *Helicobacter*. Although several of the dRNA-seq-identified *H. pylori* sRNAs are candidates for putative *trans*-encoded base-pairing RNAs, the mechanisms and functions for most are still unknown. The dRNA-seq approach revealed a highly abundant and conserved 87-nt-long sRNA, RepG (Regulator of polymeric G-repeats), which is encoded between genes of an orphan response regulator (HP1043) and a protein of unknown function (HP1044) (Fig. 8.3b). This sRNA represents the first example of a *trans*-acting antisense RNA in *Helicobacter* and represses expression of one of the four chemotaxis receptors, TlpB, in *H. pylori* strain 26695 (Sharma et al. 2010). TlpB has been suggested to sense protons and plays a role in pH-taxis, quorum sensing, and colonization and inflammation of the gastric mucosa in mice and Mongolian gerbils (McGee et al. 2005; Williams et al. 2007; Croxen et al. 2006; Rader et al. 2011). RepG regulates *tlpB* expression at the posttranscriptional level by direct interaction between the C/U-rich terminator loop of the sRNA and a homopolymeric G-repeat in the 5′ UTR of *tlpB* mRNA (Pernitzsch et al. 2014). Whereas RepG is highly conserved, the *tlpB* G-repeat length varies among diverse *H. pylori* strains, resulting in a strain-specific *tlpB* regulation. The G-repeat corresponds to one of the variable simple sequence repeats of *H. pylori*, which usually affect transcription or coding potential of genes when they are located in promoter regions or within open reading frames, respectively (Moxon et al. 2006). Modification of the G-repeat in the *tlpB* 5′ UTR within *H. pylori* strain 26695 demonstrated that the G-repeat length determines the outcome of posttranscriptional regulation (activation or repression) of *tlpB* by RepG, mainly at the translational level (Pernitzsch et al. 2014). This represents an unexpected connection of phenotypic variation through variable simple sequence repeats and sRNA-mediated regulation and shows that studying sRNAs in *H. pylori* can reveal novel twists of gene regulation. In addition, expression profiling of other sRNA candidates under various growth conditions, as well as target gene identification by whole proteome and transcriptome analysis of sRNA mutants, together with bioinformatics-based predictions, will help to understand the roles of sRNAs during stress response and/or virulence regulation of *H. pylori*.

Cis-Encoded Antisense RNAs

Naturally occurring antisense transcription is a common phenomenon in all kingdoms of life and has been considered as an important feature in creating transcriptional and thus, phenotypic complexity. *Cis*-encoded asRNAs are encoded in the chromosome or on mobile genetic elements, e.g., plasmids, transposons, or phages, and have been shown to affect gene expression by translation inhibition, transcription interference and attenuation, transcript stabilization, or degradation (Wagner et al. 2002; Brantl 2007; Waters and Storz 2009). Using tiling arrays or RNA-seq,

recent genome-wide transcriptome studies of several Gram-negative and -positive bacteria suggested that antisense transcription from the chromosome is more widespread than anticipated and revealed a wealth of *cis*-encoded antisense RNAs (reviewed in Sorek and Cossart 2010; Georg and Hess 2011; Thomason and Storz 2010). Although increasing numbers of asRNAs have been reported in numerous studies, it is still under debate whether all of these transcripts are functional or are simply the result of spurious transcription (Wade and Grainger 2014). In Gram-positive bacteria, long asRNAs derived from divergently transcribed genes have been reported to span full genes or even operons (Sesto et al. 2013). Using such an "excludon" paradigm, divergently transcribed genes of related or opposing functions can control each other's expression.

Consistent with the increasing number of studies that report widespread antisense transcription in bacteria, more than 900 *cis*-encoded asRNAs were identified in *H. pylori* strain 26695 using dRNA-seq (Sharma et al. 2010). These include *bona fide* asRNAs, as well as overlapping 5′ or 3′ regions of mRNAs from contiguous genes that are transcribed in opposite directions. Antisense transcription occurs across the entire genome of *H. pylori* independent of local GC content, and no general bias toward core or variable genes was observed. With at least one antisense TSS associated with about half of all open reading frames (46 %), the fraction of genes associated with asRNAs identified by RNA-seq in *H. pylori* is among the highest compared to other bacteria (reviewed in Thomason and Storz 2010; Georg and Hess 2011). Since overlapping transcription can affect the expression of the gene on the complementary strand by various mechanisms, posttranscriptional regulation by *cis*-encoded antisense RNAs and, for example, the double-strand-specific ribonuclease RNase III might mediate widespread gene expression control in *Helicobacter*.

Among the wealth of antisense transcripts, a new class of asRNA was discovered. These asRNAs are complementary to stable RNAs, such as ribosomal RNAs (23S and 16S rRNAs) or antisense to about one third of all *H. pylori* tRNAs. Antisense RNA targeting of stable RNA has also been reported in the chloroplast of the plant *Arabidopsis thaliana*, where a asRNA to the 5S rRNA seems to inhibit its maturation (Sharwood et al. 2011). In *H. pylori*, two small *cis*-encoded asRNAs, HPnc1880 and HPnc0260/0270, were identified that are expressed antisense to the 5′ end precursor of the 23S-5S ribosomal RNAs and to the 3′ end of tRNA-Val, respectively. Both asRNAs are induced in response to low pH, indicating that they may have a regulatory function under specific stress conditions. However, future studies will be required to investigate whether they are involved in rRNA maturation and/or degradation.

The recent characterization of a naturally occurring 292-nt-long *cis*-encoded asRNA, 5′*ureB*-sRNA, from the opposite strand of the urease operon (*ureAB*), further demonstrated functionality of asRNAs in *Helicobacter* and their potential role in acid adaptation (Wen et al. 2011). In *H. pylori* strain 43504, transcription of the 5′*ureB*-sRNA is induced under neutral pH conditions by the unphosphorylated ArsR response regulator of the acid-sensing ArsRS two-component system, which activates the expression of the *ureAB* operon in its phosphorylated form in response

to acid (Fig. 8.3c). The antisense 5′*ureB*-sRNA represses expression of the urease apoenzyme by interacting with the 5′ end of *ureB* coding region, resulting in premature transcription termination of the polycistronic *ureAB* mRNA by transcription attenuation (Wen et al. 2013). Whether the truncated *ureAB* transcript is subsequently degraded or if the reduced amount of UreB protein leads to a decrease in urease activity is still unclear. The 5′*ureB*-sRNA was not detected in the dRNA-seq study of *H. pylori* strain 26695, which might be due to low expression levels under the conditions examined or strain-specific antisense RNA expression. Different *H. pylori* strains might have evolved strain-specific sRNA/asRNA repertoires as observed in a comparative dRNA-seq analysis of multiple strains of *C. jejuni* (Dugar et al. 2013). Such unique *cis*- and *trans*-encoded RNAs could mediate strain-specific regulation and thereby determine phenotypic differences among strains and facilitate colonization of different hosts of niches.

8.3.5 Class I Toxin–Antitoxin Loci

In addition to novel sRNAs, the dRNA-seq study revealed several new small ORFs encoding hydrophobic proteins of less than 50 amino acids which had been missed during genome annotation (Sharma et al. 2010). Several of these predicted proteins resemble small toxins or antimicrobial peptides and have *cis*-encoded antisense RNAs, indicating that they may represent class I toxin–antitoxin (TA) loci in *H. pylori*. Class I TA systems are composed of a protein toxin whose translation is inhibited by an antisense RNA (Fozo et al. 2008a). A family of six structurally related ~80-nt-long asRNAs, termed IsoA1-6 (RNA inhibitor of small ORF family A), are expressed antisense to small ORFs of homologous 30 amino acid-long AapA1-6 (antisense RNA-associated peptide family A) peptides. Although there can be multiple loci encoding members of these peptide families within one *H. pylori* strain (one to nine copies per genome), the peptides belonging to family A are only conserved in two closely related *Helicobacter* species: *H. cetorum* and *H. acinonychis*. Three additional peptide families (AapB, AapC, and AapD) were identified in the *H. pylori* genome, two of which were also conserved in other bacterial species. The AapD family, for instance, displays strong homology to the Ibs type I toxin, which was first identified in *E. coli* (Fozo et al. 2008b). The corresponding peptides for each family (A, B, C, and D) were not particularly hydrophobic compared to other small hypothetical proteins previously annotated in the *H. pylori* genome. For at least one of the peptides of family A (AapA), recent work has shown that (I) overexpression of this peptide is toxic for the bacterium and (II) asRNAs expressed in antisense orientation (IsoA) prevent their synthesis in vitro and in vivo (Arnion and Darfeuille unpublished; Sharma et al. 2010). Thus, the *aapA–isoA* loci might represent first examples of class I TA systems in *H. pylori*. The physiological role of these TA loci is currently unknown and whether they might be involved in persister cell formation or antibiotics resistance as recently shown for the TisB peptide from *E. coli* (reviewed in Wagner and Unoson 2012).

8.4 Protein Factors Involved in Posttranscriptional Regulation

Throughout their life cycle, RNAs are subjected to multiple processing and regulatory steps. During their regulatory or structural function, they can interact with a variety of RNA-binding proteins (RBPs). These include ribonucleases (RNases) that govern sRNA and/or target mRNA maturation or turnover, auxiliary protein factors that mediate RNA–RNA interactions and/or stabilize sRNAs, proteins that mediate intracellular transport of RNAs, or those whose functions are modulated by the sRNA. Concordantly, sRNAs act in concert with RBPs and RNases to elicit posttranscriptional regulation of gene expression. In *H. pylori*, analysis of the urease operon transcript revealed pH-dependent transcript stabilities and processing into multiple species, indicating an extensive posttranscriptional regulation of this operon in response to acid stress (Akada et al. 2000). However, it is still unclear which sRNAs or RPBs are required for processing of this mRNA. Only few RNA–protein interactions have been studied in *H. pylori* so far. For example, the essential tmRNA has been shown to interact with its protein cofactor SmpB, and both are required for *trans*-translation and translational control (Thibonnier et al. 2008, 2010). The identification and subsequent characterization of novel *H. pylori* ribonucleoprotein complexes will identify novel RBPs and will expand our knowledge about cellular regulators and the underlying mechanisms of riboregulation in these bacteria.

8.4.1 RNA-Binding Proteins

The Sm-like RNA chaperone Hfq is considered to be a key player in posttranscriptional regulation of gene expression in many bacteria (Vogel and Luisi 2011). Deletion of *hfq* causes pleiotropic phenotypes including impaired stress response as well as reduced virulence in several bacterial pathogens (Chao and Vogel 2010). In contrast to Gram-negative bacteria, Hfq seems to be dispensable for sRNA-mediated regulation in Gram-positive bacteria (Jousselin et al. 2009). The lack of an apparent *hfq* homolog in Epsilonproteobacteria suggests two things: either a so-far unidentified RNA-binding protein replaces the role of Hfq, or sRNAs in these bacteria act independently of an RNA chaperone by novel mechanisms compared to enterobacterial Hfq-binding sRNAs.

Besides Hfq, the ubiquitous family of the CsrA (carbon storage regulator)/RsmA (repressor of secondary metabolites) proteins/regulators have been shown to act as global posttranscriptional regulators of gene expression and also virulence in bacterial pathogens (Seyll and Van Melderen 2013; Heroven et al. 2012). The CsrA protein mainly acts as a translational repressor by direct binding to GGA-rich sequences in the 5′ UTR of bacterial mRNAs and, thereby, controls global physiological phenomena such as central carbon metabolism, motility,

biofilm formation, quorum sensing, and virulence (Romeo et al. 2013). A hallmark of these systems in enterobacteria is the regulation of CsrA activity by two sRNAs, CsrB/CsrC, which mimic the RNA substrate of CsrA and thereby sequester the protein away from target RNAs like a sponge (Babitzke and Romeo 2007). The CrsA homolog of *Helicobacter* is required for oxidative stress response, virulence in mice infections, and full motility (Barnard et al. 2004). Reduced protein and mRNA levels of the FlaA and FlaB flagellins were also observed in an a nonflagellated *csrA* deletion mutant of *H. pylori* strain J99 (Kao et al. 2014). However, different effects on morphology and flagellin expression have been reported for other *H. pylori* strains, indicating that there might be a strain-specific regulation. Neither target genes of CsrA nor homologs of the CsrA-antagonizing sRNAs, CsrB/CsrC, have been identified in *H. pylori* so far. A global analysis of RNA substrates of CsrA from *C. jejuni* using a combination of co-immunoprecipitation of chromosomally epitope-tagged CsrA combined with RNA-seq of co-purified transcripts revealed that many transcripts of the flagellum or related to motility are bound by CsrA in *C. jejuni* (Dugar and Sharma unpublished). This indicates CsrA might play a potential role in the regulation of flagellar assembly. Moreover, CsrA was found to repress translation of the major flagellin FlaA in *C. jejuni*.

In addition to CsrA, the zinc-ribbon domain-containing protein HP0958 (FlgZ) has been described as a potential posttranscriptional regulator of motility genes in *H. pylori* (Douillard et al. 2008; Ryan et al. 2005). HP0958 might act as a chaperone for the alternative sigma factor RpoN, as it is required for its stability (Pereira and Hoover 2005; Pereira et al. 2011). HP0958 is essential for flagellum biogenesis and involved together with the sigma factor FliA in proper regulation of the hierarchical expression of flagellar genes. This protein was shown to bind and stabilize the major flagellin *flaA* mRNA, thereby modulating the amount of the transcript available for translation (Douillard et al. 2008). However, the exact mechanism and potential additional targets of HP0958 still need to be examined.

The recent findings that additional proteins with so far unknown functions or those unrelated to RNA metabolism are involved in posttranscriptional regulation indicate that we are far away from knowing all RNA-binding proteins and their roles in bacteria (Pandey et al. 2011; Mitobe et al. 2011). For example, in *H. pylori*, a direct interaction of the apoenzyme of aconitase (AcnB) with the 3′ UTR of the cell wall-modifying peptidoglycan deacetylase (*pgdA*) was shown to increase the stability and expression of *pgdA*, resulting in altered in vivo survival (Austin and Maier 2013). AcnB is a moonlighting protein in *E. coli* and a major enzyme of the TCA cycle under iron-rich conditions. During iron limitation, its apo form (apo-AcnB) favors binding to and stabilization of its own mRNA 3′ UTR (Tang and Guest 1999). Binding of AcnB to its own mRNA also prevents mRNA degradation by the iron-regulated sRNA RyhB under low-iron conditions, despite its repression of translation initiation through RyhB (Benjamin and Masse 2014).

Several strategies for the global investigation of RNA–protein complexes have been developed in recent years. These include, for example, co-immunoprecipitation of epitope-tagged RNA-binding proteins and identification

of RNA-binding partners by RNA sequencing (RIP-Seq). In *Salmonella*, RIP-seq analysis of FLAG-tagged Hfq revealed various sRNAs and mRNAs that are specifically bound and/or stabilized by this RNA chaperone (Sittka et al. 2008). For an unbiased, genome-wide identification of novel RNA-binding proteins, an orthogonal approach using aptamer-tagged RNAs and affinity chromatography can be used, where recovered RNA–protein complexes are analyzed by mass spectrometry (Windbichler and Schroeder 2006; Said et al. 2009). More recently, approaches that use in vitro and in vivo ultraviolet (UV) cross-linking of RNA–protein interactions and co-immunoprecipitation (CLIP) strategies have been developed that allow for investigation of RNA–protein complexes at a high resolution and specificity (König et al. 2012). In contrast to RIP-seq, these approaches are not limited to very stable RNA–protein (RNP) complexes and less prone to nonspecific interactions. In *H. pylori*, several strategies for the isolation and analysis of RNP complexes have been established (Rieder et al. 2012). Using either affinity purification of aptamer-tagged RNAs or RIP-seq, RNA–protein interactions between the ribosomal proteins S1 and various mRNAs as well as sRNAs were identified in *H. pylori* strain 26695. Ribosomal protein S1 might be a candidate protein that does not only act to facilitate translation initiation but might also act as a general RNA chaperone (Hajnsdorf and Boni 2012). Moreover, HP1334, a protein of unknown function, was shown to specifically interact with HPnc6910 and stabilize expression of this abundant sRNA (Rieder et al. 2012). Therefore, *Helicobacter* proteins of unknown function or those associated with other functions such as bacterial membrane binding, translation, cell cycle, or virulence could potentially bind RNA and play a role in posttranscriptional regulation.

8.4.2 Ribonucleases

In enterobacteria, sRNA-mediated target-mRNA decay mainly depends on the RNA degradosome, a protein complex composed of an endoribonuclease (RNase E), a 3′–5′ polynucleotide phosphorylase (PNPase), an enolase and an RNA helicase (RhlB) (Morita et al. 2005; Caron et al. 2010). Although the exact mechanism of repression is still unclear, both functionally characterized antisense RNAs in *Helicobacter* (RepG and 5′ *ureB*-sRNA; Fig. 8.3b, c) cause reduced protein levels, most likely due to transcript destabilization or active recruitment of RNases upon sRNA–target mRNA interaction. As all Epsilonproteobacteria appear to lack an RNase E homolog, it is unclear whether and which endoribonuclease participates in sRNA-mediated mRNA cleavage in these bacteria. Recent studies in *B. subtilis* and *Staphylococcus aureus* identified two functional RNase E orthologs in the RNA degradosome of Gram-positive bacteria, RNase J1 and RNAse J2 (Mathy et al. 2010; Roux et al. 2011). Furthermore, in *S. aureus* the double-strand-specific RNase III degrades, mainly in concert with the sRNA, RNAIII, several mRNAs encoding virulence factors (Huntzinger et al. 2005; Chevalier et al. 2008; Boisset et al. 2007; Lioliou et al. 2012). Only nine ribonucleases have been annotated in

Table 8.1 Homologs of ribonucleases and RNA degradation enzymes in *Helicobacter pylori*

RNase	Homolog in *H. pylori* strain 26695	Activity or substrate	Essential[a]	References
RNase Y	HP0760	Endoribonuclease	No	
RNase R/II	HP1248	3′–5′ exonuclease	No	Tsao et al. (2009)
YbeY	HP1160	Putative endoribonuclease/ RNA chaperone	Yes	
PNPase	HP1213	3′–5′ exonuclease	No	
RNase J	HP1430	5′P RNA, exo- (5′–3′) and endoribonuclease	Yes	Redko et al. (2013)
RNase III	HP0662	Double-stranded RNA	No, but strain specific	
RNase H1	HP0661	RNA in RNA/DNA hybrid	No	
RNase H2	HP1323	RNA in RNA/DNA hybrid	*n.d.*	
RNase P (protein)	HP1448	5′ end of pre-tRNA	*n.d.*	
	HP0268	DNA nicking endonuclease/ RNase activity	Yes	Lee et al. (2015)
RppH	HP1228	putative RNA pyrophospho-hydrolase, 5′PPP RNA	No	

No homolog has been identified so far for RNase E, G, T, Z, D, and PH
n.d. not determined
[a]Based on unpublished results from the Darfeuille/Sharma labs if not indicated otherwise

H. pylori so far, including potential homologs of RNase J and RNase III (Table 8.1). In *Helicobacter*, most of the mRNA degradation seems to be carried out by a minimal RNA degradosome consisting of a homolog of RNase J and the only DExD-box RNA helicase of *H. pylori*, RhpA (Redko et al. 2013). The biochemical characterization of a purified recombinant enzyme showed that it contains both 5′–3′ exonucleolytic and endonucleolytic activity similar to its *B. subtilis* ortholog (Dorleans et al. 2011). In addition to RNase J, *H. pylori* also contains a potential homolog of the RNA pyrophosphohydrolase RppH also known as NudA, (Lundin et al. 2003), which removes 5′-terminal phosphates to generate a full-length mRNA intermediate that is monophosphorylated and therefore vulnerable to rapid 5′-exonucleolytic digestion by RNase J (Richards et al. 2011). In general, *H. pylor*i seems to possess the majority of the key enzymes (RNase J, RNase Y, PNPase, and RNase III) that are known to be involved in mRNA degradation in the Gram-positive *B. subtilis* (Condon 2010). In line with their important function in the RNA metabolism of Gram-positive bacteria, *H. pylori* RNase J and RNase III are essential in the majority of *H. pylori* strains (Redko et al. 2013; Iost and Darfeuille unpublished). A large number of processes that occur during sRNA-mediated decay and rRNA maturation await characterization and assignment to specific enzymes or encoding genes. For example, the highly conserved bacterial protein, YbeY, may

play a major, previously unrecognized role in bacterial sRNA regulation (Pandey et al. 2011, 2014), and its deletion has been described to sensitize cells to various stresses and affect rRNA biosynthesis, translational fidelity, and ribosome assembly in *E. coli* and *Sinorhizobium meliloti* (Davies et al. 2010; Rasouly et al. 2009, 2010; Jacob et al. 2013; Grinwald and Ron 2013). Furthermore, there are strong genetic interactions in functions of *ybeY* and other genes encoding exoribonucleases such as RNase R and PNPase (Davies et al. 2010). Interestingly, the *ybeY* gene is essential in *H. pylori* (Iost and Darfeuille unpublished). In contrast, the two 3′–5′ exoribonucleases RNase R and PNPase are not essential, but they have been found to play an important role in controlling virulent gene expression in *H. pylori* (Rosenzweig and Chopra 2013; Tsao et al. 2009). Similarly, inactivation of the *pnp* gene in *C. jejuni* significantly altered its ability to adhere and invade gastrointestinal epithelial cells and, thus, affect chicken colonization (Haddad et al. 2012). Surprisingly, deletion of this exoribonuclease only modified the abundance of a specific set of proteins involved in virulence and motility. Moreover, the 3′–5′exoribonuclease RNase R has been shown to posttranscriptionally downregulate six virulence-related genes of *H. pylori* (Tsao et al. 2009). Whether or not RNase R is also involved in sRNA-mediated regulation needs to be clarified.

8.5 Conclusions and Outlook

The dRNA-seq-based analysis of the *H. pylori* primary transcriptome revealed a surprisingly complex transcriptional output from its small genome. The discovery of an unexpected wealth of sRNAs, as well as extensive antisense transcription, indicates a global level of posttranscriptional gene expression control in this pathogenic Epsilonproteobacterium, which might complement its relatively modest repertoire of protein transcriptional regulators. The ongoing functional characterization of *cis*- and *trans*-acting sRNA candidates along with the identification of RNA-binding proteins and RNases will help to unveil their regulatory functions and role in *H. pylori* virulence control and stress response. As shown for the *trans*-acting RepG sRNA, such studies can reveal novel mechanisms of posttranscriptional regulation independent of the RNA chaperone Hfq, and will expand our knowledge about riboregulation in emerging human pathogens.

Besides mechanistic aspects of riboregulation, research on sRNAs and antisense transcripts in *H. pylori* will help to understand phenotypes observed in previous work, including genetic screens. Global transposon screens for virulence and colonization factors revealed about 220 candidate mutants with a colonization defect in mice (Baldwin et al. 2007). Several mutants showed a transposon hit at the end of genes and intergenic regions, indicating that regulatory RNA elements such as riboswitches or *cis*- and *trans*-encoded sRNAs might be important for *H. pylori* virulence. Moreover, several virulence-associated genes could be under posttranscriptional control.

The study of riboregulation in *H. pylori* will also help to understand common themes of posttranscriptional regulation in pathogenic Epsilonproteobacteria, including *Campylobacter spp*. First sRNA candidates have also been identified in *C. jejuni* NCTC11168 based on RNA-seq (Chaudhuri et al. 2011; Porcelli et al. 2013; Taveirne et al. 2013). Furthermore, a comparative dRNA-seq analysis of multiple *C. jejuni* strains indicates that many of the sRNAs are conserved among *C. jejuni* strains (Dugar et al. 2013). However, some of these conserved sRNAs display distinct expression patterns in certain strains, indicating that they could have varying, strain-specific functions. This comparative approach revealed also strain-specific sRNAs and transcriptional output based on point mutations in promoter regions, which might underlie phenotypic differences among strains. With regard to the high genotypic and phenotypic diversity of *H. pylori* strains, such a comparative approach between different *H. pylori* isolates might help to understand differences among strains at the transcriptome level. A cross-species comparison of the primary transcriptomes of *C. jejuni* and *H. pylori* revealed a lack of conservation of operon organization, position of intragenic and antisense promoters, and leaderless mRNAs, indicating that regulatory features may have evolved after these species split from a common ancestor (Porcelli et al. 2013). While *Helicobacter* and *Campylobacter* seem to have evolved their own specific sRNAs, they harbor similar capacity for riboregulation compared to *E. coli*, respective to their smaller genome sizes.

Next-generation sequencing methods have proven to be an effective tool for uncovering transcriptome structure and identifying novel transcripts in prokaryotes as well as for single-nucleotide resolution gene expression profiling (Croucher and Thomson 2010; Sorek and Cossart 2010; Sharma and Vogel 2014). A full understanding of the infection process requires the investigation of gene expression changes in both the pathogen and the host. Parallel sequencing of infection samples (in vivo or in vitro) without physical separation of host and bacterial RNA, followed by mapping of cDNA reads to the host and pathogen genome (dual RNA-seq), will allow for simultaneous analysis of gene expression changes in host and pathogen (Westermann et al. 2012). Accordingly, RNA-seq could provide a powerful tool in analyzing transcriptomes from biopsy samples of fresh clinical *H. pylori* isolates from human patients with different clinical outcomes of infection. Furthermore, the investigation of single-cell transcriptomes using RNA-seq is emerging as a powerful tool to profile cell-to-cell variations within bacterial or host cell populations (Saliba et al. 2014). Overall, in-depth analysis of transcriptional output and its functional consequences, including sRNA functions, in *H. pylori*, as well as its cousin *C. jejuni*, together with parallel transcriptome analysis during the time course of infection will help to shed light on posttranscriptional regulation and virulence mechanisms not only in Epsilonproteobacteria but also other bacterial pathogens, including species that lack Hfq.

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Chapter 9
Genome Evolution: *Helicobacter pylori* as an Extreme Model

Ichizo Kobayashi

Abstract *Helicobacter pylori* strains have quite diverse genome sequences likely because of high mutation and recombination rates, and they may be a useful model to study genetics and evolution. Here, I discuss some features of their evolution that have emerged from comparative analyses of complete *H. pylori* genomes and methylomes. Emphasis will be placed on the roles of various modes of recombination.

The evolution of their chromosome synteny was reconstructed by analyzing inversions via four mechanisms: recombination events involving long or short sequence similarities, inversions adjacent to the insertion of a mobile element, and DNA duplications associated with inversions, a novel process of DNA duplication.

Phylogenetic trees of individual genes are often different from that of the core genome in size and topology, partly due to homologous recombination between lineages. The fine population structure of the species was inferred from an analysis of homologous recombination using a method called chromosome painting. Gene sequences often diverge between European strains and East Asian strains. The evolution of Western-type CagA to East Asian-type CagA can be explained by illegitimate recombination events, with the Amerindian type as an intermediate. Massive decay of molybdenum-related genes was found in East Asian strains.

Whole methylome decoding at single-nucleotide resolution revealed that the *H. pylori* methylome is highly variable because its many methyltransferases often change sequence specificity. Their target recognition domains may move between different genes, sometimes beyond species barriers, and they may even move within a gene (domain movement). These extremely variable methylomes, as opposed to variable genomes, might provide targets for natural selection in adaptive evolution—a hypothesis that may be called epigenetics-driven adaptive evolution.

Keywords Genome comparison • Recombination • DNA duplication • Inversion • CagA • Epigenetics • Methylome • DNA methylation

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9.1 Prologue

Helicobacter pylori may be regarded as a model organism to study genetics and evolution. Long-standing questions in biology may be answered by studying this eubacterial species that infects humans. These questions include the nature of adaptive evolution, the units and nature of natural selection, the nature of the information transferred to the next generation, the roles of epigenetics and recombination in these processes, and the nature of species. *H. pylori*'s relatively specific and defined niche (the stomach and duodenum), high mutation and mutual homologous recombination rates (Suerbaum and Josenhans 2007), and abundant DNA methylation systems (Furuta et al. 2014) underlie the idea that they may provide deep insights into the aforementioned issues.

Recent innovations in DNA sequencing and related technology now allow decoding of many whole-genome sequences of this species, as well as other prokaryotes. We can study their complete genome sequences at single-nucleotide resolution. Especially, we can study the roles of various forms of recombination in genome-wide evolution: homologous recombination involving a pair of long similar sequences, site-specific recombination involving specific sequences, and illegitimate recombination involving very short and unpredictable sequences. They can also be classified according to their relationship with a particular DNA region, such as a mobile genetic element or a particular gene, as well as whether they result in an insertion, deletion, inversion, duplication, decay, etc.

One recent advance in DNA sequencing, Single-Molecule Real-Time (SMRT) sequencing, now allows decoding of a complete methylome at single-nucleotide resolution in bacteria (Murray et al. 2012). This provides a unique occasion to evaluate the roles of epigenetics in evolution. In *H. pylori*, which encodes many DNA methyltransferases, such studies may lead to new concepts in epigenetics and adaptive evolution.

This brief sketch of *H. pylori* evolution emphasizes the roles of recombination and methylation in genome-wide evolution that have emerged from single-nucleotide resolution comparisons of multiple complete genomes/methylomes in this species. We often focus on differences between Western-type strains and Eastern-type strains.

9.2 Phylogeny and Population Structure

The current standard method for the phylogenetic classification of *H. pylori* is multi-locus sequence typing (MLST) based on concatenated sequences from seven conserved genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*). The results are archived in a large and ever-expanding database (the *H. pylori* MLST database [http://pubmlst.org/helicobacter/]), and phylogenetic trees are constructed from these data. Population structure is inferred from these sequences according to

their clustering, as determined using STRUCTURE (Falush et al. 2003a, b). The extant populations are hpAfrica2, hpAfrica1, hpNEAfrica, hpEurope, hpAsia2, hpSahul, and hpEastAsia (Falush et al. 2003b; Moodley et al. 2012). Each of these may be divided into subpopulations. For example, hpEastAsia is divided into hspAmerind, hspMaori, and hspEastAsia.

9.2.1 Genome Trees vs. Gene Trees

Starting with multiple complete genome sequences, we can obtain more information about their phylogeny. The common core genome structure of genomes can be extracted (Uchiyama 2008). The concatenated sequence of conserved genes results in a well-resolved phylogenetic tree, as shown in Fig. 9.1a (Yahara et al. 2013). Such trees are much more robust than standard MLST trees primarily because the tree is composed of a large quantity of sequence information and many more (approximately 1000) genes.

When phylogenetic trees of individual genes are examined, they turn out to be quite variable in size and topology (Fig. 9.2a) (Uchiyama 2008; Yahara et al. 2012). The incongruence with the core tree is at least partly explained by high levels of homologous recombination between lineages. Indeed, each of the global *H. pylori* genomes appears to be a mosaic of sequences transferred from other lineages (Yahara et al. 2012, 2013).

9.2.2 Inference of Population Structure from Mutual Homologous Recombination

Phylogenetic tree construction is, in principle, based on the assumption that genome evolution takes place primarily through nucleotide substitution. In organisms with low levels of mutual homologous recombination, phylogenetic tree construction will allow the identification of populations, which in turn will allow the detection of rare recombination events between such populations. The high mutual homologous recombination rate in *H. pylori* makes it difficult to infer population structure from phylogenetic trees and to identify traces of past recombination events.

Recently, a method called chromosome painting in silico was developed to overcome this problem in the human genome (Lawson et al. 2012), and this method was applied to global strains of *H. pylori* (Fig. 9.1b) (Yahara et al. 2013). This method detects the transfer of DNA sequence chunks between genomes through homologous recombination throughout the genome. Based on this, the chromosome-painting algorithm calculates the expected number of chunks imported from a donor genome to a recipient genome and then summarizes these values in a matrix (the “co-ancestry matrix”) (Fig. 9.1b) (Yahara et al. 2013).

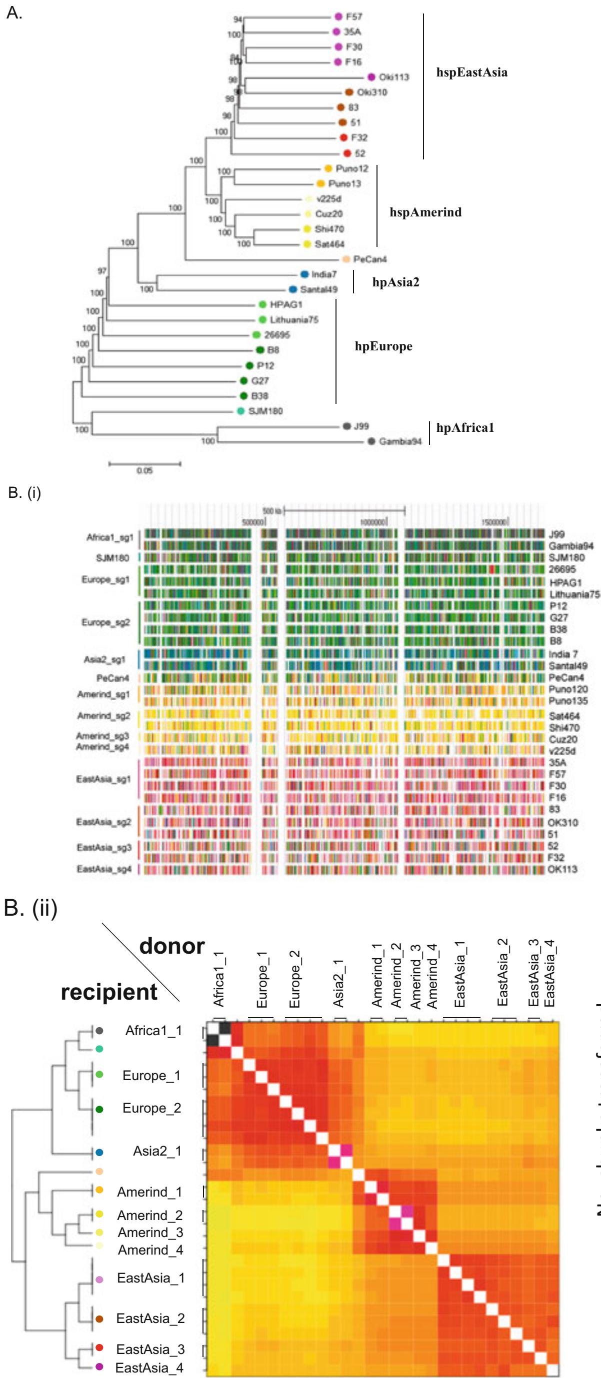

Fig. 9.1 Genome evolution in *H. pylori*. (**a**) A phylogenetic tree of *H. pylori* based on core genome sequences. *Numbers* indicate bootstrap values. The *scale bar* indicates substitutions per nucleotide. The *color in front* of a strain name indicates a subgroup identified with chromosome

Based on the matrix, individual strains were assigned to subgroups by the fineSTRUCTURE clustering algorithm (the left part of Fig. 9.1b). This new method revealed a finer population structure than a previous method that examined only seven MLST genes (Yahara et al. 2013).

An examination of the genetic flux in the co-ancestry matrix (Fig. 9.1b) showed some singleton strains to be hybrids of subgroups. For example, the 13th strain (PeCan4) is a hybrid in that has received a considerable number of chunks from the Amerindian, Africa1, and European strains. The co-ancestry matrix also revealed evident signs of population admixture in Africa, Europe, and parts of Asia (Yahara et al. 2013).

A modification of the chromosome-painting method allowed the detection of regions that have frequently transferred between lineages in multiple bacterial species (Yahara et al. 2014; Yahara et al. 2015).

9.3 Evolution of Individual Genes

The evolution of individual genes of *H. pylori* turned out to often involve various modes of recombination. Many genes have diverged between Western (= African and European) strains and East Asian strains (Kawai et al. 2011). These include many virulence genes, for example, the *cagA* oncogene (Hatakeyama 2014).

9.3.1 The **cagA** *Oncogene*

The CagA gene product is injected into epithelial cells, undergoes phosphorylation at a tyrosine (Y) residue in its EPIYA motif by host cell kinases, and perturbs host signaling pathways. CagA is known for its geographical, structural, and functional diversity in its C-terminal half, where the EPIYA host-interacting motif is repeated. The Western version carries EPIYA segment types A, B, and C, while the East Asian version carries types A, B, and D, which results in higher virulence (Hatakeyama 2014).

Fig. 9.1 (continued) painting and the fineSTRUCTURE algorithm (Modified from Figure S2 of Yahara et al. (2013)). (**b**) Chromosome painting/fineSTRUCTURE analysis. (i) Chromosome painting in silico. Each *lane* indicates the chromosome of a strain shown on the *right*. The strains are classified by fineSTRUCTURE into subgroups labeled by *colors on the left*. A *color* along the chromosome indicates that the subgroup apparently donated a chunk of single nucleotide polymorphisms (SNPs) through homologous recombination. All genomic positions are transformed to those of a reference strain (26695). (ii) Co-ancestry matrix with population structure and genetic flux. The *color* of each cell of the matrix indicates the expected number of chunks imported from a donor genome (*column*) to a recipient genome (*row*). The tree on the *left* shows the clustering of the listed population subgroups (Modified from Figures 1 and 2 of Yahara et al. (2013))

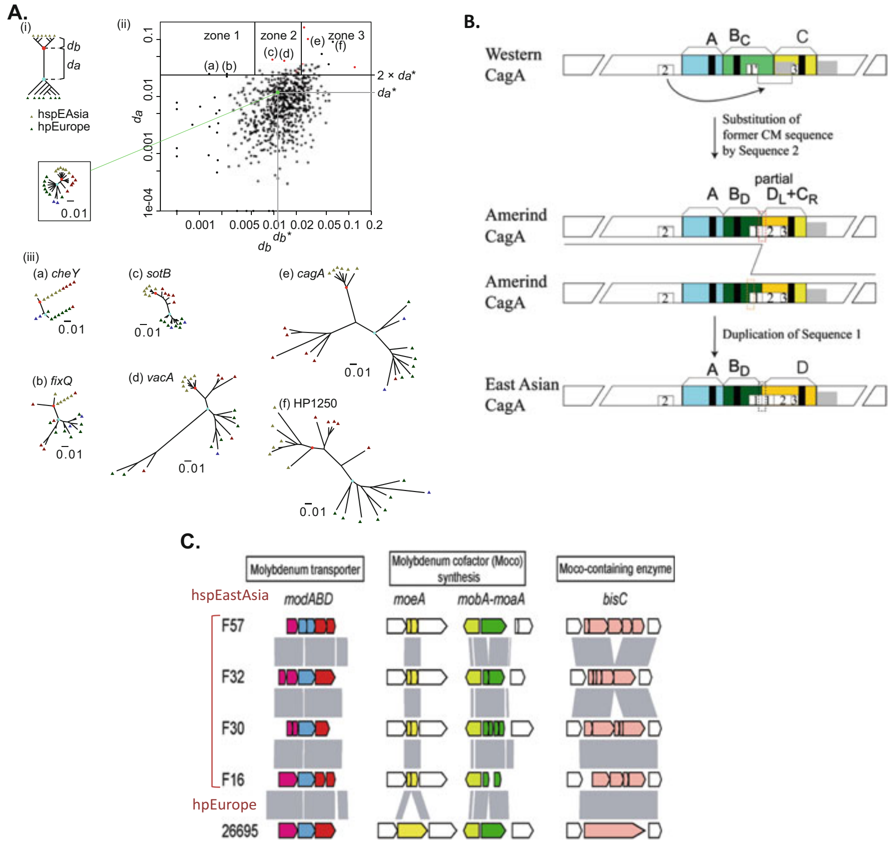

Fig. 9.2 Evolution of individual genes. (**a**) Phylogenetic trees of individual genes. (*i*) Diagram of the analysis. *Black dots*, the last common ancestors of Eastern and Western strains. d_a, the length of the branch separating the two groups; d_b, the average branch length of the Eastern strains. (*ii*) Plot of gene trees based on the two distance values. Large *green dot*, the well-defined core tree; d_a*, d_a for the well-defined core tree; d_b*, d_b for the well-defined core tree; inset box, the well-defined core tree; zone 1, d_b <0.00550; zone 2, 0.00550≤ d_b ≤0.0231; zone 3, d_b >0.0231; *red dot*, genes with positive selection resulting in amino acid changes and with $d_a > 2 \times d_a$*, that is, d_a >0.02324. $N = 692$ genes. (*iii*) Representative gene trees with high divergence between hspEastAsia and hpEurope strains. Their positions are indicated in (*ii*). Lowest common ancestor (LCA) of hspEastAsia (*red*) and hpEurope (*cyan*) is marked (Modified from Figure 8 of Kawai et al. (2011)). (**b**) CagA and its evolutionary pathway. *Black box*: the EPIYA sequence. *Gray box*: the CM sequence (Modified by Yoshikazu Furuta and the author from Figure 1 of Furuta et al. 2011c). (**c**) Decay of molybdenum-related genes in hspEastAsia strains. The *left* labels indicate strain names (Modified by Mikihiko Kawai and the author from Figure 4 of Kawai et al. (2011))

Insight into the relationships between *cagA* variants through various modes of recombination was obtained by analyzing all known *cag*A variant sequences (over 1100) in the public database at single-nucleotide resolution (Furuta et al. 2011c). The left half of the EPIYA-D segment characteristic of East Asian *cag*A was

derived from the Western-type EPIYA, with the Amerindian-type EPIYA as the intermediate, through rearrangements of specific sequences within the gene (Fig. 9.2b).

Many of their structural variants can be explained by: (i) homologous recombination between DNA sequences encoding the CM (CagA multimerization) domain; (ii) site-specific recombination between DNA sequences in the EPIYA motif; and (iii) illegitimate recombination between short similar DNA sequences (Furuta et al. 2011c).

9.3.2 Decay of Molybdenum-Related Genes

Some genes decay, via point mutations and various types of recombination, as has occurred in *H. pylori*. To closely follow such evolutionary processes, it is necessary to thoroughly characterize the gene content in each of the multiple genomes. Such phylogenetic profiling needs to determine the presence or absence of a domain, rather than a gene (a coding region), and to detect split genes, partially deleted genes, and partially duplicated genes (Uchiyama 2006). The analysis can then go to the single-nucleotide level.

Such an analysis revealed that functions related to molybdenum (Mo) were lost in hspEastAsia strains (Kawai et al. 2011). The trace element Mo is essential for nearly all organisms. After transport into the cell as molybdate, it is incorporated into metal cofactors for specific enzymes (molybdo-enzymes) that catalyze reduction–oxidation reactions mediated by two-electron transfer. At least one gene involved in each of these Mo-related functions decayed through point mutation or recombination in all hspEastAsia strains analyzed. Some Amerindian strains (B type) also lack Mo-related genes (Gressmann et al. 2005). The occurrence of apparently independent multiple mutations suggests some selection against the use of Mo.

9.3.3 Outer Membrane Proteins

Outer membrane proteins (OMPs), which are involved in host interactions, among others, are numerous and variable in *H. pylori* (Leituti and Goldberg 2012). They form many large families that show various modes of evolution via mutation and recombination. These include decay, gene conversion (Kawai et al. 2011), and duplication (see below). Some of their sequences are specific to phylogenetic groups. There are, for example, C-terminal sequences in HopMN that are characteristic of hpEastAsia strains (Kawai et al. 2011), while the *horA* gene is fragmented in hspEastAsia strains (Kawai et al. 2011).

9.4 Evolution of Chromosome Synteny

In general, inversion events, as well as insertion/deletion events, play an important role in the evolution of chromosome synteny. Large insertion and deletion events affecting synteny, especially those mediated by mobile elements such as transposons, prophages, genomic islands, and restriction-modification (RM) systems, are important but are beyond the scope of this article.

9.4.1 Inversion

An inversion relationship was identified by comparing two complete genome sequences of *H. pylori* (Alm et al. 1999). More recently, all large inversion events between ten complete genomes of global strains were identified (Fig. 9.3b) (Furuta et al. 2011a). Sequence alignments in these studies revealed four underlying mechanisms: (i) homologous recombination between long sequences of high identity in an opposite orientation; (ii) illegitimate recombination between short similar sequences in an opposite orientation; (iii) inversion adjacent to the insertion of a mobile element; and (iv) DNA duplication associated with inversion (DDAI).

9.4.2 DNA Duplication Associated with Inversion

In the last process (DDAI), a DNA segment at one chromosomal locus is copied and inserted, in an inverted orientation, into a distant locus on the same chromosome, while the entire region between these two loci is also inverted (Fig. 9.3a). DDAI was found by comparing the complete genome sequences of Western strains and East Asian strains: Gain and loss of genes (loci) for OMPs occurred at breakpoints of chromosomal inversions (Fig. 9.3a). This mode of DNA duplication was also found in other organisms (Ranz et al. 2007; Chen et al. 2013).

9.4.3 Reconstruction of Synteny Evolution

Recognition of these four modes of inversion allowed the reconstruction of synteny evolution through inversions in *H. pylori* (Fig. 9.3c). The paths of synteny evolution are related to, but not identical with, those of genome sequence evolution. For example, closely related genomes of four Japanese strains take different forms because of different inversion events.

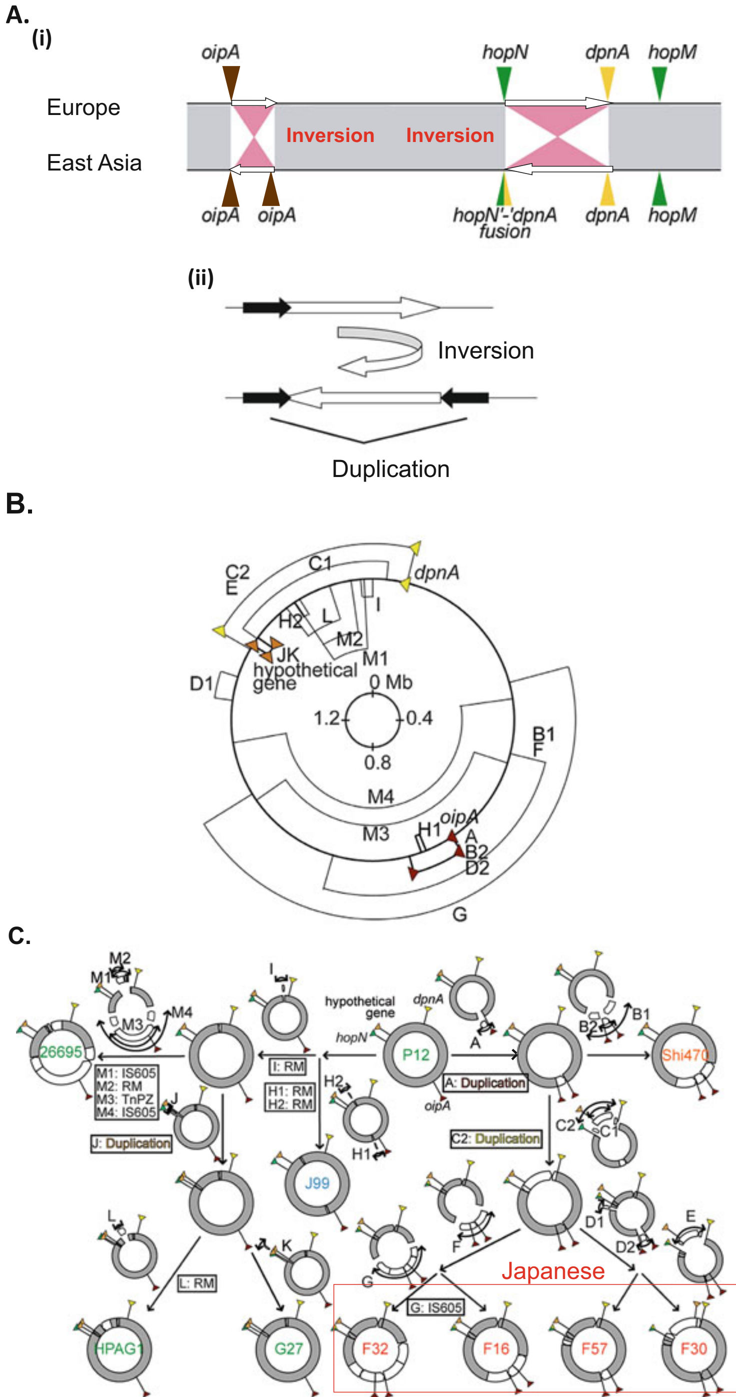

Fig. 9.3 Evolution of chromosome synteny through inversion. (**a**) DNA duplication associated with inversion (DDAI). (*i*) Linkage of *oipA* gene duplication and *hopN* gene decay with

9.5 Evolution of the Methylome

Epigenetic modifications, such as DNA methylation, have large effects on gene expression and genome maintenance in prokaryotes and eukaryotes. Although many of the epigenetic marks are reprogrammed in most cases in plants and animals, there are increasing lines of evidence for trans-generation inheritance of epigenetic status. *H. pylori* has a large number of DNA methyltransferase genes, with different strains having unique repertoires.

9.5.1 Restriction–Modification Systems

Many of the DNA methyltransferases in prokaryotes are a part of RM systems (Fig. 9.4a). In an RM system, a modification enzyme (a DNA methyltransferase) transfers a methyl group to a specific DNA sequence. A paired restriction enzyme will attack DNA lacking this ID of the self-epigenome.

RM systems behave as selfish mobile elements, just like transposons and viral genomes (Furuta and Kobayashi 2013). They are sometimes linked to genome rearrangements, such as inversion events. Because they increase genetic isolation and affect gene expression (Furuta et al. 2014; Kumar et al. 2012), they may contribute to adaptive evolution (Fig. 9.4b).

RM systems are classified into four types (Roberts et al. 2003). They differ in the location of their target recognition domain (TRD) for methylation. In Type III systems, the TRD is in the same polypeptide (Mod protein) as the DNA methyltransferase (Rao et al. 2014). In Type I systems, the TRD is on the S (specificity) subunit and consists of TRD1 and TRD2, each recognizing half of a bipartite recognition sequence (Loenen et al. 2014). In some S subunits, the number of repeats of an amino acid sequence measures the distance (the number of nonspecific nucleotide Ns) between these two half sites. A thorough description of the many RM systems in *H. pylori* is beyond the scope of this article (see REBASE Genomes, http://rebase.neb.com/rebase/rebase.html).

Fig. 9.3 (continued) chromosomal inversions between typical Western and East Asian genomes. The upper line corresponds to the P12 genome, and the lower line corresponds to an ancestral structure of four Japanese genomes (Modified from Figure 1 of Furuta et al. 2011a with a permission from PNAS). (*ii*) The concept of DDAI. Duplication of the *black arrow* region to a new site in an opposite orientation is associated with inversion of the *white arrow* region between its old site and its new site. (**b**) Inversions detected by genome comparisons. Large inversions detected by comparing ten global genomes were mapped on a *H. pylori* genome. The *outer circle* indicates the genome of P12, a European strain, whereas the *inner circle* indicates its origin and coordinates. *An arc outside the outer circle* indicates an inversion in the East Asian strains and Amerindian strain, whereas an *inside arc* indicates an inversion in European and/or West African strains. A *triangle* indicates a region generated by a DDAI event (From Figure 2 of Furuta et al. 2011a with a permission from PNAS). (**c**) Reconstruction of synteny evolution through inversion. *Triangles* indicate regions duplicated through DDAI (Modified from Figure 5 of Furuta et al. 2011a with a permission from PNAS)

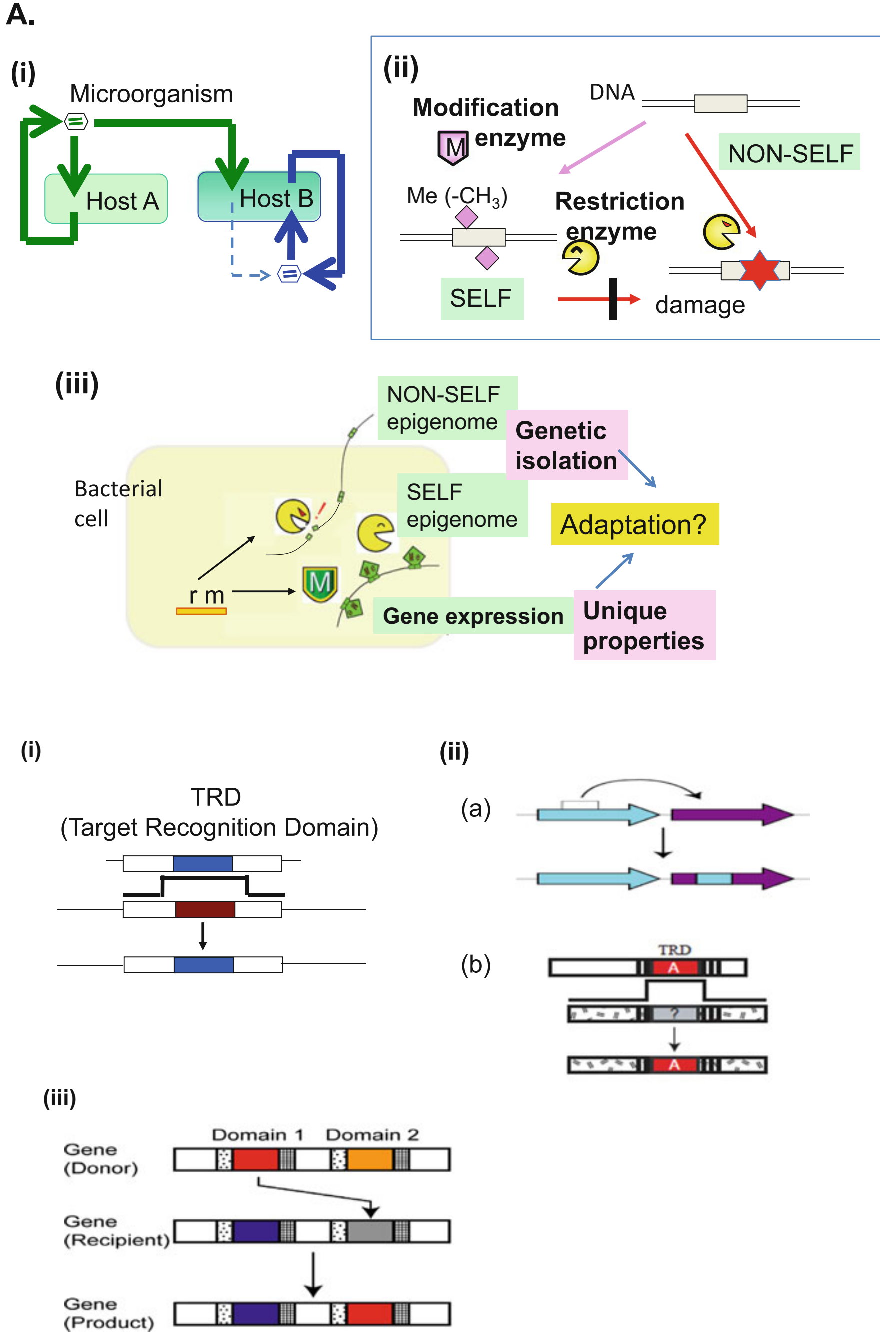

Fig. 9.4 Restriction-modification systems. (**a**) Action. (*i*) Adaptation of a microorganism to its host. The microorganism may be a bacteriophage and the host may be a bacterium. (*ii*) Distinction

9.5.2 Base-Excising Restriction Enzymes

All the restriction enzymes examined thus far hydrolyze phosphodiester bonds in the DNA backbone, while a member of their family with half-pipe fold excises a base from the recognition sequence (Miyazono et al. 2014). In other words, it is a DNA glycosylase. The resulting apurinic/apyrimidinic (AP) site is sometimes cleaved by the second activity of the enzyme, AP lyase (Fukuyo et al. 2015). Restriction enzymes may be now classified into two basic classes: the phosphodiesterase class and the glycosylase class. Its family member, R.HpyAXII, present in several *H. pylori* strains can limit transformation (Humbert and Salama 2008). The PabI family of restriction-modification system behaves as a mobile genetic element, similar to the other families of Type II restriction-modification systems. The distribution of the *hrgC* gene replacing the PabI family in the subpopulations of *H. pylori*, hspAmerind, hspEAsia and hpAsia2, corresponds to the two human migration events, one from East Asia to Americas and the other from China to Malaysia (Kojima and Kobayashi 2015) .

9.5.3 Sequence Specificity Variation in DNA Methylation Systems

Genome sequence comparisons and methylome decoding (Furuta and Kobayashi 2012b; Furuta et al. 2011b; Furuta et al. 2014) revealed that these DNA methyltransferases often change their sequence specificity, generating wide diversity in the methylome.

In Type III RM systems, the changes may be mediated by the replacement of the TRD repertoire by allelic homologous recombination (Fig. 9.4d) or by ectopic homologous recombination (gene conversion) (Fig. 9.4d). The latter takes place between distantly related genes at separate loci, taking advantage of the weakly similar DNA sequences encoding methyltransferase motifs. Some of the TRD sequences (homology groups) may move beyond the species boundary and spread throughout the bacterial world (Furuta and Kobayashi 2012b).

Fig. 9.4 (continued) between the self and non-self-epigenome. A modification enzyme transfers a methyl group to a specific DNA sequence. A cognate restriction enzyme will cleave DNA lacking this self-epigenome identification. (*iii*) Possible dual roles in adaptive evolution. Methylation of a specific sequence at many sites along the genome may provide a specific gene expression pattern, among other unique properties. These lineages are isolated from each other. (**b**) Changes in the sequence specificity of RM systems through various modes of recombination. (*i*) Allelic recombination. The same gene (locus). (*ii*) Gene conversion. Different genes (loci). *a* General scheme. *b* The case with Type III *mod* genes. (*iii*) Domain movement. Different sites in the same gene. Recombination events at the marked sequences flanking target recognition domains (TRDs) are responsible for the movement (From Figure 1 of Furuta et al. 2011b)

In the Type I S subunit, TRD sequences may replace each other by allelic homologous recombination at the same site, move between different genes, and move between different sites within a gene (DoMo = domain movement) (Fig. 9.4d). The movement between TRD1 and TRD2 likely involves shared sequences flanking the TRDs. There is evidence that a change in the number of repeats between TRD1 and TRD2 takes place within the bacteria during an infection (Andres et al. 2010).

The resulting changes in the methylome may lead to changes in gene expression and phenotypes, and they may be subject to natural selection (Furuta and Kobayashi 2012a; Furuta et al. 2014). This hypothesis of epigenetics-driven adaptive evolution awaits further experimental tests.

9.6 Epilogue

I have described how single-nucleotide resolution comparisons of complete genomes and methylomes revealed the dynamics of genome and epigenome evolution. I have not covered the emerging relationships of these genome/epigenome changes with phenotype changes, which are relevant to host interactions and adaptive evolution. Most of the description here is based on strain comparisons, but there are also interesting studies of the evolution of individual lineages. I also had to omit a recent description of the microbiome surrounding *H. pylori*.

I expect that current breakthroughs in OMICS analyses, aided by bioinformatic analysis and other experimental approaches, will lead to the emergence of a more complete picture of adaptive evolution in this model organism within a decade.

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Chapter 10
Non-*Helicobacter pylori Helicobacter* Infections in Humans and Animals

Bram Flahou, Freddy Haesebrouck, and Annemieke Smet

Abstract Since the first description of the human pathogen *Helicobacter pylori* in the early 1980s, the number of known species in the genus *Helicobacter* has increased largely. Currently, 45 different *Helicobacter* species have been identified. Bacteria belonging to this genus can roughly be divided into two major groups, gastric and enterohepatic species. Gastric helicobacters express urease at a high level which helps them to survive in the acidic environment of the stomach, whereas most enterohepatic helicobacters do not. The best-known gastric *Helicobacter* species is *H. pylori*. This chapter, however, deals with non-*H. pylori* helicobacters (NHPH). Most NHPH are animal-associated bacteria, but some of them are of zoonotic significance. First, gastric infections with these bacteria in humans are considered. Thereafter, an overview of natural and experimental gastric infections in animal hosts is given, with emphasis on the gastric helicobacters that are mainly associated with dogs, cats, and pigs. Finally, enterohepatic *Helicobacter* species are briefly discussed.

Keywords Gastric non-*Helicobacter pylori* helicobacters • Enterohepatic helicobacters • Zoonotic significance • Gastric disease • Enterohepatic disease • Animal models

10.1 Introduction

Since the original description of the human pathogen *H. pylori* in 1983, the number of known species in the genus *Helicobacter* has increased largely (Warren and Marshall 1983). Currently this genus includes 45 identified species. An overview is shown in Fig. 10.1. The *Helicobacter* bacteria can roughly be divided into gastric and enterohepatic species. Gastric *Helicobacter* species are able to survive the acidic environment in the stomach by expressing high levels of urease (Pot et al. 2007). Enterohepatic species do not normally colonize the gastric mucosa.

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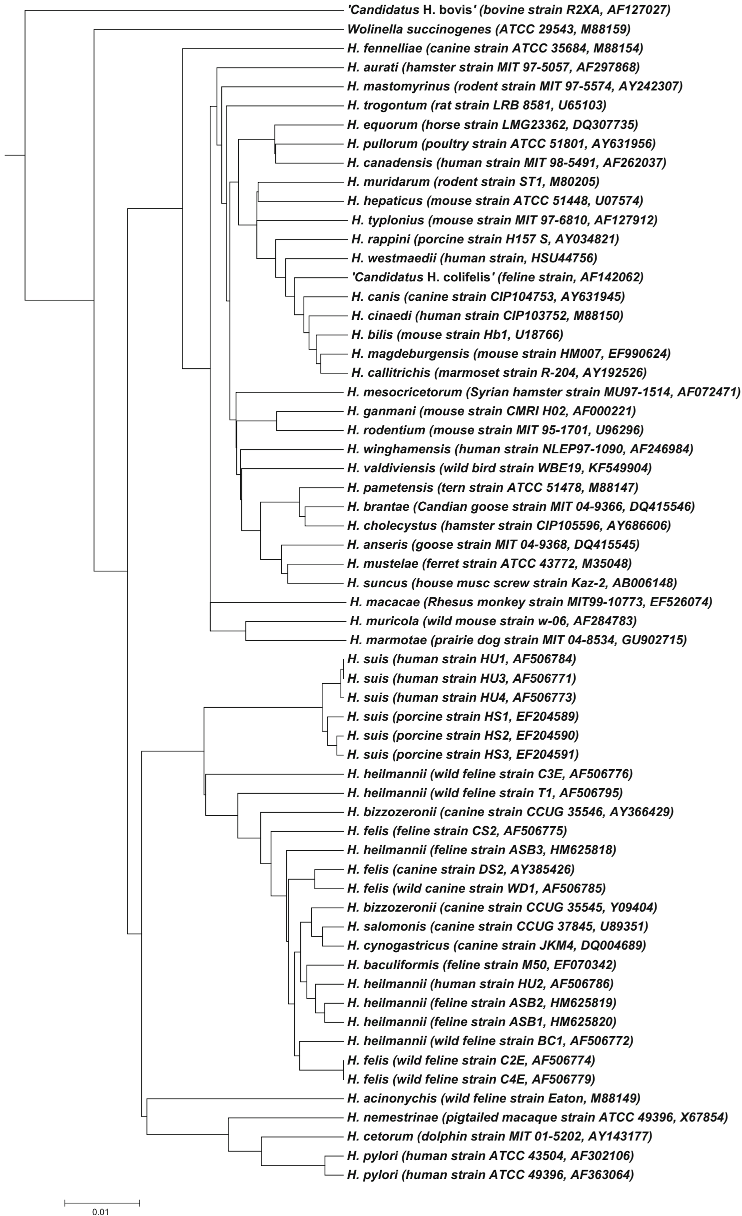

Fig. 10.1 Phylogenetic tree based on the near-complete 16s rRNA gene sequences from all gastric and enterohepatic *Helicobacter* species described so far. The alignment of the sequences and the construction of the phylogenetic tree were performed as described before (Smet et al. 2012)

Instead, they thrive at the mucosal surface of the intestinal tract and/or the liver (Sterzenbach et al. 2007). To date, *Helicobacter* spp. have been detected in nearly 150 vertebrate species, including animals from every continent and all four non-fish vertebrate taxonomic classes (Schrenzel et al. 2010). Animal-associated helicobacters, and especially the gastric species, are characterized by their extremely fastidious nature, which to date has resulted in a low number of in vitro isolates available worldwide (Haesebrouck et al. 2009). Several of these species have a pathogenic potential in different animal hosts, and some are capable of causing disease in humans (Table 10.1). The presence and diversity of helicobacters in the vertebrate fauna and their transfer possibilities between hosts are critical factors on how fast the *Helicobacter* ecology will evolve and what their impact is on animal and human health (Schrenzel et al. 2010). This chapter mainly focuses on the biology and pathogenesis of animal-associated gastric *Helicobacter* infections in humans and animals. An overview of the significance of enterohepatic *Helicobacter* spp. in human and animal disease is summarized at the end of this chapter.

10.2 Gastric *Helicobacter* Infections in Humans

In the early years of *H. pylori* research, pathologists examining human gastric biopsies already reported the presence of bacteria with a long spiral-shaped morphology. These microorganisms were similar to bacteria reported in the stomach of pigs, cats, dogs and nonhuman primates and were originally referred to as *Gastrospirillum hominis* (McNulty et al. 1989). Later on, they were renamed to *H. heilmannii* (Heilmann and Borchard 1991). Although at that time the name *H. heilmannii* had no official standing in nomenclature, it was used for many years to refer to the group of long spiral-shaped bacteria in the human stomach, which actually comprises several different *Helicobacter* species. Later on these microorganisms were reclassified into *H. heilmannii* type 1, representing *H. suis* from pigs, and *H. heilmannii* type 2, comprising a group of canine and feline *Helicobacter* spp. (Haesebrouck et al. 2009). The valid description of *H. heilmannii* further added some confusion on the nomenclature of this complex and expanding group of microorganisms (Smet et al. 2012). Therefore, the terms *H. heilmannii* (sensu lato), referring to the group of gastric non-*H. pylori Helicobacter* spp. (NHPH), and *H. heilmannii* (sensu stricto), referring to the species, have been proposed (Haesebrouck et al. 2011).

To date, these microorganisms have been associated with gastritis, gastric and duodenal ulcers, and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma in humans. Gastric NHPH have microscopically been detected in 0.2–6 % of humans with severe gastric complaints undergoing a gastroscopy, but this is most probably an underestimation of their true prevalence (Haesebrouck et al. 2009). It cannot be excluded that infections with these bacteria sometimes remain unapparent or cause mild disease signs which are often not thoroughly examined.

Table 10.1 *Helicobacter* spp. and their hosts

Taxon	Natural hosts	Zoonotic potential
Gastric *Helicobacter* spp.		
'*Candidatus* H. bovis'	Cattle	Yes
'*Candidatus* H. homininae'	Chimpanzee, gorilla	Unknown
H. acinonychis	Cheetah, tiger, lion	Unknown
H. ailurogastricus	Cat	Unknown
H. baculiformis	Cat	Unknown
H. bizzozeronii	Cat, dog	Yes
H. cetorum	Whale, dolphin	Unknown
H. cynogastricus	Dog	Unknown
H. felis	Dog, cat, cheetah, New Guinea wild dog, rabbit	Yes
H. heilmannii	Dog, cat, cheetah, bobcat, tiger, lynx, leopard, puma	Yes
H. mustelae	Ferret	Unknown
H. pylori	Human	/
H. salomonis	Cat, dog, rabbit	Yes
H. suis	Pig, mandrill monkey, rhesus macaque, crab-eating macaque	Yes
Enterohepatic *Helicobacter* spp.		
'*Candidatus* H. colifelis'	Cat	Unknown
H. anseris	Goose	Unknown
H. aurati	Hamster	Unknown
H. bilis	Mouse, rat, gerbil, dog, cat, sheep	Yes
H. brantae	Goose	Unknown
H. callitrichis	Marmoset	Unknown
H. canadensis	Bird, pig	Yes
H. canis	Dog, cat	Yes
H. cholecystus	Hamster	Unknown
H. cinaedi	Hamster, rat, cat, dog, rhesus monkey, baboon	Yes
H. equorum	Horse	Unknown
H. fennelliae	Dog	Yes
H. ganmani	Mouse	Yes
H. hepaticus	Mouse, gerbil	Yes
H. macacae	Rhesus monkey, baboon	Unknown
H. marmotae	Woodchuck, cat	Unknown
H. magdeburgensis	Mice	Unknown
H. mastomyrinus	Rodents	Unknown
H. mesocricetorum	Hamster	Unknown
H. muricola	Wild mouse	Unknown
H. muridarum	Mouse, rat	Unknown
H. pamatensis	Bird, pig, cat	Yes
H. pullorum	Poultry	Yes
H. rappini	Mouse, sheep, dog	Yes

(continued)

Table 10.1 (continued)

Taxon	Natural hosts	Zoonotic potential
H. rodentium	Mouse, rat	Unknown
H. suncus	House musk shrew	Unknown
H. trogontum	Rat, pig, sheep	Unknown
H. typhlonius	Mouse, rat	Unknown
H. valdiviensis	Wild birds	Unknown
H. westmeadii	Human	/
H. winghamensis	Human, wild rodents	Yes

Clinical symptoms associated with gastric NHPH infections include acute or chronic epigastric pain, nausea, dyspepsia, reflux esophagitis, heartburn, vomiting, hematemesis, abdominal pain, irregular defecation frequency and consistency, and dysphagia, often accompanied by a decreased appetite (Haesebrouck et al. 2009).

In patients undergoing an endoscopy, a variety of lesions can be observed ranging from a normal to slightly hyperemic mucosa, mucosal edema, nodular inflammation, and the presence of ulcerations in the antrum of the stomach or in the duodenum (Haesebrouck et al. 2009; Sykora et al. 2003). Histopathological examination of gastric biopsies reveals infiltration with lymphocytes and plasma cells, sometimes organized in lymphocytic aggregates (Joosten et al. 2013b). Other lesions, such as intestinal metaplasia, have occasionally been described in patients infected with NHPH, and some of these patients were also infected with *H. pylori* (Stolte et al. 1997; Yakoob et al. 2012). Compared to an *H. pylori*-associated gastritis, gastritis associated with NHPH is often less active and less severe. On the other hand, the risk of developing MALT lymphoma is higher with NHPH than with *H. pylori* (Haesebrouck et al. 2009).

Tests to rapidly diagnose infection with gastric NHPH are currently unavailable. Urea breath tests, used to diagnose infection with *H. pylori*, are often negative in patients infected with animal-associated *Helicobacter* spp. (Matsumoto et al. 2014). This can be explained by the fact that infections with these bacteria are, in contrast to *H. pylori* infections, more often focal and predominantly found in the antrum of the stomach (Stolte et al. 1997). Due to the fastidious nature of gastric NHPH, isolation of these bacteria is not an option for routine diagnostic purposes. Until now, only *H. bizzozeronii* (Andersen et al. 1999; Kivisto et al. 2010) and *H. felis* (Wüppenhorst et al. 2013) have been cultured from gastric biopsies. Therefore, analysis of gastric biopsies by molecular microbiological methods and histology is so far the only way to determine infections with these microorganisms. A German study analyzed 89 human gastric biopsies, previously shown to be NHPH positive (Trebesius et al. 2001). Eighty percent of these samples were positive for *H. suis*, 17 (19 %) samples were positive for *H. heilmannii*, and 5 (6 %) hybridized with a probe specific for *H. felis*, *H. bizzozeronii*, and *H. salomonis*. De Groote and co-workers (2005) screened paraffin-embedded gastric biopsies from 101 patients

with confirmed gastric NHPH infection. Fourteen samples were positive for *H. suis*, whereas 49 were infected with helicobacters from cats and dogs. Another Belgian study showed similar results. *H. suis* was the most prevalent species (37 %), followed by *H. salomonis* (21 %), *H. felis* (15 %), *H. heilmannii* (8 %), and *H. bizzozeronii* (4 %) (Van Den Bulk et al. 2005b). A Polish study evaluated the incidence of gastric NHPH infection in dyspeptic children at the age of 4–18 years and found a prevalence of 0.2 % (Iwanczak et al. 2012). Another study focused on the association between coinfection with canine and feline NHPH and *H. pylori* and gastric pathology in patients with dyspepsia. *H. pylori* was found in 67 % of the patients, and only 6 % and 4 % of them were coinfected with *H. heilmannii* and *H. felis*, respectively (Yakoob et al. 2012). Recently, a remarkably high prevalence (27 %) of *H. suis* DNA was found in gastric biopsies from human patients with idiopathic parkinsonism. The putative significance of this bacterium in Parkinson's disease requires further investigation (Blaecher et al. 2013).

Evidence is accumulating that pigs, cats, and dogs constitute reservoir hosts for gastric *Helicobacter* spp. with zoonotic potential (Haesebrouck et al. 2009). *Helicobacter* DNA has been detected in saliva from cats, dogs, and pigs, indicating that the oral cavity of these animals may act as source of NHPH infection for humans (Ekman et al. 2013; Casagrande Proieti et al. 2010; Shojaee Tabrizi et al. 2010). Fecal-oral transmission has also been suggested as a possible route for infection in cats (Ghil et al. 2009). Besides direct contact with animals, other routes of transmission of NHPH should not be neglected. It has been shown that *H. felis* is able to survive in water for several days, highlighting the possible role for water in the transmission of this species (Azevedo et al. 2008). Recently, De Cooman and co-workers (2013) demonstrated that *H. suis* can be present on and survive in minced pork. This indicates that raw or undercooked pork may also constitute a source of *H. suis* infection for humans. Nowadays, the prevalence of *H. pylori* in humans from the Western world is decreasing from generation to generation, leaving a niche for possible infection with these animal-associated gastric *Helicobacter* spp.

For patients with severe clinical symptoms and pathology, treatment is necessary. There is, however, a lack of clinical trials, and only a few reports deal with antimicrobial susceptibility and acquired resistance of gastric NHPH (Vermoote et al. 2011b; Van den Bulck et al. 2005a). Triple therapy using the combination of a proton-pump inhibitor and two antimicrobial agents, like clarithromycin, metronidazole, amoxicillin, or tetracycline, may be effective in most cases but not always. A Finnish patient infected with *H. bizzozeronii* received a triple therapy of tetracyclines, metronidazole, and lansoprazole for 1 week. The symptoms subsided, but the infection was not cleared and the patient continued to suffer from mild nausea. *H. bizzozeronii* was isolated from the stomach, and determination of its antimicrobial susceptibility showed resistance against tetracycline and metronidazole (Schott et al. 2012). Furthermore, it was demonstrated that acquired resistance to metronidazole in *H. bizzozeronii* was due to the contingency nature of an oxygen-insensitive NAD(P)H-nitroreductase. This phenomenon was also described for *H. heilmannii* (Kondadi et al. 2013).

10.3 Natural and Experimental Gastric *Helicobacter* Infections in Animal Hosts

*10.3.1 Gastric Non-*H. pylori Helicobacter *spp. Associated with Dogs and Cats*

10.3.1.1 Prevalence in Dogs and Cats

In pet animals, gastric *Helicobacter* spp. have been frequently described with a prevalence of 67–86 % in clinically healthy dogs, 61–100 % in dogs presenting chronic vomiting, and 41–100 % in healthy cats as well as cats showing chronic vomiting (Haesebrouck et al. 2009; Shojaee Tabrizi et al. 2010). Ghil and colleagues (2009) reported that the prevalence of *Helicobacter* spp. in feral cats was approximately twofold higher than in domestic cats. Often cats and dogs are naturally infected with multiple gastric *Helicobacter* spp. (Haesebrouck et al. 2009). The first *Helicobacter* species isolated from the stomach of cats and dogs was *H. felis* (Lee et al. 1988). Later on, *H. bizzozeronii*, *H. salomonis*, and *H. cynogastricus* were isolated from the canine gastric mucosa, whereas H. baculiformis, H. heilmannii and H. ailurogastricus isolates were obtained from the stomach of cats (Haesebrouck et al. 2009; Smet et al. 2012; Joosten et al. 2015). It has been shown that *H. bizzozeronii* is the most predominant species in the canine stomach, whereas *H. felis* and *H. heilmannii* are the predominant *Helicobacter* spp. in cats (Priestnall et al. 2004; Svec et al. 2000; Wiinberg et al. 2005). The prevalence of *H. cynogastricus* and *H. baculiformis* in pet animals as well as their zoonotic potential is so far unknown. Only few data is available on the transmission of NHPH infections in dogs and cats. Transmission of *H. salomonis* from a dam to her puppies, as well as between infected and noninfected pups, has been described (Hänninen et al. 1998). It has been suggested that in this case, transmission occurred through oral-oral or gastric-oral contact, as nursing dogs have very close contact with their offspring and puppies eat material vomited by the dam (Hänninen et al. 1998).

10.3.1.2 Role of *Helicobacter* spp. in the Development of Gastric Pathologies in Dogs and Cats

In general, canine and feline *Helicobacter* spp. have been associated with chronic active gastritis (Haesebrouck et al. 2009). Histological changes in the lamina propria include mild mononuclear inflammatory infiltration, the presence of lymphoid follicles, fibrosis, and glandular degeneration in the stomach of cats naturally infected with *H. heilmannii* (Takemura et al. 2009). One study reported a correlation between *Helicobacter* infection and the presence of feline lymphoma (Bridgeford et al. 2008). Gastric and duodenal ulcers have been rarely reported among cats and dogs, and an association with *Helicobacter* infections has not been

made (Haesebrouck et al. 2009). To study the pathogenesis of NHPH infections in dogs and cats, several experimental infection studies have been performed. A mononuclear infiltration throughout the gastric mucosa, with follicular organization of the inflammatory cells, has been demonstrated in the stomach of *H. felis*-infected-specific pathogen-free (SPF) cats (Scanziani et al. 2001). Another experimental infection study with *H. felis* in young gnotobiotic dogs described lymphoid hyperplasia in the fundus and body of the stomach (Diker et al. 2002). On the contrary, Simpson and co-workers (1999) found a similar degree of inflammation in both *H. felis*-infected SPF dogs and uninfected control dogs. These contradictory results may be explained by differences in virulence between *H. felis* strains. The review by Haesebrouck and colleagues (2009) suggested that the pathogenic significance of gastric helicobacters in cats and dogs may be related to (1) the species expressing its own virulence which may be increased in cases of mixed infections due to synergistic effects, (2) differences within strains from the same species, or (3) host differences. An Italian study investigated the localization of *Helicobacter* spp. in the fundic mucosa of laboratory beagle dogs. They demonstrated that *H. bizzozeronii* was present in the superficial and basal portions of the fundic glands, while *H. felis* was only detected in the superficial portions of the glands. Additionally, these helicobacters were also located free in the cytoplasm or within lysosomes of parietal cells (Lanzoni et al. 2011). The urea breath test, widely used for rapid and noninvasive detection of *H. pylori* infection in humans, has recently been applied on laboratory beagle dogs. A sensitivity and specificity of 89 % was reported suggesting the usefulness of this technique to monitor gastric *Helicobacter* infections in dogs (Kubota et al. 2013).

10.3.1.3 Genomics, Genetics, and Experimental Studies in Rodents on the Pathogenesis of Canine and Feline *Helicobacter* Infection

Recently the genomes of *H. felis*, *H. bizzozeronii*, and *H. heilmannii* have been sequenced (Arnold et al. 2011; Schott et al. 2011; Smet et al. 2013). These genomes contain genes encoding homologues of known *H. pylori* virulence factors discussed in Chaps. 3, 4, 5, 6, and 7. These genomes possess a complete *comB* system conferring natural competence, but they all lack the cytotoxin-associated gene pathogenicity island (*cag*PAI), a functional vacuolating cytotoxin A (VacA), and the *H. pylori* adhesins identified so far. Amorim and colleagues (2014) showed that several canine and feline helicobacters, like *H. felis* and *H. heilmannii*, are able to adhere to the canine gastric mucosa. Which adhesins are involved is so far unknown.

Besides SPF pets, rodent models have also been shown to be useful experimental infection models to obtain insights into the pathogenesis of gastric NHPH infections in animals and humans (O'Rourke et al. 2004a). *H. felis* infection in mice is often used as an animal model to study *H. pylori*-related gastric pathology in humans. Due to the lack of several important *H. pylori* virulence factors, researchers must be

aware that *H. felis* might not always have similar outcomes in pathogenicity compared to *H. pylori*. Extrapolation of data obtained in an *H. felis* infection mouse model to *H. pylori* infections in humans should therefore be done with caution. To date, many reports studied the pathogenesis of canine and feline *Helicobacter* infection in rodent models. One of the very first steps in the pathogenesis of gastric infections caused by these microorganisms is colonization of the gastric mucosa in which binding to mucins (MUC) plays an important role. MUC1, MUC5AC, and MUC6 are the major mucins covering the gastric mucosa. Liu and colleagues (2014) investigated the gastric mucin expression pattern in the stomach of *H. heilmannii*-infected BALB/c mice. They showed a remarkable increased expression of Muc6 and Muc13 in the first 9 weeks postinfection. Since Muc6 is expressed by the gastric glands and, unlike *H. pylori*, *H. heilmannii* was mainly localized in the deep glands of the gastric mucosa, the potential role of Muc6 in *H. heilmannii* colonization was suggested. The MUC13 mucin is normally not expressed in a healthy stomach and has so far only been described as a marker for gastric cancer in humans. The increased expression already in the early stages of infection highlights its role in *H. heilmannii* colonization (Liu et al. 2014). The mucin Muc1 is constitutively expressed by the gastric mucosa and is likely the first point of contact between the host stomach and adherent pathogens. It has been shown that Muc1 limits *H. felis* binding to gastric epithelial cells. However, it does not limit colonization and gastric pathology following infection (Every et al. 2008). The pathological changes in the mouse stomach infected with *H. salomonis*, *H. bizzozeronii*, or *H. felis* have been evaluated as a means of distinguishing different NHPH species in terms of virulence. *H. salomonis* was not able to colonize the murine stomach. *H. bizzozeronii* showed moderate pathological changes, while *H. felis* induced the most severe inflammatory changes (De Bock et al. 2005). Another study by the same research group demonstrated that *H. felis* and *H. bizzozeronii* induce gastric parietal cell loss in Mongolian gerbils, highlighting the preference of these bacteria for parietal cells as was also demonstrated in their natural host (De Bock et al. 2006). Takaishi and co-workers (2009) studied the effect of gastrin on *H. felis*-associated gastric carcinogenesis using hypergastrinemic, gastrin-deficient, and wild-type C57BL/6 mouse models. Gastrin is released by G cells mainly in the antrum of the stomach in response to food intake and stimulates the secretion of gastric acid by parietal cells. Severe corpus dysplasia with mild gastric atrophy was noted in *H. felis*-infected hypergastrinemic (INS-GAS) mice, while mild to moderate antral dysplasia was seen in the gastrin-deficient and wild-type mice. Gastrin deficiency did not result in an alteration of *H. felis* colonization, but it was shown that gastrin is an essential cofactor for the development of gastric dysplasia in *H. felis*-infected C57BL/6 mice. Joosten et al. (2013a) demonstrated that chronic *H. heilmannii* infection in Mongolian gerbils was associated with decreased gastric acid secretion and increased gastrin mRNA levels stimulated by interleukin-1 beta (IL-1β). This latter finding could be considered as a reaction to the *H. heilmannii*-induced hypochlorhydria. Another study investigated the role of *H. felis* infection in the etiology of iron deficiency in INS-GAS C57BL/6 mice. Decreased serum iron concentrations were associated

with a concomitant reduction in the number of parietal cells, strengthening the association between hypochlorhydria and gastric *Helicobacter*-induced iron deficiency. Additionally, the marked changes in gastric iron concentrations and the reduced number of parietal cells following *Helicobacter* infection may be relevant to the more rapid development of carcinogenesis in *H. felis*-infected INS-GAS mice (Thomson et al. 2012). The loss of parietal cells can lead to two distinct types of mucous metaplasia: intestinal metaplasia and spasmolytic polypeptide-expressing metaplasia (SPEM). It has been suggested that intestinal metaplasia induced by *Helicobacter* infection develops in the presence of preexisting SPEM, supporting the role of SPEM as a neoplastic precursor in the carcinogenesis cascade (Liu et al. 2014; El-Zaatari et al. 2013). Several reports uniformly showed that *H. felis* predominantly causes a T helper (Th)1/Th17 immune response in mice, as has also been described for *H. pylori* (Ding et al. 2013; Obonyo et al. 2011; Sayi et al. 2011; Stoicov et al. 2009, Hitzler et al. 2012). Other gastric NHPH, like *H. heilmannii*, have been shown to cause a predominant Th2/Th17 host immune response in BALB/c mice which clearly differs from what is observed during *H. felis* infection (Liu et al. 2014). In BALB/c mice chronically infected with *H. heilmannii*, MALT lymphoma-like lesions were observed (Liu et al. 2014; O'Rourke et al. 2004a). Similar pathological findings have also been described in BALB/c mice chronically infected with *H. felis* suggesting that this mouse model can be seen as a critical model to study gastric MALT lymphoma (Stolte et al. 2002).

*10.3.2 Gastric Non-*H. pylori Helicobacter *spp. Associated with Large Felines*

Besides domesticated cats, *Helicobacter* spp. like *H. felis* and *H. heilmannii* have occasionally been found in the stomach of wild felines, such as lynx, leopards, pumas, bobcats, tigers, and cheetahs (Hamir et al. 2004; Mörner et al. 2008; O'Rourke et al. 2004b; Luiz de Camargo et al. 2011). In cheetahs, gastritis caused by these bacteria is characterized by the infiltration of lymphocytes and plasma cells in the epithelium and lamina propria with gland destruction and parietal cell loss. In some cases lymphoid follicles were observed, especially in captive animals and less frequently in wild animals (Terio et al. 2005; Munson et al. 2005). Terio and colleagues (2012) further investigated the local immune response in cheetahs with varying degree of *Helicobacter*-associated gastritis. The type of cells involved was similar among all types of gastritis with the exception that a large number of lamina proprial activated CD79a^{+}CD21-B cells and plasma cells were only seen in cheetahs with severe gastritis (Terio et al. 2012). Another and more frequently found *Helicobacter* species in big cat predators is *H. acinonychis*. This species has been associated with severe chronic gastritis in cheetahs, tigers, and lions (Tegtmeyer et al. 2013). Cattoli and co-workers (2000) demonstrated that eradication of this bacterium from the stomach using antimicrobial treatment resulted in

the resolution of gastric lesions in tigers. *H. acinonychis* has been described as the most closely related species to *H. pylori* (Tegtmeyer et al. 2013). Eppinger and colleagues (2006) sequenced the *H. acinonychis* genome and showed that this species arose after a single host jump of *H. pylori* from humans to large felines approximately 43,000–56,000 years ago. Comparison between the *H. acinonychis* and *H. pylori* genomes revealed that both species share a high number of core genes (Tegtmeyer et al. 2013). The *H. acinonychis* genome also possesses unique features that confirmed the direction of the host jump from humans to large felines. Interestingly, *H. acinonychis* lacks a *cag*PAI and a functional VacA as has been described for other animal-associated gastric helicobacters as well. Additionally, the *H. acinonychis* genome contains a high number of fragmented genes due to frameshifts and/or stop codons which are probably caused by niche changes and host specialization (Gressmann et al. 2005). So far, only little information is available about the interaction of *H. acinonychis* with its host. Dailidiene and co-workers (2004) showed that *H. acinonychis* was able to infect mice and to coexist and recombine with *H. pylori* in the stomach.

10.3.3 Gastric Helicobacter *Infection in Pigs*

In 1990, large spiral-shaped bacteria were described for the first time in the gastric mucus layer and on the mucosal surface of pig stomachs (Mendes et al. 1990; Queiroz et al. 1990). Initially, *Gastrospirillum suis* was proposed as a name, but subsequent characterization showed that this organism in fact belonged to the genus *Helicobacter* (De Groote et al. 1999b). A new name, '*Candidatus* Helicobacter suis', was then proposed, and despite numerous attempts worldwide, the first successful in vitro isolate was only obtained in 2007, by using a new biphasic culture method, which finally led to the description of *H. suis* as a new species (Baele et al. 2008). Sequence analysis of 16S rRNA, 23S rRNA, partial *hsp60*, and partial *ureAB* gene sequences confirmed that *H. suis* is identical to the previously called *H. heilmannii* type 1. Besides pigs and humans, this species also colonizes the stomach of nonhuman primates, such as mandrills, macaques, and baboons (O'Rourke et al. 2004b).

10.3.3.1 Prevalence of *Helicobacter suis* in Pigs

The reported prevalence of *H. suis* infection in pigs depends on the study. In general, this bacterium is detected in more than 60 % of the European, Asian, and South and North American pigs at slaughter age (Barbosa et al. 1995; Grasso et al. 1996; Cantet et al. 1999; Park et al. 2004; Hellemans et al. 2007b; Kopta et al. 2010). Most likely, *H. suis* is transmitted via the oral-oral route through saliva or via the gastric-oral route through vomiting or regurgitation, but this remains to be investigated. Despite a very high prevalence in adult pigs, far lower degrees of

infection have been shown in younger animals. Hellemans and co-workers (2007b) detected a prevalence of only 2 % in suckling piglets, which however increased rapidly after weaning.

So far, *H. suis* has not been detected in the stomach of wild boars. In a Polish study, the authors did find gastric *Helicobacter* spp., but interestingly, these bacteria were shown not to be *H. suis* (Fabisiak et al. 2010). More worldwide studies are, however, required to draw firm conclusions on the presence of *H. suis* in these wild ancestors of domesticated pigs.

10.3.3.2 *Helicobacter suis* and Its Role in the Development of Gastric Pathology in Pigs

H. suis has been shown to cause gastritis in experimentally and naturally infected pigs, mainly in the antrum (Mendes et al. 1991; Grasso et al. 1996; Queiroz et al. 1996; Park et al. 2000; Hellemans et al. 2007a; De Bruyne et al. 2012). Besides an occasional neutrophilic infiltrate, the inflammation mainly composes of a diffuse lymphocytic/plasmacytic infiltration as well as lymphocytic aggregates and lymphoid follicles. In experimentally infected pigs, this gastritis shows a spatial association with the main sites of colonization: the antrum and fundus (Hellemans et al. 2007a; De Bruyne et al. 2012). Interestingly, diffuse lymphocytic infiltration and lymphoid follicles have been shown to be present in the stomach, and mainly in the cardiac region, of newborn piglets without *Helicobacter* infection (Driessen et al. 2002; Mazzoni et al. 2011). Most likely, germinal centers in lymphoid follicles are formed in the presence of an antigenic stimulus, for instance, during *H. suis* infection.

Besides the strong association with gastritis, *H. suis* infection has also been associated with ulceration of nonglandular stratified squamous epithelium of the *pars esophagea* of the stomach, although *H. suis* bacteria probably do not colonize this specific stomach region (Barbosa et al. 1995; Queiroz et al. 1996; Choi et al. 2001; Roosendaal et al. 2000; Hellemans et al. 2007b; De Bruyne et al. 2012). Other research groups did not find this association (Grasso et al. 1996; Cantet et al. 1999; Melnichouk et al. 1999; Park et al. 2000; Szeredi et al. 2005), so the exact role of *H. suis* in the development of these lesions remains to be elucidated. Sapierzyński and colleagues (2007) demonstrated that *H. suis* infection in pigs results in an increased number of gastrin-producing cells and a decreased number of somatostatin-producing cells in the antrum. Since gastrin stimulates and somatostatin inhibits the secretion of hydrochloric acid by parietal cells, this may influence gastric acid production, which may also be altered due to the tropism of this bacterium for parietal cells (Hellemans et al. 2007a). An altered gastric acid secretion could in turn be involved in the development of ulceration of the *pars esophagea*. The discrepancies found in literature may be due to differences in laboratory techniques, different sampling practices, or differences in virulence between *H. suis* strains. In any case, hyperkeratosis and ulceration of the nonglandular part of the stomach have been reported in many countries. Up to

80 % of the market pigs in Australia (Robertson et al. 2002) and 60 % of the sows (Hessing et al. 1992) in the Netherlands have been described to be affected.

The development of lesions in the *pars esophagea* is most likely a process involving different factors, including stress, transport, the presence in the stomach of short-chain fatty acids, and pelleting and fine grinding of the feed (Haesebrouck et al. 2009; Argenzio and Eisemann 1996). The latter factor may have an influence on the fluidity of gastric contents (Elbers and Dirkzwager 1994), leading to an increased contact of the stratified squamous epithelium of the nonglandular region with the luminal content of the distal part with acid, bile (refluxed from the duodenum), and pepsin.

Ulceration of the porcine gastric nonglandular mucosa may result in decreased feed intake, a decrease in daily weight gain, and even sudden death due to fatal hemorrhage (Ayles et al. 1996; Haesebrouck et al. 2009), thus leading to significant economic losses. There is also little doubt that this disease can cause pain and discomfort. Interestingly, a decrease in daily weight gain of up to 10 % has been observed in pigs experimentally infected with *H. suis*, however, without a clear association with the development of lesions in the nonglandular part (De Bruyne et al. 2012).

Besides *H. suis*, other *Helicobacter* species have been described in the stomach of pigs, including an *H. pylori*-like bacterium, responsible for ulceration of the *pars esophagea* in gnotobiotic piglets (Krakowka and Ellis 2006), *H. bilis* and *H. trogontum* (Hänninen et al. 2003, 2005). The main site of colonization of the latter two is most probably the lower intestinal tract, and it remains to be determined whether these bacteria are able to colonize the porcine stomach and cause gastric pathology.

10.3.3.3 Genomics, Genetics, and Experimental Studies on the Pathogenesis of *H. suis* Infection

Genome sequencing of *H. suis* (Vermoote et al. 2011a) revealed the presence of homologues of *H. pylori* genes involved in acid acclimation, motility, chemotaxis, oxidative stress resistance, and adhesion to gastric epithelial cells. These include genes encoding urease A and B subunits and urease accessory proteins, genes encoding different components of the flagellar apparatus, several *che* and *tlp* genes, *katA*, *sodB*, genes encoding several members of the major *H. pylori* outer membrane families, and *hpaA*. In addition, *H. suis* harbors a near complete *comB* type IV secretion system and homologues of the *H. pylori* neutrophil-activating protein and γ-glutamyl transpeptidase, but it lacks most members of the *cag*PAI as well as a functional VacA.

In literature, several studies describe experimental infection of mice with *H. heilmannii*. Sometimes, however, this name might be misleading, as several of these studies have in fact used *H. suis* bacteria, obtained from mucus, or homogenized gastric tissue of infected mice, pigs, or nonhuman primates, because of the lack of in vitro isolated strains. Infection of mice with these inocula induces

inflammation, already 7 days after inoculation (Moura et al. 1993). During long-term infection studies of up to 2 years, infiltration of the gastric mucosa with lymphocytes and plasma cells with subsequent development of lymphoid follicles has been observed, both in studies using mucosal homogenates and pure in vitro isolated *H. suis* strains (Park et al. 2008; Flahou et al. 2010). Conflicting reports have been published regarding the immune response underlying *H. suis*-induced gastritis. Several authors have described the inflammation and formation of gastric lymphoid follicles to be mainly driven by a Th1 response (Cinque et al. 2006; Mimura et al. 2011). Others have described a mixed Th1/Th2 response (Park et al. 2008), whereas studies using pure in vitro isolated *H. suis* strains revealed that experimental *H. suis* infection causes an upregulation of IL-17, IL-10, and IL-4 expression, in the absence of interferon (IFN)-γ upregulation (Flahou et al. 2012), which also clearly contrasts to the immune response elicited by *H. pylori* in this same animal model. Recently, however, Liang and colleagues (2015) described a strong upregulation of IFN-γ expression in *H. suis*-infected Mongolian gerbils.

The resulting chronic gastritis has been shown not to depend upon the presence of Peyer's patches in the small intestine, in contrast to what has been described for *H. pylori* (Nobutani et al. 2010). Often, this chronic gastritis evolves to more severe histopathological lesions. In BALB/c mice experimentally infected with different *H. heilmannii* bacteria, "isolated" in vivo in mice and originating both from humans and animals, gastric MALT lymphoma has been shown to develop starting from 18 months postinfection (O'Rourke et al. 2004a). In this study, the most severe pathological changes were seen in mice infected with in vivo "isolates" from a human patient, a bobcat, a crab-eating macaque, and mandrill monkeys. Except for the strain from the bobcat, these strains were in fact shown to be *H. suis*. Similar lesions were observed in C57BL/6 mice infected for at least 6 months with an in vivo "isolate" of '*Candidatus* H. heilmannii', which in fact was shown to belong to the species *H. suis* (Nakamura et al. 2007; Haesebrouck et al. 2009), as well as in Mongolian gerbils infected with an in vitro isolated strain of *H. suis* (Flahou et al. 2010). These histopathological changes resemble inflammation-related changes in the stomach of humans infected with NHPH, including *H. suis*.

In addition to the involvement of several cytokines, including IL-4 and chemokine (C-X-C motif) ligand 13 (CXCL-13) (Flahou et al. 2012; Yamamoto et al. 2014), several mechanisms have been described to stimulate lymphomagenesis in this setting. An overexpression of miR-142-5p and miR-155, as well as increased lymphangiogenesis and angiogenesis, has been shown to be involved in *H. suis*-induced gastric MALT lymphoma (Saito et al. 2012; Nakamura et al. 2008). The latter changes are accompanied by an increased expression of vascular endothelial growth factors (VEGF), such as VEGF-A and VEGF-C, and some of its receptors, including Flt-1 and Flt-4 (Nishikawa et al. 2007; Nakamura et al. 2007, 2008, 2010). In addition, the formation of peripheral lymph node addressin (PNAd)- and mucosal addressin cell adhesion molecule 1 (MadCAM-1)-expressing high endothelial venule-like vessels plays a role (Suzuki et al. 2010). These alterations may cause a sustained "homing" of lymphocytes to the gastric mucosa. Interestingly, the administration of antibodies raised against certain of the

abovementioned factors, including CXCL-13, Flt-1, and Flt-4, induces a marked reduction of the size of the region affected by gastric MALT lymphoma, which may have implications for the future treatment guidelines of *Helicobacter*-induced gastric MALT lymphoma (Nakamura et al. 2010, 2014; Yamamoto et al. 2014).

Besides inflammation-related pathological changes, *H. suis* also interacts with the mucosal epithelium. In infected humans, the majority of *H. pylori* bacteria remain in the mucus layer, whereas a limited number adhere to gastric epithelial (mucus-secreting) cells (Lindén et al. 2008). *H. suis*, on the other hand, is most often observed in the vicinity of or inside the canaliculi of acid-producing parietal cells in pigs, humans, and experimentally infected mice and Mongolian gerbils (Hellemans et al. 2007a; Joo et al. 2007; Flahou et al. 2010). Regularly, these parietal cells show signs of degeneration or oncosis. Many questions remain unanswered with regard to the mechanisms underlying the epithelium-related changes observed during *H. suis* infection. As described above, several important *H. pylori* virulence factors, such as the *cag*PAI and VacA, are absent or nonfunctional in *H. suis* (Vermoote et al. 2011a). So far, the *H. suis* γ-glutamyl transpeptidase (GGT) is the only virulence factor from *H. suis* with a confirmed role in death of gastric epithelial cells. In vitro research using a human gastric epithelial cell line (AGS) has shown that the enzyme causes apoptosis or necrosis, depending in part on the amount of reactive oxygen species (ROS) generated through degradation of reduced glutathione (GSH), an important antioxidant (Flahou et al. 2011). Besides its role in death of epithelial cells, the same enzyme has been shown to impair lymphocyte function, which is in line to what has been published for *H. pylori* (Zhang et al. 2013). Supplementation of GGT-treated cells with known substrates of the enzyme was shown to modulate the observed effects: glutamine restored normal proliferation of lymphocytes, whereas supplementation with reduced glutathione strengthened *H. suis* GGT-mediated inhibition of proliferation. Most likely, depletion of glutamine and the generation of reactive oxygen species through degradation of GSH are involved. In this same study, *H. suis* outer membrane vesicles were identified as a possible delivery route of *H. suis* GGT to lymphocytes residing in the deeper mucosal layers.

Parallel to the cell death it induces, *H. suis* infection also causes an increased proliferation of progenitor cells in the isthmus of gastric glands (Flahou et al. 2010). A direct effect of the bacteria may be involved (Fast et al. 2011), or alternatively, this may reflect a compensatory hyperproliferation due to increased epithelial cell loss (Shirin and Moss 1998).

10.3.4 Gastric **Helicobacter** *Infection in Nonhuman Primates*

Captive rhesus macaques are commonly infected with *H. pylori* (Drazek et al. 1994). The anatomy and physiology of the stomach resemble that of humans, which suggests that the rhesus monkey model can serve as a good experimental

model to study *H. pylori* infection. For instance, it has been shown that socially housed rhesus monkeys rapidly acquire *H. pylori* infection, particularly during the peripartum period (Solnick et al. 2003, 2006). Once acquired, infection is associated with chronic gastritis resembling that seen in humans.

Besides *H. pylori*, nonhuman primates can be naturally infected with gastric NHPH. Although an exact species designation of the colonizing *Helicobacter* species is sometimes lacking, *H. suis* seems to be the species involved (O'Rourke et al. 2004b; Martin et al. 2013; Nakamura et al. 2007; Matsui et al. 2014). In the stomachs of rhesus monkeys, gastric NHPH which were not identified to the species level have been observed in the mucus covering the surface epithelial cells, as well as in the lumina of gastric glands. These microorganisms were shown to be able to invade and on occasion damage parietal cells, which was however often accompanied by an apparent hyperchlorhydria. This contrasted with *H. pylori* infection in this model, which appeared to cause a more pronounced gastritis, while apparently not modifying the acid output (Dubois et al. 1991). In another study, *H. suis* was shown to be present in the stomach of all rhesus monkeys that were used for determining the effects of an experimental *H. pylori* infection on the gastric microbiota. Interestingly, the populations of *H. suis* and *H. pylori* were shown to be highly dynamic, and a potential competitive inhibition/exclusion was observed between both species (Martin et al. 2013).

NHPH infection in baboons has been associated with the development of gastritis by Mackie and O'Rourke (2003) but not by others (Curry et al. 1989). NHPH have been described to be naturally present in the stomachs of cynomolgus monkeys from different geographic regions (Reindel et al. 1999; Drevon-Gaillot et al. 2006). Again, *H. suis* seems to be the species involved (O'Rourke et al. 2004b), and these microorganisms can be found in the gastric pits, in the superficial glands, or on the surface epithelium. In one study, no correlation was observed between these bacteria and the infiltration of lymphoplasmacytic cells and inflammatory lesions in these gastric tissues (Drevon-Gaillot et al. 2006).

Recently, a putative new gastric *Helicobacter* species was detected in wild chimpanzees and gorillas, which was provisionally named '*Candidatus* H. homininae' (Flahou et al. 2014).

10.3.5 Gastric Helicobacters Associated with Ruminants and Horses

'*Candidatus* Helicobacter bovis' has been demonstrated in the pyloric part of the abomasum of calves and adult cattle. So far, this bacterium has not been cultivated in vitro (De Groote et al. 1999a), and its involvement in bovine gastric disease is unknown. Although gastric ulcers regularly occur in calves and adult cattle (Haringsma and Mouwen 1992; Jelinski et al. 1995; Ok et al. 2001), no association between gastric ulceration and *Helicobacter* colonization was observed in a recent

study, since no *Helicobacter* bacteria could be detected in the animals that were sampled (Valgaeren et al. 2013). *H. pylori* has been demonstrated in the stomach of sheep (Dore et al. 2001), and so far, no helicobacters have been demonstrated in the stomach of goats (Gueneau et al. 2002; Momtaz et al. 2014). Interestingly, *H. pylori* has also been detected in milk from cows, sheep, and other ruminants (Quaglia et al. 2008; Angelidis et al. 2011; Rahimi and Kheirabadi 2012). Although Rahimi and colleagues claim to have isolated *H. pylori* bacteria, most studies used polymerase chain reaction or fluorescence in situ hybridization to detect *H. pylori*. Further studies are therefore needed to assess whether *H. pylori* or *H. pylori*-like bacteria are involed. In addition, it needs to be confirmed whether milk consumption can serve as a route of transmission for *H. pylori*.

Although some studies describe the absence of *Helicobacter* spp. in the stomach of horses (Husted et al. 2010; Perkins et al. 2012), others describe the presence of *Helicobacter*-like organisms or their DNA in this niche. So far, however, helicobacters have not yet been cultivated from the equine stomach (Scott et al. 2001; Contreras et al. 2007), so their possible role in the development of gastric ulcers, which are common in horses (Haesebrouck et al. 2009), remains speculative.

10.3.6 Gastric Helicobacters Associated with Other Animal Species

10.3.6.1 Marine Mammals

Urease-positive helicobacters have been isolated from the main stomach or feces of various cetaceans, including stranded wild Atlantic white-sided dolphins and captive Pacific white-sided dolphins, Atlantic bottlenose dolphins, and a beluga whale (Harper et al. 2002, 2003a). In 2002, these bacteria were characterized and described as *H. cetorum* (Harper et al. 2002). The results of a health study, in which 20 wild Atlantic bottlenose dolphins were sampled, showed that the prevalence in these animals was at least 50 % (Harper et al. 2003a). In addition to the studies described above, helicobacters with a high homology to *H. cetorum* have been isolated from or detected in fecal samples from captive seals and sea lions from Australia, South American fur seals, the stomach of an Atlantic spotted dolphin, gastric fluids, dental plaques, saliva and gastric tissue of captive dolphins and a killer whale from Argentina, fecal material from wild and captive Yangtze finless porpoises, the stomach of common dolphins, an Atlantic white-sided dolphin and a striped dolphin from European waters, and the aquatic environment of captive dolphins (Oxley and McKay 2005; Goldman et al. 2009a, b, 2011; Suárez et al. 2010; McLaughlin et al. 2011; Davison et al. 2014).

Some of the captive animals from which *H. cetorum* was recovered showed clinical signs, such as intermittent inappetence, lethargy, or chronic regurgitation. Endoscopic or gross examination revealed the presence of esophageal and

forestomach ulcers, as well as gastric mucosal hemorrhages (Harper et al. 2002; Davison et al. 2014). Cytological examination of gastric fluid of some animals indicated the presence of inflammation in the stomach (Harper et al. 2002). This was confirmed by histopathological analysis, revealing *Helicobacter* colonization of the epithelial surface accompanied by a diffuse lymphoplasmacytic gastritis in the main stomach and to a lesser extent in the pyloric stomach, and a mild distortion of the adjacent glands (Harper et al. 2002; Suárez et al. 2010).

In a recent study, two genomes of *H. cetorum* were sequenced: one strain originated from a dolphin and one strain isolated from a captive Beluga whale (Kersulyte et al. 2013). Although these genomes, differing markedly from one another in gene content, appeared to be larger than *H. pylori* genomes, the strains were shown to be more closely related to *H. pylori* and *H. acinonychis* than to other known species. They lack the *cag*PAI but do possess novel alleles of the *vacA* gene. In addition, they reveal an extra triplet of *vacA* genes, metabolic genes distinct from *H. pylori*, as well as genes encoding both an iron- and nickel-cofactored urease.

Besides *H. cetorum*, other putative novel *Helicobacter* spp., distinct from *H. cetorum*, have been detected in the gastric fluids, gastric mucosa, or dental plaque from dolphins, harp seals, and a sea lion with chronic gastritis (Harper et al. 2003b; Oxley et al. 2004, 2005; Goldman et al. 2011).

10.3.6.2 Ferrets

Not so long after the discovery and description of *H. pylori* in humans, spiral organisms were isolated from a gastric ulcer of a ferret and from the gastric mucosa of two healthy ferrets (Fox et al. 1986). In 1989, these organisms were named *Helicobacter mustelae* (Goodwin et al. 1989). Only a minority of ferrets younger than 6 weeks are colonized by this bacterium, in contrast to the vast majority of adult ferrets (Fox et al. 1988), indicating that widespread colonization and persistence occur after weaning (Fox et al. 1991a; Forester et al. 2000). Keeping in mind the ease by which ferrets vomit, oral-oral and gastric-oral contact most likely play a role in transmission of *H. mustelae* (Fox et al. 1991a). Fecal-oral transmission has, however, also been suggested. *H. mustelae* has indeed been isolated successfully from feces of ferrets, and successful isolation correlated with periods of transient hypochlorhydria, which may allow larger numbers of bacteria to exit the stomach (Fox et al. 1992).

H. mustelae colonizes the mucosal surface in the corpus region, which often induces only a superficial gastritis (Marini and Fox 1999). In the antrum, however, *H. mustelae* colonizes the surface, gastric pits, and superficial portion of the glands, leading to the development of a diffuse mononuclear gastritis with inflammatory cells often occupying the full thickness of the mucosa (Fox et al. 1991b).

The incidence of gastric ulceration in ferrets varies between 1.4 and 35 % (Andrews et al. 1979). Both gastric and duodenal ulcerations have been reported in ferrets infected with *H. mustelae* (Fox et al. 1986, 1990). Given the high prevalence of *H. mustelae* in adult ferrets, long-term observations of experimentally

infected pathogen-free ferrets are needed to elucidate the exact role of *H. mustelae* infection in the development of peptic ulcer disease. An increased epithelial cell proliferation has been detected in the gastric mucosa of ferrets infected with *H. mustelae*, which may play a role in the development of gastric tumors (Yu et al. 1995). Indeed, gastric adenocarcinoma has been described in the pyloric mucosa of two ferrets infected with *H. mustelae* (Fox et al. 1997). In both cases, the invasion of neoplastic tubules into the deep submucosa was described. Gastric MALT lymphoma, accompanied by destruction of the gastric glands, has also been described in the antrum of ferrets infected with *H. mustelae* (Erdman et al. 1997). For both tumor types, however, evidence remains so far circumstantial (Solnick and Schauer 2001).

H. mustelae adheres firmly to the gastric epithelium, and only a few bacteria are seen lying in the mucus (O'Rourke et al. 1992). In *H. mustelae* infected ferrets, the gastric mucosal hydrophobicity is reduced, which correlates with the degree of mucosal inflammation (Gold et al. 1996). This may promote the attachment of *H. mustelae*, which is thought to be mainly hydrophilic. *H. mustelae* binds to the same receptor lipids as *H. pylori*, in particular phosphatidylethanolamine (Gold et al. 1995). Like other gastric helicobacters, *H. mustelae* possesses a urease enzyme and flagella, consisting of a body, hook, and flagellar filament composed of FlaA and FlaB subunits. Clyne and coworkers (2000) showed that these flagella do not play a direct role in promoting adherence of *H. mustelae* to gastric epithelial cells. Double mutants of *H. mustelae* in *flaA* and *flaB* genes were shown to be completely nonmotile and unable to colonize the ferret, whereas single-gene *flaA* and *flaB* mutants have a decreased motility but are still able to colonize the ferret's stomach (Andrutis et al. 1997; Josenhans et al. 1995). An isogenic urease-negative mutant of *H. mustelae* was shown to fail to colonize the ferret stomach (Andrutis et al. 1995; Solnick et al. 1995). In addition to the standard nickel-dependent UreAB, *H. mustelae* has been shown to possess a second, nickel-independent urease (Stoof et al. 2008). Instead, this UreA2B2 is activated with ferrous ions in the absence of auxiliary proteins. This unique metalloprotein is sufficient for the bacteria to survive an acid shock in the presence of urea, and it is thought to play a role in survival of the bacteria in the stomach of carnivores, with a diet rich in iron and low in nickel (Stoof et al. 2008; Carter et al. 2011, 2012).

H. mustelae produces an array of surface rings, which seem to be unique to this *Helicobacter* species. They are composed of the *Helicobacter* surface ring (Hsr) protein, comprising approximately 25 % of the total envelope protein of *H. mustelae* (O'Toole et al. 1994). These surface rings have been shown to play a role in bacterial colonization and the pathogenesis of *H. mustelae* infection, as shown by reduced numbers of bacteria recovered from mutant-dosed ferrets (Patterson et al. 2003). Moreover, animals inoculated with the Hsr-negative strain show less inflammation compared to ferrets infected with the wild-type strain. Like other animal-associated gastric *Helicobacter* species, *H. mustelae* lacks a *cag*PAI and VacA.

10.3.6.3 Hamsters and Mice

H. aurati has been isolated from the inflamed stomachs and ceca of adult Syrian hamsters (Patterson et al. 2000a). Certain features, including the fusiform shape and the presence of periplasmic fibrils, distinguish it from other enterohepatic *Helicobacter* species detected in hamsters, such as *H. cholecystus*, *H. mesocricetorum*, and *H. cinaedi* (Solnick and Schauer 2001; Whary and Fox 2004; Ceelen et al. 2007a). Although *H. aurati* possesses urease activity, the fact that bacteria were recovered from cecal samples more often than from antral samples indicates that the preferential colonization site of *H. aurati* in hamsters is probably the intestinal tract (cecum) with subsequent spreading of this bacterial agent to the stomach in some animals, possibly due to the coprophagic behavior of hamsters (Patterson et al. 2000b). The precise role of *H. aurati* in gastric diseases in hamsters has not yet been fully elucidated, although the organism has been identified in hamsters suffering from chronic gastritis and intestinal metaplasia (Patterson et al. 2000a, b). In the stomach of these same hamsters, the authors reported the presence of another helical, urease-negative *Helicobacter* species, as well as a smaller, urease-negative *Campylobacter* species. Likewise, natural infection with different *Helicobacter* species, including *H. aurati*, was reported in a Syrian hamster with gastritis-associated adenocarcinoma (Nambiar et al. 2005). There are no indications that *H. aurati* is of zoonotic significance.

Also in mice, urease-positive helicobacters have been described in the stomach. In line with what has been described for *H. aurati* infection in Syrian hamsters, *H. muridarum* has occasionally been detected in the stomach, with or without concurrent inflammation, although these bacteria are found more frequently in the ileal and cecal mucosa of the animals (Lee et al. 1992). *H. suncus* has been isolated from the stomach of house musk shrews with chronic gastritis (Goto et al. 1998).

10.3.6.4 Rabbits

Only two reports describe the presence of *H. felis* and *H. salomonis* DNA in the stomach of rabbits. So far these bacteria have not been cultivated from rabbits, and their pathogenicity toward these animals is unknown (Haesebrouck et al. 2009; Van den Bulck et al. 2006).

10.4 Enterohepatic *Helicobacter* Species

The NHPH species described above are mainly found colonizing the stomach of their hosts. A large number of *Helicobacter* species, however, prefer to settle in the lower intestinal tract or the liver of a wide variety of mammalian, reptilian, avian, or amphibian host species as well as humans (Schrenzel et al. 2010; Hansen et al. 2011).

Several enterohepatic *Helicobacter* species are found in rodents with or without signs of intestinal or hepatic disease, including *H. bilis*, *H. hepaticus*, *H. cholecystus*, *H. muridarum*, *H. mastomyrinus*, *H. typhlonius*, *H. rodentium*, *H. mesocricetorum*, *H. magdeburgensis*, *H. aurati*, *H. cinaedi*, *H. ganmani*, *H. trogontum*, and *H. muricola* (Lee et al. 1992; Fox et al. 1994, 1995; Patterson et al. 2000a; Vandamme et al. 2000; Robertson et al. 2001; Solnick and Schauer 2001; Won et al. 2002; Shen et al. 2005; Traverso et al. 2010). Several models of experimental enterohepatic *Helicobacter* infection, including *H. hepaticus* infection in immunodeficient IL-$10^{-/-}$ knockout mice, are used to model the development of colitis/inflammatory bowel disease in humans (Solnick and Schauer 2001; Hansen et al. 2011).

H. canis has been detected in or isolated from healthy dogs and cats as well as dogs suffering from endemic diarrhea or necrotic hepatitis (Solnick and Schauer 2001; Shen et al. 2001). Several other enterohepatic *Helicobacter* species have been demonstrated in dogs and cats, including *H. bilis*, *H. cinaedi*, '*Candidatus* H. colifelis', *H. marmotae*, and *H. fennelliae* (Solnick and Schauer, 2001; Fox et al. 2002; Misawa et al. 2002; Hänninen et al. 2005; Rossi et al. 2008; Otte et al. 2012; Castiglioni et al. 2012). The pathogenic significance of these microorganisms for dogs and cats remains uncertain.

H. equorum has been isolated from the feces of healthy horses (Moyaert et al. 2007a, b). The prevalence of this microorganism in different adult horse populations varies between 0.8 % and 7.9 % but is much higher (66 %) in 1- to 6-month-old foals (Moyaert et al. 2009). Experimental infections revealed that this microorganism colonizes the cecum, colon, and rectum of adult horses without causing apparent pathological changes (Moyaert et al. 2007c).

Several *Helicobacter* spp., including *H. trogontum*, *H. bilis*, and *H. canis*, have been identified or isolated from sheep, and infection with the first two species has been associated with abortion and the presence of hepatic necrosis in aborted lambs (Dewhirst et al. 2000; Solnick and Schauer 2001; Hänninen et al. 2003, 2005; Swennes et al. 2014). Enterohepatic *Helicobacter* species have not been described in other ruminants, including cattle and goats.

H. pamatensis (Seymour et al. 1994), *H. trogontum* (Hänninen et al. 2003), *H. bilis* (Hänninen et al. 2005), and atypical *H. canadensis* strains (Inglis et al. 2006) have been isolated from the gastrointestinal tract or feces of pigs, but their pathogenic significance for this animal host is not known.

H. pullorum has been isolated from the ceca and feces of apparently healthy chickens, as well as from laying hens with vibrionic hepatitis (Stanley et al. 1994). In two in vivo studies, experimentally infected chickens remained clinically healthy although mild lesions were observed in the ceca (Neubauer and Hess 2006; Ceelen et al. 2007b). *H. pullorum* or its DNA have also been detected in other bird species, including turkeys (Zanoni et al. 2011) and a parakeet suffering from diarrhea (Ceelen et al. 2006b). Other *Helicobacter* species detected in birds include *H. canadensis*, which has mainly been associated with geese but also with chickens, guinea fowl, and pheasants (Fox et al. 2000; Nebbia et al. 2007; Robino et al. 2010). *H. anseris* and *H. brantae* have been isolated from the feces of resident Canada

geese (Fox et al. 2006), and still now, new species are being discovered, including *H. valdiviensis*, isolated from wild bird fecal samples (Collado et al. 2014).

Also in monkeys, enterohepatic helicobacters have been detected, including *H. callitrichis* in common marmosets, *H. cinaedi* and *H. macacae* in rhesus macaques and baboons, and several enterohepatic *Helicobacter* spp. in gorillas and chimpanzees (Fernandez et al. 2002; García et al. 2006; Fox et al. 2007; Won et al. 2007; Flahou et al. 2014). It has been suggested that infection with *H. macacae* plays a role in the development of chronic idiopathic colitis and intestinal adenocarcinoma in rhesus macaques (Lertpiriyapong et al. 2014).

Several enterohepatic *Helicobacter* species have been associated with disease in humans. These reports include associations between *H. canis*/*H. winghamensis* and gastroenteritis; *H. hepaticus* and cholecystitis, liver carcinogenesis, or chronic pancreatitis; *H. bilis* and chronic cholecystitis, biliary duct, and gallbladder cancer; *H. ganmani* and liver disorders; *H. pullorum* and enteritis or diarrhea; *H. canadensis* and enteritis; and *H. cinaedi*/*H. fennelliae* and chronic diarrhea, enteritis, proctitis, or proctocolitis in homosexual men (Totten et al. 1985; Stanley et al. 1994; Steinbrueckner et al. 1997; Fox et al. 2000; Melito et al. 2001; Solnick and Schauer 2001; Matsukura et al. 2002; Murata et al. 2004; Tolia et al. 2004; Kobayashi et al. 2005; Apostolov et al. 2005; Nilsson et al. 2006; Zhang et al. 2006; Hamada et al., 2009). Enterohepatic *Helicobacter* species have also been associated with the various forms of inflammatory bowel disease (Hansen et al. 2011), and several species, including *H. cinaedi*, *H. fennelliae*, *H. canadensis*, *H. canis*, *H. westmaedii*, and *H. rappini*, have been isolated from (immunosuppressed) patients with bacteremia (Solnick and Schauer 2001; Tee et al. 2001, Matsumoto et al. 2007; Prag et al. 2007; Abidi et al. 2013; Rimbara et al. 2013).

Most enterohepatic *Helicobacter* species do not contain an urease enzyme, although there are exceptions, including *H. hepaticus* and *H. bilis*. A large number of enterohepatic *Helicobacter* species contain a bacterial toxin called the cytolethal distending toxin (CDT). The toxic effects of this major virulence factor involve cellular distension, actin cytoskeleton remodeling, G2/M cell cycle arrest, and cytolethality (Ceelen et al. 2006a; Varon et al. 2014). CDT is typically composed of three subunits: CdtA, CdtB, and CdtC, which are all required for a maximal cytotoxic activity (Liyanage et al. 2013; Varon et al. 2014). Other factors involved in host-bacteria interactions include a type VI secretion system, which is expressed by several enterohepatic helicobacters, including *H. hepaticus*, *H. pullorum*, *H. cinaedi*, and *H. trogontum* (Chow and Mazmanian 2010; Goto et al. 2012; Kaakoush et al. 2013; Sirianni et al. 2013).

10.5 Conclusions and Outlook

There are clear indications that gastric NHPH species can cause disease in humans. Some distinct features, such as the association with gastric MALT lymphoma, indicate that these zoonotic bacteria should not just be considered as a “light”

version of *H. pylori*. There are clear indications that domestic animals constitute reservoir hosts for these gastric *Helicobacter* species with zoonotic potential. A correct diagnosis to the species level remains sometimes problematic. Therefore, diagnostic methods enabling the correct identification of these bacteria are needed to help clarify the epidemiology and pathology of these infections in humans. The successful in vitro isolation of several of these species has opened new perspectives for understanding the pathogenesis of non-*H. pylori Helicobacter* associated gastric pathology in their natural hosts as well as humans. An increased knowledge may in the end contribute to the development of new treatment and prevention measures.

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Part II
Immune Responses and Host Factors

Chapter 11
Animal Models of *Helicobacter pylori* Infection

Jay V. Solnick, Kathryn A. Eaton, and Richard M. Peek Jr.

Abstract Experimentation in animal models is essential to fully understand the biology of *H. pylori*. Although a variety of animals have been successfully infected with *H. pylori*, current studies most commonly use either mice, Mongolian gerbils, or nonhuman primates, each of which has strengths and weaknesses. The mouse is inexpensive and convenient, and the elegant genetics permits dissection of the host response to infection. However, the function of the *H. pylori* type IV secretion system—the best-known virulence factor—is commonly lost during colonization of mice. This occurs less frequently in the gerbil, which also has the major advantage that some *H. pylori* strains cause gastric adenocarcinoma during relatively short-term colonization. But there is no genetics available in the gerbil and there are fewer reagents available than for the mouse. Nonhuman primates are physiologically most similar to humans and are naturally infected with *H. pylori*, but are limited by high cost and availability. Choice of the most appropriate model depends on the question, resources, and the availability of local expertise.

Keywords *Helicobacter pylori* • Animal model • Mouse • Gerbil • Nonhuman primate

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11.1 Introduction

Despite recent controversy over the relatively few animal models that dominate biomedical research, and the extent to which they recapitulate disease processes in humans (Bolker 2012; Seok et al. 2013; Couzin-Frankel 2013), animal experimentation remains fundamental to our understanding of human health. Because of cost, convenience, and particularly the availability of reagents and genetic tools, the laboratory mouse is most often the model of choice. But for many infectious diseases, mice cannot be readily infected with any given human pathogen, so investigators must use either a different animal or a surrogate pathogen that is similar to that which infects humans. Such was the case soon after the discovery of *Helicobacter pylori*, when early attempts to infect mice and rats were unsuccessful, even if they were germ-free (Cantorna and Balish 1990; Ehlers et al. 1988). The first animal experimental infection was by Krakowka and coworkers, who demonstrated that neonatal gnotobiotic piglets were susceptible to infection with *H. pylori* (then *Campylobacter pylori*), which reproduced many of the histologic features found in chronically infected humans (Krakowka et al. 1987). At about the same time, natural *H. pylori* infection was identified in captive rhesus monkeys (Baskerville and Newell 1988), and closely related species were cultured from domestic and laboratory animals, including *H. felis* in cats (Lee et al. 1990) and *H. mustelae* in ferrets (Fox et al. 1990). For the first 10 years after the discovery of *H. pylori*, animal experimentation was predominantly performed using these surrogate models—*H. felis* in mice and *H. mustelae* in ferrets—with a few studies of *H. pylori* in pigs and nonhuman primates.

Although the first experimental rodent infection with *H. pylori* was described by Karita and colleagues in 1991 (Karita et al. 1991), the mouse model was largely ignored until 1995 when a seminal paper was published in *Science* (Marchetti et al. 1995). Initially, many investigators had difficulty reproducing these findings. The trick appeared to be that only some strains would robustly colonize mice and that they needed to be fresh human clinical isolates, ideally adapted to the mouse by repeated in vivo passage. This was validated in 1997 with the introduction of Sydney Strain 1 (SS1), a mouse adapted strain that soon became the international standard for animal experimentation with *H. pylori* (Lee et al. 1997). In addition to the mouse, some investigators use the Mongolian gerbil because it is also a model of gastric adenocarcinoma, or the nonhuman primate because it physiologically and anatomically most closely resembles humans. Here, we critically review these three model systems—mouse, gerbil, and nonhuman primate—and briefly consider the human challenge model that has been developed for studies of *H. pylori* immunization. Other models such as dogs (Rossi et al. 1999), cats (Fox et al. 1995), pigs (Kronsteiner et al. 2013), guinea pigs (Shomer et al. 1998), rats (Elseweidy et al. 2010), and even *Drosophila* (Wandler and Guillemin 2012) are sometimes used, but they are relatively uncommon and are not considered further.

11.2 Mouse Model

11.2.1 Early Studies

Because mouse colonization was initially unsuccessful, the gnotobiotic piglet model was developed (Krakowka et al. 1987) and used to investigate *H. pylori* virulence factors (Eaton et al. 1991, 1997), vaccination strategies (Eaton and Krakowka 1992; Eaton et al. 1998), and host immune responses. But the piglet model was complex and expensive, so other helicobacter species were examined as surrogates for *H. pylori*. In 1989, Adrian Lee's group isolated *Helicobacter felis*, which caused severe gastritis and preneoplastic proliferation (Lee et al. 1990). *H. felis* was the first model used in genetically engineered mice to investigate the role of host and bacterial factors in gastritis and cancer, and it provided a major advance in pathogenesis studies.

11.2.2 **H. felis** *in Mice*

H. felis in mice has been used principally to study the host immune response (Sayi et al. 2009, 2011; Lee et al. 1990; Mohammadi et al. 1997; Roth et al. 1999) and carcinogenesis (Fox et al. 1996; Wang et al. 2000; Houghton et al. 2004; Stoicov et al. 2009). *H. felis* was also the first helicobacter species used in the adoptive transfer mouse model to demonstrate the importance of helper T cells (Th1) in disease (Mohammadi et al. 1996). *H. felis* lacks some *H. pylori* virulence factors such as the *cag* pathogenicity island (*cag*PAI) and the vacuolating cytotoxin (VacA), but the model has the advantage of robust colonization and induction of a severe local inflammatory response that leads to gastric epithelial damage and preneoplastic lesions. For this reason, *H. felis* continues to be an excellent model organism to study the host immune response to gastric helicobacter, as well as examination of host and environmental factors that promote gastric neoplasia.

11.2.3 **H. pylori** *in Mice*

The mouse model was not commonly used until 1997 when strain SS1 was introduced (Lee et al. 1997). SS1 is an *H. pylori* isolate from a human patient that was adapted to mouse colonization by serial in vivo passage. It colonizes mice well, persists indefinitely in most mouse strains, causes histological gastritis, and is genetically tractable, allowing mutational analysis of bacterial colonization and virulence factors. SS1 rapidly became the standard for use in mouse models. It has been used to confirm the roles of urease, flagella, chemoreceptors, and other virulence factors in gastric colonization (Eaton et al. 2002; Kim et al. 1999;

Andermann et al. 2002) and is commonly used for vaccine development (Pappo et al. 1999; Garhart et al. 2003; Akhiani et al. 2002; Moss et al. 2011) and studies of host immune responses (Gray et al. 2013; Eaton et al. 2001, 2006). Since the isolation of SS1, several laboratories have isolated other mouse-colonizing strains or have adapted strains by serial passage in mice, but SS1 remains a commonly used strain for in vivo studies.

In 2002, a controversy arose regarding the use of SS1 in a mouse disease model. This was based on the finding that while SS1 contained the *cag*PAI, it did not appear to have a functional type IV secretion system (T4SS) as determined in cell culture (Crabtree et al. 2002; Philpott et al. 2002). To address this limitation, investigators have used pre-mouse SS1 (PMSS1), the original human isolate that has a functional T4SS and gave rise to SS1 after mouse passage (Arnold et al. 2010). Infection of mice with PMSS1 can produce T4SS-dependent gastritis, gastric atrophy, epithelial hyperplasia, and metaplasia. Some have interpreted this to suggest that PMSS1 is unique and does not undergo loss of T4SS function during mouse passage. However, recent experiments show that loss of T4SS function in *H. pylori* PMSS1 is not unusual following gastric colonization, both in mouse and nonhuman primate models. The mechanism appears to be in-frame recombination in *cagY*, which encodes an essential protein in the *H. pylori* T4SS (Barrozo et al. 2013). This is perhaps not surprising, since *H. pylori* is known to be genetically polymorphic and subject to adaptive changes in vivo (Linz et al. 2014). There are likely also other genomic changes in the *cag*PAI or elsewhere in the genome during in vivo mouse passage. Thus, SS1 remains a useful strain for examination of host immune response and gastritis due to *H. pylori*, but not for the effects of the T4SS, which can only be examined in the mouse model during short-term infection (typically less than 8 weeks) before the loss of T4SS function.

11.2.4 Gastritis in Helicobacter Mouse Models

Gastritis due to helicobacter infection in mice is similar but not identical to gastritis in humans. In both host species, gastritis is mild and develops slowly in most individuals, although individual human patients and immune-modulated mouse strains may develop more severe manifestations of disease. The inflammatory infiltrate in both mice and humans consists of a mixture of neutrophils (referred to as "active" gastritis) and mononuclear inflammatory cells that are rarely specifically identified, but are likely a mixture of lymphocytes, plasma cells, and macrophages or dendritic cells. In humans, chronic infection is sometimes associated with loss of gastric glands and fibrosis (atrophic gastritis) and replacement of gastric-type glandular epithelium with intestinal type epithelium, complete with absorptive and goblet cells (intestinal metaplasia). Gastric cancer is thought to develop as a result of intestinal metaplasia progressing to dysplasia and neoplasia (Correa and Houghton 2007).

Disease due to gastric helicobacter in mice is somewhat bacterial and host strain dependent. Most commonly, *H. pylori* causes mild slowly progressive chronic gastritis and lymphofollicular hyperplasia. Mice do not develop atrophy or intestinal metaplasia. Instead, chronic colonization leads to epithelial hyperplasia and a type of metaplasia known as spasmolytic polypeptide-expressing metaplasia (SPEM) (Weis and Goldenring 2009). In chronically infected C57BL/6 mice, chronic active gastritis begins at the junction of pyloric and fundic epithelium and spreads into the fundus. SPEM develops late in disease and eventually progresses to grossly apparent gastric hypertrophy. Proliferative lesions may become extensive and severe, eventually involving most of the glandular gastric mucosa and sometimes herniating through the gastric muscularis mucosae. SPEM is rarely described in humans, but it is the most common type of gastric metaplasia in mice where it is thought to be a preneoplastic lesion (Nomura et al. 2004; Weis and Goldenring 2009). *H. felis* causes lesions that are similar to those caused by *H. pylori*, but are more severe and rapidly progressive (Court et al. 2002). Because it causes extensive gastric epithelial proliferation in mice, *H. felis* is commonly used in conjunction with cocarcinogens and/or genetically engineered mice to investigate the progression to neoplasia.

Progression and severity of gastric lesions in helicobacter-infected mice are enhanced by adoptive transfer of CD4+ T cells or by absence or suppression of T-cell regulatory cytokines such as IL-10 (Eaton et al. 2001) or T-regulatory cell subsets (Gray et al. 2013). Gastritis and secondary epithelial lesions are enhanced by Th1 and Th17 cytokines, particularly interferon gamma IFN-γ (Sayi et al. 2009), which appears to be a principal inducer of gastritis and metaplasia in mouse models (Syu et al. 2012). Other pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (Thalmaier et al. 2002) and IL-1β (Huang et al. 2013), have also been associated with disease in mouse models.

One experimental strategy that has proven useful in investigation of both bacterial and host immune contributions to disease is the adoptive transfer of CD4+ T cells from C57BL/6 mice to helicobacter-infected congenic T- and B-cell-deficient (SCID or $RAG^{-/-}$) mice, which results in severe, rapidly progressive proliferative gastritis (Gray and Eaton 2012; Eaton et al. 1999). Use of specific T-cell subsets or T cells from cytokine-deficient mice can be used to isolate the role of specific host factors in disease (Eaton et al. 2001, 2006; Raghavan et al. 2003; Roth et al. 1999; Lucas et al. 2001; Sayi et al. 2009). The importance of Th1 T cells, IFN-γ, TNF-α, and IL-17 in gastritis and the role of T-regs in regulating host responses and gastritis were all discovered using the adoptive transfer *H. pylori* model.

11.2.5 Cancer Models

Long-term infection by either *H. pylori* or *H. felis* causes gastric epithelial hyperplasia, SPEM-type metaplasia, and dysplasia, but *H. pylori* infection alone does not

appear to cause neoplastic transformation in mice. Proliferative and dysplastic lesions are more pronounced in mice infected with *H. felis*, and several laboratories have interpreted advanced lesions as neoplastic (Cai et al. 2005; Houghton et al. 2004), but functional characteristics of neoplastic transformation are rarely examined, precluding definitive diagnosis. In one study, neoplastic cells isolated from proliferative lesions in gastrin-deficient mice demonstrated anchorage-independent growth (Zavros et al. 2005), but this has not been demonstrated in helicobacter-associated proliferative lesions in mice. Regardless of differences in interpretation of morphologic lesions, SPEM and dysplasia are generally considered preneoplastic in mice (Weis and Goldenring 2009), and for this reason, infection with *H. felis* (and to a lesser extent *H. pylori*) in mice has been used to model cancer progression, cocarcinogenic agents, and the role of genetic mutations in tumor development.

The mouse glandular gastric mucosa is relatively resistant to agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-Nitroso-N-methylurea (MNU) that cause gastric adenocarcinoma in rats, though several studies have used these agents as potential cocarcinogens in helicobacter-infected mice. Because of the increasing availability of genetically engineered strains, mice offer the best opportunity to evaluate the role of specific genes in cancer progression. Several models have been used for this purpose, including p53 hemizygous transgenic mice (Fox et al. 1996), adenomatous polyposis coli (APC)-gene mutant mice (Fox et al. 1997), and knockout mice with deletions in trefoil factor 2 (Fox et al. 2007), p27 (cyclin-dependent kinase inhibitor) (Kuzushita et al. 2005), iNOS (Nam et al. 2004), p53 (Fox et al. 2002), and MyD88 (Banerjee et al. 2014), among others. Insulin-gastrin (INS-GAS) transgenic mice, which express gastrin under control of the insulin promoter, are predisposed to development of gastric proliferative lesions when colonized with *H. felis* (Wang et al. 2000) and *H. pylori* (Lofgren et al. 2011). Gastric microbiota other than helicobacter species may also contribute to the development of neoplasia, since INS-GAS mice with a complex gut microbiota progress more rapidly to neoplasia following *H. pylori* infection than germ-free mice colonized with *H. pylori* alone (Lofgren et al. 2011). Even colonization with three members of the altered Schaedler flora (ASF) is sufficient to recapitulate the more aggressive pathology found in conventional INS-GAS mice (Lertpiriyapong et al. 2014), which suggests that there is not a single or even a few unique species that, together with *H. pylori*, promote neoplasia. Nevertheless, if a cancer-promoting microbial fingerprint could be identified by bacterial phylogeny or metagenomics, this might provide another biomarker that could be used to identify *H. pylori*-infected patients who are at greatest risk of gastric cancer (Cooke et al. 2013).

11.3 Mongolian Gerbil Model

11.3.1 Background

The first published description of the gerbil (*Meriones unguiculatus*) model appeared in 1991, when Yokota and coworkers reported that gerbils developed mild gastritis 2 months after *H. pylori* oral gavage (Yokota et al. 1991). *H. pylori* colonizes deep within the gastric mucus gel layer near the epithelia, similar in topography to where the bacteria are found in human gastric specimens (Schreiber et al. 2004). Gerbils infected with *H. pylori* can develop gastric ulcer and intestinal metaplasia in the stomach (Hirayama et al. 1996), as well as gastric carcinoma following the administration of chemical carcinogens such as MNU or MNNG (Tokieda et al. 1999). In 1998, a groundbreaking article demonstrated that long-term (>1 year) infection of Mongolian gerbils with *H. pylori* led to gastric adenocarcinoma in approximately one-third of infected animals, without the coadministration of carcinogens, which was subsequently confirmed by others (Watanabe et al. 1998; Ogura et al. 2000). Carcinomas that developed in *H. pylori*-infected gerbils typically occurred in the distal stomach and the pyloric region, and contained well-differentiated intestinal-type epithelium, reflecting many of the features of intestinal-type gastric adenocarcinoma in humans.

One caveat of using rodent models of gastric cancer such as Mongolian gerbils is that there has been some difficulty in distinguishing herniation of nonneoplastic mucosa from invasive carcinoma, particularly gastrointestinal neoplasia that arises in the setting of inflammation. To address this, guidelines for interpretation of these lesions have been established by a multidisciplinary working group (Boivin et al. 2003). Originally formulated for evaluation of intestinal tumors, these guidelines have also been applied to gastric lesions (Hagiwara et al. 2011; Houghton et al. 2004; Rogers et al. 2005). In many studies on *H. pylori*-induced cancer in gerbils, several features are used to distinguish invasive carcinoma from mucosal herniation (Table 11.1).

11.3.2 Host Factors

Although somewhat limited due to their outbred nature, Mongolian gerbils have been used to study the effects of some host constituents on the development of gastric cancer. IL-1β is a Th1 cytokine that inhibits acid secretion and is increased within gastric mucosa of *H. pylori*-infected persons (Noach et al. 1994). In humans, polymorphisms in the IL-1β gene cluster (*IL-1β31* and *IL-1β511*) are associated with increased IL-1β production and a significantly increased risk for hypochlorhydria, gastric atrophy, and distal gastric adenocarcinoma, but only among those infected with *H. pylori* (El-Omar et al. 2000; Figueiredo et al. 2002). Takashima and colleagues examined these relationships in greater

Table 11.1 Features of invasive carcinoma in the Mongolian gerbils

Features
Higher grades of cytologic dysplasia than the overlying mucosa
Desmoplasia unassociated with a prominent inflammatory infiltrate
Irregular, sharp, or angulated, rather than rounded glands
Invading glands spread laterally to the surface component
Invading glands lack circumferential lining by epithelial cells
Invading glands lack full investment of basement membrane
More than two glands in the submucosa or deeper gastric wall

depth in gerbils infected with *H. pylori* by quantifying changes in gastric acidity and then defining the role that IL-1β played in this response (Takashima et al. 2001). Compared to uninfected animals, gerbils infected for 6 or 12 weeks with *H. pylori* developed significantly increased levels of inflammation and IL-1β expression within the gastric mucosa, which was accompanied by a loss of acid secretion. More definitive experiments demonstrated that treatment of *H. pylori*-infected gerbils with an IL-1β antagonist abolished the loss of acid secretion, implicating this cytokine in the development of achlorhydria within an *H. pylori*-infected stomach (Takashima et al. 2001).

Other groups have also developed innovative reagents specific for Mongolian gerbils. Primers targeting pro-inflammatory cytokines including IL-1β, IL-4, IL-6, IL-10, IL-12, KC, IFN-γ, TNF-α, and TGF as well as somatostatin, COX-2, iNOS, and H^+K^+ATPase have been developed based on species-specific gerbil cDNA. Crabtree et al. used such reagents to demonstrate that long-term *H. pylori* infection increases the levels of IL-12 and IFN-γ expression within gerbil gastric mucosa (Crabtree et al. 2004). *H. pylori* infection of gerbils has also been shown to increase serum levels of gastrin, which can promote cell growth, and increased gastrin levels are directly related to heightened gastric epithelial cell proliferation (Peek et al. 2000). Nozaki and coworkers developed a gastric cell line from a gerbil gastric cancer specimen, which was used to demonstrate that *H. pylori* can activate transcription factor NF-κB signaling in a *cag*PAI-dependent manner (Nozaki et al. 2005).

11.3.3 Bacterial Factors

Compared to gerbils infected with wild-type *H. pylori*, gerbils colonized with *cag*PAI mutant strains develop significantly less severe gastritis (Ogura et al. 2000; Akanuma et al. 2002; Israel et al. 2001; Saito et al. 2005). Rieder and coworkers investigated alterations not only in the intensity but also the topography of inflammation in gerbils infected with wild-type *H. pylori* or isogenic *cagA* or *cagY* mutants (Rieder et al. 2005). Inactivation of *cagA* or *cagY* resulted in an inflammatory response that was primarily restricted to the gastric antrum, largely sparing the acid-secreting corpus. Consistent with these histologic changes,

intragastric pH values were increased only in gerbils challenged with the wild-type *H. pylori* strain (Rieder et al. 2005). These results indicate that a functional *cag*PAI T4SS is required to induce corpus-predominant gastritis, a precursor lesion in the progression to intestinal-type gastric adenocarcinoma. *H. pylori* possesses polar flagella consisting of two major subunits, FlaA and FlaB (O'Toole et al. 2000), and deletion of *flaA* results in flagellar truncation and decreased motility (Josenhans et al. 1995). Using signature-tagged mutagenesis, Kavermann and coworkers identified *flaA* as an essential gene for colonization of gerbils by *H. pylori*, along with several other genes involved in either flagellar assembly or that encode structural components of flagella (Kavermann et al. 2003). Other essential colonization genes that were identified using this technique include those encoding outer membrane proteins, secretion and transport systems, and mediators of chemotaxis, acid survival, and stress responses (Kavermann et al. 2003).

Although gerbils can develop gastric cancer in response to *H. pylori* alone, the prolonged time course required for transformation in earlier studies precluded large-scale analyses that comprehensively evaluate mediators that are critical in the carcinogenic cascade (Watanabe et al. 1998). Since serial passage of *H. pylori* in rodents increases colonization efficiency, the Peek lab investigated whether in vivo adaptation of a human *H. pylori* strain (B128) would enhance its carcinogenic potential. A gerbil infected with *H. pylori* strain B128 was sacrificed 3 weeks post-challenge, and a single-colony output derivative (strain 7.13) was used to infect an independent population of gerbils (Franco et al. 2005). The kinetics and intensity of inflammation induced by strain 7.13 were similar to those induced by parental strain B128. However, after 8 weeks of infection with strain 7.13, gastric dysplasia and adenocarcinoma developed in approximately 75 % and 60 %, respectively, while these lesions were not present in any gerbils infected with the progenitor *H. pylori* strain B128 (Franco et al. 2005). *H. pylori* strain 7.13 also activated β-catenin to a significantly higher level in gastric epithelial cells than B128, and isogenic inactivation of *cagA* in strain 7.13 revealed that β-catenin activation was mediated by CagA. Mimicking the outcome of human infection, gerbils infected with *H. pylori* wild-type strain 7.13 developed gastric cancer to a greater degree than those infected with a 7.13 *cagA* deletion mutant (Franco et al. 2008).

To identify additional proteins that may mediate the development of *H. pylori*-induced gastric cancer, these investigators then combined two-dimensional difference gel electrophoresis (2D-DIGE) with mass spectrometry to identify differentially expressed membrane and cytosolic proteins from the non-carcinogenic *H. pylori* strain B128 and its carcinogenic derivative, strain 7.13. Twenty-eight significant expression changes in proteins were detected. A novel modification in FlaA was also detected, which was due to an amino acid substitution that resulted in altered motility (Franco et al. 2009). Another virulence determinant of *H. pylori* that is important for adherence is OipA. Expression of OipA has been associated with increased secretion of the pro-inflammatory cytokine IL-8 in vitro, and infection of gerbils with *H. pylori oipA* mutants has revealed a role for this outer membrane protein in inflammation and gastric cancer (Franco et al. 2008).

11.3.4 Dietary Factors

Diet is likely an important risk factor for gastric cancer, particularly diets high in salt (Tsugane and Sasazuki 2007). However, epidemiologic studies of diet in humans are subject to many limitations, and it is difficult to determine whether dietary parameters are causally linked to gastric cancer or merely represent markers for other factors that are important in gastric cancer pathogenesis. To further investigate potential relationships between diet and gastric cancer risk, several studies have examined the role of diet in the gerbil model of *H. pylori*-induced gastric cancer. *H. pylori* infection and a high-salt diet can independently induce gastric atrophy and intestinal metaplasia in Mongolian gerbils (Bergin et al. 2003), but they appear to be synergistic when animals also receive a chemical carcinogen (Kato et al. 2006; Nozaki et al. 2002). CagA-dependent gastric adenocarcinoma was detected in a significantly higher proportion of *H. pylori*-infected gerbils maintained on a high-salt diet compared to infected animals on a regular diet (Gaddy et al. 2013). Increased *cagA* transcription within the gastric mucosa was also detected in *H. pylori*-infected gerbils fed a high-salt diet compared to those on a regular diet (Gaddy et al. 2013), suggesting that high-salt diets potentiate the carcinogenic effects of *cagA*$^{+}$ *H. pylori* strains.

Dietary iron depletion may also promote *H. pylori*-induced gastritis and gastric cancer in gerbils infected with *cagA*-positive *H. pylori* (Noto et al. 2013). A low-iron diet did not produce these effects in animals infected with an isogenic *cagA* mutant strain, and these mutants exhibited a significant decrease in colonization density compared to wild-type *H. pylori* (Tan et al. 2011). To define mechanisms through which low iron concentrations may augment virulence, *H. pylori* strains cultured from gerbils fed a low-iron diet or a regular diet were compared using 2D-DIGE and mass spectrometry. Multiple proteins differed in abundance when comparing *H. pylori* strains isolated from iron-deplete versus iron-replete gerbils, including proteins that mediate survival, microbial adherence, and function of the *cag* T4SS (Noto et al. 2013). *H. pylori* FlaA and FlaB, the major flagellin subunits, were also significantly upregulated in bacteria cultured from iron-deplete animals. *H. pylori* strains isolated from iron-depleted gerbils also expressed significantly higher levels of CagA (Noto et al. 2013). Strains isolated from iron-depleted gerbils showed more T4SS pili, translocated more CagA, and induced higher levels of IL-8 when compared to strains isolated from iron-replete gerbils (Noto et al. 2013), effects that reversed during passage in vitro (Noto et al. 2015). These experiments indicate that exposure of *H. pylori* to low-iron conditions enhances the ability of the bacteria to induce responses with carcinogenic potential in gastric epithelial cells.

11.3.5 Role of the Gastric Microbiota

Although *H. pylori* infection is the strongest identified risk factor for gastric cancer, recent studies in humans have indicated that other residents of the gastric microbiome may influence malignant transformation (Abreu and Peek 2014). One clinical trial reported that treatment of *H. pylori* was associated with a significant reduction in gastric cancer incidence rates, yet only 47 % of treated persons remained free of *H. pylori* at the time of evaluation (Ma et al. 2012). This suggests that antibiotic therapy alters other microbial species that could affect the development of gastric cancer.

Few studies have examined the gastric microbiota in gerbils. The most commonly represented phyla of the gerbil gastric microbiome include Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes (Fox and Sheh 2013; Yang et al. 2013). Similar to mice, the genus *Lactobacillus* dominates the gastric microbiota of uninfected gerbils (Fox and Sheh 2013; Yang et al. 2013). One study that used molecular techniques to compare differences in the gerbil gastric microbiota before and after *H. pylori* infection demonstrated a reduction in the diversity of Lactobacillus following *H. pylori* infection (Sun et al. 2003). Another group utilized quantitative PCR to follow the relative abundance of 15 microbial species in the gerbil stomach (Osaki et al. 2012). In uninfected gerbils, the most abundant genera were *Lactobacillus* and *Enterococcus*, followed by equal levels of *Atopobium* and *Clostridium*. In gerbils that were successfully infected with *H. pylori*, the relative abundance of *Clostridium coccoides* increased, compared to uninfected gerbils. In gerbils that were challenged with *H. pylori* but were not successfully colonized, the proportion of *C. coccoides*, *C. leptum*, and *Bifidobacterium* was reduced. Gerbils that were challenged with *H. pylori* but not colonized represented the only group that harbored members of the *Eubacterium cylindroides* and *Prevotella* species (Osaki et al. 2012). However, the importance of differences in microbial composition to the development of gastric cancer in the gerbil model has yet to be determined.

11.4 Nonhuman Primate Model

11.4.1 Background

Spiral bacteria were first observed in the stomach of rhesus monkeys early in the twentieth century (Doenges 1938, 1939) and described again in 1982 (Sato and Takeuchi 1982). However, the bacteria found in rhesus monkeys at that time were almost certainly not *H. pylori*, but rather the large tightly coiled spiral organisms that morphologically resemble those seen in a variety of animal hosts (Haesebrouck et al. 2009), and which today would probably be identified as either *Helicobacter suis* or *Helicobacter heilmannii*. Subsequently, several groups reported cultivation

of *H. pylori* from naturally infected rhesus (*Macaca mulatta*), cynomolgus (*M. fasicularis*), and pigtailed (*M. nemestrinae*) macaques (Brondson and Schoenknecht 1988; Baskerville and Newell 1988; Newell et al. 1988; Reindel et al. 1999; Perry et al. 2010). The isolate from pigtailed macaques was originally designated a novel species (Brondson et al. 1991), but was later found to be a heterotypic synonym of *H. pylori* (Suerbaum et al. 2001). More extensive phenotypic testing and sequencing of the 16S rRNA gene confirmed that *H. pylori* isolated from naturally infected macaques was indistinguishable from strains that infect humans (Drazek et al. 1994; Doi et al. 2005). Comparative genomic hybridization also suggests that *H. pylori* isolates from naturally infected rhesus monkeys are not distinguishable from human strains, though they cluster together, probably because they were collected from the same primate center (Joyce et al. 2002). Most *H. pylori* isolates from naturally infected cynomolgus (Doi et al. 2005) and rhesus (Solnick unpublished observations) monkeys appear to contain the *cag*PAI. Whether *H. pylori* from naturally infected macaques is autochthonous is unknown, because samples from feral primates without human contact are difficult to obtain. To address this, we analyzed the DNA sequences of seven housekeeping genes from five *H. pylori* strains cultured from rhesus macaques at the California National Primate Research Center. Comparison to a large sequence database used to characterize strains from human populations worldwide (Falush et al. 2003; Linz et al. 2007) showed that the rhesus *H. pylori* strains belong to the hpEurope clade, strongly suggesting that they were acquired from humans (Solnick and Achtman unpublished observations).

11.4.2 Strengths and Limitations of the Nonhuman Primate Model

Nonhuman primates provide the opportunity to study *H. pylori* in an animal that is anatomically and physiologically most similar to humans. For example, nonhuman primates have a gastric anatomy and pH similar to humans, while the rodent stomach has a higher pH and is divided into two anatomical regions, the large forestomach lined by squamous epithelium and the more acidic glandular portion (Kararli 1995). Other than one report in a single colony of experimental cats (Handt et al. 1995a), natural *H. pylori* infection has been described only in primates. While *H. pylori* is probably not autochthonous in feral nonhuman primates, socially housed captive macaques are often naturally infected, typically in the first year of life (Solnick et al. 2003), a pattern that resembles the epidemiology of human infection in countries with a high prevalence (Bardhan 1997). Natural transmission can be readily demonstrated experimentally among cohoused animals (Solnick et al. 2006). There are also extensive resources available for studies of nonhuman primates. For example, multiple genomes have been sequenced, including the rhesus macaque; transcriptome analyses of rhesus and other nonhuman primate

species are in progress. Many reagents for studies in nonhuman primates are also readily available and can be found at the National Primate Reagent Resource maintained by the National Institutes of Health (http://www.nhpreagents.org). From a practical perspective, gastric samples can be obtained repeatedly by endoscopic biopsy to follow the course of infection over time.

But the nonhuman primate model also has disadvantages, particularly cost and availability. The US national primate research centers (NPRCs) funded by the National Institutes of Health are intended to support investigators who receive their primary research project funding from NIH either at the home institution or across the country, but they may also be used by investigators funded by other agencies, foundations, and the private sector. Together, the NPRCs have more than 26,000 nonhuman primates representing more than 20 species, predominantly macaques. Primate research is also conducted on a smaller scale at many other universities and private centers. In the European Union, the European Primate Network (EUPRIM-Net) brings together nine European primate centers that combine research and breeding to form a virtual European Primate Center. Costs vary across centers, but at the California NPRC, some representative charges in 2016 are approximately $7,000 for animal purchase, $3,500 use fee for non-terminal experiments, and $8.00 for indoor housing per diem, though this can range from $4.00 for outdoor housing in large field cages to $44.00 in the newborn nursery. Another limitation of the nonhuman primate model of *H. pylori* is that it is typically not a disease model. Gastric diseases associated with *H. pylori* in humans—peptic ulcers, gastric adenocarcinoma, and MALT lymphoma—have been seen in rhesus monkeys naturally colonized with *H. pylori* (D.R. Canfield, personal communication; Kimbrough 1966; Parker et al. 1981). However, in typical short-term experiments lasting a few weeks or months, one typically sees only histologic gastritis. Ethical and safety issues unique to research with nonhuman primates should also be considered.

11.4.3 Experimental Infection

The rhesus macaque is the most common nonhuman primate used in experimental research in the USA (Conlee et al. 2004), and it is typically the species of choice for studies of *H. pylori*. Identification of animals without prior *H. pylori* infection is challenging, largely because infection is so common (Dubois et al. 1994; Solnick et al. 1999). Noninvasive testing by enzyme-linked immunosorbent assay (ELISA) with human or rhesus-derived strains has been variably successful (Handt et al. 1995b, 1997); our experience is that it is sensitive but not specific. In a prospective study of socially housed rhesus macaques, 8 of 20 newborn monkeys (40 %) were culture positive for *H. pylori* by 12 weeks of age, reaching 90 % by 1 year (Solnick et al. 2003). In older animals, the frequency of positive cultures declines, though they almost always remain seropositive (Solnick et al. 2003). This discordance might reflect the limited sensitivity of culture (typically about 10^2

CFU/g of tissue) or that infection is multifocal. It could also be that animals sometimes clear the infection yet remain serofast, perhaps because of frequent encounters that fail to produce a persistent infection. Concomitant infection with *H. suis* or *H. heilmannii* might also confound the results of *H. pylori* serology, as well as the urea breath test (Solnick et al. 1999, 2002), since these large gastric spiral organisms are very common and are urease positive (Solnick et al. 1994). Specific pathogen-free (SPF) rhesus monkeys lacking *H. pylori* can be derived by removing them from the dam within a few hours of birth and raising them in a nursery (Solnick et al. 1999), though this is obviously expensive. This approach is also limited by the difficulty of performing endoscopy in newborn macaques, which typically cannot be done easily before about 4–6 months of age, and requires a pediatric bronchoscope with limited biopsy capability. SPF monkeys derived at the NIH-sponsored NPRCs are unlikely to be free of *H. pylori* because they are screened predominantly for viruses, most notably herpes B virus, but not *H. pylori* (Kanthaswamy et al. 2010). Fortunately, depending on the question being addressed, it may be unnecessary to use monkeys that have never been infected with *H. pylori*, since the infection can be treated with antibiotics and the animals reinfected (Dubois et al. 1999), albeit with a lower bacterial load (Dubois et al. 1999; Solnick et al. 2001). Experimental infection of SPF rhesus macaques with *H. pylori* closely recapitulates the gastritis seen in human infection (Fig. 11.1).

Many different *H. pylori* strains have been used successfully for experimental challenge of nonhuman primates, which is typically performed by gastric gavage. Early studies suggested that *H. pylori* strains derived from monkeys might colonize better than those from humans (Euler et al. 1990). While this may be true, there is no doubt that many human strains will colonize as well, though mixed infections suggest strongly that some strains are better suited to monkeys than others (Dubois et al. 1999; Solnick et al. 2001). Like in mouse and gerbil models, it is important that the strains be freshly isolated from their host with minimal laboratory passage. Many investigators have attempted to increase the likelihood of experimental infection by suppressing gastric acid and using a large inoculum (e.g., 10^9 colony-forming units (CFU)), though neither is necessary if the strain is well suited to monkeys (Solnick et al. 2001). Inoculation of as few as 10^4 CFU has been successful (Solnick et al. 2001). Both *cag*PAI-positive and *cag*PAI-negative strains will colonize, the latter producing less inflammation and higher colonization density (Hornsby et al. 2008). As in the mouse model, strains isolated from experimentally infected monkeys lose function of the T4SS, which is mediated by recombination in the highly repetitive region of *cagY* (Barrozo et al. 2013). Interestingly, *cagY*-mediated gain of function in the T4SS is also seen in macaques (Barrozo et al. 2013). Since *cagY* codons are under strong positive selection, and recombination can both up- and downmodulate T4SS function in a graded fashion, it seems likely that this is a strategy to “tune” or optimize the host inflammatory response to achieve a homeostatic balance. Colonization in rhesus macaques also results in loss of expression of the *babA* adhesin, either by phase variation or by gene conversion with the *babB* paralog (Solnick et al. 2004; Styer et al. 2010). Loss of T4SS function and expression of *babA* typically occur during the first 4–8 weeks

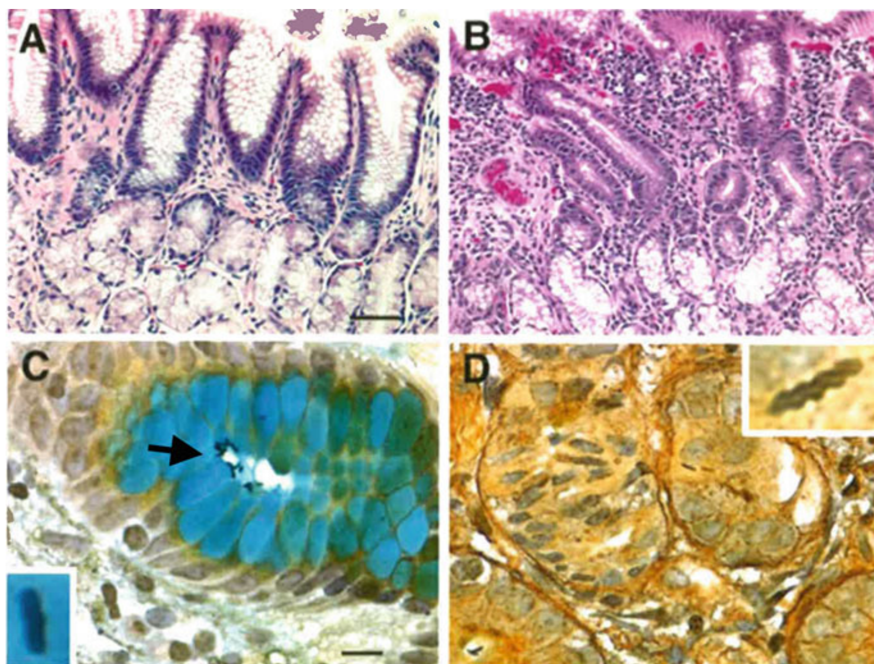

Fig. 11.1 Helicobacter sp. infection in rhesus macaques. (**a**) Normal uninfected pyloric antrum with only a few scattered inflammatory cells in the lamina propria (H&E stain, *scale bar* = 50 μ). (**b**) Pyloric antrum illustrating characteristic gastritis secondary to *H. pylori* infection. The superficial lamina propria is expanded by inflammation comprising mainly plasma cells with lesser numbers of lymphocytes and scattered neutrophils (H&E stain). (**c**) Higher magnification of *H. pylori* organisms characteristically located on the luminal surface of epithelial cells lining gastric pits (modified Steiner silver stain, *scale bar* = 20 μ); *inset* shows higher power magnification of *H. pylori*. (**d**) In contrast to *H. pylori*, *H. heilmannii* inhabits the gastric corpus, generally extending deeper into glands and often residing inside parietal cells, and elicits little or no inflammation. Inset illustrates the corkscrew spiral shape of the larger organisms, which are likely either *H. heilmannii* or *H. suis*. Photomicrograph courtesy of D.R. Canfield, DVM

after challenge, which appears to be a period of rapid change in the *H. pylori* genome during acute infection in both rhesus macaques and humans (Linz et al. 2014).

Once established, infection in a well-adapted strain is typically persistent, though some strains will show transient infections and others will not colonize at all. The outcome can be readily monitored by serial endoscopic biopsies, which can be processed for quantitative CFU, histopathology, host (Huff et al. 2004) and bacterial (Boonjakuakul et al. 2005) gene expression, bacterial genomic evolution (Linz et al. 2007; Solnick et al. 2004), and other assays. Colonization density is typically 10^5–10^6 CFU/g of gastric tissue, but may decline somewhat after the immune response develops in the first few weeks after infection.

11.4.4 How Has the Nonhuman Primate Model Been Used?

The nonhuman primate model has been used to address a number of important questions in fundamental and clinical aspects of *H. pylori* biology. Here, we give a few illustrative examples. Studies in socially housed macaques suggest that natural *H. pylori* transmission occurs very early in life, probably by the oral-oral route (Solnick et al. 2003, 2006). An early study found that immunization with urease and *Escherichia coli* LT appeared to protect rhesus macaques from natural infection (Dubois et al. 1998), but the infection status of the animals before immunization was only examined by serology. Subsequent studies under more controlled conditions were disappointing (Lee et al. 1999; Solnick et al. 2000). The macaque model has also provided an opportunity to examine host and bacterial gene expression (Boonjakuakul et al. 2005; Huff et al. 2004; Suerbaum and Josenhans 2007), and the development of *H. pylori* genomic diversity, which is perhaps greater than that in any other bacterial species known (Suerbaum and Josenhans 2007). The results suggest that there is a mutation burst early during acute infection (Linz et al. 2014), with rapid genomic changes that alter expression of outer membrane proteins (OMPs) and the T4SS (Barrozo et al. 2013; Solnick et al. 2004; Styer et al. 2010). Alterations in *H. pylori* OMPs likely reflect a complex cross talk between expression of bacterial lectins and changes in host glycosylation (Cooke et al. 2009; Linden et al. 2008; Mahdavi et al. 2002; Wirth et al. 2006). Although not typically a disease model, the rhesus macaque has also been used to study the effects of *H. pylori* and diet on the gastric cancer cascade. Macaques experimentally infected with *cag*PAI-positive *H. pylori* and chronically fed *N*-ethyl-*N*-nitrosoguanidine (ENNG) developed gastric intestinal metaplasia starting at 2 years (Liu et al. 2009). Untreated control monkeys and monkeys treated singly with either *H. pylori* or ENNG showed no neoplastic changes. These data demonstrate the synergistic effects of *H. pylori* infection with a dietary carcinogen that is present in East Asian diets.

11.4.5 Human Challenge Model

Two clinical studies have been published describing the results of experimental *H. pylori* infection in *H. pylori*-naive humans, which demonstrated successful colonization in most individuals (Aebischer et al. 2008; Graham et al. 2004). Both studies used the *cag*PAI-negative, antibiotic-susceptible Baylor strain (BCS100; ATCC BAA-945) isolated from a patient with mild gastritis. At least two other human trials have been performed but remain unpublished (David Graham, personal communication). Although potentially useful in vaccine studies for which the human challenge model was developed, safety and regulatory issues will likely preclude its widespread use.

11.5 Conclusions and Outlook

Animal models are essential for a full understanding of *H. pylori* pathogenesis and for vaccine development. Cost, convenience, and the ever-increasing availability of genetically engineered mice will ensure that the mouse remains the dominant model for in vivo studies of *H. pylori*, using either *H. felis* to examine inflammatory and carcinogenic mechanisms, or *H. pylori* to examine vaccines and specific bacterial virulence factors. The gerbil is arguably the best disease model, though it is more expensive than mice, and there are no genetically engineered animals and relatively few reagents. The nonhuman primate remains the model most closely resembling humans, but cost and availability limit its use to specialized centers.

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Chapter 12
Helicobacter pylori and the Host Immune Response

Anne Müller and Mara L. Hartung

Abstract *Helicobacter pylori* is an ancient companion of humans and has coevolved with the human race at least since its migration out of Africa. Consequently, it is well adapted to its exclusive niche, the mucosal surface of the human stomach, and has developed elaborate strategies to evade or suppress immunity and establish persistent infection. This chapter will discuss the most recent findings regarding innate immune recognition of *H. pylori* and the mechanisms that allow the bacteria to avoid detection and subsequent killing by antimicrobial peptides and other first-line defense mechanisms. Additional topics focus on the differences in adaptive immune responses that may explain the broad spectrum of disease outcomes in *H. pylori*-infected individuals, which range from a completely asymptomatic carrier state to often fatal gastric cancer development. The immune cell compartments driving *H. pylori* infection-associated immunopathology are discussed along with their main effector mechanisms. Additionally, several strategies employed by *H. pylori* to block the clonal expansion of specific effector T cells, and to preferentially induce the differentiation of regulatory T cells, are introduced in light of their putative role in establishing and maintaining persistent infection. A final topic deals with the consequences of *H. pylori*-specific immunomodulation for the risk of the carrier to develop immune-related disorders such as chronic inflammatory diseases of the lower bowel and certain allergic disease manifestations. A potential protective effect of *H. pylori* on such immune disorders is discussed with regard to the latest epidemiological findings in humans as well as experimental studies in animal models.

Keywords *Helicobacter pylori* • Type IV secretion • Immunomodulation • Adaptive immunity • Innate immune recognition • Virulence determinants • Persistence

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12.1 Introduction

Helicobacter pylori exclusively infects the human gastric mucosa, where it colonizes the mucus and binds to gastric epithelial pit cells (Salama et al. 2013). The mucus layer and gastric epithelial cell monolayer of the stomach mucosa thus form the first host defense barrier against *H. pylori*. The host/*H. pylori* interaction at the mucosal surface of the stomach is characterized by a fine balance of both pro- and anti-inflammatory responses, which, in ~80 % of carriers, permits persistent infection while at the same time preventing clinically overt disease. *H. pylori* expresses pathogen-associated molecular patterns (PAMPs) that evade detection by the host innate immune system yet retain their biological function as structural components of the bacterial cell wall and motility apparatus. Other evolutionary adaptations allow the bacteria to suppress anti-*Helicobacter* immunity by directly interfering with effector T cell activation, proliferation, and function and by indirectly blocking T cells via the induction of highly suppressive regulatory T cells. The development of peptic ulcer disease and gastric premalignant and malignant lesions is now widely viewed to be the consequence of a misbalance in effector and regulatory T cell responses to the infection that arises due to a specific genetic or lifestyle predisposition of the host or a mismatch between the genetic makeup of host and pathogen. The preferential induction of regulatory over effector T cells, which is a hallmark of persistent (asymptomatic) *H. pylori* infection, exerts not only local but also systemic effects that remain poorly understood. One of the consequences of the systemic immunomodulation by *H. pylori* is the relative protection of the infected fraction of the population from allergic and chronic inflammatory diseases such as allergen-induced asthma, hay fever, ectopic dermatitis, inflammatory bowel diseases, and celiac disease, all of which are immune-related disorders. The purpose of the following chapters is the detailed discussion of the elaborate molecular adaptations of *H. pylori* that allow it to evade and manipulate host immune responses and to thereby establish persistent infection. The local and systemic consequences of this host/pathogen interaction are also discussed.

12.2 Innate Immune Responses to *H. pylori*

As mentioned above, the gastric epithelial cell monolayer of the stomach mucosa forms the first innate immune defense barrier against *H. pylori* and therefore has been studied in great detail. The direct interaction of *H. pylori* with epithelial cells results in the assembly of the type IV secretion system (T4SS) pilus (for more details, see Chap. 4) and the subsequent exposure to virulence factors encoded on the Cag pathogenicity island (*cag*PAI); among these, the CagL/integrin α5β1 interaction has proven to be directly responsible for the activation of NF-κB and production of the neutrophil chemoattractant interleukin-8 (IL-8) (Gorrell et al. 2012; Jimenez-Soto et al. 2009; Kwok et al. 2007; Shaffer et al. 2011). In

line with the well-known pro-inflammatory/immunostimulatory activity of the *H. pylori* T4SS, mutants lacking this activity colonize mice and gerbils at higher levels than the corresponding wild-type strains because they are controlled less efficiently; *cag*PAI mutants further cause significantly less preneoplastic immunopathology in rodent models (Arnold et al. 2011b; Rieder et al. 2005). In human carriers, *cag*PAI-positive strains (often identified serologically by their expression of the *cag*PAI-encoded CagA protein) are clearly associated with more severe disease and higher gastric cancer risk than *cag*PAI/CagA-negative strains (Blaser et al. 1995; Huang et al. 2003). Several studies suggest that in humans, the expression of *cag*PAI proteins appears to confer a colonization advantage through the downregulation of antimicrobial peptides: for example, it has been demonstrated that *H. pylori* reduces the expression of human β-defensin 1 in a T4SS- and NF-κB-dependent manner, with colonization and gastric β-defensin 1 levels being inversely correlated in *H. pylori* carriers (Patel et al. 2013). Another human antimicrobial peptide, β-defensin 3, which is known to be highly active against *H. pylori*, is initially induced by the infection in vitro in an epidermal growth factor receptor (EGFR)- and mitogen-activated protein (MAP) kinase-dependent manner but is then stably shut down through CagA-mediated activation of the Src homology domain containing protein tyrosine phosphatase 2 (SHP2) and downmodulation of the EGFR signaling pathway (Bauer et al. 2012a). The downregulation of β-defensin 3 protein could indeed be confirmed in the gastric mucosa of *H. pylori*-infected subjects (Bauer et al. 2012b). In line with the critical role of the *cag*PAI-encoded T4SS in pro-inflammatory and immune escape mechanisms, its expression and function have been shown to be fine-tuned by DNA rearrangements at direct repeats in the coding sequence of the CagY protein, an essential component of the T4SS, which is subject to immune-driven selective pressure in both mice and nonhuman primates (Barrozo et al. 2013).

Whereas *cag*PAI-induced responses are best characterized and understood in gastric epithelial cells, most non-PAI-mediated interactions of *H. pylori* with the host have been studied in cells of the innate immune system. Innate immune recognition of *H. pylori* is unique in that it predominantly results in anti- rather than pro-inflammatory responses; several pathogen-associated molecular patterns (PAMPs) of *H. pylori* have further evolved to specifically avoid excessive inflammation (Fig. 12.1). The lipopolysaccharide (LPS) constituent of the bacterial outer membrane, which in enteropathogenic bacteria has strong immunostimulatory properties and readily activates inflammatory signaling via toll-like receptor 4 (TLR4), has evolved in *H. pylori* to avoid TLR4 recognition (Cullen et al. 2012; Moran et al. 1997). The relative bio-inactivity of *H. pylori* LPS has been attributed to its tetra-acylation (whereas *E. coli* LPS is hexa-acylated) (Moran et al. 1997) and to the removal of phosphate groups from the 1′ and 4′ positions of the lipid A backbone, which generates LPS that has less negative charge, escapes detection by TLR4, and resists binding by antimicrobial peptides (Cullen et al. 2012). Mutants lacking the phosphatases required for lipid A modification consequently fail to colonize mice (Cullen et al. 2012). A similarly elaborate mechanism of immune evasion has been reported for *H. pylori* flagellin, which is

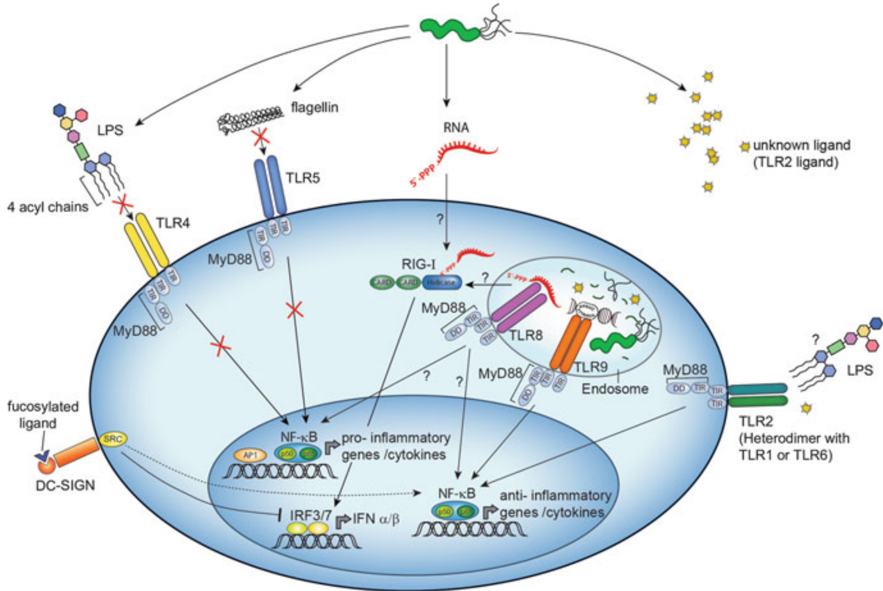

Fig. 12.1 *H. pylori* subverts and evades innate immune recognition. *H. pylori* produces several PAMPs that have evolved to evade detection by pro-inflammatory TLRs. Examples include *H. pylori*'s tetra-acylated LPS, which is bioinactive due to specific lipid A modifications that prevent detection by TLR4. *H. pylori* flagella cannot be detected by TLR5 due to mutations in the TLR5 binding site of flagellin. *H. pylori* DNA, enriched for the immunoregulatory sequence TTTAGGG, as well as an as yet uncharacterized PAMP (and possibly *H. pylori* LPS) are detected by TLRs 9 and 2, respectively; these TLRs preferentially activate anti-inflammatory signaling pathways and IL-10 expression. 5′ triphosphorylated RNA is detected by the cytosolic receptor RIG-I, which activates the transcription factors IRF3 and IRF7 to induce type I IFN expression; *H. pylori* RNA is likely also detected by TLR8 in endosomes. *H. pylori*'s fucosylated DC-SIGN ligands suppress activation of the signaling pathways downstream of DC-SIGN and activate anti-inflammatory genes. Note that not all depicted pattern recognition receptors are necessarily expressed by the same cell type; a generic cell is shown here for simplicity. DD, death domain; TIR, toll/interleukin-1 receptor domain; CARD, caspase activation and recruitment domain; MyD88, myeloid differentiation primary response gene 88; DC-SIGN, DC-specific intercellular adhesion molecule-3-grabbing non-integrin; SRC, steroid receptor coactivator

mutated in exactly those N-terminal positions that mediate binding to TLR5 in *Salmonella enterica* flagellin (Andersen-Nissen et al. 2005; Gewirtz et al. 2004). Additional interactions of *H. pylori* PAMPs with host innate immune receptors include the detection of *H. pylori* DNA by the endosomally localized TLR9 (Otani et al. 2012) and the binding of an as yet unidentified ligand to TLR2 (Fig. 12.1). The interactions via TLR9 and TLR2 result in predominantly anti-inflammatory responses. Lipofection of dendritic cells (DCs) with *H. pylori* DNA activates TLR9 (Rad et al. 2009), with documented anti-inflammatory consequences in the first months of infection in a mouse model (Otani et al. 2012). The putative immunoregulatory properties of *H. pylori* DNA have in fact even been exploited successfully for therapeutic purposes: oral administration of purified DNA

alleviates experimentally induced inflammatory bowel disease in mice (Luther et al. 2011), a finding that has been attributed to the unique immunoregulatory sequence 5′TTTAGGG that is overrepresented in the *H. pylori* genome (Owyang et al. 2012). The predominant TLR activated by *H. pylori* is TLR2, which drives an anti-inflammatory signature characterized by high expression levels of the regulatory cytokine interleukin-10 (IL-10) in DCs (Rad et al. 2009) (Fig. 12.1). Whereas the *H. pylori* ligand for TLR2 remains unknown, it is clear from various studies conducted in vitro and in vivo that TLR2 signaling counteracts *H. pylori* clearance and promotes immune tolerance (Sayi et al. 2011; Sun et al. 2013). TLR2-deficient mice control *H. pylori* and related *Helicobacter* species efficiently develop more severe and accelerated immunopathology (Sayi et al. 2011; Sun et al. 2013). TLR2 proficiency of B cells and DCs is required for the differentiation of regulatory T cells, the *H. pylori*-induced production of IL-10 and other tolerogenic responses in vitro and in vivo, and likely accounts for the phenotype of $TLR2^{-/-}$ mice (Rad et al. 2009; Sayi et al. 2011; Sun et al. 2013). In addition to the mentioned PAMPs and their receptors, *H. pylori* RNA has also been shown to mediate innate immune recognition. 5′-triphosphorylated RNA of *H. pylori* is sensed by the endosomal TLR8, as well as the cytoplasmic retinoic acid-inducible gene (RIG)-like helicase receptor RIG-I, the latter leading to the production of type I interferons (IFNs) (Rad et al. 2009). Whether this pathway of innate immune detection contributes to *H. pylori* control or pathogenesis is currently not known.

In addition to the described immune escape and immunomodulatory mechanisms involving TLRs, *H. pylori* has further evolved to bind the C-type lectin receptor DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN); however, in contrast to other pathogens expressing (mannosylated) DC-SIGN ligands, the fucosylated DC-SIGN ligands of *H. pylori* fail to activate the signaling cascade downstream of this receptor and instead dissociate the respective signaling complexes to suppress pro-inflammatory signaling (Gringhuis et al. 2009). Anti-inflammatory consequences have also been reported of the *H. pylori*-induced activation of the inflammasome. It is now clear that *H. pylori* activates caspase-1 and induces the proteolytic processing of caspase-1-dependent cytokines in an NLRP3- and ASC-dependent manner (Hitzler et al. 2012b; Kim et al. 2013). Mature IL-1β and IL-18 are produced and secreted by DCs that have been exposed to *H. pylori* (Hitzler et al. 2012b; Kim et al. 2013); transcriptional activation of pro-IL-1β appears to depend on the *cag*PAI (Kim et al. 2013), whereas pro-IL-18 is known to be constitutively expressed. Interestingly, the two caspase-1-dependent cytokines fulfill opposing roles in the context of *H. pylori* control and pathogenesis. Whereas IL-1β is absolutely required for the differentiation and function of Th1 and Th17 cells and thus for the control of experimental infection as well as for the development of gastritis and preneoplastic immunopathology (Hitzler et al. 2012b; Kim et al. 2013), IL-18 is dispensible for immunity to *H. pylori* (Hitzler et al. 2012b). The phenotypes of IL-1R and IL-1β-deficient mouse strains are nicely in line with observations in humans, where promoter polymorphisms in the IL-1β gene leading to increased steady-state production of the cytokine predispose to an increased gastric cancer risk (El-Omar et al. 2000).

Quite to the contrary, mice lacking IL-18 or its receptor control *Helicobacter* infections more efficiently because they fail to generate FoxP3-positive regulatory T cells (Tregs) and to develop immune tolerance to the infection and, as a consequence, exhibit overly active Th17 cells (Hitzler et al. 2012b; Oertli et al. 2012). The defect in Treg differentiation of IL-18/IL-18R-deficient mice has been attributed to the failure of IL-$18^{-/-}$ DCs to induce Treg differentiation (Oertli et al. 2012). The differential properties of caspase-1-dependent cytokines in *H. pylori* control and immunopathology are reminiscent of the functions of both cytokines in the lower GI tract, especially in models of experimentally induced colitis, which are driven in large part by IL-1β (Coccia et al. 2012) and restricted by IL-18 (Zaki et al. 2010).

Collectively, the data generated in recent years on the various innate immune responses to (and their manipulation by) *H. pylori* suggest that the bacterium effectively prevents its clearance and promotes its persistence by both evading innate immune detection and subsequent killing and by skewing innate immune responses toward anti-inflammatory and regulatory signals. The postulated >60,000 years of coexistence of *H. pylori* with its human host have provided the selective pressure necessary to drive these evolutionary processes; the remarkable genetic variability, high mutation rate, and natural competence of *H. pylori* provide the genetic setting facilitating its host adaptation.

12.3 Anti-*Helicobacter* Adaptive Immune Responses Differ in Symptomatic and Asymptomatic Carriers

Whereas approximately one-half of humanity is infected with *H. pylori*, only a fraction of human carriers will develop *H. pylori*-related gastric or duodenal disease. The remaining ~80 % of the infected population remain asymptomatic for life and in fact may never know that they are colonized. Host genetic traits (see Chap. 14 on host gene polymorphisms and their effects on disease outcome) and the specific virulence factors expressed by the infecting *H. pylori* strain (see Chaps. 3, 4, 5, 6, and 7) have been discussed as critical modulators influencing disease outcome. One important predictor and driver of disease that has emerged in recent years from studies conducted in human carriers as well as in mouse models is the severity and polarization of gastric *H. pylori*-specific T helper cell responses (Arnold et al. 2011b; Harris et al. 2008; Robinson et al. 2008). Individuals with peptic ulcer disease (PUD) have a threefold higher anti-*H. pylori* Th1 response and sixfold higher Th2 response than asymptomatic carriers (Robinson et al. 2008). The PUD patients at the same time exhibited a twofold lower Treg response than asymptomatic carriers and significantly reduced IL-10 and transforming growth factor (TGF)-β levels in the gastric mucosa; the authors concluded from this imbalance that an inadequate Treg response was associated with and possibly responsible for the development of *H. pylori*-associated disease (Robinson

et al. 2008). Another study with a similar objective and approach compared the anti-*H. pylori* responses of children and adults and also found an inverse correlation between the degree of gastritis and the numbers of gastric Tregs and production of Treg-derived cytokines (Harris et al. 2008). These two seminal studies have confirmed and extended previous work showing that Tregs home to and accumulate in the gastric mucosa of infected but not uninfected individuals, where they suppress *H. pylori*-specific memory T cell responses (Lundgren et al. 2003, 2005a, b). Taken together, the observational studies in humans imply that asymptomatic (healthy) carriers predominantly launch Treg responses to *H. pylori* infection, which effectively control immunopathology and promote persistent infection, whereas symptomatic carriers presenting with disease exhibit T-effector-dominated responses (Fig. 12.2). The studies in humans are corroborated by experimental studies in mice, where Treg depletion improves infection control by deregulating Th1 and Th17 responses and at the same time promotes the development of chronic infection-associated gastritis, atrophy, and intestinal metaplasia (Arnold et al. 2011b; Hitzler et al. 2011). Treg-derived IL-10 and TGF-β are each critical for preventing T-effector cell-driven immunopathology (Arnold et al. 2011b), suggesting that the expression levels of these cytokines in the gastric mucosa are excellent indicators of disease outcome. Interestingly, the lack or neutralization of IL-10 signaling is sufficient to clear *H. pylori* and to induce strong T cell-dependent immunopathology (Ismail et al. 2003; Sayi et al. 2011).

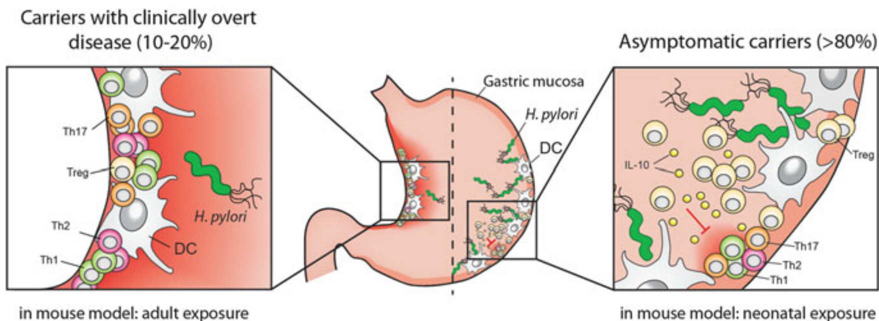

Fig. 12.2 Symptomatic and asymptomatic carriers differ in terms of their anti-*H. pylori* T cell responses. Observational studies comparing the T cell responses of peptic ulcer disease patients and asymptomatic carriers (Robinson et al. 2008) and children vs. adults with relatively mild and severe gastritis, respectively (Harris et al. 2008), have revealed that Treg/T-effector cell ratios correlate with disease outcome. Asymptomatic carriers predominantly generate Treg responses to the infection, which suppress Th1 and Th17 cells through the production of soluble cytokines IL-10 and TGF-β and other immunosuppressive mechanisms. This scenario is modeled in C57BL/6 mice neonatally infected with *H. pylori* (Arnold et al. 2011b) In contrast, the gastric mucosa of symptomatic carriers is infiltrated by Th17 and Th1 cells and exposed to high levels of the signature cytokines IFN-γ, TNF-α, and IL-17, which drive chronic gastritis and promote disease progression toward chronic atrophic gastritis and hyperplasia, intestinal metaplasia, ulcers, and gastric cancer. Infection of adult C57BL/6 mice mirrors the scenario found in symptomatic carriers. *H. pylori* colonization levels are generally higher in asymptomatic vs. symptomatic carriers and directly proportional to gastric mucosal Treg numbers

H. pylori is typically acquired during early childhood, with the mother serving as the main source of infection in populations with low prevalence (Weyermann et al. 2009). The infection is generally contracted within the first 2 years of life (Rothenbacher et al. 2000), i.e., at a time when the immune system is immature and predisposed to develop immune tolerance rather than immunity to foreign dietary and environmental antigens and the newly acquired microbiota (Arnold et al. 2005). Animal models that take into account and aim to reflect the age at the time of *H. pylori* acquisition have revealed that exposure to *H. pylori* during the neonatal period leads to the development of Treg-mediated immune tolerance to *H. pylori* (Arnold et al. 2011b). Although anti-*H. pylori* effector T cell responses are generated normally by the neonatally infected murine host, these are under the tight control of Tregs (Fig. 12.2). Neonatally infected animals are protected against infection-induced gastric immunopathology, not just in the weeks and months following experimental infection but apparently for life (Arnold et al. 2011b). The hallmarks of the neonatal infection model are reminiscent of the Treg-predominant responses of infected children (Harris et al. 2008); it is interesting to note in this context that children not only suffer less frequently from the consequences of gastric colonization with *H. pylori* but also appear to benefit more in terms of their reduced allergy risk (see below). Understanding the relative role of T-effector *vs*. Treg responses in the balance of *H. pylori* clearance and immunopathology is of immediate practical relevance in *H. pylori* vaccinology, as protective immunity can only be achieved by vaccination strategies aimed at overcoming immune counter-regulation (Becher et al. 2010; Hitzler et al. 2011) and should ideally be sterilizing (see Chap. 24 on vaccine development against *H. pylori*).

12.4 Gastric Preneoplastic Immunopathology Is Driven by T Helper Cell Responses and T Cell-Derived Cytokines

It is now well accepted due to work conducted in experimental infection and vaccine-induced protection models that the host control of *H. pylori* colonization depends on $CD4^+$ effector T cells, but not on B cells or antibodies (Akhiani et al. 2002, 2004a, b; Ermak et al. 1998; Hitzler et al. 2011; Sayi et al. 2009; Velin et al. 2005, 2009; Velin and Michetti 2010). Human volunteer infections confirm that strong T cell responses and *H. pylori* clearance are tightly correlated and possibly causally associated (Aebischer et al. 2008). Out of a total of 58 volunteers who were challenged with live *H. pylori* in a seminal study published in 2008, 13 managed to clear the infection as judged by urea breath test; eight of these had been vaccinated against *H. pylori* using the *Salmonella typhimurium* vaccine strain Ty21a expressing *H. pylori* urease and five had cleared the challenge infection spontaneously (Aebischer et al. 2008). *H. pylori*-specific T helper cells were detected in 9 of the 13 volunteers who cleared the infection successfully, but only in

6 of 45 who did not. While the study disappointingly provided little evidence for vaccination-induced protective immunity, it did show convincingly that anti-*H. pylori* T cell responses correlate well with clearance (Aebischer et al. 2008).

It is evident from studies using T cell-deficient mouse strains that $CD4^+$ $TCR\alpha/\beta^+$ T cells are required for the control of *H. pylori* as well as for the induction of immunopathology: mice specifically lacking $CD4^+$ $TCR\alpha/\beta^+$ T cells either due to a deletion of the major histocompatibility complex (MHC) II or to lack of the TCR β−chain fail to control experimental infections (in vaccinated as well as naive mice) and are at the same time protected against infection-associated immunopathology (Hitzler et al. 2011, 2012a; Akhiani et al. 2002; Ermak et al. 1998). It has been postulated that the $CD4^+$ T helper cell subsets and signature cytokines that contribute to infection control in experimental models at the same time promote the immunopathology that is a hallmark of immunocompetent hosts and that manifests histologically as chronic (atrophic) gastritis (Hitzler et al. 2012a; Horvath et al. 2012; Sayi et al. 2009; Shi et al. 2010; Stoicov et al. 2009). Considerable early evidence suggests that Th1-polarized T cells are critical mediators of *H. pylori* control and immunopathology. Th1 cells and their signature cytokine IFN-γ have been implicated in vaccine-induced protective immunity and in infection-associated gastritis: mice lacking the p40 subunit of the Th1-polarizing cytokine IL-12 or the receptor for the Th1 signature cytokine IFN-γ fail to control *H. pylori* upon challenge infection and IFN-$\gamma R^{-/-}$ mice further fail to develop gastritis (Akhiani et al. 2002). Similarly, mice lacking Th1 cells due to the genetic ablation of the lineage-defining transcription factor T-bet are protected from gastric atrophy and the development of gastric cancer later in life (Stoicov et al. 2009). The adoptive transfer of wild-type but not IFN-γ-deficient $CD4^+$ T cells controls the infection and induces preneoplastic pathology in *H. pylori*-infected T cell-deficient recipients (Sayi et al. 2009). In hindsight, some of the early work must be interpreted with caution: for example, it is now clear that the p40 subunit of IL-12 is shared by the related cytokine IL-23, which is generally accepted to drive Th17 but not Th1 responses (Cua et al. 2003). Consequently, $p40^{-/-}$ mice lack Th17 as well as Th1 cells (Cua et al. 2003). More recent studies have addressed the relative contribution of Th1 and Th17 cells to infection control; neither $p19^{-/-}$ nor $p35^{-/-}$ mice – lacking the specific subunits of IL-23 and IL-12, i.e., deficient for either Th17 or Th1 cells – were protected upon vaccination with the gold standard cholera toxin adjuvant whole cell extract vaccine (Hitzler et al. 2011); the contribution of Th17 cells to *H. pylori* control was also confirmed by other investigators (Horvath et al. 2012). In addition to their role in *H. pylori* control, Th17 cells also contribute to infection-induced immunopathology, as assessed in mice lacking the IL-23-specific p19 subunit (Hitzler et al. 2012a; Horvath et al. 2012) Whether the best-studied Th17 signature cytokine IL-17 mediates the effects of Th17 cells remains controversial; neutralizing antibodies to IL-17 prevents *H. pylori* control and immunopathology in vaccination models (Velin et al. 2009) but have been reported to promote colonization in non-vaccinated, experimentally infected mice (Shi et al. 2010). In line with the known reciprocal negative regulation of Th1 and Th17 subsets, this effect has been attributed by some

investigators to higher Th1 cytokine expression in the absence of IL-17 signaling (Otani et al. 2009). In summary, the work of many groups has documented beyond doubt that the control of *H. pylori* and the resulting infection-induced gastric immunopathology preceding the development of gastric cancer are inseparably linked, at least in mouse models, and mediated by both Th1 and Th17 subsets of T cells. Public health strategies aimed at reducing gastric cancer risk by eradicating *H. pylori* in high-risk individuals or populations thus might benefit from combining antibiotic treatment with T cell-targeting immunomodulatory therapies to accelerate mucosal healing and improve treatment outcome also in individuals with preexisting atrophy and metaplasia that do not currently benefit sufficiently from eradication therapy alone (Rokkas et al. 2007; Wong et al. 2004).

12.5 *H. pylori* Suppresses Effector T Cell Responses to Achieve Persistent Infection

Given the critical role of effector T cells (Th1 and Th17 subsets) in controlling *H. pylori*, it is not surprising that the bacteria have evolved elaborate mechanisms of suppressing human T cell activity, proliferation, and clonal expansion. One key virulence factor/persistence determinant with T cell inhibitory properties is the vacuolating cytotoxin VacA. VacA was initially identified due to its ability to induce massive vacuolation in primary gastric epithelial cells and certain gastric epithelial cell lines (Smoot et al. 1996; Harris et al. 1996) and to its association with peptic ulcer disease (Atherton et al. 1995) (see Chap. 5 on VacA). It is also known to induce apoptosis in gastric epithelial cells (Cover et al. 2003), presumably via insertion into mitochondrial membranes followed by cytochrome C release (Domanska et al. 2010). Its vacuolating and pro-apoptotic activity requires a stretch of N-terminally encoded hydrophobic amino acids, which allow VacA to form hexameric pores in artificial lipid bilayers as well as in endosomal, lysosomal, and mitochondrial membranes of epithelial cells and phagocytes (McClain et al. 2001; Czajkowsky et al. 1999). VacA is expressed by all *H. pylori* isolates in the form of either the m1 or m2 allele, which differ in expression levels, vacuolating activity, and association with disease (Atherton et al. 1999). Mouse studies have demonstrated that VacA is not only a virulence but also a colonization factor, as mutants lacking VacA are rapidly outcompeted by the wild type in mixed infections (Salama et al. 2001) and colonized at significantly lower levels in single infections (Oertli et al. 2013). In addition to its vacuolating and pro-apoptotic effects on epithelial cells and in line with its critical role in colonization, VacA has been shown to inhibit the activation and proliferation of T and of B cells (Fig. 12.3) (Torres et al. 2007; Boncristiano et al. 2003; Gebert et al. 2003; Sundrud et al. 2004). In human primary T cells, activation upon TCR engagement is blocked at the level of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and the nuclear translocation of the transcription factor nuclear factor of activated T cells

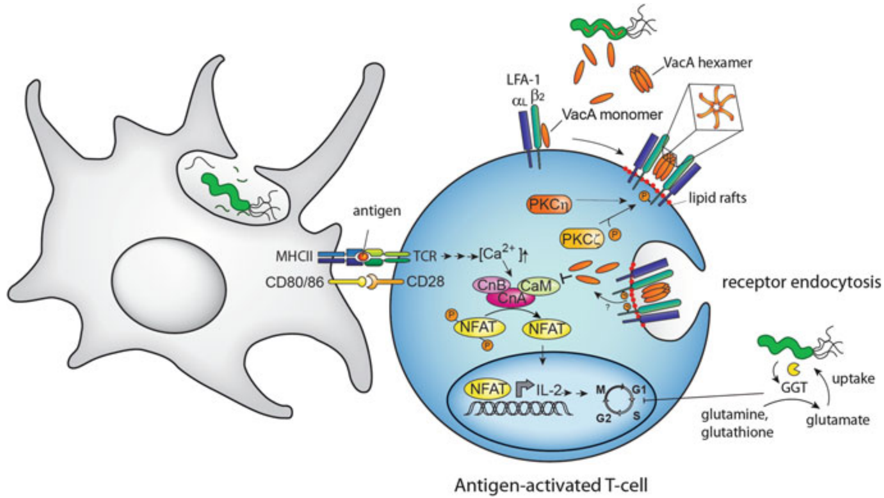

Fig. 12.3 *H. pylori* impairs T cell-mediated immunity through the production and secretion of VacA and GGT. All strains of *H. pylori* express the secreted virulence factors VacA and GGT to directly inhibit T cell activation, proliferation, and effector functions. Hexameric VacA binds to the β2 integrin subunit of the heterodimeric transmembrane receptor LFA-1; the receptor complex is internalized upon protein kinase C-mediated serine/threonine phosphorylation of the β2 integrin cytoplasmic tail. Cytoplasmic VacA prevents nuclear translocation of NFAT by inhibiting its dephosphorylation by the $Ca2^{+}$/calmodulin-dependent phosphatase calcineurin and thereby blocks IL-2 production and subsequent T cell activation and proliferation. GGT arrests T cells in the G1 phase of the cell cycle and thus prevents their proliferation. Note that the direct effects of VacA on T cells appear to be human specific. LFA-1, lymphocyte function-associated antigen-1; NFAT, nuclear factor of activated T cells; GGT, γ-glutamyl transpeptidase; CnA, B, calcineurin A and B subunits; CaM, calmodulin

(NFAT) (Boncristiano et al. 2003; Gebert et al. 2003). VacA activity on T cells requires the same N-terminal hydrophobic region that also mediates vacuolization (Sundrud et al. 2004) and binding to a surface receptor, the β2 integrin (CD18) (Sewald et al. 2008), which associates with CD11a/αL to form the heterodimeric lymphocyte function-associated antigen-1 (LFA-1) receptor (Fig. 12.3). VacA is taken up as LFA1 is recycled in a PKC-mediated, phosphorylation-dependent manner (Sewald et al. 2010). It seems that CD18 must be directly phosphorylated by either PKCη or PKCζ in its cytoplasmic tail to initiate VacA endocytosis and inhibition of NFAT target gene transactivation (Fig. 12.3) (Sewald et al. 2010). Murine T cells are resistant to VacA because they do not express the receptor (Sewald et al. 2008; Algood et al. 2007); rather, it appears that VacA promotes persistence in mice through a mechanism involving its interaction with DCs (see below) (Oertli et al. 2013). In humans, VacA's inhibitory activity on T cells likely prevents the clonal expansion of *H. pylori*-specific, antigen-activated T cells, thereby interfering effectively with a critical branch of adaptive immune defense against this infection.

In addition to VacA, all strains of *H. pylori* produce and secrete a second immunomodulatory molecule known to interfere with T cell proliferation, the gamma-glutamyl transpeptidase (GGT). GGT has enzymatic activity and catalyzes the transfer of the γ-glutamyl moiety of glutamine or glutathione to amino acids, allowing *H. pylori* to convert glutamine and glutathione into glutamate, which can be taken up and incorporated into the TCA cycle. *H. pylori* mutants lacking GGT fail to colonize mice (Chevalier et al. 1999; Oertli et al. 2013), and this phenotype has been linked to the ability of GGT to prevent T cell proliferation (Gerhard et al. 2005; Schmees et al. 2007) (Fig. 12.3). Other parameters of T cell activation (NFAT translocation, cytokine production) are not affected by GGT; its inhibitory effect on proliferation could be linked to cell cycle arrest in the G1 phase and to the enzymatic activity of GGT (Gerhard et al. 2005; Schmees et al. 2007). Similar to VacA, GGT also exerts strong immunomodulatory effects on DCs, which acquire tolerogenic activity upon exposure to GGT-proficient *H. pylori* or the recombinant protein (discussed in more detail below) (Oertli et al. 2013). The inclusion of both VacA and GGT in experimental *H. pylori* vaccines (Malfertheiner et al. 2008) indicates that the neutralization of *H. pylori*'s immunomodulatory properties is widely seen as a promising intervention strategy. Further details on GGT function can be obtained in Chaps. 6 and 7.

12.6 *H. pylori* Promotes Tolerogenic DC Functions and Induces Regulatory T Cells

DCs were long known mostly for their essential role in immunity to intracellular and extracellular pathogens, to which they contribute through their unique ability to prime naive T cells to differentiate, to proliferate, and to acquire effector functions such as cytotoxicity and cytokine production. It has become clear in recent years, however, that DCs are also critically involved in the development of immune tolerance to autoantigens, allergens, and harmless antigens of the commensal human microflora (Maldonado and von Andrian 2010; Yogev et al. 2012). A major pathway driving DC-mediated immune tolerance to self-, dietary, or environmental antigens involves the thymus-independent, "peripheral" induction of highly suppressive, "inducible" Tregs, which, like their thymus-derived "natural" counterparts, typically express the lineage-defining transcription factor FoxP3, the surface marker CD25, and an array of secreted and surface-exposed regulatory molecules that may include IL-10, TGF-β, CTLA-4, PD1, GITR, and others. Tregs efficiently block effector T cell responses by direct and indirect mechanisms, and their prolonged dysregulation results in severe and often fatal autoimmunity (Bollrath and Powrie 2013; Harrison and Powrie 2013). Persistent pathogens, such as *Mycobacterium tuberculosis* and certain helminths, have evolved to selectively recruit or activate Tregs to or in their preferred niche to promote chronicity (McBride et al. 2013; Ottenhoff 2012; Maizels and Smith 2011). The same is true

for *H. pylori*, which preferentially induces Tregs (Robinson et al. 2008) and relies on Treg-mediated immunosuppression to promote persistent infection (Arnold et al. 2011b) (also see above). Although difficult to study meaningfully in humans, the process of Treg induction and DC/Treg-mediated immune tolerance and suppression has received substantial attention lately and several key mechanisms have been elucidated. Multiple studies have shown in vitro and in vivo that *H. pylori* specifically targets DCs to promote their tolerogenic (i.e., Treg-inducing) properties and to at the same time subvert their immunogenic functions. Cultured murine and human DCs that have been exposed to and have phagocytosed live *H. pylori* fail to prime Th17 and Th1 responses and instead preferentially induce FoxP3 expression and suppressive activity in cocultured naive T cells (Kao et al. 2010; Kim et al. 2011; Oertli et al. 2012) (Fig. 12.4). This tolerogenic activity has been attributed to the failure of *H. pylori*-exposed DCs to mature, i.e., to express high levels of MHCII and co-stimulatory markers such as CD80, CD86, and CD40, as well as T helper cell-differentiating and T helper cell-activating cytokines such as IL-12 or IL-23 (Kaebisch et al. 2013; Oertli et al. 2012) (Fig. 12.4). This is true even in the presence of strong maturation signals delivered via TLR-4 or TLR-9 engagement, e.g., by *E. coli* LPS or CpG oligonucleotides (Oertli et al. 2012). The semi-mature DCs that result from *H. pylori* exposure are characterized by high expression of MHCII yet virtually no or low expression of co-stimulatory markers; similarly, these DCs fail to produce IL-12, IL-6, and TNF-α and instead secrete large amounts of the anti-inflammatory cytokine IL-10 (Oertli et al. 2012) (Fig. 12.4). Semi-mature and immature DCs have been implicated in Treg induction and tolerogenic immune responses (Maldonado and von Andrian 2010); indeed, *H. pylori*-exposed, semi-mature DCs are excellent inducers of Treg differentiation in vitro and in vivo (Kao et al. 2010; Oertli et al. 2012), and their forced maturation by LPS treatment is sufficient to break tolerance in vivo (Oertli et al. 2012). Consistent with the experimental results, the gastric mucosa of *H. pylori*-infected human carriers is populated by semi-mature DCs lacking co-stimulatory markers (Oertli et al. 2012). The *H. pylori* virulence and persistence factors required for the bacteria's tolerogenic activity on DCs are under intense investigation, whereas two secreted factors, the vacuolating cytotoxin VacA and the γ-glutamyl transpeptidase GGT, have been implicated in the inhibition of murine DC maturation and tolerogenic reprogramming (Kim et al. 2011; Oertli et al. 2012); the T4SS of *H. pylori* seems to be equally or more important in human DCs (Kaebisch et al. 2013).

Several studies have attempted to functionally address the contribution of (tolerogenic) DCs to immune tolerance and to *H. pylori*-specific immunity in experimental models (Kao et al. 2010). Interestingly, the depletion of $CD11c^+$ DCs in a genetic model taking advantage of the CD11c-driven expression of the diphtheria toxin receptor improves clearance of *H. pylori* upon challenge infection of vaccinated mice (Hitzler et al. 2011). Whereas prior vaccination with *H. pylori* extract in conjunction with either the gold standard cholera toxin adjuvant or a novel mycobacteria-derived adjuvant (CAF01) slashed *H. pylori* burdens by two orders of magnitude, this reduction could be further improved by another one to two

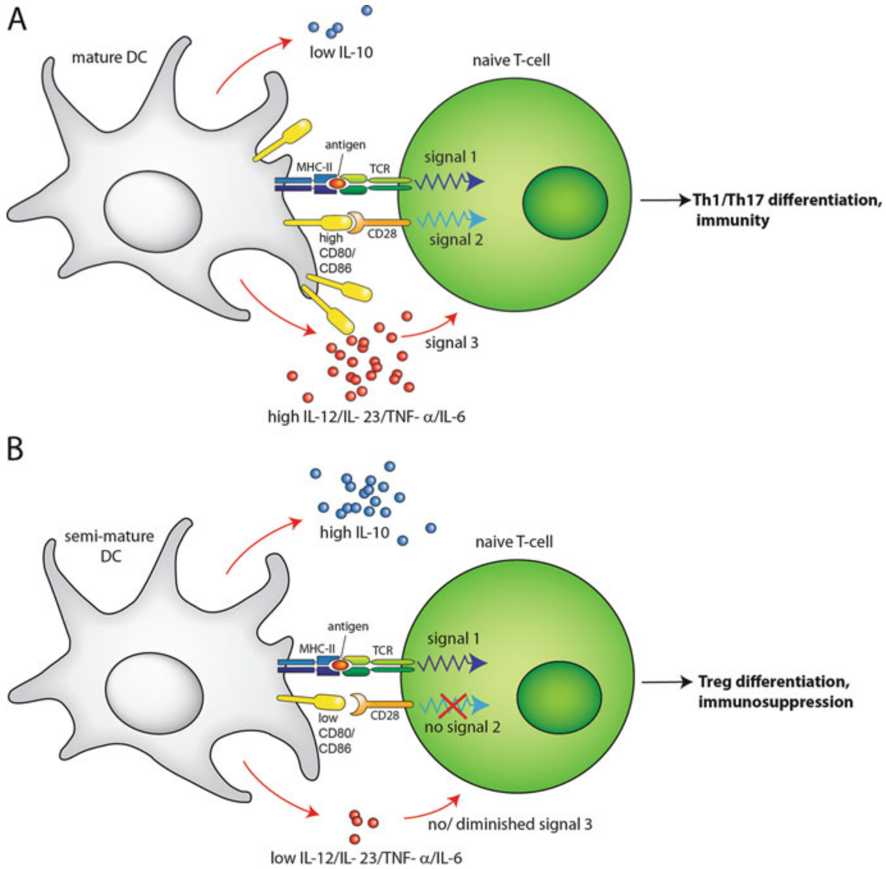

Fig. 12.4 The maturation status of dendritic cells (DCs) directs T helper cell differentiation. Immature DCs with high phagocytic activity follow chemokine gradients to sites of microbial colonization, where they actively sample antigen, which is then processed and presented in the context of MHC class II molecules on the cell surface. (**a**) Simultaneous stimulation of phagocytic DCs by recognition of PAMPs via cytosolic or membrane-bound pattern recognition receptors promotes DC maturation, which results in upregulation of maturation markers and co-stimulatory molecules (CD40, CD80, CD86) on the cell surface, as well as the production of T cell-differentiating and T cell-activating, as well as other pro-inflammatory cytokines (IL-12, IL-23, TNF-α, IL-6). Three signals (antigen recognition via the T cell receptor, co-stimulatory signals, and soluble cytokine signals) are required for the differentiation of naive T cells into Th1 or Th17 cells, with high levels of IL-12 driving Th1 and high levels of IL-23 driving Th17 differentiation. Both subsets are required for *H. pylori* control. (**b**) *H. pylori* exposure generates semi-mature DCs with high MHC class II expression but no or little co-stimulation and low levels of Th1/Th17 cell-differentiating cytokines, resulting in Treg differentiation and immunosuppression. An additional marker of semi-mature, tolerogenic DCs is their expression and secretion of IL-10

orders of magnitude by the depletion of DCs (Hitzler et al. 2011). Similar effects were obtained in experimental *H. pylori* infection models not involving prior immunization (Hitzler et al. 2011; Oertli et al. 2012). In both settings, the depletion

of DCs not only reduced bacterial burdens but also boosted all relevant correlates of (vaccine-induced) protective immunity, such as the recruitment of memory T cells, mast cells, and neutrophils to the gastric mucosa, the priming of *H. pylori*-specific Th1 and Th17 cells in the draining mesenteric lymph nodes, and the local production of protective cytokines including IFN-γ and IL-17 (Hitzler et al. 2011; Oertli et al. 2012). The results suggest that DCs are dispensible for immunity to *H. pylori* and instead are an essential element of immunoregulation, preventing control of the infection. Similar observations have been made in models of autoimmunity, in which the depletion of DCs invariably aggravates rather than improves disease severity (Yogev et al. 2012). A model of adoptive bone marrow-derived DC transfer into *H. pylori*-infected mice also showed that the transfer of DCs that had been exposed to *H. pylori* ex vivo, but not of naive DCs, efficiently induced Treg differentiation in vivo (Kao et al. 2010). Tregs induced in vivo upon Hp-DC transfer in turn suppressed anti-*H. pylori*-specific Th17 responses and their depletion or the depletion of IL-10 and/or TGF-β, promoted *H. pylori* clearance (Kao et al. 2010). All available in vivo data thus suggest that *H. pylori* effectively directs DCs to acquire tolerogenic properties, which drive Treg differentiation and anti-inflammatory cytokine production, suppress T-effector cell functions, and promote persistent infection (Fig. 12.4).

12.7 *H. pylori* Protects Against Allergic and Chronic Inflammatory Diseases Through the Induction of Treg-Mediated Immune Tolerance

Public health in developed countries has been dominated by two major trends since the second half of the twentieth century. The incidence of infectious diseases has declined sharply in that time frame, whereas immunological disorders such as multiple sclerosis (MS), inflammatory bowel disease (IBD), allergic asthma and other allergic diseases, and type I diabetes have dramatically increased in incidence over the same time period (Bach 2002). The incidence of infections with *H. pylori* has paralleled those of other infectious agents, with childhood acquisition rates dropping in the USA from >50 to 10 % between the beginning and the end of the twentieth century (Blaser and Falkow 2009). In line with the carcinogenic properties of *H. pylori* infection (Parsonnet et al. 1991), a beneficial effect of this trend has been the steady decline in gastric cancer rates and associated mortality in countries from which *H. pylori* has disappeared (Forman 2005). The downsides (some confirmed, some debated) of the loss of *H. pylori* in developed countries are (a) the increase in esophageal diseases such as esophagitis, Barrett's esophagus, and esophageal cancer with the latter having increased in incidence by over sixfold in the years from 1975 to 2000 alone (Pohl and Welch 2005) and (b) the increase in asthma and allergies (Eder et al. 2006). Numerous epidemiological studies have shown an inverse association of *H. pylori* infection with asthma and other allergies

with respiratory tract manifestations, which was particularly strong in children and adolescents and in individuals with early onset allergies and asthma (Amberbir et al. 2011; Blaser et al. 2008; Chen and Blaser 2007, 2008; Reibman et al. 2008). Meta-analyses examining 14 and 19 published studies on the topic have confirmed the general trend (Wang et al. 2013; Zhou et al. 2013). The chronic inflammatory skin disease atopic dermatitis/eczema has also been inversely linked to *H. pylori* infection in studies including over 3000 German schoolchildren and almost 2000 Japanese university students (Herbarth et al. 2007; Shiotani et al. 2008). This trend was recently confirmed in a longitudinal study conducted on over 1000 Ethiopian children (Amberbir et al. 2014). Similarly, *H. pylori* infection seems to confer protection against IBD, as suggested by a recent meta-analysis of 23 articles examining such a possible link (Luther et al. 2011). Only 27 % of IBD patients had evidence of infection with *H. pylori* compared to 41 % of patients in the control group (Luther et al. 2011). A large epidemiological survey conducted on 136,000 patients in the USA found a decreased risk of celiac disease in individuals infected with *H. pylori* (Lebwohl et al. 2013).

Following up on the various observational studies in human populations, possible protective effects of experimental *H. pylori* infection have been examined in animal models of allergic asthma and IBD (Fig. 12.5). In a murine model of allergic asthma induced by ovalbumin or house dust mite antigen sensitization and challenge, *H. pylori* infection conferred protection against the airway hyperresponsiveness, bronchoalveolar eosinophilia, lung inflammation, and goblet cell metaplasia that are hallmarks of asthma in humans and mice (Arnold et al. 2011a). The protective effects were evident in animals that had been experimentally infected during the neonatal period (Arnold et al. 2011a), i.e., at an age when humans typically contract the infection from their mothers (Weyermann et al. 2009). Asthma protection conferred by *H. pylori* was abolished by antibiotic eradication therapy prior to allergen challenge and depended critically on Tregs (Fig. 12.5); the systemic depletion of Tregs abrogated asthma protection, and pure populations of Tregs were sufficient to transfer protection from neonatally infected donors to naive recipients (Arnold et al. 2011a). These results are in line with the finding that neonatal infection with *H. pylori* induces Treg-mediated immune tolerance to the bacteria (Arnold et al. 2011b) and that children predominantly launch Treg responses to *H. pylori* infection (Harris et al. 2008). Experimental models of colitis in mice also confirm the trends seen in human IBD patients (Fig. 12.5); for example, *H. pylori* infection effectively reduced the Th17-driven colitis induced by *Salmonella typhimurium* infection (Higgins et al. 2010). The protective agent of *H. pylori* in colitis appears to be its DNA, which exhibits a strongly biased ratio of immunoregulatory-to-immunostimulatory sequences and – when administered orally – protects against the development of acute or chronic DSS-induced colitis (Luther et al. 2011). The protective effects of *H. pylori* DNA were attributed to its activity on DCs, which prevents IL-12 and type I IFN production and generally favors anti- over pro-inflammatory responses (Luther et al. 2011; Owyang et al. 2012). Overall, the combined results from observational

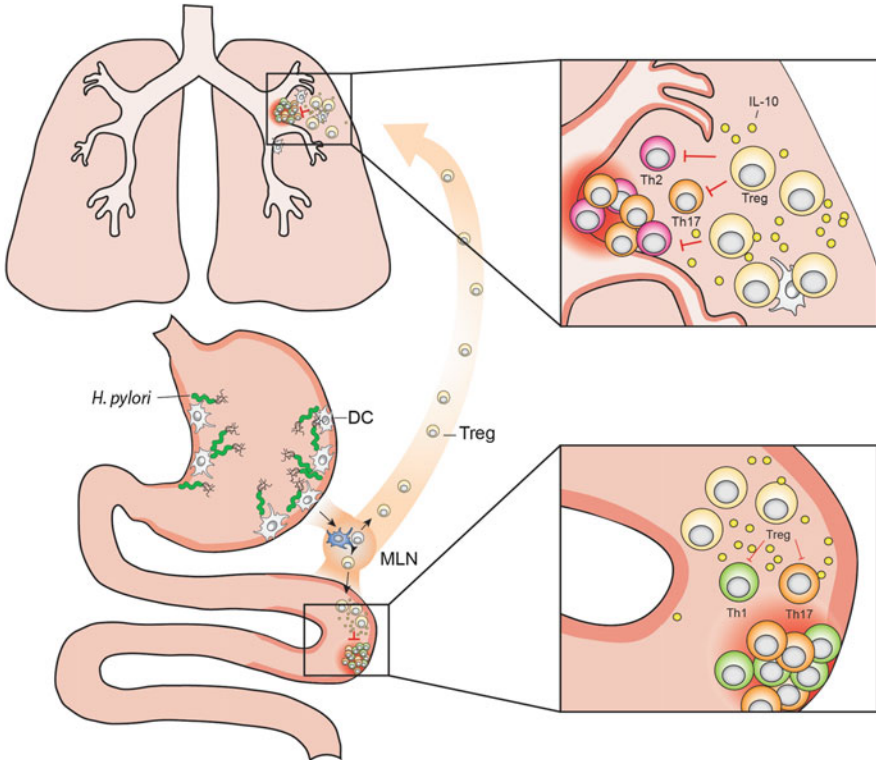

Fig. 12.5 *H. pylori* exerts systemic immunomodulatory effects. *H. pylori* exclusively inhabits the gastric mucosa, but has systemic immunomodulatory effects that manifest in the airways and lower gastrointestinal tract. Tissue-resident DCs can sample *H. pylori* antigens in the gastric mucosa and subsequently migrate to the stomach-draining and mesenteric lymph nodes (MLNs), where they prime T cell (in particular Treg) responses. Alternatively, soluble antigens can be transported via the lymph to the MLNs for presentation by resident DC populations. MLN-derived, *H. pylori*-specific Tregs enter the circulation and accumulate not only in the gastric mucosa but also at other mucosal surfaces of the body, such as those of the airways and lower bowel. According to current models, pathogenic effector T cell populations (allergen-specific Th17 and Th2 cells and colitogenic Th1 and Th17 cells) are suppressed by *H. pylori*-induced Tregs via soluble mediators (such as IL-10) and contact-dependent mechanisms. Abbreviations used: Th1/2/17, T helper cell subsets; Treg, regulatory T cell

studies in humans and interventional studies in mice suggest strongly that *H. pylori* infection can protect against experimentally induced allergic asthma and IBD; the protective mechanisms appear to involve Tregs and DCs, which are actively induced and "tolerized" by *H. pylori*, respectively. It is likely that the protection against allergic and chronic inflammatory diseases that is conferred by *H. pylori* is a by-product of its immunomodulatory activity, which allows the bacteria to suppress Th1 and Th17 responses and to establish and maintain persistent infection. Similar effects have been reported for other persistent pathogens including helminths and

Mycobacterium tuberculosis, which have been proposed to suppress allergen-specific immune responses in asthma through the induction of Tregs or through immune deviation (Obihara et al. 2007; Barlan et al. 2006). The potential use of microbial products and live attenuated bacteria or parasites in the treatment of allergies and IBD has received increasing attention lately, and some (especially helminth-derived) compounds are in clinical development (Torres et al. 2013). Whether *H. pylori* may be exploited in a similar fashion remains to be seen in the future.

12.8 Conclusions and Outlook

Despite having received substantial attention for three decades, several aspects of the *H. pylori*/host interaction remain poorly understood. Among them is the manipulation of innate and adaptive immunity by *H. pylori*, a feature that is central to the persistence of the bacteria and to the chronicity of *H. pylori*-associated diseases. Much attention has focused on gaining a better understanding of the prerequisites of successful vaccination aiming to prevent the primary infection (see Chap. 24), whereas less is known about the steady-state interaction of an established *H. pylori* infection with the host immune system. The asymptomatic carrier state in particular is vastly understudied, and the genetic and lifestyle parameters affecting the risk of developing *H. pylori*-associated gastric disease remain largely enigmatic. Although it is now clear that gastric infection-induced lesions are immunopathological in nature and at least in animal models can be attributed to the detrimental effects of T helper cells and their signature cytokines on the gastric mucosa, it appears likely that *H. pylori* toxins contribute to the mucosal damage. Experimental research into the pathomechanisms active during *H. pylori* infections is limited by the failure of most strains to colonize small rodents persistently and to the differences in gastric physiology and immunology that exist between humans and all currently used animal models (maybe with the exception of rhesus macaques). Future avenues of research in the field of *H. pylori* immunobiology will likely explore the relative contribution of direct (bacterially mediated) and indirect (immunopathological) effects to gastric carcinogenesis and will shed more light on the differential disease risk within and across human populations. Exciting new insights are expected as more elaborate model systems (such as gastric organoids, humanized mice, etc.) become available to the broader research community. Finally, future research directions will certainly take into account that *H. pylori* – beside its role as a gastric pathogen – is also an ancient member of our gastric microbiota that, together with other constituents of the microbiota of the gastrointestinal tract, has shaped the evolution of the human mucosal immune system.

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Chapter 13
The Role of Inflammatory Responses in Mouse Gastric Tumorigenesis

Hiroko Oshima, Mizuho Nakayama, and Masanobu Oshima

Abstract It has been established that chronic inflammation plays an important role in cancer development. The expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostaglandin biosynthesis, is induced in most cancer tissues and plays a key role in tumorigenesis. *Helicobacter pylori* infection causes atrophic gastritis, which is associated with the induction of COX-2 expression and its downstream product, prostaglandin E_2 (PGE_2), biosynthesis. Transgenic mice expressing COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in the gastric mucosa show the generation of an inflammatory microenvironment via the activation of the COX-2/PGE_2 pathway. Notably, simultaneous activation of canonical Wnt signaling and the COX-2/PGE_2 pathway causes intestinal-type gastric tumor development, although Wnt activation alone is not sufficient for tumor formation. These results suggest that *H. pylori* infection-associated chronic inflammation contributes to gastric tumorigenesis through activation of the COX-2/PGE_2 pathway. Using a gastric tumor mouse model (*Gan* mice), we found that the inflammatory microenvironment induces the activation of epidermal growth factor receptor (EGFR) signaling and promotes canonical Wnt signaling. Moreover, infiltrated macrophages express tumor necrosis factor-α (TNF-α) in gastric tumors, which plays an important role in tumor promotion through the induction of NADPH oxidase organizer 1 (NOXO1) expression. NOXO1 contributes to the production of reactive oxygen species (ROS) by the NOX1 complex, which is thought to be important for the maintenance of stem cell properties. These studies indicate that chronic inflammation promotes gastric tumorigenesis through a variety of mechanisms. Accordingly, targeting an inflammatory microenvironment should be an effective therapeutic or preventive strategy for gastric cancer.

Keywords Gastric cancer • Inflammation • COX-2 • PGE_2 • EGFR • TNF-α • Wnt signaling

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13.1 Introduction

It has been reported that the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of developing gastrointestinal cancer (Thun et al. 1991). The target molecules of NSAIDs are cyclooxygenase (COX)-1 and COX-2, the rate-limiting enzymes for prostaglandin biosynthesis. COX-2 expression is induced in inflammatory lesions and cancer tissues, whereas COX-1 is constitutively expressed in variety of organs. COX-2-derived PGE_2 plays a key role in both inflammation and cancer development, while COX-1-dependent PGE_2 plays a housekeeping role such as protection of gastrointestinal mucosa. We previously demonstrated that COX-2 expression is induced predominantly in stromal cells including fibroblasts of human and mouse benign intestinal polyps (Oshima et al. 1996; Sonoshita et al. 2002). Moreover, disruption of the genes encoding COX-2 or the one of four PGE_2 receptors (called EP2) in *Adenomatous polyposis coli* (*Apc*) gene knockout mice ($Apc^{\Delta 716}$ mice) resulted in significant suppression of intestinal polyposis (Oshima et al. 1996; Sonoshita et al. 2001). Number of intestinal polyps in the COX-2 gene-disrupted $Apc^{\Delta 716}$ mice decreased to about 20 % of the COX-2 wild-type $Apc^{\Delta 716}$ mice. These results indicate that COX-2/PGE_2 signaling through the EP2 receptor plays a key role in intestinal tumor development. In the intestinal tumors of $Apc^{\Delta 716}$ mice, PGE_2 signaling contributes to angiogenesis by inducing the expression of angiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Seno et al. 2002). It has also been reported that PGE_2 signaling activates peroxisome proliferator-activated receptor δ (PPARδ) in the intestinal tumors of another *Apc* gene-mutant mice (Apc^{Min} mice), resulting in suppression of the apoptosis of tumor cells (Wang et al. 2004). They also demonstrated that treatment of Apc^{Min} mice with PGE_2 caused significant promotion of intestinal tumor development. Moreover, it has been shown that PGE_2 signaling induces DNA methylation in intestinal tumor epithelial cells, which can contribute to tumorigenesis by silencing expression of tumor suppressor genes (Xia et al. 2012). Recently, it has been demonstrated that PGE_2 induces expression of chemokine ligands with CXC motifs, CXCL1 and CXCL2, which further recruit myeloid-derived suppressor cells (MDSCs) to intestinal tumors. MDSCs then suppress antitumor immunity, resulting in the promotion of inflammation-associated tumorigenesis in colon (Katoh et al. 2013). Taken together, these findings indicate that the COX-2/PGE_2 pathway promotes intestinal tumorigenesis through a variety of mechanisms.

In contrast to colon cancer, the role of the COX-2/PGE_2 pathway in gastric tumorigenesis is still not fully understood, although COX-2 expression is widely found in gastric cancer tissues (Saukkonen et al. 2001). Since *Helicobacter pylori* infection induces COX-2 expression and PGE_2 production, it is possible that the COX-2/PGE_2 pathway is one of the important mechanisms underlying *H. pylori* infection-induced gastric tumorigenesis. To investigate the mechanism(s) by which the COX-2/PGE_2 pathway is involved in gastric tumorigenesis, we constructed a

gastric tumor mouse model, *Gan* (Gastric neoplasia) mice. *Gan* mice develop intestinal-type gastric tumors in the glandular stomach by the simultaneous activation of canonical Wnt signaling and the COX-2/PGE_2 pathway in gastric mucosa (Oshima et al. 2006, 2009; Oshima and Oshima 2010). Although genetic alterations that activate Wnt signaling are not common in human gastric cancer cells (The Cancer Genome Atlas Research Network 2014), activation of the Wnt signaling is found in more than 50 % of cases suggesting a role of Wnt signaling in gastric tumorigenesis (Oshima et al. 2006). Wnt signaling plays an important role in maintenance of epithelial stem cells in undifferentiated status by increasing "stemness." In this chapter, we will review the role of COX-2/PGE_2-associated inflammation in gastric tumorigenesis, which was uncovered using the *Gan* mouse model.

13.2 The Development of a Gastric Tumor Model: *Gan* Mice

The eradication of *H. pylori* results in a significant reduction of COX-2 expression and improvement of gastric atrophy, indicating that *H. pylori* infection induces the COX-2/PGE_2 pathway in the gastric mucosa (Wambura et al. 2004). On the other hand, β-catenin accumulation was detected in about 50 % of gastric cancers (Oshima et al. 2006). In the Wnt signaling-activated epithelial cells, β-catenin is stabilized in cytoplasm and translocated to nuclei-forming complex with T-cell factor (TCF), which leads to induction of Wnt-target gene expression. Based on this information, we constructed compound transgenic mice, *K19-Wnt1/C2mE* mice (*Gan* mice), that express Wnt1, COX-2, and mPGES-1 simultaneously in gastric epithelial cells using the cytokeratin 19 gene (Krt19) promoter (Oshima et al. 2006, 2009; Oshima and Oshima 2010). K19 promoter is transcriptionally active in gastric epithelial cells including undifferentiated epithelia (Brembeck et al. 2001; Oshima et al. 2004). Wnt1 is a ligand for the Frizzled receptor that activates canonical Wnt signaling, resulting in acquisition of stem cell property. An inducible PGE-converting enzyme, mPGES-1, is expressed both in inflamed and cancer tissues together with COX-2, resulting in increased levels of PGE_2. Accordingly, Wnt signaling and the COX-2/PGE_2 pathways are activated simultaneously in the *Gan* mouse gastric mucosa (Oshima et al. 2006, 2009; Oshima and Oshima 2010). The activation of Wnt signaling alone in *K19-Wnt1* mice that express only Wnt1 in gastric epithelial cells causes the development of small preneoplastic lesions consisting of dysplastic epithelial cells in the gastric mucosa, suggesting a role of Wnt signaling activation in initiation of gastric tumorigenesis (Oshima et al. 2006) (Fig. 13.1). On the other hand, the induction of the COX-2/PGE_2 pathway in the stomach in *K19-C2mE* mice results in the infiltration of inflammatory cells including macrophages into the gastric mucosa and the development of mucous cell metaplasia (Oshima et al. 2004). Consistently, induction of COX-2/PGE_2 signaling

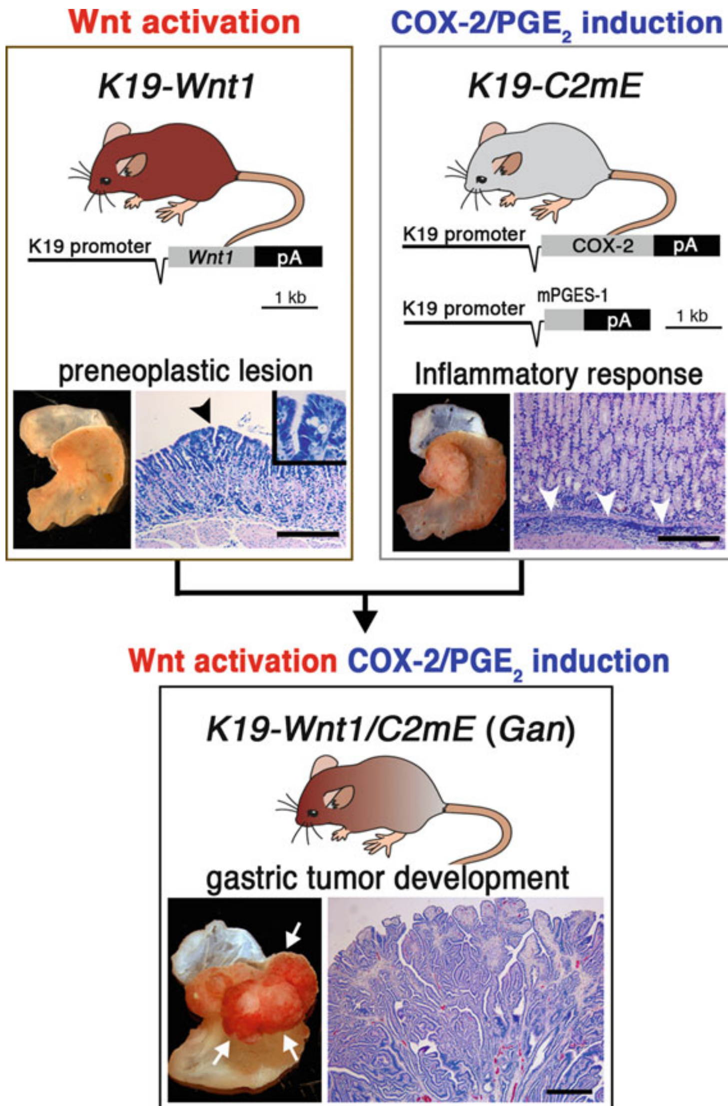

Fig. 13.1 Transgenic mouse models of gastric tumorigenesis. The transgenic vectors and representative macroscopic and microscopic photographs of the stomach are shown for each *transgenic line*. *K19-Wnt1/C2mE* (*Gan*) mice are compound transgenic mice bred from *K19-Wnt1* to *K19-C2mE* mice that express Wnt1 and COX-2/mPGES-1, respectively. The *arrowhead* and inset in the *K19-Wnt1* figure show a preneoplastic lesion initiated by Wnt signaling activation. *The arrowheads* in the *K19-C2mE* figure show PGE_2-induced inflammatory infiltration to submucosal. The *arrows* in *K19-Wnt1/C2mE* indicate gastric tumors. The *bars* indicate 100 μm (Reproduced from Oshima et al. Cancer Sci, 2009 with permission from Wiley-Blackwell)

in intestinal mucosa causes activation of infiltrated macrophages, which contributes to generation of inflammatory microenvironment (Wang et al. 2014). Because COX-2/PGE_2 pathway is activated in most of gastrointestinal cancer tissues, it is possible that COX-2/PGE_2 signaling is responsible for elicitation of inflammatory responses in these tumors. Importantly, simultaneous activation of Wnt signaling and the COX-2/PGE_2 pathway in the *Gan* mice causes intestinal-type gastric tumor development with 100 % incidence (Oshima et al. 2006) (Fig. 13.1). These results indicate that the cooperation of Wnt signaling activation and COX-2/PGE_2 pathway-induced inflammatory responses causes gastric tumor development. Microarray analyses indicated that the gene expression profiles of *Gan* mouse tumors are similar to those of human intestinal-type gastric cancer (Itadani et al. 2009). Accordingly, *Gan* mice recapitulate human intestinal-type gastric cancer from the molecular mechanism to the characteristics of tumor histopathology and gene expression profiles.

13.3 Tumor-Associated Macrophages in *Gan* Mouse Gastric Tumors

Macrophages in tumor tissues are called as "tumor-associated macrophages" or TAMs, and accumulating evidence has indicated that TAMs play an important role in cancer development and malignant progression (Pollard 2009; Qian and Pollard 2010). In the *Gan* mouse gastric tumors, macrophage-trophic CC motif chemokines, CCL2 and CCL8, are induced, and macrophages are accumulated in gastric tumor stroma. These macrophages are activated and express inflammatory cytokines including TNF-α and the interleukins IL-1β and IL-6 (Oshima et al. 2011a). Expression of CCL2 and CCL8 and macrophage infiltration into the gastric mucosa are also found in the gastritis tissues of COX-2/mPGES-1 transgenic (*K19-C2mE*) mice (Oshima et al. 2004). Accordingly, it is possible that PGE_2-signaling plays a key role in TAM recruitment in gastric mucosa. Interestingly, when *Gan* mice were maintained under germfree conditions, the inflammatory responses and macrophage infiltration were significantly suppressed, although COX-2/PGE_2 pathway is continuously activated (Oshima et al. 2011a). Moreover, gastric tumorigenesis was also suppressed in germfree *Gan* mice. These results indicate that activation of COX-2/PGE_2 pathway is not sufficient for generation of inflammatory microenvironment, but both COX-2/PGE_2 pathway and innate immune response to bacterial infection are required. Moreover, such microenvironment is important for promotion of tumor development.

Bacterial infection is recognized by toll-like receptors (TLRs) that activate innate immune responses. It has been shown that disruption of the *Tlr2*, *Tlr4*, or *Myd88* gene encoding the toll-like receptors TLR2 and TLR4 and the adapter protein Myd88, respectively, results in significant suppression of the repair of the injured intestinal mucosa, indicating that the innate immune response through

TLR signaling plays an important role in intestinal mucosal homeostasis (Rakoff-Nahoum et al. 2004). Furthermore, disruption of *Myd88* gene in Apc^{Min} mice as well as chemically induced hepatocellular carcinoma (HCC) mouse model suppressed tumor development in these models, along with suppression of COX-2 and cytokine expression (Rakoff-Nahoum and Medzhitov 2007; Naugler et al. 2007). Recently, several reports have shown a role for bacterial infection in intestinal tumorigenesis. For example, *Fusobacterium* infection promotes Apc^{Min} mouse intestinal tumorigenesis by inducing inflammatory responses, and IL10$^{-/-}$ mice showed more severe intestinal inflammation and a tumor phenotype when treated with the chemical mutagen, azoxymethane (Allen-Veroce and Jobin 2014). Accordingly, it is possible that the innate immune responses to bacterial infection mediated by TLR/Myd88 signaling are also important for gastric mucosal homeostasis and tumorigenesis. Epidemiological studies also showed that polymorphisms in TLR4 gene were associated with increased risk of gastric cancer development, suggesting a role of innate responses in gastric tumorigenesis (Hold et al. 2007; El-Omar et al. 2008).

In the *Gan* mouse tumor tissues, macrophages are activated by innate immune responses, and these express cytokines, chemokines, and growth factors, which contribute to tumor cell proliferation. Macrophage depletion in *Gan* mouse tumors by treatment with clodronate liposomes resulted in the induction of gastric tumor cell apoptosis (Oshima et al. 2011a). The depletion of macrophages in $Apc^{\Delta 716}$ mice by crossing them with colony stimulating factor 1 gene (*Csf1*) mutant mice (*op*/*op* mice: a mutant strain that shows decreased mature macrophages in peripheral tissues because of *Csf1* gene mutation) also resulted in significant suppression of intestinal tumorigenesis (Oguma et al. 2008). Accordingly, it is possible that the innate immune response triggers macrophage infiltration into the gastric mucosa, which promotes tumor development.

Importantly, it has been established the role of innate immunity also in epithelial cells in tumorigenesis. Expression of TLR2 in gastric tumors of *Gan* mice and another gastric tumor model, gp130$^{F/F}$ mice, was increased significantly. Gp130 is a co-receptor of IL-6 and IL-11, and gp130$^{F/F}$ mutation causes constitutive activation of Stat3 signaling. Importantly, disruption of *Tlr2* in gp130$^{F/F}$ mice resulted in significant suppression of gastric tumorigenesis, indicating a role of TLR2 signaling in tumor formation (Tye et al. 2012). More recently, it has been demonstrated that TLR2-MyD88 signaling in the intestinal stem cells is required for maintenance of stemness (Scheeren et al. 2014). Accordingly, it is possible that innate immune responses in macrophages promote tumorigenesis through generation of inflammatory microenvironment, whereas those in epithelial cells also accelerate tumor development by acquirement of stemness.

13.4 The Mechanisms by Which Inflammation Promotes Tumor Formation

13.4.1 EGFR Signaling Activation

cDNA microarray analyses of *Gan* mice and other transgenic mouse lines indicated that the expression of ligands for the epidermal growth factor receptor (EGFR), such as amphiregulin, epiregulin, and heparin-binding EGF-like growth factor (HB-EGF), is significantly upregulated in both *Gan* mouse gastric tumors and in *K19-C2mE* mouse gastritis tissues, indicating that these EGFR ligands are induced via a COX-2/PGE_2-associated inflammation-dependent mechanism (Oshima et al. 2011b). Importantly, members of the ADAM (a disintegrin and metalloproteinase) family proteases (ADAM8, ADAM9, and ADAM10) are also upregulated in both *Gan* mouse tumors and *K19-C2mE* mouse gastritis. These ADAM family members are important for tumorigenesis through the activation of EGFR signaling by shedding from membrane-bound forms of precursor EGFR ligands (Mochizuki and Okada 2007). Accordingly, one of the mechanisms by which PGE_2-associated inflammation promotes tumor formation is the activation of EGFR signaling through the simultaneous induction of EGFR ligands and ADAM proteases, which leads to acceleration of tumor cell proliferation (Fig. 13.2). Consistently, treatment of *Gan* mice with an EGFR inhibitor significantly suppressed gastric tumorigenesis, and combination treatment with an EGFR inhibitor and a COX-2 inhibitor resulted in complete regression of gastric tumors (Oshima et al. 2011b). These results suggest that such combination treatment may be an effective strategy to prevent the development of inflammation-associated gastric cancer or to treat existing gastric cancer.

13.4.2 Wnt Signaling Activation

Wnt signaling is important for the stem cell properties of epithelial cells, and thus, constitutive activation of Wnt signaling causes gastrointestinal tumorigenesis. It has been shown that β-catenin accumulation, a hallmark of Wnt signaling activation, is significantly increased in the invasion front and metastasized tumor cells of human colon cancer, suggesting that increased Wnt signaling activation leads to the malignant progression of colon cancer (Fodde and Brabletz 2007). In the gastric preneoplastic lesions of the *K19-Wnt1* mice that express Wnt signaling ligand Wnt1 in gastric epithelial cells, macrophages are infiltrated into the stroma surrounding dysplastic epithelial cells, and β-catenin nuclear accumulation is enhanced in dysplastic epithelial cells (Oguma et al. 2008). These results suggest that epithelial Wnt signaling activity in preneoplastic lesions is enhanced by infiltrated macrophages. Activated macrophages express variety of inflammatory cytokines including TNF-α, IL-1β, and IL-6. Importantly, stimulation of gastric cancer cells with the

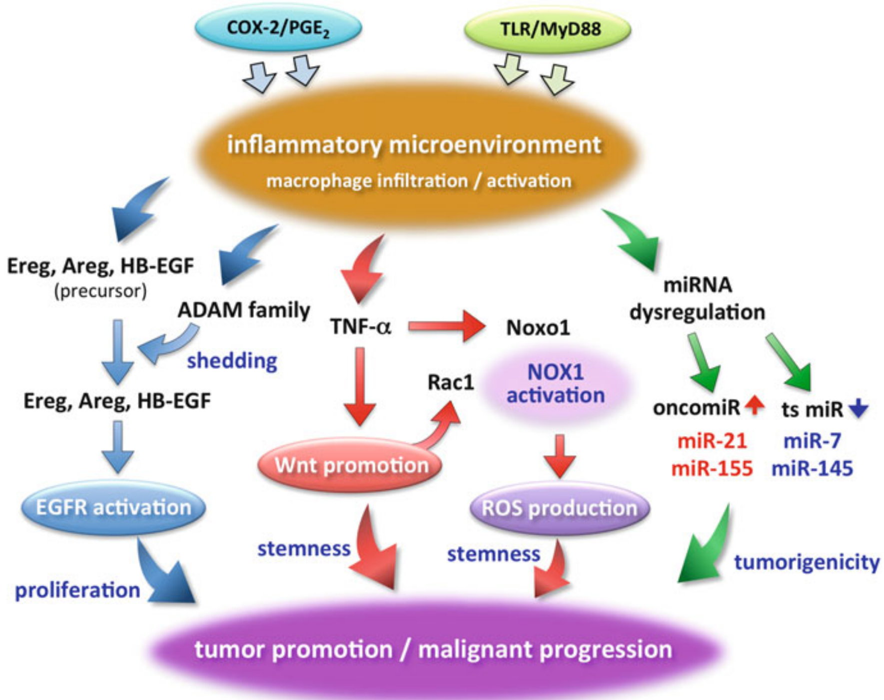

Fig. 13.2 Possible mechanisms by which the inflammatory microenvironment promotes gastric tumorigenesis. COX-2/PGE$_2$ pathway and TLR/Myd88 signaling cooperatively generate inflammatory microenvironment by recruitment of macrophages and their activation. Such microenvironment promotes tumorigenesis through a variety of mechanisms, including EGFR activation (*left*), Wnt signaling promotion (*center left*), ROS production (*center right*), and modulation of oncomiR and tumor suppressor microRNA expression (*right*)

conditioned medium from lipopolysaccharide (LPS)-treated activated macrophages caused increased Wnt signaling activity, which was mimicked by treating cells with TNF-α (Oguma et al. 2008). Moreover, the infection of the stomachs of *K19-Wnt1* mice with *Helicobacter felis* resulted in tumor development with increased Wnt signaling activity in the tumor epithelial cells. These results indicate that activated macrophages in tumor microenvironment promote the Wnt activity level of tumor epithelial cells via TNF-α expression, which further contributes to tumor development and progression (Fig. 13.2) (Oshima et al. 2004; Oguma et al. 2010).

In the inflammatory environment, macrophages express not only TNF-α but also a variety of cytokines. Notably, other inflammatory cytokines have also been shown to promote gastrointestinal tumorigenesis. For example, transgenic expression of IL-1β in the mouse stomach leads to gastritis and gastric tumor development that correlates with the recruitment of myeloid-derived suppressor cells (Tu et al. 2008). Moreover, it has been reported that activation of Stat3, a transcription factor that

plays an important role in inflammatory responses, in intestinal epithelial cells promotes colitis-associated tumorigenesis in mouse models (Bollrath et al. 2009; Grivennikov et al. 2009). Stat3 is activated by IL-6 and IL-11 signaling through their co-receptor gp130. Although IL-6 has shown to be responsible for Stat3-dependent tumor promotion in these studies, it has been shown recently that IL-11 has a strong correlation with elevated Stat3 activation in gastrointestinal cancers, and IL-11 has a prominent role during the progression of colon and gastric cancers (Putoczki et al. 2013). Therefore, it is possible that the pro-inflammatory cytokine network comprising TNF-α, IL-1β, IL-6, and IL-11 in the tumor microenvironment promotes gastrointestinal tumorigenesis through a variety of mechanisms.

13.4.3 Noxo1 Expression and ROS Production

A polymorphism at the −308 position of the TNF-α gene promoter, the TNF-α-308A allele, is associated with an increased risk of gastric cancer, with an odds ratio of 1.9, suggesting a role for TNF-α in gastric tumorigenesis (El-Omar et al. 2002; Machado et al. 2003). To further examine the role of TNF-α in gastric cancer development other than via Wnt activation, we crossed *Gan* mice with the TNF-α gene (*Tnf*) knockout mice and examined the gastric phenotype. Importantly, gastric tumor development was significantly suppressed in $Tnf^{-/-}$ *Gan* mice, and the mean gastric tumor volume in $Tnf^{-/-}$ *Gan* mice was decreased to 18 % of that in $Tnf^{+/+}$ *Gan* mice (Fig. 13.3) (Oshima et al. 2014). The major source of TNF-α in the tumor tissue is bone marrow-derived cells (BMDCs), including macrophages. Bone marrow transplantation from $Tnf^{+/+}$ mice into $Tnf^{-/-}$ *Gan* mice restored the gastric tumor phenotype, with significant infiltration of BMDCs in the tumor stroma (Oshima et al. 2014). These results indicate that TNF-α signaling activation in BMDCs, possibly macrophages, is important for gastric tumor promotion.

By cDNA microarray analyses using $Tnf^{+/+}$ *Gan* and $Tnf^{-/-}$ *Gan* mouse tumors, about 150 genes were selected as candidate TNF-α-dependent tumor-promoting genes. Interestingly, characteristic genes for intestinal stem cells, including CD44 a cell-surface glycoprotein and known as a receptor for hyaluronic acid, Prom1, Sox9, and EphB3 (Itzkovitz et al. 2012), were included in the gene list. These genes are expressed in intestinal stem cells, and some of their products have shown to play a role in maintenance of stem cell property. Accordingly, it is possible that TNF-α plays an important role in the maintenance tumor cells in undifferentiated status. We thus compared TNF-α-dependent genes with the list of Lgr5-expressing gastric stem cell-specific genes (Barker et al. 2012) and found Noxo1 that is upregulated in both tumor cells and normal stem cells. Noxo1 is a NADPH oxidase (NOX)-organizing protein 1, which is a component of the NOX1 complex, which generates reactive oxygen species (ROS) (Adachi et al. 2008). It has been shown that Nox1 expression is required for the transformation of Ras-activated cancer cells, and Noxo1 is upregulated in colon cancers (Mitsushita et al. 2004; Juhasz et al. 2009). Moreover, it has been reported that the small Rho GTPase Rac1,

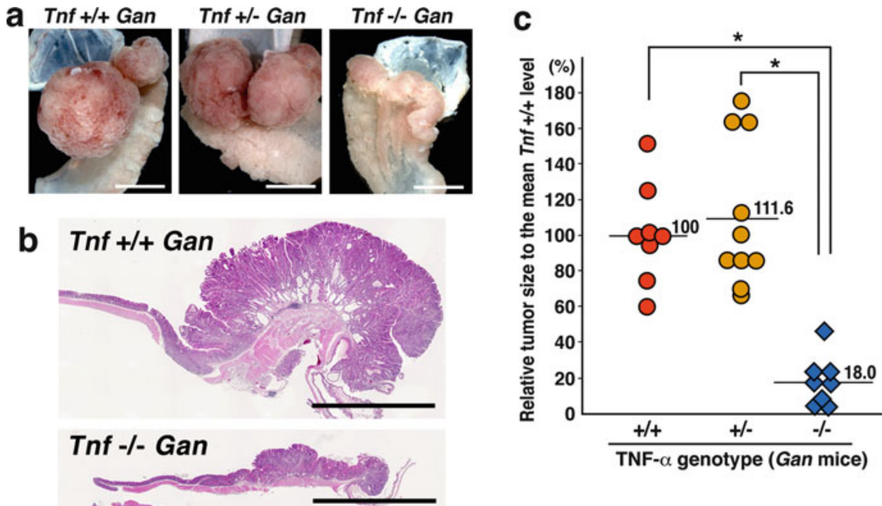

Fig. 13.3 Suppression of gastric tumor development in *Gan* mice by TNF-α gene (*Tnf*) disruption. **a** Representative macroscopic photographs of *Tnf* $^{+/+}$, *Tnf* $^{+/-}$, and *Tnf* $^{-/-}$ *Gan* mouse gastric tumors at 50 weeks of age. The *bars* indicate 5 mm. **b** Representative histological photographs of the whole views of *Tnf* $^{+/+}$ *Gan* (*top*) and *Tnf* $^{-/-}$ *Gan* mouse (*bottom*) gastric tumors. Note that gastric tumor development was significantly suppressed by disruption of TNF-α gene. **c** The gastric tumor size of the *Tnf*$^{+/+}$ *Gan*, *Tnf* $^{+/-}$ *Gan* and *Tnf* $^{-/-}$ *Gan* mice relative to the mean size of *Tnf* $^{+/+}$ *Gan* mouse tumors (set at 100 %). *Asterisks*, $P < 0.05$ (Reproduced from Oshima et al. Oncogene, 2014 with permission from Nature Publishing Group)

another component of the Nox1 complex, is activated by cooperation of Wnt signaling and NF-κB activation in *Apc*^{Min} mouse intestinal tumor cells, and activated Rac1 promotes ROS production of the NOX1 complex (Myant et al. 2013). Increased ROS level by NOX1 complex has shown to be important for the stem cell property of intestinal tumor cells. Taken together, these results suggest that TNF-α signaling activates the NOX1 complex through induction of Noxo1 expression, resulting in increased ROS production, which contributes to the maintenance of tumor cells in undifferentiated status (Fig. 13.2).

On the other hand, it has been shown that a variant form of CD44 (CD44v) is induced in *Gan* mouse gastric tumor cells by a PGE_2-dependent mechanism (Ishimoto et al. 2010), and CD44v is required for gastric tumorigenesis by the suppression of ROS production by glutathione synthesis (Ishimoto et al. 2011). Consistently, CD44 gene disruption in *Gan* mice resulted in significant suppression of the gastric tumorigenesis. Accordingly, it is possible that strict regulation of the ROS level is important for the stemness and survival of tumor cells, i.e., a basal ROS level is required for stemness, but a high ROS level is toxic and decreases tumor cell survival. However, the precise mechanisms underlying the involvement of ROS in tumor development are still being investigated.

13.4.4 Other Mechanisms, MicroRNA

In *Gan* mouse gastric tumor tissues, expression of vascular endothelial growth factor (VEGF) is increased significantly and angiogenesis is enhanced. We found that VEGF expression is induced in stromal fibroblasts of *Gan* mouse tumor tissues and that tumor cell-derived factors stimulate bone marrow-derived stromal cells to express VEGF (Guo et al. 2008). It is possible that inflammatory signaling is involved in angiogenesis in tumors.

It is known that expression of some microRNAs is affected by inflammatory responses through activation of NF-κB or Stat3. MicroRNAs are single-stranded small noncoding RNAs that regulate gene expression by posttranscriptional interference of specific mRNAs (Ambros 2004). Interestingly, several oncogenic microRNAs, such as miR-21 and miR-155, are upregulated in both *Gan* mouse gastric tumors and *K19-C2mE* gastritis tissues, suggesting that these microRNAs are induced by inflammation-dependent manner (Kong et al. 2012). On the other hand, tumor suppressor microRNAs, such as miR-7 and miR-145, are downregulated in both *Gan* mouse tumors and *K19-C2mE* gastritis, suggesting inflammation-dependent downregulation. Expression of miR-7 induces differentiation of epithelial cells by inhibition of EGFR expression. Thus, downregulation of miR-7 contributes to maintenance of epithelial cells in undifferentiated status. Accordingly, it is possible that inflammatory responses promote gastric tumorigenesis by both inducing oncogene microRNAs (oncomiRs) and suppressing the expression of tumor suppressor microRNAs (ts miRs) (Fig. 13.2).

13.5 Conclusions and Outlook

H. pylori infection induces chronic gastritis, which further induces expression of COX-2 and mPGES-1, resulting in enhanced PGE_2 biosynthesis in the gastric mucosa. *Gan* mice develop inflammation-associated gastric tumors by simultaneous activation of Wnt signaling and COX-2/PGE_2 pathway, which recapitulates molecular mechanisms of human gastric tumorigenesis. Wnt signaling activation alone induces only limited preneoplastic lesions. Accordingly, *H. pylori*-induced COX-2/PGE_2 pathway plays an essential role in gastric tumorigenesis through generation of inflammatory microenvironment. Using *Gan* mouse model, it has been demonstrated that both COX-2/PGE_2 pathway and innate immune responses are cooperatively responsible for the generation of an inflammatory microenvironment with macrophage infiltration and cytokine expression. Such a microenvironment promotes gastric tumorigenesis through the activation of EGFR signaling that causes increased proliferation, promotion of Wnt signaling activation, and enhancement of NOX1 complex-dependent ROS production. Wnt activation and ROS production are important for stemness and thus contribute to maintenance of tumor cells in undifferentiated status. These results strongly suggest that targeting

the COX-2/PGE_2 pathway-dependent inflammatory microenvironment will be an effective therapeutic and preventive strategy for gastric cancer.

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Chapter 14
Influence of Host Gene Polymorphisms on Development of Gastroduodenal Diseases

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Abstract *Helicobacter pylori* infection remains the commonest chronic bacterial infection in the world and is associated with a variety of clinical outcomes that range from simple asymptomatic gastritis to more serious conditions such as peptic ulcer disease and gastric cancer. The key determinants of these outcomes are the severity and distribution of *H. pylori*-induced gastritis. Host genetic factors play an important role in influencing disease risk, but identifying candidate genes is a major challenge that has to stem from a profound understanding of the pathophysiology of the disease. In the case of *H. pylori*-associated disease, the initial search focused on candidate genes that attenuate gastric physiology and lead to a destructive chronic inflammatory response against the infection. In particular, certain cytokine and innate immune response gene polymorphisms appear to influence risk of gastric cancer and its precursor conditions. More recent genome-wide association studies have identified novel genetic markers that show impressive associations with gastric cancer risk but whose function remains unclear. Very recently, there has been progress in identifying genetic risk markers for acquisition of *H. pylori* infection, but there remains a lack of suitable markers for risk of peptic ulcer disease. Future research agenda should focus on identifying the full genetic risk profile for *H. pylori*-induced gastroduodenal disease. This will help target the population most at risk by directing eradication therapy and closer follow-up to the affected individuals.

Keywords *Helicobacter pylori* • Host genetics • Genetic polymorphisms • Gastric cancer • MALT lymphoma • Peptic ulcer disease • Chronic inflammation

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14.1 Introduction

Helicobacter pylori (*H. pylori*) is associated with a variety of clinical outcomes that range from simple asymptomatic gastritis to serious diseases such as peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. The basic pathogenesis of *H. pylori*-induced disease is the establishment of chronic gastritis. The severity and distribution of this gastritis determines the clinical outcome. There are three main gastric phenotypes that result from chronic *H. pylori* infection: (1) the commonest by far is a mild pangastritis that does not affect gastric physiology and is not associated with significant human disease; (2) a corpus-predominant gastritis associated with gastric atrophy, hypochlorhydria and increased risk of gastric cancer (the gastric cancer phenotype) (El-Omar et al. 1997); and (3) an antral-predominant gastritis associated with high gastric acid secretion and increased risk of duodenal ulcer disease (the DU phenotype) (El-Omar et al. 1995). What is most curious is that the DU outcome is actually protective against the gastric cancer outcome, and this conundrum needs to be explained.

There is accumulating evidence that acid secretory capacity is crucial in determining the distribution and natural history of *H. pylori* infection (El-Omar et al. 1995, 1997; Amieva 2008). In hosts with low secretory capacity (genetically determined or secondary to pharmacologic inhibition), the organism is capable of colonising a wider niche than would be possible in the presence of high volumes of acid. Colonisation of a wider niche, including the corpus mucosa, leads to corpus gastritis with resultant functional inhibition of acid secretion. This inhibition is mediated by *H. pylori*-induced inflammatory cytokines such as the interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), and the net effect is the establishment of a more aggressive gastritis that accelerates the development of gastric atrophy. Once atrophy develops, acid secretion is not only attenuated by the functional inhibition caused by inflammatory mediators but by a more permanent morphological change that is harder to reverse. This situation is very relevant to the subgroup of humans who develop the gastric cancer phenotype in the presence of chronic *H. pylori* infection.

The inflammatory response to *H. pylori* infection is modulated by cofactors, including bacterial and host genes, and environmental factors. In this chapter, we review the evidence for the role of host genetic factors in determining the risk of gastroduodenal disease. We start by discussing the known host genetic factors involved in acquiring the infection. We then discuss the published literature on host genetic factors that predispose to specific disease outcomes such as peptic ulcer disease, gastric cancer and MALT lymphoma.

14.2 Host Genetics and Risk of *Helicobacter pylori* Infection

Genetic association studies have been instrumental in aiding clinicians and researchers in the study of infectious diseases, ranging from linkage mapping to candidate gene studies and genome-wide association studies (GWAS). There are numerous studies that have assessed genetic risk factors for HIV, malaria, leprosy and tuberculosis. In this section, we summarise the most notable studies on the role of host genetic factors relevant to *H. pylori* infection, an infection that is by far the commonest globally. We start by discussing the candidate gene approach followed by the more recent GWAS studies.

14.2.1 Candidate Gene Studies of **Helicobacter pylori** *Infection*

At an epidemiologic level, there is little doubt that genetic factors influence susceptibility to *H. pylori* infection. In a landmark study, Malaty and co-workers assessed *H. pylori* status in 269 pairs of twins, including 36 monozygotic twin pairs reared apart, 64 monozygotic twin pairs reared together, 88 dizygotic twin pairs reared apart and 81 dizygotic twin pairs reared together (Malaty et al. 1994). The probandwise concordance rate for *H. pylori* infection was higher in monozygotic twin pairs (81 %) than in dizygotic twin pairs (63 %) ($P = 0.001$). For twins reared apart, the probandwise concordance rates for *H. pylori* infection were 82 % and 66 % for monozygotic and dizygotic twins, respectively ($P = 0.003$). The correlation coefficient was 0.66 for monozygotic twins reared apart, and it provides the best single estimate of the relative importance of genetic effects (heritability) for variation in the acquisition of *H. pylori* infection (Mandell et al. 2004).

Despite this genetic influence, the study of this heritability and its clinical consequences is hampered by the fact that the *H. pylori* infection is acquired during childhood, and it is almost impossible to prove exposure or acquisition with any precision. This is largely due to the fact that the infection may be asymptomatic or may be associated with a mild illness that could easily be confused with any common childhood ailment. To complicate matters, it is never clear if absence of the infection in an individual is due to lack of exposure or due to genetically determined resistance to the infection. Unfortunately, these are difficult confounding factors to dissect or control.

Tseng and colleagues attempted to study genetic susceptibility to *H. pylori* infection using the Jamaica Mother Infant Cohort, a prospective follow-up of children, designed to examine the transmission and natural history of certain infections (Mandell et al. 2004; Tseng et al. 2006). Between January 1989 and August 1990, 339 expectant mothers were enrolled from two prenatal clinics in Kingston, Jamaica. Expectant mothers provided demographic, reproductive and

medical history data and blood samples at the time of delivery. For children, physical examination and phlebotomy were performed at birth and repeated every 6 weeks for the first 6 months, then every 3 months until age 2 and every 6 months thereafter. Single-nucleotide polymorphisms (SNPs) at 17 loci in 11 cytokine genes (*IL1A*, *IL1B*, *IL2*, *TNF*, *TLR4*, *IL4*, *IL6*, *IL10*, *IL10RA*, *IL12A* and *IL13*) were analysed. The only positive finding was that the *IL1A*-889 T allele, known to express a higher level of cytokine IL-1α, was associated with a lower risk of *H. pylori* infection among these children. The finding supports the hypothesis that an upregulation of specific pro-inflammatory cytokines may protect against *H. pylori* colonisation (Smith et al. 2003). However, a further study by Liou and co-workers appears to contradict this hypothesis. They studied the role of promoter polymorphisms in the interleukin-1β gene (*IL1B*) in healthy individuals with and without *H. pylori* infection (Liou et al. 2007) and reported that a pro-inflammatory genetic make-up increased the risk of having *H. pylori* infection. Overall, the findings of these studies suggest that other host genetic factors, particularly in genes related to the initial handling of *H. pylori* within the gastric mucosa, may prove more relevant to the pathogenesis of this infection.

14.2.2 Tumour Necrosis Factor Alpha (TNF-α)

One of the most commonly studied genes regarding the acquisition of *H. pylori* infection is the tumour necrosis factor-α (*TNFA*) gene. Hamajima and co-workers studied 1374 Japanese subjects and reported a reduced odds ratio (OR) for *H. pylori* seropositivity in individuals with *TNFA*-1031CC genotype as compared to those with the *TNFA*-1031TT genotype (OR = 0.43, 95 % CI = 0.20–0.91), with the OR adjusted for sex, age and recruitment source. In the same study, subjects with *TNFA*-857CC and the aforementioned *TNFA*-1031CC genotypes showed the lowest *H. pylori* seropositivity (38.2 % of 34 subjects), whereas those with the *TNFA*-857TT and the *TNFA*-1031TT genotypes showed the highest seropositivity (66.7 % of 42 subjects) (Hamajima et al. 2003).

However, in a similar study conducted in 2006 on a population of 963 Japanese Brazilian individuals investigating the relationship between the *TNFA*-857TT and *TNFA*-1031TT genotypes and *H. pylori* seropositivity, no significant association was found between the two factors (Atsuta et al. 2006). Saijo and colleagues studied 410 Japanese transit company employees and showed that contrary to other studies, the *TNFA*-857TT genotype could actually have a protective effect against chronic *H. pylori* infection, with subjects with *TNFA*-857TT having a significantly lower odds ratio for *H. pylori* seropositivity (OR = 0.15, 95 % CI = 0.03–0.59, $P = 0.007$) (Saijo et al. 2007). In yet another study by Abdiev and co-workers on an Uzbek population of 167 participants, it was shown that subjects with the *TNFA*-1031TC genotype had a significantly increased risk for anti-*H. pylori* IgG seropositivity (OR = 2.82, 95 % CI = 1.05–7.57), as compared to *TNFA*-1031TT (Abdiev et al. 2010), an interesting contradiction to the results from the Hamajima study.

The *TNFA*-308G/A promoter polymorphism has also been associated with infection stratified by the cytotoxin-associated gene A (*cagA*) subtype of *H. pylori*. A study conducted on a Korean population of 83 patients with known gastric disease and 113 healthy controls showed that *H. pylori* infection was strongly associated with the *TNFA*-308G/A polymorphism as compared to healthy controls (OR = 2.912, 95 % CI = 1.08–7.84; $p = 0.034$) (Yea et al. 2001). The same polymorphism had an even stronger correlation with the *H. pylori cagA*-positive infection as compared to the polymorphism in *cagA*-negative *H. pylori* infection (OR = 8.757, 95 % CI = 1.413–54.262; $p = 0.019$) and healthy controls (OR = 3.683; 95 % CI = 1.343–10.101, $p = 0.011$). However, these results were not replicated in the Abdiev study, which showed no association between the *TNFA* polymorphisms −857C/T and −1031C/T and the risk of anti-CagA IgG seropositivity or gastric atrophy (Abdiev et al. 2010).

From the data above, it can be concluded that the *TNFA* gene plays an important role in deciding an individual's susceptibility to *H. pylori* infection. However, the conflicting results highlight the need for larger and adequately powered studies.

14.2.3 Toll-Like Receptors (TLRs)

The TLRs represent one of the major groups of pattern recognition receptors (PRRs) within the host innate immune system, allowing cells of the innate immune system to recognise pathogens by detecting molecules expressed across large numbers of different pathogen species, the pathogen associated molecular patterns (PAMPs).

14.2.3.1 TLR4

TLR4 is known to be involved in signal transduction pathways initiated by lipopolysaccharides (LPSs) mostly from Gram-negative bacteria. In a 2000 study conducted by Arbour and co-workers, it was demonstrated that common missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of the TLR4 receptor can cause a reduced response to inhaled LPS in humans and that the Asp299Gly mutation can also cause disruptions in LPS signalling as mediated by TLR4 (Arbour et al. 2000). The above results clearly demonstrate that the varying levels of LPS responsiveness between individuals can be attributed to TLR4 and that genetic mutations can have an effect on an individual's degree of response to any form of environmental exposures. In 2001, a study by Maeda et al. showed that macrophages from C3H/HeJ mice carrying point mutations in the *Tlr4* gene showed decreased activation of transcription factor nuclear factor kappa B (NF-κB) and TNF-α secretion compared with C3H/HeN mouse macrophage when infected with *H. pylori* (Maeda et al. 2001). This illustrates the importance of TLR4 mutations in

H. pylori-induced NF-κB activation in macrophages, which are crucial in mediating the body's inflammatory response to *H. pylori* infection. Additionally, Kawahara and colleagues also showed that TLR4 is the primary receptor responsible for the initiation of the gastric mucosal response to *H. pylori* infection because of the large amount of TLR4 protein found on the plasma membrane of gastric pit cells in guinea pigs as compared to the lack of TLR2 and TLR9 transcripts and insignificant amounts of TLR2 protein present in the same region (Kawahara et al. 2001).

14.2.3.2 TLR2 and TLR5

In more recent years, various studies have identified other TLR proteins as the primary factors for the initiation of the gastric mucosal response to *H. pylori* infection. Bäckhed and co-workers showed that while all cell lines within the gastric mucosa expressed TLR4, *H. pylori* itself could not be recognised by TLR4 (Backhed et al. 2003). It has also been shown that macrophages from wild-type and TLR4-deficient mice could produce a strong cytokine response involving IL-6 and monocyte chemoattractant protein-1 (MCP-1) when stimulated by intact *H. pylori* (Mandell et al. 2004), thus casting doubts on the popular hypothesis that TLR4 is instrumental in the initiation of a host inflammatory response against *H. pylori* infection.

Additionally, a study conducted by Smith and co-workers in 2003 on gastric epithelial cells showed that *H. pylori* infection of the cultures caused NF-κB activation in both HEK293 cells and MKN45 gastric epithelial cells transfected with TLR2 and TLR5, but not TLR4 (Smith et al. 2003). These results were replicated in a 2004 study by Mandell and colleagues on transfected HEK293 cells, where it was shown that human TLR2 expression was sufficient in inducing a response to intact *H. pylori* bacteria, while TLR4 transfection was proven to be insufficient (Mandell et al. 2004). It was also demonstrated in the same study that macrophages from TLR2-deficient mice had a significant lack of response to stimulation by intact *H. pylori*, as shown by the host's failure to secrete cytokines at 100:1 bacterium-to-macrophage ratios. Furthermore, HEK293 cells transfected with TLR2 and TLR5 expression plasmids showed an induction of chemokine gene expression by *H. pylori* infection. These results suggest that TLR2 and TLR5 have a more significant role to play in influencing the ability of the host to recognise and respond to *H. pylori* infection as compared to TLR4 as previously believed.

14.2.4 *Susceptibility to* H. pylori *Infection and GWAS Studies*

The first GWAS study that explored the relationship between *H. pylori* seropositivity and host genetics was published by Mayerle and co-workers in 2013 (Mayerle et al. 2013). In this study, two independent GWAS studies were conducted on two independent population-based cohorts from Northeastern Germany (Study of Health in Pomerania, $n = 3830$) and the Netherlands (Rotterdam Study, $n = 7108$). The main underlying objective of the study was to identify genetic loci associated with *H. pylori* seroprevalence and as a result discover the pathophysiology behind this association. *H. pylori* seroprevalence, used as an indicator for previous or current infection, was defined as an anti-*H. pylori* IgG equal to or greater than 20 U/mL. Individuals with the highest 25 % of the IgG titre distribution were considered as case patients, and those in the lower 75 % of the IgG titre distribution made up the control group in this study. In total, there were 2736 case patients and 8202 control patients, largely of European ancestry. Faecal *H. pylori* antigen testing was used to determine the presence of infection in these individuals. GWAS meta-analysis identified two genome-wide significant loci in terms of their association to *H. pylori* seropositivity, namely, the *TLR* locus on 4p14 and the *FCGR2A* locus on 1q23.3. The lead SNP on the *TLR* locus with the lowest p value was rs10004195 (OR = 0.70, 95 % CI = 0.65–0.76), closely followed by rs4833095 (OR = 0.70, 95 % CI = 0.65–0.76). For the *FCGR2A* locus, the lead SNP was rs368433 (OR = 0.73, 95 % CI, 0.65–0.81).

Three different TLRs are located along the 4p14 region: TLR1, TLR6 and TLR10. In an additional study conducted on 1763 participants from both cohorts, analysis of whole-blood RNA gene expression profiling showed that among the three TLR genes, only TLR1 was differentially expressed in relation to the rs10004195 genotype (in the presence of the rs10004195-A allele [beta = −0.23, 95 % CI = −0.34 to −0.11]). Furthermore, analysis of TLR1, TLR6 and TLR10 mRNA amounts also showed that there was a specific and genotype-independent transcriptional upregulation of TLR1 in the presence of *H. pylori*. These results imply that the increase in TLR1 mRNA expression as a result of the rs10004195 SNP is strongly associated with an increased risk of *H. pylori* seropositivity.

14.2.4.1 Effects of TLR1 on *H. pylori* Infection

The mechanism for the relationship between increased TLR1 expression and a higher *H. pylori* seroprevalence remains unexplained. However, TLR1 has been shown to interact with TLR2 to form a heterodimer (Jin et al. 2007), which is responsible for the initiation of cell signalling in response to the recognition of tri-acylated lipopeptides from Gram-negative bacterial cell wall (Takeuchi et al. 2002). This is particularly relevant to *H. pylori* infection as tri-acylated

lipopeptides can be found in the structure of *H. pylori* ligand A, allowing it to be recognised by the TLR1-TLR2 complex. It has been suggested that the resulting activation of the immune cascade could reduce the anti-inflammatory response of the host against *H. pylori* and allow a persistent infection (El-Omar 2013). Another explanation proposed recently is that the SNP at the TLR1 gene causes less effective anti-inflammatory signalling initiated by the TLR1-TLR2 complex in response to the presence of *H. pylori*, thus increasing the risk of persistent infection (Guan et al. 2010).

The second genome-wide significant locus was identified as the *FCGR2A* locus located on chromosome 1q23.3, the lead SNP being rs368433 A/G. This SNP is located in an intron of FCGR2A encoding the Fcγ receptor 2a. Whole-blood RNA gene expression profiling showed that this SNP was associated with a decrease in FCGR2A expression in individuals carrying one or more minor alleles, thus increasing the risk of *H. pylori* infection. However, further analysis of mRNA amounts failed to show any significant statistical association between *H. pylori* bacterial load and the expression of FCG2RA in whole blood.

14.2.4.2 Effect of FCGR2A on *H. pylori* Infection

As mentioned above, the rs368433 SNP at the *1q23* region has also been associated with an increased risk of *H. pylori* seroprevalence. While the results were not as significantly defined as that of the rs10004195 SNP and the mechanism of this SNP's effects is still unclear, there is evidence showing that polymorphisms in the *FCGR2A* gene could possibly affect the individual's ability to phagocytose bacterial cells. Homozygotes for allelic polymorphisms in the *FCGR2A* gene were shown to have polymorphonuclear leukocytes and monocytes which could less effectively phagocytose and internalise erythrocytes coated with human IgG2 (Salmon et al. 1992). It is possible that the same effect can be applied to *H. pylori* infection, where homozygotes for the rs368433 SNP could result in less effective phagocytosis IgG2-opsonised bacteria by neutrophils, resulting in an increased risk of infection.

While the study by Mayerle and co-workers is indeed ground-breaking, there remain many unanswered questions in this field. Firstly, the study primarily focused on *H. pylori* seropositivity, whereas *H. pylori* colonisation and symptomatic disease were not assessed. As such, it is crucial that more studies are conducted to assess if the same genetic polymorphisms are associated with symptomatic *H. pylori* infection, and these studies also have to fulfil the difficult task of measuring exposure levels to *H. pylori* in the populations under study. Western populations have a low *H. pylori* exposure pressure compared to South American and African populations (Miwa et al. 2002; Prinz et al. 2006). Therefore, *H. pylori* seronegativity in the reported results could have been due to the lack of exposure to *H. pylori* instead of a protective genetic effect.

The translational benefits from the study of host genetics of *H. pylori* infection are potentially impressive. For example, better understanding of the genetic basis of

risk might inform better development of antibiotics or indeed vaccines against the infection.

14.3 Genetic Susceptibility to *H. pylori*-Induced Peptic Ulcer Disease

The study of genetic susceptibility to peptic ulcers is restricted by their heterogeneity: (1) Gastric ulcers do not share the pathophysiology of duodenal ulcers. The latter is associated with the classic antral-predominant pattern of gastritis and acid hypersecretion, while the former is associated with a variety of phenotypes ranging from DU-like (e.g. prepyloric ulcers) to gastric cancer-like (with gastric atrophy and hypochlorhydria). Studies that have failed to take into account this rigorous definition of phenotype may have been compromised. (2) Most studies published to date have failed to exclude non-steroidal anti-inflammatory drug (NSAID)-induced ulcers from both groups. The pathogenesis of NSAID ulcers is different from peptic ulcers, and failing to exclude them may introduce significant bias. Finally, classic *H. pylori*-induced peptic ulcers are now extremely rare, and very few research units are able to muster adequately powered studies to address the issue. Perhaps a more worthwhile research agenda would be to concentrate on understanding the genetic determinants of NSAID-induced ulceration. These drugs are very commonly prescribed, and their side effects continue to cause significant morbidity and mortality. Very little is known about the host genetic factors that influence risk of side effects.

14.4 Role of Host Genetic Factors in Gastric Cancer

14.4.1 Single-Nucleotide Polymorphisms (SNPs) in Candidate Genes

There are many examples in the literature of polymorphic genes increasing susceptibility to gastric cancer (El-Omar et al. 2008). This role was initially investigated within chosen candidate genes based on knowledge of gastrointestinal disease pathophysiology, with particular focus on gastric cancer as the most serious outcome of *H. pylori* infection. Advances in technology for genetic sequencing now allow simultaneous assessment of multiple previously unidentified SNPs within a large number of genes, and this has revealed exciting novel insights into gastric carcinogenesis. It is clear that genetic polymorphisms vary in accordance to ethnicity and, along with exposure to environmental risk factors, are a key player in individual susceptibility to the development of gastric cancer.

SNPs within host inflammatory genes are a prime example of a candidate gene approach, with emphasis on host response to *H. pylori* infection and pathological

consequences of this. IL-1β was the first pro-inflammatory cytokine gene to be identified as an important candidate gene in gastric cancer susceptibility. It was specifically chosen for its influence on gastric physiology, namely, upregulation in response to *H. pylori* infection and potent inhibition of gastric acid secretion (El-Omar 2001). Subsequent evidence of the pivotal role of this cytokine in gastric tumourigenesis came from a transgenic mouse model displaying promoter-driven targeted IL-1β overexpression in the stomach. This model provided evidence for a crucial role for IL-1β in gastric premalignant pathology to overt gastric cancer in the absence of *H. pylori* infection (Tu et al. 2008). When *H. pylori* colonisation was introduced into this model, the pathological consequences were accelerated reinforcing the importance of host-environment interaction.

Indeed, polymorphisms in the *IL1* gene cluster (encoding IL-1α, IL-1β and IL-1RN, the naturally occurring receptor antagonist) were shown to be associated with increased risk of developing premalignant gastric atrophy and hypochlorhydria in response to *H. pylori* infection within a Caucasian population of gastric cancer relatives (El-Omar et al. 2000). These premalignant gastric pathologies were associated with carriage of the *IL1B*-31*C or *IL1B*-511*T and *IL1RN**2/*2 genotype. This positive association remained true for the development of non-cardia gastric cancer, with an estimated odds ratio of 1.6 (95 % CI, 1.2–2.2) and 2.9 (95 % CI, 1.9–4.4) for carriage of *IL1B*-31*C/*IL1B*-511*T and *IL1RN**2/*2, respectively. This association has subsequently been validated in various populations with different ethnicities (Kimang'a 2012; Zhao et al. 2012). However, there are numerous accounts of interethnic and geographical variation with conflicting reports of association in the literature. Overall, several large-scale meta-analyses confirmed the positive association between *IL1* markers and risk of gastric cancer, especially in Caucasian populations (Camargo et al. 2006; Vincenzi et al. 2008; Loh et al. 2009; He et al. 2011).

Following this finding, over the last decade, other potential candidate inflammatory cytokine genes, such as *IL-8*, *IL-10* and *TNFA*, have been studied in the context of gastric cancer risk. Most recently, this was examined in a Human Genome Epidemiology (HuGE) systematic review and meta-analysis (Persson et al. 2011). Persson and colleagues conducted a series of meta-analyses using a predefined protocol and looked at the most studied polymorphisms in inflammatory genes, including *IL1B*, *IL1RN*, *IL8*, *IL10* and *TNFA* in the literature published over a 16-year period (1990–2006). Gastric cancer was stratified on histological subtype and anatomic subsite, *H. pylori* infection status and geographical location (Asian or non-Asian study population) and by a quantitative index of study quality. There was a consistent positive association between carriage of *IL1RN*2* and increased risk of gastric cancer, specific to non-Asian populations. This risk was seen for both intestinal and diffuse cancers and, particularly, for distal cancers. In Asian populations, reduced risk was observed in association with *IL1B*-31C carrier status. When considering all the conflicting associations in the literature, it is essential to recognise the importance of the tumour factors mentioned above in addition to *H. pylori* infection status and ethnic origin of the population under study. Many of

these discrepant studies could be explained by variations in study design and laboratory techniques.

Most recently, another cytokine gene under focus as a candidate gene was *IL-17*. The IL-17 family of pro-inflammatory cytokines (IL-17A-F) is produced from mainly Th17 cells but also other cell types in the inflammatory milieu, such as NK T cells, neutrophils and $CD8^+$ cytotoxic T (Tc17) cells. IL-17A has been implicated in many inflammatory driven diseases, including autoimmunity and cancer (Zou and Restifo 2010). Interest in the association between IL-17 gene polymorphisms and gastric cancer arose in response to emerging evidence implicating IL-17 in gastric cancer pathogenesis (Maruyama et al. 2010; Iida et al. 2011; Meng et al. 2012; Zhuang et al. 2012). Several studies have now been published assessing risk of gastric cancer in association with several polymorphic loci in the *IL17* genes in multiple ethnic populations, and overall, G to A transition at position-187 in the IL17-A gene (rs22759133) has been associated with an increased risk (Shibata et al. 2009; Qinghai et al. 2014; Rafiei et al. 2013; Zhang et al. 2014). It is still unclear whether genetic variation in *IL17A* could impact host response to *H. pylori* infection and be a driver for gastric cancer development. Overall, it has been shown that IL-17-producing cells play a varied and important role in host response to *H. pylori* infection (Kabir 2011). Do IL-17 polymorphisms impact susceptibility to *H. pylori* infection? Current reports are conflicting, ranging from no link between allelic carriage and *H. pylori* status (Rafiei et al. 2013) to a recent report of a positive interaction with polymorphisms at multiple sites in the *IL17A* gene and *H. pylori* infection in association with increased gastric cancer risk (p for interaction 0.036).

14.4.2 Genome-Wide Association Studies

Novel susceptibility loci offering new molecular insights into gastric cancer development have been identified using genome-wide association studies (GWAS). A significant association between diffuse gastric cancer and polymorphic genetic variation (rs2294008 and rs2976392) that fell within exon 1 of the PSCA (prostate stem cell antigen) gene was identified using this technology, in a large Japanese population (Sakamoto et al. 2008). Allelic differences at this site, G>A and C>T at rs2976392 and rs2294008, respectively, interfered with transcription initiation and conferred risk of diffuse gastric cancer with an adjusted odds ratio of 4.18 (95 % CI of 2.88–6.21, $p = 1.5 \times 10^{17}$), and this association was subsequently confirmed within a Korean population of over 450 diffuse-type gastric cancer patients. The authors went on to show that the PSCA protein is expressed in areas of the glandular crypts. Crucially, this expression is in close proximity to stem cell progenitors, the proposed initiator cell for this particular histological subtype of gastric cancer. Furthermore, PSCA was downregulated at both the gene and protein level in gastric cancer tissue specimens. The role for PSCA in the inhibition of epithelial cell proliferation was highlighted by a series of in vitro experiments. This molecular

association was unexpected and highlights the power of newer genetic sequencing technologies in contrast to the candidate gene approach. This association has now been validated in several Asian population-based case-control studies (Matsuo et al. 2009; Lu et al. 2010; Zeng et al. 2011; Wu et al. 2009). In a Caucasian population, carriage of the rs2294008 T allele was associated with risk of chronic atrophic gastritis (OR 1.5 (95 % CI 1.1–1.9)) in addition to overt non-cardia gastric malignancy (OR 1.9 (95 % CI 1.3–2.8)) (Lochhead et al. 2011). Data from the EPIC (European Prospective Investigation into Cancer and Nutrition) cohort confirmed positive association of similar strength (OR 1.42 (95 % CI; 1.23–1.66) with carriage of the T allele and both intestinal and diffuse histological subtypes of gastric cancer (Sala et al. 2012). The relationship between gastric cancer risk and polymorphic alleles in the PSCA gene remains true in several meta-analyses (Qiao and Feng 2012; Shi et al. 2012; Wang et al. 2012; Zhang et al. 2012; Gu et al. 2014). Does carriage of the PSCA risk T allele impact on patient outcome? Two previous studies assessed this and reported conflicting data. Wang and co-workers (Wang et al. 2011) reported that carriage of the T allele conferred 25 % increased survival rate (HR 0.75 (5 % CI 0.59–0.96)) for diffuse subtype gastric cancer, whereas the other study reported a significantly worse survival outcome (OR 2.2 (95 % CI 1.22–3.69) (Lochhead et al. 2011). It is clear that further analysis is required in a larger, more varied patient cohort to further explore this possibility.

The intestinal glycocalyx mucus layer is composed of tightly packed glycosylated proteins from the mucin family that provides a physical barrier protection between luminal contents and the host. The large group of mucin proteins (MUC1-21) is either membrane bound or can be secreted. The physiological role of MUC1 in particular has been of interest in the pathogenesis of gastrointestinal disease as this protein is not simply an inert barrier. Instead MUC1 can profoundly influence many important cell functions, such as cell growth, inhibition of apoptosis via mitochondrial influences, influences on cell-cell adhesion, stimulation of kinase-driven cell signalling pathways and interaction with several transcription factors, such as the STATs and NF-κB, to impact on target downstream expression. As such MUC1 has been termed an oncoprotein and has been implicated in a number of cancers (Kufe 2009, 2012; Senapati et al. 2010; Boltin and Niv 2013). Interruption of the interaction between MUC1 and other proteins such as β-catenin, ICAM and EGFR has been explored for therapeutic gain in the treatment of cancer. As an alternative strategy, anti-MUC1 anticancer immunotherapy has also been developed, reflecting its pivotal role in cancer pathogenesis (Kufe 2012; Mukhopadhyay et al. 2011; Silva et al. 2001). Therefore, as one may expect, the relevance of MUC1 in gastric cancer has been widely investigated, and it is accepted that aberrant expression of this protein occurs in malignant gastric tissue. Indeed, several studies have investigated *MUC1* as an important gene associated with gastric cancer risk in different geographical and ethnic populations, from both a candidate gene approach and detection within susceptibility loci in GWAS studies (Silva et al. 2001; Xu et al. 2009; Abnet et al. 2010; Jia et al. 2010; Saeki et al. 2011; Zhang et al. 2011; Palmer et al. 2012; Song et al. 2014). Overall, the consensus from these studies is that rs4072037 (G>A) polymorphism in exon 2 confers risk.

Specifically, carriage of the G allele at this site is protective. Presence of the G allele was associated with a 28 % reduced risk of gastric cancer within a recent meta-analysis that included over 6000 gastric cancer cases and 10,000 controls across several geographical and ethnic populations (Zheng et al. 2013). From a functional perspective, carriage of the A allele was associated with reduced protein expression in gastric cancer tissue (Xu et al. 2009). Specifically, this polymorphism affects gene promoter function and influences splice variants in gastric epithelium (Saeki et al. 2011). With particular relevance to this review, it has been shown that epithelial cell expression of the mucin proteins in the stomach inhibits the binding of *H. pylori* to the host epithelial layer, therefore protecting the host from chronic inflammatory activity in the underlying gastric mucosa (Guang et al. 2010; He et al. 2014). MUC1 also negatively regulates signalling via TLRs in the respiratory mucosa, and therefore this phenomenon is not confined to the gastrointestinal tract (Ueno et al. 2008). In addition, it has been shown that *muc1* regulates host inflammatory response through inhibition of pro-inflammatory mediators in response to *H. pylori* infection (Sheng et al. 2012). The emerging hypothesis is that carriage of the A allele reduces protein expression of MUC1, allowing *H. pylori* to directly interact with the gastric epithelium. This then creates a chronic and persistent, unchecked inflammatory response in the host that could positively influence cancer initiation and progression. However, this is not fully understood. Indeed, Marin and colleagues showed that genetic variation in *MUC* family member genes did not impact on clinical outcome and progression to overt gastric malignancy in individuals with premalignant phenotype followed up over at least a 12-year period (Marin et al. 2012). Interestingly, carriage of more than one of these susceptibility-associated SNPs confers a cumulative risk effect, with greater than eightfold increased risk of gastric cancer with carriage of both *MUC1* and *PSCA* risk alleles (Saeki et al. 2011). This may account for the high incidence of gastric cancer in the Japanese population, despite relatively low *H. pylori* colonisation, where more than 60 % of the population is believed to carry one or both susceptibility SNPs (Saeki et al. 2013).

GWAS studies continue to reveal susceptibility loci associated with gastric cancer risk (Abnet et al. 2010; Shi et al. 2011; Kang et al. 2014). Ongoing analysis will no doubt uncover additional important polymorphic genes associated with gastric cancer susceptibility. The crucial question is how these will fit functionally into the pathogenesis of this disease and how this genetic susceptibility interacts with environmental factors, such as *H. pylori* infection status to initiate and promote this disease.

14.5 Genetic Associations with MALT Lymphoma

Marginal zone B-cell lymphomas of mucosa-associated lymphatic tissue (MALT), also known as MALT lymphomas or MALTomas, are the predominant lymphoma occurring within the stomach (Fischbach 2013). Infection with *H. pylori* is known

to be almost essential in the development of this rare malignancy, with 98.5 % of cases being seropositive for *H. pylori* in one study (Eck et al. 1997). This relationship is further strengthened by the fact that gastric MALT lymphoma can often be successfully treated with *H. pylori* eradication therapy (Fischbach 2013). Inflammation is thought to be the key mechanism by which *H. pylori* drives gastric MALT lymphoma pathogenesis (Fischbach 2013). However, the majority of patients with *H. pylori* infection do not develop gastric MALT lymphoma, and host genetic factors are being recognised as increasingly important risk factors in determining which patients with *H. pylori* infection go on to develop this rare malignancy. This section summarises recent studies into host genetic factors associated with gastric MALT lymphoma and the insights they offer into its pathogenesis.

14.5.1 Detoxification and Antioxidant Genes

The glutathione S-transferases (GST) are a major family of enzymes involved in the conjugation of substrates to glutathione for the purpose of detoxification. They also have an antioxidant function and neutralise ROS protecting against DNA damage (Saeidnia and Abdollahi 2013). In a study published in 2003, Rollinson and colleagues proposed that gene deletions at the GST T1 and GST M1 loci resulting in a lack of the active protein would be associated with an increased risk of gastric MALT lymphoma (Rollinson et al. 2003). They compared 66 cases of patients with gastric MALT lymphoma from the North of England against 163 healthy controls and found that the GST T1 null genotype was significantly associated with gastric MALT lymphoma (57.6 % of cases versus 13.5 % of controls, OR 9.51, 95 % CI 4.57–19.81). This finding was replicated in a study published in 2004 by Wu and co-workers, who compared 75 cases with MALT lymphoma against 321 healthy controls of a Han Chinese background (Wu et al. 2004b). They also found a significant association between the GST T1 null genotype and the risk of gastric MALT lymphoma (57.3 % cases versus 43.0 % of controls, OR 1.8, 95 % CI 1.1–3.0), albeit with a reduced effect size. With regard to the GST M1 null genotype, no significant associations were found in either Rollinson's study (62.1 % cases versus 54.5 % controls, OR 1.10, 95 % CI 0.59–2.07) or Wu's study (60 % cases versus 52 % controls, no statistics available).

The positive associations between the GST T1 null genotype show very different effect sizes, with an odds ratio of 9.51 in the North of England cohort and an odds ratio of 1.8 in the Han Chinese cohort. The likely explanation for this is the difference in carrier rates for the GST T1 null genotype in the control populations. The Han Chinese controls had a higher frequency of the GST T1 null genotype at 43 % compared to a carrier rate of 13.5 % in the North of England controls. This is consistent with existing studies which show a higher carrier rate for this genotype in Han Chinese populations (Wu et al. 2004b; Strange and Fryer 1999). These findings offer interesting insights into gastric MALT lymphoma pathogenesis. As the glutathione S-transferases play a major role in protecting DNA against damage, it

has been proposed that impaired antioxidant functioning in individuals carrying the GST T1 null genotype would be subjected to higher rates of DNA damage enhancing the rate of MALT lymphoma pathogenesis. Supporting this theory are studies showing an association between the GST T1 null genotype and other cancers, including astrocytoma, meningioma, myelodysplasia (Rebbeck 1997) and other lymphoproliferative disorders (Strange and Fryer 1999).

14.5.2 Cytokine and Cytokine Receptor Genes

14.5.2.1 Interleukin-1

Rollinson and co-workers first studied the association of *IL1RN* with gastric MALT lymphoma. They compared 66 cases of gastric MALT lymphoma from the North East of England against 163 healthy controls and found that the *IL1RN* 2/2 genotype was significantly associated with gastric MALT lymphoma (33.9 % cases versus 8 % controls, OR 5.51, 95 % CI 2.16–14.07). A subsequent study by Wu et al. found no associations between the *IL1RN* gene and gastric MALT lymphoma although there were no cases positive for the 2/2 genotype and only three positive controls (Rollinson et al. 2003). A further larger study by Hellmig and co-workers comparing 153 MALT lymphoma patients with 344 controls all from the German-Austrian Lymphoma Study group had more participants carrying the 2/2 genotype (9/153 cases versus 27/344 controls) but also failed to find any association between the *IL1RN* 2/2 genotype and gastric MALT lymphoma (Hellmig et al. 2004). Hellmig et al. suggested that the use of gastric biopsies as a source of DNA in Rollinson et al.'s study could have resulted in contamination with tumour material leading to an increased frequency of the 2/2 genotype in their case population (Hellmig et al. 2004). Therefore, the evidence for the role of the *IL1RN* 2/2 genotype in gastric MALT lymphoma is conflicted at present with the largest study available showing no significant association.

With regard to the *IL1B* gene, two single-nucleotide polymorphisms of the C>T type at positions −31 and −511 of the *IL1B* gene have been associated with increased production of *IL1B*, and the *IL1B* −511 T allele has been associated with an increased risk of gastric adenocarcinoma (El-Omar et al. 2000). These polymorphisms have also been studied in gastric MALT lymphoma patients with multiple case-control studies of the IL1B-31(Rollinson et al. 2003; Hellmig et al. 2004; Wu et al. 2004a) and IL1B-511 (Wu et al. 2004a, b) gene polymorphisms failing to identify any significant associations.

14.5.2.2 Tumour Necrosis Factor (TNF)

The tumour necrosis factor (TNF) family of cytokines are another group of pro-inflammatory cytokines, of which the best studied is TNF-α. TNF-α binds

two receptors: TNFR1 present on most tissues and TNFR2 present on cells of the immune system. Polymorphisms in these genes have been associated with a range of inflammatory conditions, and in a study published in 2004, Wu and co-workers studied the associations of TNF-α, *TNFR1* and *TNFR2* gene polymorphisms with gastric MALT lymphoma. In this case-control study, 70 patients of Han Chinese background were compared with 210 healthy controls. The *TNFA* -857 T polymorphism was found to be significantly underrepresented in patients with gastric MALT lymphoma (6.4 % cases versus 14.3 % controls, OR 0.33, 95 % CI 0.15–0.75) (Wu et al. 2004a). *TNFA* polymorphisms at positions -308, -863 and -1031 were not found to be associated with gastric MALT lymphoma (Wu et al. 2004a). Similarly, polymorphisms in position -383 of the *TNFR1* gene and codon 196 of the *TNFR2* gene were not associated with gastric MALT lymphoma (Wu et al. 2004a). These findings have not been replicated in other studies. However, they are consistent with our existing understanding of gastric MALT lymphoma pathogenesis. The *TNFA*-857 T gene polymorphism has previously been associated with reduced TNF-α expression in vitro as well as with a reduced risk of Crohn's disease (van Heel et al. 2002), one form of inflammatory bowel disease, suggesting that it may be protective against gastric MALT lymphoma by reducing the inflammatory response to *H. pylori*.

14.5.3 Innate Immunity Genes

14.5.3.1 Toll-Like Receptor 4 and CD14

TLR4 is one of the best studied TLRs and binds lipopolysaccharide, an important component of the bacterial cell wall. TLR4 has been recognised to play an important role in *H. pylori*-induced gastritis. Gene polymorphisms have therefore been proposed to play a role in the host genetics of gastric MALT lymphoma pathogenesis. In a 2005 case-control study by Hellmig and co-workers, 87 patients with gastric MALT lymphoma from the German-Austrian Lymphoma Study group were compared against 344 controls positive for *H. pylori* (Hellmig et al. 2005). They proposed that the *TLR4* Asp299Gly allele, which leads to a diminished inflammatory response to lipopolysaccharide, would be protective against gastric MALT lymphoma and successfully demonstrated a protective association in this cohort (4.6 % cases versus 11.6 % controls, $p = 0.019$, OR 0.37, 95 % CI 0.13–1.03). A follow-up study by Wu in 2006 attempted to replicate this finding but failed to identify any Asp299Gly variants (Wu et al. 2006). A smaller study by Türe-Ozdemir and colleagues published in 2008 studied 56 patients with gastric MALT lymphoma and 51 *H. pylori*-infected controls and found no significant association between carriers of the Asp299Gly allele and MALT lymphoma (32.1 % cases versus 23.5 % controls, OR 1.54, 95 % CI 0.65–3.63) (Ture-Ozdemir et al. 2008). Although the findings of Hellmig and co-workers have not been

replicated, they do have biological plausibility based on current understanding of gastric MALT lymphoma pathogenesis.

Genetic polymorphisms of the gene coding for CD14, another pattern recognition receptor that acts as a co-receptor with TLR4 for lipopolysaccharide, have also been investigated for associations with gastric MALT lymphoma. A case-control study by Wu et al. investigated the -159C/T polymorphism of the *CD14* gene. In this study of 70 gastric MALT lymphoma patients and 210 healthy controls, no significant association was found between the -159C/T polymorphism and gastric MALT lymphoma (Wu et al. 2006). However, a smaller study by Türe-Ozdemir later found a marginally significant association of the -159 T allele with gastric MALT lymphoma (42.9 % cases versus 23.5 % controls, p 0.042, OR 2.44, 95 % CI 1.06–5.62) (Ture-Ozdemir et al. 2008). They proposed that the *CD14* -159C/T variant, which has been shown to lead to increased CD14 expression and TNF-α production (Lin et al. 2005), leads to enhanced inflammatory reactions, which in the case of *H. pylori* infection could lead to an increased risk of gastric MALT lymphoma. This hypothesis has biological plausibility and remains consistent with current understanding of gastric MALT lymphoma pathogenesis.

14.5.3.2 NOD1 and NOD2 Receptors

The NOD-like receptors are another important group of pattern recognition receptor. NOD1 has been shown to be important in the immune response against *H. pylori* infection (Viala et al. 2004). Subsequently Rosenstiel and colleagues investigated the role of NOD1 and NOD2 gene polymorphisms in susceptibility to gastric MALT lymphoma (Rosenstiel et al. 2006). In this study published in 2006, 83 patients with low-grade *H. pylori*-associated gastric lymphoma from the German-Austrian Lymphoma Study group were compared with 428 *H. pylori*-infected controls. They found that the rare R702W allele of the NOD2 gene was associated with an increased risk of gastric MALT lymphomas (10.8 % cases versus 4.9 % controls, p 0.044, OR 2.4, 95 % CI 1.0–5.6) (Rosenstiel et al. 2006). However, a smaller follow-up study by Türe-Ozdemir failed to replicate this finding (7.1 % cases versus 11.8 % controls, OR 0.57, 95 % CI 0.15–2.18) (Ture-Ozdemir et al. 2008). Rosenstiel and co-workers subsequently found, as part of the same study, that this variant led to reduced NF-κB activation which would be consistent with the proposal that this polymorphism is protective by limiting *H. pylori*-induced gastritis (Rosenstiel et al. 2006).

14.5.3.3 Adaptive Immunity Genes

Studies have suggested that *H. pylori*-specific T cells may play an important role in gastric MALT lymphoma pathogenesis. Genetic polymorphisms in genes coding for important T-cell signalling proteins could therefore have a role to play in determining which individuals with *H. pylori i*nfection go on to develop gastric

MALT lymphoma. Cytotoxic T lymphocyte antigen 4 (CTLA4), present on the surface of T cells, negatively regulates T-cell activation by binding B7 molecules on antigen-presenting cells. CD28, also present on the surface of T cells, is structurally related to CTLA4 and binds B7 but on binding produces a strong co-stimulatory signal leading to T-cell activation. Inducible co-stimulator (ICOS) is another molecule expressed by T cells which is part of the CD28/CTLA4 family and is also thought to play an important role in regulating T cells.

In a case-control study published by Cheng and co-workers, 62 patients with gastric MALT lymphoma were compared against 250 healthy controls to investigate the role of CTLA4, CD28 and ICOS gene polymorphisms in gastric MALT lymphoma pathogenesis (Cheng et al. 2006). They found that the *CTLA4* -318C/T genotype was associated with a significantly lower risk of gastric MALT lymphoma (4.8 % cases versus 16.0 % controls, OR 0.3, 95 % CI 0.1–0.9) and that the *CTLA* 49G/G genotype was associated with a significantly higher risk of gastric MALT lymphoma (54.8 % cases versus 47.6 % controls, OR 4.1, 95 % CI 0.9–18.2) (Cheng et al. 2006). However, when these polymorphisms were studied again comparing against *H. pylori*-positive controls, only the CTLA 49G/G genotype remained significantly associated with gastric MALT lymphoma (OR 6.4, 95 % CI 1.0–50.2) (Binion et al. 1997). No gene polymorphisms of the CD28 or ICOS genes were found to be associated with gastric MALT lymphoma (Binion et al. 1997). They propose that decreasing T-cell inhibition associated with the CTLA4 49G allele leads to an enhanced inflammatory response to *H. pylori* resulting in an increased susceptibility to gastric MALT lymphoma.

14.5.3.4 MALT1

Gastric MALT lymphomas are commonly found to have the t(11;18)(q21;q21) translocation which involve genes *MALT1* on chromosome 18 and API2 on chromosome 11. This creates a fusion protein thought to be important in MALT lymphoma pathogenesis, suggesting a possible role for germ line mutations of *MALT1* in gastric MALT lymphoma pathogenesis. A study by Hellmig and co-workers published in 2009 compared 54 patients with high-grade lymphoma against 344 healthy controls and found that the rare allele G of SNP 3 in the *MALT1* gene was protective against high-grade gastric MALT lymphoma (OR 0.2, 95 % CI 0.1–0.6). However, as part of the same study, they failed to replicate the finding in an independent cohort and concluded that there was no evidence that germ line mutations in the *MALT1* gene have a role to play in gastric lymphoma pathogenesis (Hellmig et al. 2009).

The common thread in all these findings is that the main driving force in gastric MALT lymphoma pathogenesis is inflammation, and that genetic polymorphisms that reduce or enhance the inflammatory response to *H. pylori* will subsequently reduce or enhance the risk of gastric MALT lymphoma.

14.6 Conclusions and Outlook

The genetic revolution over the past decade has enabled scientists for the first time to examine afresh a multitude of unanswered clinical problems. Defining host genetic factors that control basic physiological processes will explain many of the seemingly divergent phenotypic expressions of disease. Therefore, the most important benefit to the study of host genetics is the better understanding of disease pathogenesis. In the case of *H. pylori* infection, host genetics has helped in confirming two very important facts: first is the essential role of the initial insult in the form of the microbial challenge (in this case *H. pylori*), and second is the important role of chronic inflammation with its long-term deleterious effects on gastric physiology. As such, the most sensible and practical conclusions from this knowledge are to either avoid getting the infection in the first place or to remove it or ameliorate its effects once it is established.

The other benefit to studying host genetic factors is in being able to predict clinical outcomes following certain exposures (microbial, chemical, dietary, pharmacological, etc). For example, if we could define who might develop an atrophic, hypochlorhydric response to *H. pylori* infection, this could form the basis for genetic screening so that these individuals could be offered eradication therapy. As things stand, this approach offers little benefit over simply checking for *H. pylori* infection itself. The reason is that the currently identified genetic risk markers are very common in the population and are not specific enough to act as predictors of gastric cancer risk. It may be that future advances in affordable high-throughput genotyping could uncover a much more extensive genetic profile that satisfies the criteria for a screening test. If such a development were feasible, we must ensure that our governments enact laws that protect individuals from being discriminated against on the basis of their genetic heritage. These issues require a mature debate that has to start now.

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Part III
Helicobacter pylori-Associated Diseases

Chapter 15
Helicobacter pylori and Nonmalignant Diseases

Doron Boltin and Yaron Niv

Abstract Infection with *Helicobacter pylori* initially leads to superficial gastritis and usually progresses to chronic active gastritis. Although the vast majority of chronically infected subjects remain asymptomatic, somewhere between 10 % and 15 % develop peptic ulcer disease. Most commonly, *H. pylori* infection is located in the gastric antrum, where it acts to inhibit somatostatin release and thus stimulate acid secretion, causing duodenal ulceration. Patients with pangastritis are predisposed to developing gastric ulceration. Although population-based studies show that patients with gastroesophageal reflux disease have a lower likelihood of *H. pylori* infection, there is no robust evidence suggesting that eradication of *H. pylori* leads to erosive esophagitis. Patients who lack endoscopic evidence of disease such as peptic ulceration (i.e., functional dyspepsia) experience a greater reduction in their dyspeptic symptoms following eradication of *H. pylori* as compared to placebo. The pathophysiology of *H. pylori*-mediated dyspepsia in these patients probably involves increased acid, decreased ghrelin, and altered gastric emptying. Eradication of *H. pylori* in patients with functional dyspepsia has the added benefit of preventing future peptic ulcer disease, especially in Asian populations. *H. pylori* has been weakly linked to various nonmalignant conditions ranging from halitosis to coronary heart disease. However, evidence in support of actively seeking and treating *H. pylori* exists only for idiopathic thrombocytopenic purpura and iron deficiency anemia. In epidemiological studies, *H. pylori* infection has been shown to be inversely associated with Crohn's disease and asthma.

Keywords *Helicobacter pylori* • Peptic ulcer • Gastroesophageal reflux • Dyspepsia

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15.1 Introduction

Helicobacter pylori has been implicated as a precipitating factor, a perpetuating factor, or a protective factor, in various nonmalignant diseases. *H. pylori* may exert its effect via direct toxicity or via indirect paracrine, endocrine, neurocrine, or immune pathways. Foremost, *H. pylori* is associated with the formation, complication, and recurrence of peptic ulcer disease, both as a solitary factor and in conjunction with nonsteroidal anti-inflammatory drugs (NSAIDs). Testing for *H. pylori* and subsequent eradication is therefore essential in patients with a history of ulcer bleeding or prior to commencing long-term NSAID therapy. On the other hand, evidence of a role for *H. pylori* in causing functional dyspepsia is less robust, and the likelihood of symptomatic improvement following eradication varies greatly across geographic region. *H. pylori* infection offers theoretical protection against gastroesophageal reflux disease (GERD), owing to the hypochlorhydria typically manifest in the setting of pangastritis. Indeed, the prevalence of infection observed in subjects with both erosive and nonerosive GERD is reduced. Nevertheless, there is no evidence that GERD may develop following *H. pylori* eradication nor is there evidence that preexisting GERD or Barrett's esophagus may worsen. Epidemiological studies have found an inverse relationship between *H. pylori* infection and atopic disease and Crohn's disease, and various candidate immune mechanisms have been explored. As yet, no causal relationship has been established.

15.2 Peptic Ulcer Disease

15.2.1 Pathophysiology

Peptic ulcer disease (PUD) occurs when mucosal defense mechanisms are overwhelmed by the destructive effects of acid and pepsin and most commonly result from *H. pylori* infection or NSAIDs (Table 15.1). Although acute infection with *H. pylori* causes hypochlorhydria, chronic *H. pylori* infection may cause either hypo- or hyperchlorhydria. Whether *H. pylori* ultimately increases or decreases gastric acid secretion depends upon the severity and distribution of gastritis (Schubert and Peura 2008). Pangastritis usually results in decreased acid secretion. This is probably mediated by inflammatory cytokines and bacterial toxins including the vacuolating cytotoxin (VacA) gene product and cytotoxin-associated gene A (CagA) product (Fig. 15.1). This pattern of gastritis is seen in approximately 85 % of subjects with chronic *H. pylori* infection and predisposes to gastric ulceration. Conversely, 15 % of subjects with chronic *H. pylori* infection may develop antral predominant gastritis, which is characterized by reduced antral secretion of somatostatin. This in turn results in elevated basal and stimulated

Table 15.1 Etiology of peptic ulcer disease

Common
H. pylori infection
NSAIDs/aspirin
Uncommon
Hypersecretion
Zollinger–Ellison syndrome
Systemic mastocytosis
Medications (usually in combination with NSAIDs)
Bisphosphonates
Corticosteroids
Potassium chloride
5-Fluorouracil
Sirolimus
Mycophenolate mofetil
Infiltrative disease
Carcinoma/lymphoma
Sarcoidosis
Crohn's disease
Infections
Helicobacter heilmannii
Herpes simplex virus
Cytomegalovirus
Tuberculosis
Syphilis
Ischemia
Atherosclerosis/embolic disease
Cameron ulcer
Cocaine
Severe systemic disease

gastrin secretion and increased corporal acid production. This pattern of gastritis predisposes to duodenal ulceration.

15.2.2 Epidemiology

The population-based prevalence of peptic ulcer disease depends greatly on ethnic, geographic, and socioeconomic factors, as well as the method of data collection. A recent meta-analysis with pooled data from the USA, Europe, and Israel found an annual PUD incidence of 0.03–0.19 % and a 1-year prevalence of 0.1–1.5 % (Sung et al. 2009). The occurrence of PUD has been decreasing over the past few decades in most Western populations. The prevalence of *H. pylori* infection in duodenal ulcers was reported to be 84 % in studies published from 1999 to 2003 (Fig. 15.2),

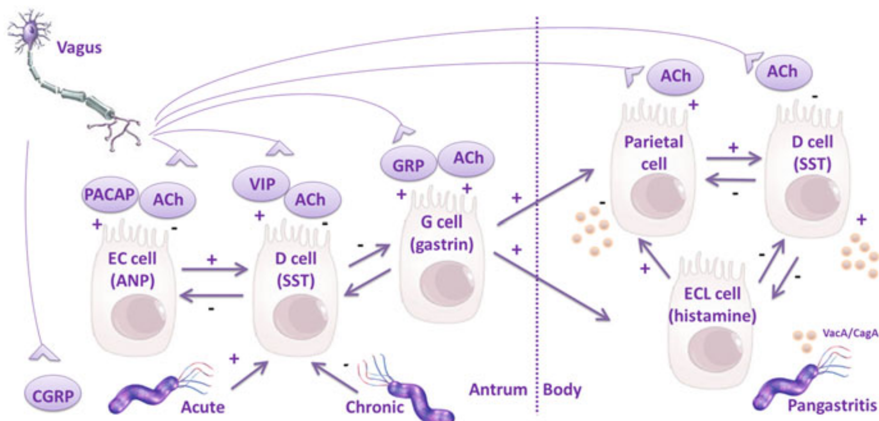

Fig. 15.1 Regulation of gastric acid secretion. Acute infection with *H. pylori* activates CGRP neurons to stimulate SST and thus inhibit gastrin secretion. In duodenal ulcer patients with chronic antral *H. pylori* infection, the organism or cytokines released from the inflammatory infiltrate inhibit SST and thus stimulate gastrin and hence acid secretion. When *H. pylori* colonizes the gastric body, bacterial toxins such as VacA and CagA act to decrease acid secretion; however, the precise mechanism is unknown

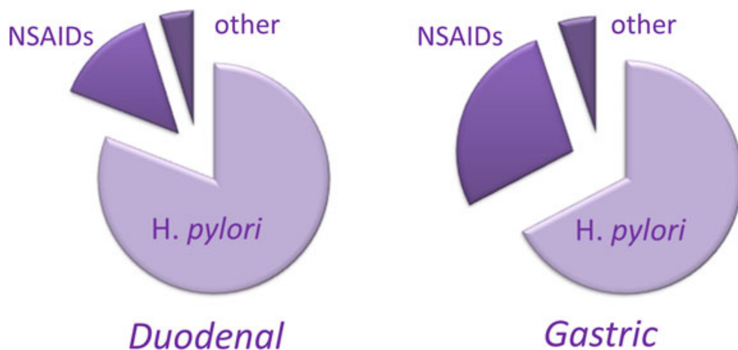

Fig. 15.2 Pie charts depicting the etiology of peptic ulcer disease. The percentages shown are based on studies conducted in Western populations. Such a representation is clearly simplistic, as *H. pylori* and NSAID use often coexist. Similarly, the relative proportion of each factor varies according to age, ethnicity, and socioeconomic status

whereas in studies published from 2004 to 2008, the prevalence of *H. pylori* was 77 % (Gisbert and Calvet 2009). However, this trend might not be apparent in some Asian populations, where the incidence of *H. pylori* gastric ulcer could be increasing (Jang et al. 2008).

The declining incidence of PUD has occurred in parallel to a decline in *H. pylori* infection rates. In areas of high *H. pylori* prevalence (e.g., in Asia), *H. pylori* causes almost all uncomplicated duodenal ulcers and more than 80 % of gastric ulcers, especially if the previous use of NSAIDs has been excluded (Gisbert et al. 1999). In

low-prevalence areas such as the USA, *H. pylori* accounts for a smaller proportion of PUD (Table 15.2). However, evidence supporting this is derived from retrospective cohorts, which do not fully consider NSAID and proton pump inhibitor (PPI) use (Jyotheeswaran et al. 1998; Ciociola et al. 1999).

15.2.3 Treatment of PUD

Eradication of *H. pylori* infection undoubtedly alters the natural history of PUD. Several studies have shown that ulcer recurrence is very low or nonexistent 1 year following eradication. This is in stark comparison with the natural recurrence rate of over 70 % (Marshall et al. 1988). Following failure of *H. pylori* eradication, ulcer recurrence may exceed 50 % (Fiocca et al. 1991). The details of medical treatment for *H. pylori* infection are covered in Chap. 20.

15.2.4 Complications of PUD

15.2.4.1 Bleeding

Complications of PUD develop in 20–25 % of patients and include hemorrhage, perforation, penetration, and obstruction. Bleeding is the most frequent complication and accounts for approximately 70 % of complicated PUD. Bleeding is the major cause of PUD-associated morbidity and mortality. *H. pylori*-associated ulcers are thought to confer a lower risk of bleeding compared to NSAID or idiopathic ulcers (Vaira et al. 1997; Boltin et al. 2012). Gisbert and coworkers reviewed 32 studies and found *H. pylori* in 80 % of bleeding peptic ulcers which was lower than the positivity rate in non-bleeding ulcers (Gisbert and Abraira 2006). However, when delayed detection techniques are employed to determine *H. pylori* infection, sensitivity is greatly improved, and the prevalence of *H. pylori* in bleeding ulcers approaches that of non-bleeding ulcers. This was shown in an analysis of 71 studies including 8496 subjects, where *H. pylori* was found to increase the risk of ulcer bleeding by a factor of 1.79 (Sánchez-Delgado et al. 2011). CagA-positive strains of *H. pylori* confer an even higher risk of ulcer bleeding (Stack et al. 2002).

Prospective long-term data indicate that the risk of rebleeding is virtually eliminated following *H. pylori* eradication (Gisbert et al. 2012). Persistence of H. *pylori* appears to be one of the most important factors causing rebleeding in patients with ulcer bleeding. For this reason, PPI treatment is recommended until the documented healing of a gastric ulcer, or until *H. pylori* eradication for duodenal ulcers, but not beyond (Malfertheiner et al. 2012).

Table 15.2 Large studies ($N \geq 100$) performed in Western countries evaluating *Helicobacter pylori* prevalence in patients with duodenal ulcer (1999–2013)

Author	Year	Country	Number of patients	Prevalence of *H. pylori* (%)
Ciociola et al.	1999	The USA	2394	73
Gisbert et al.	1999	Spain	774	95
Higuchi et al.	1999	Japan	330	96
Tsuji et al.	1999	Japan	120	96
Aoyama et al.	2000	Japan	111	98
Arakawa et al.	2000	Japan	368	96
Meucci et al.	2000	Italy	317	92
Nishikawa et al.	2000	Japan	152	99
Bytzer et al.	2001	Denmark	276	88
Lutgen et al.	2001	France	152	79
Spaziani et al.	2001	Italy	240	75
Sugiyama et al.	2001	Japan	151	99
Xia et al.	2001	Hong Kong	599	79
Palli et al.	2002	The UK	275	90
Kamada et al.	2003	Japan	464	97
Arents et al.	2004	The Netherlands	254	89
Arroyo et al.	2004	Spain	472	96
Kato et al.	2004	Japan	100	83
Chu et al.	2005	Hong Kong	1343	70
Xia et al.	2005	Hong Kong	271	90
Ong et al.	2006	The UK	288	69
Pietroiusti et al.	2008	Italy	608	93
Chen et al.	2010	Taiwan	626	88.7
Ortega et al.	2010	Chile	5664	86.6
Li et al.	2010	China	1030	92.6
Musumba et al.	2012	The UK	386	66
Cekin et al.	2012	Turkey	222	84.9
Buzás et al.	2013	Hungary	4647	59.1

15.2.4.2 Perforation

H. pylori eradication is similarly beneficial following perforation of a peptic ulcer. A large meta-analysis found that *H. pylori* eradication combined with operative management resulted in a lower rate of ulcer recurrence at 1 year, compared to surgery followed by PPI therapy alone (5.2 % vs. 35.2 %) (Tomtitchong et al. 2012). Therefore, after surgical management of perforated peptic ulcer, *H. pylori* eradication is recommended in all infected patients in order to prevent ulcer relapse.

15.2.4.3 Gastric Outlet Obstruction

In a shift from the traditional approach of managing obstructing duodenal ulcers surgically, most current guidelines recommended conservative treatment with *H. pylori* eradication. A nonsurgical approach may also combine enteral nutritional support and endoscopic dilatation. Surgery should be reserved for salvage treatment in patients unresponsive to medical therapy (Gisbert and Pajares 2002).

15.2.5 **H. pylori*, Aspirin, and NSAIDs***

Both *H. pylori* and NSAIDs are established risk factors for PUD and ulcer-related bleeding. When these two factors are simultaneously present, the potential for complications is compounded, even though it is impossible to know precisely the individual contribution of each factor (Huang et al. 2002). *H. pylori* increases the risk of NSAID-related mucosal injury (Lanza et al. 2009). So too, patients with complicated PUD who continue to receive NSAIDs/aspirin following *H. pylori* eradication have an increased incidence of rebleeding.

Naïve patients, without prior PUD, who are set to commence long-term therapy with NSAIDs/aspirin clearly benefit from *H. pylori* eradication. This is based on large, well-designed randomized controlled trials (Chan et al. 1997). In subjects already receiving long-term NSAIDs/aspirin, there is no obvious benefit in treating *H. pylori*. A meta-analysis concluded that long-term PPI is more effective than *H. pylori* eradication for the prevention of ulcer bleeding in patients already receiving NSAIDS (Vergara et al. 2005). In this study, 2.6 % of subjects developed PUD following eradication, whereas none of the patients receiving PPI developed an ulcer, despite persistent *H. pylori* infection.

Patients receiving low-dose aspirin are at minimal risk of PUD and ulcer-related bleeding. A prospective 10-year cohort study including 904 subjects receiving low-dose aspirin compared three patient groups: *H. pylori*-positive patients with bleeding ulcers who resumed aspirin following eradication, *H. pylori*-negative patients with bleeding ulcers who resumed aspirin following ulcer healing, and new users of aspirin without a history of ulcers (average risk) (Chan et al. 2013). None of the patients received PPI treatment. The rate of ulcer bleeding in patients with previous ulcer bleeding following eradication was similar to the average-risk group (0.97 % and 0.66 %, respectively). Recurrent ulcer bleeding was highest in aspirin users without prior *H. pylori* infection (5.22 %). In keeping with these findings, the Maastricht IV/Florence Consensus Report recommends that patients with a history of PUD should be tested for *H. pylori* and following eradication these patients do not require long-term PPI (Malfertheiner et al. 2012). *H. pylori*-negative patients should receive adequate antisecretory therapy if they have a history of ulcer bleeding, since they are prone to ulcer bleeding with aspirin use.

15.3 Gastroesophageal Reflux Disease

15.3.1 Pathophysiology

The most commonly cited cause for GERD is transient relaxation of the lower esophageal sphincter. Additional causes include a hypotensive sphincter, hiatal hernia, and acid hypersecretion. Chronic *H. pylori* infection is commonly associated with hypochlorhydria, and therefore patients harboring *H. pylori* are theoretically protected from GERD and may even exhibit an augmented response to proton pump inhibitors. Following eradication of *H. pylori*, acid hypersecretion may occur for up to 8 weeks due to increased parietal and ECL cell masses; however, these changes are short-lived and GERD does not generally ensue (Gillen et al. 1999).

15.3.2 Epidemiology

GERD affects 25–40 % of the population. Population-based studies have consistently found a lower prevalence of *H. pylori* infection among patients with GERD, especially CagA-positive strains. A systematic review including 20 studies and 4134 patients found a lower prevalence of *H. pylori* in GERD subjects compared to controls (OR 0.60, 95 % confidence interval, 0.47–0.78) (Raghunath 2003). This disparity was most profound in Asian populations where subjects with GERD had an even lower prevalence of *H. pylori* compared to their Western counterparts, despite their higher background prevalence. Data from 1611 African American patients with endoscopic evidence of esophagitis found that the prevalence of *H. pylori* was 4 %. After adjusting for age and gender, the odds ratio of *H. pylori* infection in erosive esophagitis was 0.06 (95 % confidence interval, 0.01–0.59; $p = 0.01$) (Ashktorab et al. 2012).

An association between *H. pylori* eradication and the subsequent development GERD remains unsubstantiated (Table 15.3). A meta-analysis of ten randomized controlled trials, comparing patients who received either *H. pylori* eradication or placebo, found no significant difference in the incidence of reflux symptoms (17 % vs. 23 %) or erosive esophagitis (5 % vs. 5.1 %) following treatment. In fact, a sub-analysis found a significantly lower incidence of reflux symptoms in the eradicated versus the placebo group (14 % vs. 25 %) (Saad et al. 2012). These findings are consistent with current recommendation not to refrain from treating helicobacter in patients with GERD (Malfertheiner et al. 2012).

Table 15.3 Randomized controlled trials comparing *Helicobacter pylori* treatment with no treatment in symptomatic adults with gastroesophageal reflux disease (GERD)

				GERD symptoms following *H. pylori* eradication, n (%)	
Author	Year	Country	Number of patients	Treatment group	Placebo
Befrits et al.	2000	Sweden	145	22/79 (27.8)	29/66 (43.9)
Bytzer et al.	2000	Denmark	276	7/83 (8.4)	5/85 (5.9)
Hamada et al.	2000	Japan	572	36/286 (12.6)	1/286 (0.3)
Vakil et al.	2000	The USA	242	41/178 (23.0)	12/64 (18.8)
Kim et al.	2001	Korea	452	26/233 (11.2)	8/144 (5.6)
Moayyedi et al.	2001	The UK	178	15/85 (17.6)	15/93 (16.1)
Schwizer et al.	2001	Switzerland	29	7/13 (53.8)	15/16 (93.8)
Laine et al.	2002	The USA	1165	33/361 (9.1)	27/172 (15.7)
Malfertheiner et al.	2002	Germany	1362	121/993 (12.2)	88/369 (23.8)
Harvey et al.	2004	The UK	1558	169/787 (21.5)	170/771 (22.0)
Kuipers et al.	2004	The Netherlands	231	30/111 (27.0)	27/120 (22.5)
Wu et al.	2004	Hong Kong	104	15/53 (28.3)	8/51 (15.7)
Ott et al.	2005	Brazil	157	8/73 (11.0)	7/60 (11.7)
Pilotto et al.	2006	Italy	61	6/31 (19.4)	6/30 (20.0)
Jonaitis et al.	2010	Lithuania	181	17/119 (14.3)	2/31 (6.4)
Nam et al.	2010	Korea	10,102	25/548 (4.6)	22/1635 (1.3)
Rodrigues et al.	2012	Brazil	32	7/9 (77.8)	12/13 (92.3)

15.3.3 Proton Pump Inhibitors

Patients with chronic *H. pylori* infection typically exhibit low basal and stimulated acid output compared to noninfected subjects (Fig. 15.1). Holtmann and coworkers demonstrated that patients with *H. pylori* infection have accelerated healing of erosive esophagitis when treated with PPIs, compared to uninfected patients. After 4 weeks of treatment, complete healing of esophageal erosions was seen in 86.6 % of H. *pylori*-positive patients and 76.3 % of *H. pylori*-negative patients, $p < 0.01$ (Holtmann et al. 1999). The mechanism, via which *H. pylori* augments healing of esophagitis in the presence of PPI, has not been explored. There is currently no evidence that lower PPI doses are required in *H. pylori*-infected GERD patients in order to induce or maintain remission of erosive esophageal disease (Schenk et al. 1999).

Long-term acid suppression with proton pump inhibition, commonly prescribed for GERD, causes the progressive loss of parietal glands. Patients with *H. pylori* infection who are treated with PPIs may develop a corpus predominant atrophic gastritis. This pattern of inflammation is distinct from the pangastritis typically seen in infected patients who are not receiving PPIs (Moayyedi et al. 2000b; Kuipers et al. 1996). In *H. pylori*-infected animal models, PPIs have been shown to

accelerate the progression of gastric cancer, although data in humans are lacking. The Maastricht IV/Florence Consensus Report recommends eradication of *H. pylori* in patients receiving chronic PPIs in order to heal gastritis and prevent atrophic changes (Malfertheiner et al. 2012).

15.3.4 Complications of GERD

15.3.4.1 Barrett's Esophagus

Barrett's esophagus is a premalignant lesion of the distal esophagus, related to chronic acid exposure. Histologically, Barrett's esophagus is characterized by metaplastic columnar epithelium which replaces the stratified squamous epithelium which normally lines the distal esophagus. Seven studies including 1621 patients with Barrett's esophagus found a significantly lower prevalence of *H. pylori* (49.1 %) compared to matched controls (57.7 %). The pooled odds ratio for *H. pylori* infection in Barrett's esophagus was 0.64 (95 % CI, 0.43–0.94; $p = 0.03$) (Rokkas et al. 2007). A negative correlation between *H. pylori* infection and Barrett's esophagus is similarly observed for CagA-positive strains (35.6 % vs. 51.5 %; OR, 0.39; 95 % CI, 0.21–0.76; $p < 0.01$). Nevertheless, there is no evidence to support withholding eradication of *H. pylori* in patients with Barrett's esophagus.

15.3.4.2 Esophageal Adenocarcinoma

It is tempting to attribute the recent increase in esophageal adenocarcinoma (EAC) in Western countries to the declining prevalence of *H. pylori*. A review of 10 studies, including 737 patients with EAC, found that the prevalence of *H. pylori* in these patients was lower compared to controls (34.3 % vs. 50.1 %; OR, 0.52; 95 % CI, 0.37–0.73; $p < 0.01$) (Rokkas et al. 2007). A negative correlation between *H. pylori* infection and EAC is similarly observed for CagA-positive strains (26 % vs. 40 % CagA positivity in EAC and controls, respectively; OR, 0.51; 95 % CI, 0.31–0.82; $p < 0.01$). There is no evidence to support withholding eradication of *H. pylori* in patients following resection of EAC.

15.4 Functional Dyspepsia

15.4.1 Classification

The Rome III committee defines functional dyspepsia (FD) as "the presence of one or more dyspepsia symptoms that are considered to originate from the

gastroduodenal region, in the absence of any organic, systemic or metabolic disease that is likely to explain the symptoms" (Drossman 2006). Dyspeptic symptoms can be broadly characterized into two subgroups: the postprandial distress syndrome or the epigastric pain syndrome. According to the Rome III consensus, *H. pylori* infection does not preclude a diagnosis of FD, despite the fact that *H. pylori* is an undisputed cause of mucosal inflammation. This has prompted a debate whether it is really appropriate that *H. pylori*-associated dyspepsia be considered a functional disease (Sugano 2011). Adding fuel to this debate, emerging endoscopic technologies enable the reliable diagnosis of *H. pylori*-associated chronic gastritis at the time of endoscopy.

15.4.2 Pathophysiology

The precise mechanism via which *H. pylori* may cause postprandial distress or epigastric pain is unknown; however, several pathways have been suggested:

1. Increased acid secretion. Patients with isolated antral *H. pylori* gastritis manifest increased gastric acid secretion. This is probably mediated by a reduction in somatostatin, a negative regulator of gastrin release. *H. pylori*-infected subjects with FD manifest greater acid secretion in response to gastrin-releasing peptide, compared to those without FD (el-Omar et al. 1993).
2. Decreased ghrelin secretion. Ghrelin is a peptide hormone produced by the P/D1 cells of the gastric fundus and is involved in hunger sensation, acid secretion, and motility. Both gastric expression and serum levels of ghrelin are significantly lower in *H. pylori*-positive subjects (Boltin and Niv 2012). Activation of ghrelin receptors leads to increased levels of neuropeptide Y (NPY) and agouti-related peptide which promote appetite. Low levels of ghrelin, as seen in *H. pylori* infection, possibly mediate symptoms of early satiety and postprandial fullness.
3. Altered gastric emptying. Chronic *H. pylori* infection leads to downregulation of muscle-specific miRNA expression. In murine models this causes hyperplasia of the muscularis mucosa leading to reduced gastric accommodation and accelerated gastric emptying (Saito et al. 2011).
4. Mast cells. Increased numbers of antral mast cells have been noted in *H. pylori*-infected subjects with FD. Although this may be involved in the pathogenesis of FD, mast cell proliferation is unlikely to be mediated by *H. pylori*, as an increased number of mast cells is also observed in FD patients without *H. pylori* (Hall et al. 2003).

15.4.3 Epidemiology

About 20–30 % of the population reports persistent dyspepsia each year. Only a minority of these patients is fully investigated; however, an organic cause is not usually identified. Therefore, the remainder can be considered to have FD. The incidence of FD is estimated at 1/100 person-years (Agréus et al. 1995).

15.4.4 Treatment

A recent systematic review identified 21 randomized controlled trials which have examined the efficacy of *H. pylori* eradication for the treatment of FD. Overall, *H. pylori* eradication is associated with a relative risk reduction of 10 %, for dyspeptic symptoms, compared to placebo. The number needed to treat to cure one case of dyspepsia is 14 (Moayyedi et al. 2011). Nevertheless, studies performed in Western populations have inconsistent results, with eradication having a variable impact on dyspeptic symptoms (Table 15.4) (Mazzoleni et al. 2011; Talley et al. 1999; Hsu et al. 2001). Eradication may, however, be beneficial in preventing the subsequent development of PUD in patients with epigastric pain. Studies in Asian populations are more likely to show a benefit for H. *pylori* treatment in FD (Gwee et al. 2009; Des Bruley Varannes et al. 2001; Lan et al. 2011). The cost-effectiveness of eradication in FD depends upon the background prevalence of H. *pylori*, the cost of treatment, as well as other factors. Therefore, although eradication may be cost-effective in Asia and Europe, this is not necessarily true in the USA (Moayyedi et al. 2000a).

15.5 Other Gastrointestinal Disease

15.5.1 Inflammatory Bowel Disease

Patients with Crohn's disease (CD) have a disproportionately low prevalence of *H. pylori* (Luther et al. 2010). The estimated relative risk of *H. pylori* infection in CD is 0.64 (95 % CI, 0.54–0.75). Over the past few decades, the declining rates of H. *pylori* carriage have mirrored the increasing prevalence of inflammatory bowel disease. This cannot be fully explained by socioeconomic or other environmental factors, since a similar relationship is not observed with ulcerative colitis or other chronic diseases which feature immune dysfunction. Subjects with *H. pylori* infection exhibit a blunted T_{h1}/T_{h17} immune response and have low tissue and serum levels of proinflammatory cytokines such as interferon-γ (IFN-γ) (Fig. 15.3) (Luther et al. 2011). On the other hand, patients with CD have an exaggerated T_{h1}/T_{h17} immune response. The factors involved in defining the nature of an

Table 15.4 Randomized controlled trials comparing *Helicobacter pylori* treatment with no treatment in adults with functional dyspepsia

						Results		
Author	Year	Country	Number of patients	Duration of follow-up	Outcome measure	Treatment group n (%)	Control n (%)	*p*
Talley et al.	1999	Australasia and Europe	278	12 months	Near-total relief of epigastric pain	32/133 (24)	31/142 (22)	ns
Hsu et al.	2001	Taiwan	161	12 months	Symptom resolution	47/81 (58.0)	44/80 (55.0)	ns
Des Bruley Varannes et al.	2001	France	253	12 months	Symptom resolution	55/129 (43)	38/124 (31)	0.048
Gwee et al.	2009	Singapore	82	12 months	Symptom resolution	10/41 (24.4)	3/41 (7.3)	0.02
Lan et al.	2011	China	195	3 months	Reduction rate (pretreatment–posttreatment scores)/pretreatment score × 100	36/98 (36.7)	19/97 (19.6)	<0.05
Mazzoleni et al.	2011	Brazil	404	12 months	50 % symptom reduction	94/192 (49.0)	72/197 (36.5)	0.01

This table includes principal studies performed and is not intended to be exhaustive

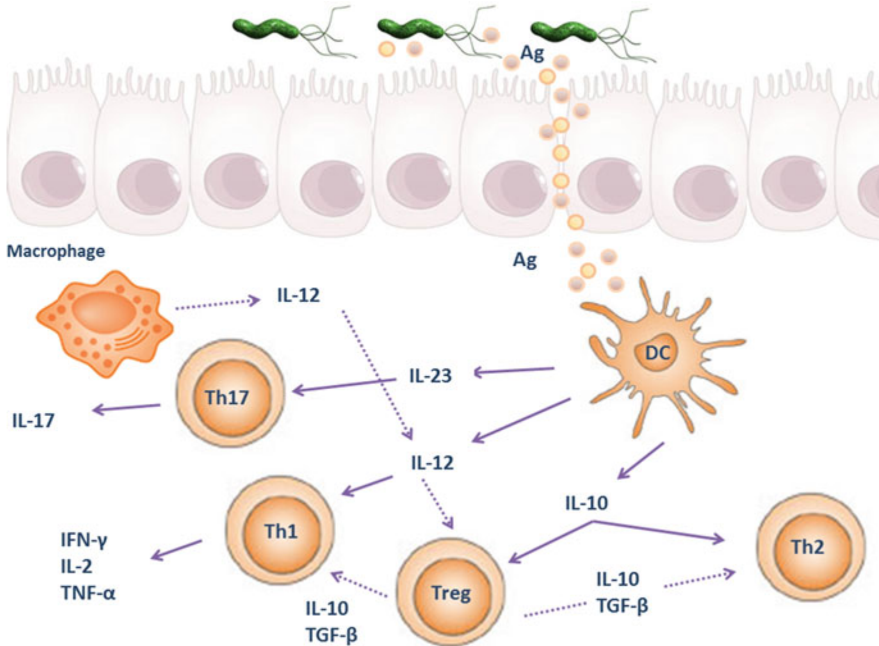

Fig. 15.3 A proposed model of *H. pylori*'s effect on host immune regulation. Dendritic cells (DCs) sample *H. pylori* antigens directly. DCs, in turn, secrete cytokines such as IL-10 to upregulate Foxp3-positive regulatory T cells (Tregs). This upregulation skews the host immunologic tone away from the IL-12 and IL-23-mediated inflammatory Th1/Th17 responses and leads to decreased production of proinflammatory cytokines. This may theoretically confer protection from the development of CD

individual's T_{h1}/T_{h17} immune response remain elusive. An individual's particular immune response might be modified by H. *pylori* at the time of infection, or it might be an inherited trait which predisposes to either to chronic gastritis (following exposure to *H. pylori*) or to future CD, at the two ends of the spectrum. Animal models suggest the former – mice colonized with *H. pylori* are protected from developing dextran sodium sulfate (DSS) – colitis, indicating that *H. pylori* modulates immune function. As more data becomes available on the immunoregulatory function of *H. pylori*, we may find additional evidence to advocate postponing eradication of *H. pylori* in children, with the added benefit of decreasing the incidence of CD.

15.5.2 Halitosis

In the vast majority of cases (>90 %), halitosis is attributable to oral or nasopharyngeal pathology. A connection between halitosis and either upper gastrointestinal

symptoms or endoscopic findings has not been conclusively proven (Tas et al. 2011). Nevertheless, a theoretical basis exists, as well as abundant empirical data, linking *H. pylori* to halitosis. Following Marshall's historic ingestion of *H. pylori* in his quest to prove Koch's postulate, oral malodor was noted by his colleagues (Marshall et al. 1985). Volatile sulfur compounds including hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH), which are produced by certain strains of *H. pylori*, are also the major components of oral malodor (Lee et al. 2006). Katsinelos et al. reported that *H. pylori* eradication in FD led to a sustained resolution of halitosis during long-term follow-up (Katsinelos et al. 2007). Nevertheless, in the absence of robust data, *H. pylori* cannot be considered a treatable cause of halitosis.

15.6 Non-gastrointestinal Disease

15.6.1 Asthma and Allergy

As described previously with respect to CD, *H. pylori* has the ability to influence the maturation and direction of host immune pathways. *H. pylori* infection can induce dendritic cells to generate regulatory T cells (Tregs) which subsequently protect against asthma (Fig. 15.3) (Oertli and Müller 2012). The *H. pylori* virulence factor, neutrophil-activating protein A (NapA), might protect against asthma by inhibiting polarization of T helper (T_h)-1 and inhibiting the allergic T_{h2} response. NapA and Tregs are under investigation as novel asthma treatments. A meta-analysis of case–control and cross-sectional studies by Zhou and coworkers, including over 28,000 subjects across three continents, found a slightly lower rate of *H. pylori* infection in subjects with asthma (OR, 0.84; 95 % CI, 0.73–0.96; $p = 0.01$) (Zhou et al. 2013). Subgroup analysis, however, found that a significant difference in *H. pylori* prevalence between asthmatic and non-asthmatic subjects exists only in the USA and not in Europe or Asia. Wang and coworkers published a similar meta-analysis but also included cohort studies (Wang et al. 2013). Pooled data from all studies revealed a significant inverse association between *H. pylori* infection and asthma for both adults (OR, 0.88; 95 % CI, 0.71–1.08; $p < 0.05$) and children (OR, 0.81; 95 % CI, 0.72–0.91; $p < 0.05$). In both meta-analyses, CagA-positive *H. pylori* strains seem to be even more protective against asthma than CagA-negative strains. For more details we refer to Chap. 12.

15.6.2 Idiopathic Thrombocytopenic Purpura

The mechanism through which *H. pylori* causes idiopathic thrombocytopenic purpura (ITP) probably involves cross mimicry between *H. pylori* and platelet

antigens. This may be specifically related to CagA strains of *H. Pylori*. It has been demonstrated that platelet-associated IgG in ITP patients recognizes the CagA antigen (Franchini and Veneri 2006). Another mechanism could involve the interaction between *H. pylori* and platelets through von Willebrand factor and IgG anti-*H. pylori* antibody, leading to chronic platelet consumption. In a review of 16 studies involving 1126 subjects with ITP, *H. pylori* prevalence was 64 %. Following successful eradication, a platelet response was seen in 53 %. However, the studies included were heterogeneous and included mainly uncontrolled and anecdotal data (Franchini and Veneri 2006). The Maastricht IV/Florence Consensus Report advocates seeking and treating *H. pylori* in the setting of ITP (Malfertheiner et al. 2012).

15.6.3 Iron Deficiency Anemia

Possible pathogenic mechanisms for *H. pylori* causing iron deficiency anemia include: occult blood loss secondary to erosive gastritis, decreased iron absorption secondary to *H. pylori*-induced hypochlorhydria, and increased iron uptake and utilization by *H. pylori* (Dubois and Kearney 2005). Although epidemiologic studies support an association between *H. pylori* infection and low ferritin, only a few small, uncontrolled case series and one small, randomized trial have shown improvement in anemia following *H. pylori* treatment (Choe et al. 1999). The available evidence suggests that *H. pylori* is most likely to be associated with anemia in patients who are anyway predisposed to iron deficiency, such as premenopausal women and children.

15.6.4 Others

H. pylori has been epidemiologically linked to a wide range of diseases including Alzheimer's disease, Parkinson's disease, Raynaud's phenomenon, scleroderma, idiopathic urticaria, acne rosacea, migraines, thyroiditis, Guillain–Barré syndrome, and coronary artery disease. The proposed mechanisms leading to these various conditions range from systemic immune reactions, cross-reactivity of bacterial and host proteins, and events secondary to gastric mucosal injury (Goodman et al. 2006). Of these associations, the strongest link is for ischemic heart disease. Specifically, CagA seropositivity has been significantly associated with acute coronary events (OR, 1.34; 95 % CI, 1.15–1.58; $p < 0.01$) (Franceschi et al. 2009). Nevertheless, the available evidence is still insufficient to make a clear causal or therapeutic link.

15.7 Conclusions and Outlook

There is a wealth of information regarding the epidemiology and pathophysiology of *H. pylori* in the setting of nonmalignant diseases. The future direction of study in the area of peptic ulcer disease is likely to involve emerging molecular technologies such as micro-RNAs. Such research may focus on RNA silencing and posttranslational regulation of the genes for proteins which mediate *H. pylori* virulence, evasion of gastric mucosal defense apparatus, and subsequent ulcer formation. Further study will also investigate the complex interaction between *H. pylori* and the host immune system. This may lead to the recognition of a host "immunophenotype" which predisposes to the development of peptic ulcer disease in the presence of *H. pylori* or asthma or Crohn's disease in its absence. Study of *H. pylori* virulence factors will continue, including their role in peptic ulcer formation. For example, the structure of the *H. pylori* proton-gated urea channel was recently described. Future research into this channel might investigate gene polymorphisms which affect function, interaction with host immunity, and even therapeutic targeting of the channel. Another rapidly developing field of research relates to the effect of *H. pylori* infection on the composition of the gastric microbiome and intestinal microbiome as a whole. The functional role of the gastric microbiome is entirely unclear, as is the effect of *H. pylori*-associated gastritis on the composition of the intestinal microbiome. A better understanding of the interaction between *H. pylori* and the intestinal microbiome may be particularly relevant in the setting of functional dyspepsia. In the area of GERD, future study may be directed at identifying patients with a high risk of esophageal adenocarcinoma, in whom *H. pylori* eradication might be withheld. Undeniably, *H. pylori* is relevant to all of the highly prevalent nonmalignant diseases discussed. For this reason, *H. pylori* in nonmalignant disease will continue to be the subject of research for many years to come.

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Chapter 16
Helicobacter pylori and Gastrointestinal Polyps

Robert M. Genta and Richard H. Lash

Abstract Polyps of the gastrointestinal tract are mucosal elevations that may have a mechanical, developmental, inflammatory, or neoplastic pathogenesis. Therefore, it is not surprising that there is no specific relationship between gastrointestinal polyps and *H. pylori* infection. In the stomach, inflammatory and proliferative responses, irrespective of their etiology, seem to favor the development of inflammatory polyps. Intact oxyntic glands, unaffected by inflammatory changes, seem to be more vulnerable to focal dilatations induced by proton pump inhibitors, hence the strong negative association of these nonneoplastic, noninflammatory polyps with *H. pylori* gastritis. Gastric adenomas, precursors of adenocarcinoma, are likely related to *H. pylori* infection by the same mechanisms that link this infection to gastric cancer. In the colon, the indisputable association of *H. pylori* gastritis with both hyperplastic and neoplastic polyps remains largely unexplained.

Keywords *Helicobacter pylori* • Fundic gland polyps • Gastric hyperplastic polyps • Gastric adenomas • Gastric inflammatory fibroid polyps • Gastric neuroendocrine tumors • Carcinoids • Duodenal adenomas • Colon polyps

16.1 Introduction

The aphorism attributed to Charles Mayo in 1933 “gastric cancer does not arise in a healthy stomach” (Bockus 1968) could be paraphrased to “gastric polyps do not arise in a healthy stomach.” An inflammatory background has traditionally been considered necessary to favor the development of what (until two or three decades ago) represented the vast majority of gastric polyps: hyperplastic-inflammatory polyps, adenomas, and neuroendocrine proliferations (then referred to as carcinoids). These three types of polyps were particularly likely to be found in patients with atrophic gastritis: hyperplastic polyps and adenomas were more common in

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multifocal atrophic gastritis and carcinoids in autoimmune atrophic gastritis. As long-standing *Helicobacter pylori* infection emerged as the cause of multifocal atrophic gastritis, the former two lesions – one inflammatory and one neoplastic – became intimately tied to *H. pylori* gastritis. Approximately 40 years ago, the German pathologist Karl Elster described cystic lesions resulting from the dilatation of oxyntic glands and proposed the name "fundic gland polyps." These polypoid lesions were soon found to be highly prevalent in patients with familial polyposis syndromes, while their sporadic occurrence in the absence of familial polyposis was rare. In the last two decades, however, not only have sporadic fundic gland polyps become the most common type of gastric polyp but they have also been found to arise almost exclusively in healthy uninfected stomachs. As *Helicobacter* was being alternatively related and unrelated to gastric polyps, another more intriguing association has emerged: colonic polyps of all types appear to be more prevalent in patients with *Helicobacter* gastritis than in uninfected subjects.

This chapter will explore the associations between *H. pylori* infection and polyps of the gastrointestinal tract. We will discuss both inflammatory and neoplastic polyps; however, we will not address the malignant transformation of polyps, as this subject is covered in other chapters of this book.

16.2 Gastric Hyperplastic Polyps

Hyperplastic polyps are proliferations of the gastric foveolar cells and associated stroma (lamina propria) (Shaib et al. 2013). Because of their prominent inflammatory stromal component and because they do not resemble the serrated, stroma-poor hyperplastic polyps of the colon, they have also been referred to as inflammatory polyps. However, the use of two different names for the same entity has created confusion, especially with inflammatory fibroid polyps; therefore, here we shall adhere to the currently standard terminology of hyperplastic polyps. The typical features of hyperplastic polyps, depicted in Fig. 16.1, include elongated, grossly distorted, branching, and dilated hyperplastic foveolae lying in an edematous stroma rich in vasculature and inflammatory cells with small, haphazardly distributed smooth muscle bundles. Hyperplastic polyps are equally common in men and women and typically occur in the sixth and seventh decades (median age 66 years). They are most frequently found in the antrum; are often multiple, sessile, or pedunculated; and often appear erythematous endoscopically, occasionally with erosions or even ulceration. Mutations of the tumor suppressor p53 gene, chromosomal aberrations, and microsatellite instability have all been detected in these polyps, and between 1 % and 20 % of hyperplastic polyps have been reported to harbor foci of dysplasia (Lauwers et al. 1993; Nogueira et al. 1999). The overall prevalence of carcinoma in hyperplastic polyps is <2 %, and it is more frequent in polyps larger than 2 cm (Murakami et al. 2001; Yao et al. 2002).

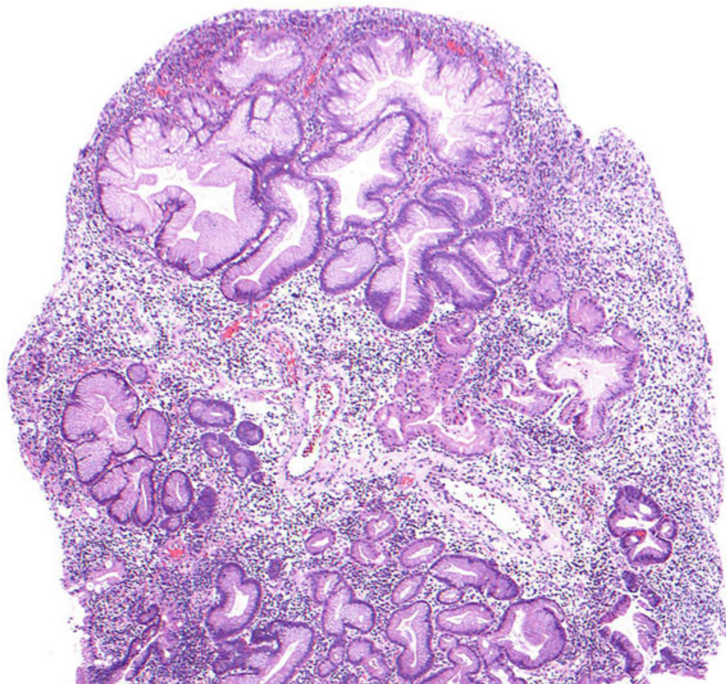

Fig. 16.1 Gastric hyperplastic polyp. These lesions are characterized by elongated, grossly distorted, branching, and dilated hyperplastic foveolae lying in an edematous stroma rich in vasculature and inflammatory cells with small, haphazardly distributed smooth muscle bundles. As in the polyp depicted here, the surface is frequently eroded. Hematoxylin and eosin, original magnification 10×

16.3 Association with *H. pylori* Infection

The classic association of gastric hyperplastic polyps has been with mucosal atrophy, and gastric carcinomas have been known to be more likely to develop in a stomach containing hyperplastic polyps. In an attempt to elucidate the reasons for this association, Dirschmid and coworkers used the updated Sydney System (Dixon et al. 1996) to evaluate the gastritis phenotype in 244 patients with hyperplastic polyps (Dirschmid et al. 2006). In none of the 244 patients was the gastric mucosa found to be normal. The most common disorder was autoimmune atrophic gastritis (56 %). *H. pylori* gastritis was seen in 37 % of the patients, 56 % of whom had corpus-predominant gastritis. Other forms of gastritis were rarely reported. The authors interpreted autoimmune gastritis and all forms of *H. pylori* gastritis (present in 89 % of their patients) as precancerous conditions and concluded that hyperplastic polyps represent a strong predictor of the synchronous presence of preneoplastic lesions. In an earlier study of 160 patients with hyperplastic polyps, in which more rigorous criteria for the diagnosis of autoimmune atrophic gastritis were applied,

Abraham and colleagues evaluated the histopathologic background of the gastric mucosa surrounding the polyps: 37 % of the patients had at least focal intestinal metaplasia, while adenoma or low-grade flat epithelial dysplasia was found in 2 % and synchronous or metachronous adenocarcinoma in 6 %. *H. pylori* gastritis accompanied hyperplastic polyps in 25 % of the patients, reactive or chemical gastropathy in 21 %, autoimmune gastritis in 12 %, and *H. pylori*-associated multifocal atrophic gastritis in 8 %. Thus, a direct association with *H. pylori* or atrophy was detected in 33 % and 20 % of the patients, respectively (Abraham et al. 2001). Although these two studies disagree on the relative percentages, both conclude that an inflammatory or reactive background greatly increases the risk of developing a hyperplastic polyp.

Foveolar hyperplasia, long recognized as a prominent feature of chemical (or reactive) gastropathy and, to a lesser extent, of *H. pylori* gastritis, initiates as a hyper-proliferative response to tissue injury (erosions, perhaps even micro-erosions, or ulcers) accompanied by increased cellular exfoliation (Dixon et al. 1986). The initial step in the formation of hyperplastic polyps is foveolar hyperplasia. Intermediate polyp forms known as "polypoid foveolar hyperplasia" (Fig. 16.2) consist of localized elongated and prominent foveolae that stand out as mucosal nodules, but without the edematous stroma, dilated glands, erosions, and mixed inflammatory infiltrates that characterize classic hyperplastic polyps (Carmack et al. 2009a). In the past two decades, in part because of the decline of *H. pylori* infection and in part because of the widespread use of acid-suppressing

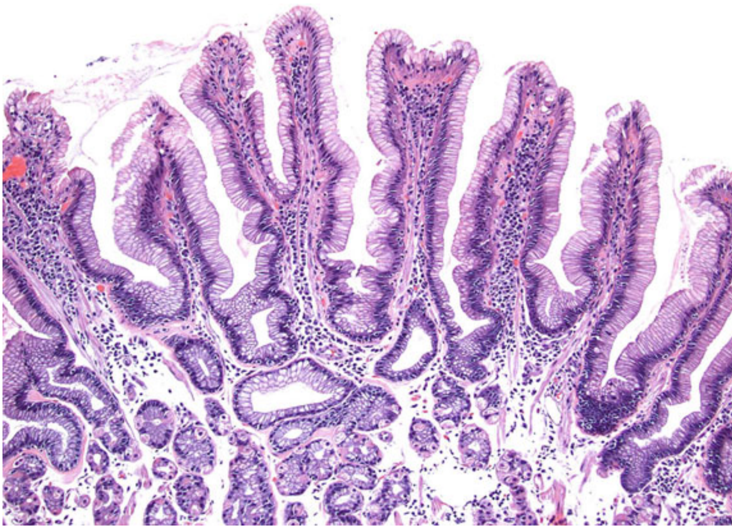

Fig. 16.2 Polypoid foveolar hyperplasia. Polypoid foveolar hyperplasia is frequently the initial step in the formation of hyperplastic polyps and consists of localized elongated and prominent foveolae that stand out as mucosal nodules, but without the edematous stroma, dilated glands, erosions, and mixed inflammatory infiltrates that characterize classic hyperplastic polyps. Hematoxylin and eosin, original magnification 10×

medications (histamine-2 receptor antagonists and proton pump inhibitors), the prevalence of chronic gastritis has been declining, particularly in the industrialized countries of Europe and North America and in the emerging economies of East Asia, while reactive gastropathy appears to be on the rise. In a 2011 survey of more than 500,000 North American patients who had gastric biopsy specimens evaluated at a single pathology laboratory, only 20.1 % of the patients had gastritis (with or without *H. pylori*), whereas 15.6 % had reactive gastropathy. The prevalence of reactive gastropathy increased steadily with age, from 2 % in children younger than 10 years to more than 20 % in patients older than 80 years (Maguilnik et al. 2012). In parallel with these shifting gastric pathologies, hyperplastic polyps have slipped from being the most common type of gastric polyp encountered at endoscopy to comprising less than 20 %,(Carmack et al. 2009b), and an increasing proportion of hyperplastic polyps is found in the background of a reactive gastric mucosa with no evidence of current or prior *H. pylori* infection. Taken together, hyperplastic polyps are related to reactive and hyper-proliferative states of the gastric mucosa, but are not specifically or even preferentially induced by *H. pylori* infection. Their association with malignancy is low.

16.4 Gastric Adenomas (Raised Intraepithelial Neoplasia)

The most common neoplastic polyp of the stomach is an epithelial dysplastic growth still commonly referred to as adenoma, in spite of the new nomenclature (raised intraepithelial neoplasia) suggested by the World Health Organization (WHO) (Lauwers et al. 2010). In Western Europe and North America, sporadic gastric adenomas have become rare, accounting for <1 % of all gastric polyps (Carmack et al. 2009b). This contrasts markedly with some East Asian regions, where the incidence of gastric cancer remains high and gastric adenomas still constitute approximately a quarter of all gastric polyps (Lauwers and Srivastava 2007; Nakamura and Nakano 1985).

Gastric adenomas occur with similar frequency in men and women, most commonly in the sixth and seventh decade. Although they can be found anywhere in the stomach, they are more often located in the antrum. Endoscopically they have a velvety lobulated appearance and are usually solitary. Histologically, gastric adenomas consist of elevated aggregates of dysplastic epithelial cells (Fig. 16.3), hence the WHO's term "raised intraepithelial neoplasia."

16.5 Association with *H. pylori* Infection

Intestinal-type gastric cancer is a consequence of chronic *H. pylori* infection, which is believed by some researchers to be a necessary but insufficient causative factor in gastric carcinogenesis (Graham 2014; Hanada and Graham 2014). While the

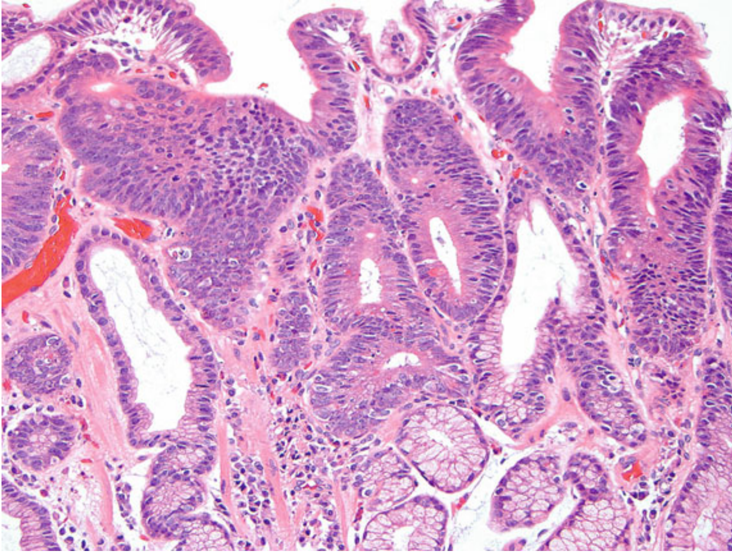

Fig. 16.3 Gastric adenoma. These tumors consist of elevated aggregates of dysplastic epithelial cells, typically starting at the surface. The currently suggested term by the World Health Organization Classification of Tumors is "raised intraepithelial neoplasia." Hematoxylin and eosin, original magnification 20×

connotation of "necessary" is arguable, it is incontrovertible that decades of *H. pylori* chronic active inflammation, epigenetic changes resulting in genetic instability, and mucosal atrophy prepare the terrain for carcinogens or spontaneous mutation to induce neoplasia. Carcinogens may be environmental, but a major source is provided by the biotransformation of ingested or secreted products by the overgrowth of other bacteria that are made possible by hypochlorhydria and achlorhydria (Correa et al. 1979).

Gastric adenomas tend to arise in a background of atrophy and intestinal metaplasia, similar to that typically associated with either *H. pylori* infection or autoimmune gastritis (Abraham et al. 2001; Laxen et al. 1982). As in the colon, gastric adenomas can be viewed as part of a sequence leading from dysplasia to carcinoma. The larger the adenomatous polyp, the greater the probability it contains foci of adenocarcinoma. Synchronous gastric adenocarcinomas have been reported in up to 30 % of patients with adenomas containing foci of adenocarcinoma (Abraham et al. 2001; Laxen et al. 1982). Finally, and perhaps most importantly, gastric dysplasia and adenomas parallel the epidemiologic patterns of gastric adenocarcinoma. In summary, gastric adenomas are neoplastic proliferations with a high predictive value for the development of gastric intestinal-type adenocarcinoma. Although gastric adenomas may arise in the context of other conditions (familial polyposis, autoimmune atrophic gastritis), there is sufficient evidence to implicate *H. pylori* infection as the most important risk factor for the development

of these polyps (Carmack et al. 2009b; Gotoda et al. 1999; Han et al. 2011; Haziri et al. 2010; Kim et al. 2013; Laxen 1981; Lee et al. 2012; Nakamura and Nakano 1985; Saito et al. 2000).

16.6 Gastric Neuroendocrine Tumors (Carcinoids)

Carcinoids are neuroendocrine tumors derived from enterochromaffin-like (ECL) cells. The term "carcinoid" was discarded in the most recent (2010) WHO classification of tumors in favor of "neuroendocrine tumor" (Solcia et al. 2010). In two large studies (Germany in 1994 and the USA in 2008), gastric neuroendocrine tumors comprised <2 % of gastric polypoid lesions (Carmack et al. 2009b; Stolte et al. 1994).

Gastric neuroendocrine tumors are classified in three distinct types (Cockburn et al. 2013). Type I represents 70–80 % of all gastric endocrine tumors. They are associated with hypergastrinemia resulting from atrophic gastritis. Therefore, they are more commonly found in elderly patients, particularly women with autoimmune (corpus-restricted) pernicious anemia, and may also be found in patients with long-standing severe *H. pylori*-related multifocal atrophic gastritis. The pathogenesis of Type I neuroendocrine tumors begins with the destruction (or significant loss) of corpus/fundus acid-secreting parietal cells, causing reduced gastric acid production and loss of feedback inhibition of gastrin secretion by antral G cells. The resulting hypergastrinemia stimulates the proliferation of ECL cells, progressing from microscopic hyperplasia to multiple endoscopically visible nodules. These tumors (Fig. 16.4) are small (<1 cm), confined to the oxyntic (corpus and fundic) mucosa, and tend to be multiple, usually coexisting with background enterochromaffin-like cell hyperplasia. Type I gastric neuroendocrine tumors tend to be found incidentally, often in patients undergoing esophagogastroduodenoscopy (EGD) as part of an evaluation for anemia. Histologically, they consist of nests or ribbons of endocrine cells (small polygonal cells with round central nuclei featuring "salt-and-pepper" chromatin) with a very low proliferation index (Cockburn et al. 2013; Solcia et al. 1998).

Type II gastric neuroendocrine tumors are associated with hypergastrinemia resulting from a gastrin-secreting tumor. They are frequently detected as part of the workup for MEN-1 syndrome or for Zollinger-Ellison syndrome, and they are the least common type, representing only 5–8 % of gastric neuroendocrine tumors (Cockburn et al. 2013; Solcia et al. 1998). The unregulated secretion of gastrin by the primary tumors causes a secondary neuroendocrine tumor (or tumors). The prognosis of Type II tumors is intermediate, with metastasis in about 30 %.

Type III (sporadic) neuroendocrine tumors are not associated with hypergastrinemia or any other known predisposing condition, are generally solitary, arise in otherwise healthy gastric mucosa, and are not accompanied by ECL-cell hyperplasia. These tumors, which represent approximately 20 % of all gastric neuroendocrine tumors, are usually detected when they become symptomatic, either

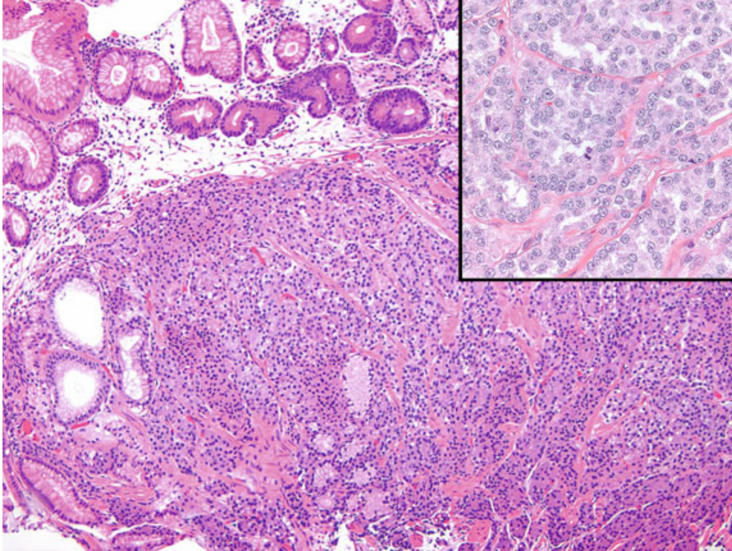

Fig. 16.4 Type I gastric neuroendocrine tumor. These small lesions consist of nests or ribbons of endocrine cells localized at the interface between mucosa and submucosa. The high-magnification insert in the upper right corner shows the details of the small polygonal cells with round central nuclei featuring “salt-and-pepper” chromatin and a very low proliferation index that forms the nests and ribbons. Hematoxylin and eosin, original magnification 20×; insert 40×

secondary to mucosal erosion and blood loss or metastasis. They can occur anywhere in the stomach, are usually solitary, and have a generally poor prognosis, with metastases around 70 % when larger than 2 cm (Rindi et al. 1999).

16.7 Association with Gastritis and *H. pylori* Infection

Type II and III neuroendocrine tumors have no relationship with gastritis. Type I represents the progression of atrophy-induced ECL-cell hyperplasia. Autoimmune gastritis is the condition that most commonly results in a degree of oxyntic mucosal atrophy sufficient to alter the acid-gastrin axis and induce ECL-cell hyperplasia and neoplasia (Neumann et al. 2013). However, a downregulation of gastric secretion involving alterations of somatostatin and gastrin production may occur in advanced stages of multifocal atrophic gastritis, a consequence of long-standing *H. pylori* infection (Chu and Schubert 2013). Taken together, the pathogenesis of Type I neuroendocrine proliferations is directly related to atrophy of the oxyntic mucosa with severe hypochlorhydria or achlorhydria. An indirect relationship with *H. pylori* exists only inasmuch as long-standing infection may cause extensive atrophy that involves the gastric corpus.

16.8 Fundic Gland Polyps

Fundic gland polyps (FGPs) are the most common type of polyps detected at EGD in Western countries. In a large 2008 pathologic study, fundic gland polyps were diagnosed in approximately 6 % of patients who had an EGD, representing 74 % of all gastric polyps submitted for histopathologic evaluation (Carmack et al. 2009b). More recent data from the same database, with more than one million patients who underwent EGD, shows a prevalence close to 8 % (Sonnenberg and Genta 2014). Fundic gland polyps are usually multiple and small (less than 1 cm) and have a smooth, glassy, sessile appearance under endoscopy. Histologically, they consist of aggregates of dilated oxyntic glands lined by gastric foveolar epithelium (Fig. 16.5). When first discovered, fundic gland polyps were believed to be hamartomatous (Elster 1976); however, their association with proton inhibitor use, confirmed in a number of studies, suggests that mechanisms related to the suppression of acid secretion by proton pump inhibition may be involved in their pathogenesis (el-Zimaity et al. 1997; Graham and Genta 2008; Raghunath et al. 2005).

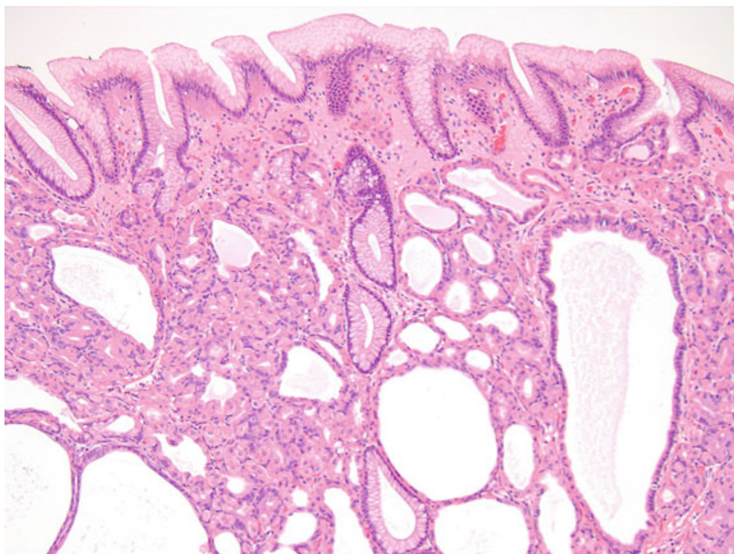

Fig. 16.5 Fundic gland polyp. These velvety elevations occur in the oxyntic mucosa and consist of aggregates of dilated oxyntic glands with flattened parietal cells. The overlying surface comprises normal gastric foveolar epithelium (Fig. 16.5). When first discovered, fundic gland polyps were believed to have a hamartomatous origin. Hematoxylin and eosin, original magnification 20×

16.9 Association with *H. pylori* Infection

The relative rarity of fundic gland polyps in patients with *H. pylori* infection has been noted in numerous studies (Cao et al. 2014; Dickey et al. 1996; Genta et al. 2009; Kishikawa et al. 2014; Sakai et al. 1998; Samarasam et al. 2009; Shand et al. 2002; Tazaki et al. 2011). In our database, the prevalence of *H. pylori* in 61,295 patients with fundic gland polyps was 0.3 %, or 36 times *less common* than in patients without fundic gland polyps. A report from Japan goes even further in support of this inverse relationship by reporting that two patients with multiple FGPs in normal fundic mucosa without inflammatory changes or atrophy experienced a complete disappearance of their polyps after the acquisition of *H. pylori* infection (Watanabe et al. 2002). Another Japanese study examined the clinical importance of sporadic fundic gland polyps in a new light. Whereas most such studies had focused on the positive predictive value of fundic gland polyps with respect to the development of some other condition (and have regularly failed to detect any such relationship), Kishikawa and colleagues correlated the presence of fundic gland polyps to the serum pepsinogen levels, *H. pylori* antibody concentration, and gastric juice pH in 375 subjects. Low risk for gastric cancer was defined as normal serum pepsinogen and negative *H. pylori* antibodies. Fundic gland polyps were found in 44 patients. The prevalence of a "low-risk" stomach in subjects with and without FGPs was 98 % and 48 %, respectively. Multivariable logistic regression analysis indicated three variables as independent factors positively associated with "low-risk" stomachs: FGPs ($OR = 38.6$), reflux esophagitis ($OR = 4.8$), and age <60 years ($OR = 1.89$). Gastric juice pH, which is associated with mucosal atrophy, was significantly lower in subjects with than without FGPs. Sporadic FGPs tend to be related to the least atrophic mucosa among non-gastric atrophy subjects without *H. pylori* infection and can be used as predictors of a low risk of gastric carcinogenesis. In summary, sporadic fundic gland polyps occur in otherwise healthy gastric mucosa and, irrespective of their possible relationship with the use of proton pump inhibitors, have a strong inverse association with *H. pylori* and cancer.

16.10 Inflammatory Fibroid Polyps

Inflammatory fibroid polyps (also known as Vanek tumors) are rare lesions that represent <0.1 % of all gastric polyps (Carmack et al. 2009b). Endoscopically they are usually firm; solitary, sessile, or pedunculated; and often ulcerated. The histologic features of these polyps are distinctive: they consist of submucosal proliferations of spindle cells, small vessels, and a conspicuous inflammatory infiltrate with a predominance of eosinophils. Hence, these polyps have been occasionally (and inaccurately) referred to as "eosinophilic granulomas" (Shaib et al. 2013).

16.11 Relationship with *H. pylori* Infection

Since the gastric mucosa adjacent to these polyps has consistently been reported as being normal, it would seem unlikely that there is a relationship with *H. pylori* gastritis. There are, however, case reports from Japan suggesting that inflammatory fibroid polyps either disappeared or decreased in size after the successful eradication of *H. pylori* infection (Hirasaki et al. 2007; Nishiyama et al. 2003). These authors speculate on but clearly fail to prove a possible role for *H. pylori* in the pathogenesis of these polyps. In summary, no convincing association between *H. pylori* infection and gastric inflammatory fibroid polyps has been established.

16.12 Other Gastric Polyps

Gastrointestinal stromal tumors, leiomyomas, and submucosal lipomas are uncommon polyps that may occur anywhere in the gastrointestinal tract, including the stomach. To our knowledge, no study has investigated their possible association with *H. pylori* infection. Since these lesions do not arise in the mucosa, a relationship with *H. pylori* would have little biological plausibility.

16.13 Duodenal Polyps

Although gastric foveolar metaplasia in the duodenum may have the endoscopic appearance of a polyp, this lesion, whose association with *H. pylori* gastritis has been suggested and later disputed (Genta et al. 2010; Tovey et al. 2004; Voutilainen et al. 2003), is not a true proliferation, does not meet the histopathologic criteria for polyp, and will not be discussed here.

Duodenal adenomas occur in patients with familial polyposis syndromes and as sporadic lesions, usually in elderly patients. Sporadic duodenal adenomas are diagnosed in approximately 0.1 % of patients who have duodenal biopsies, and approximately 1 % of these have high-grade dysplasia. A recent large study has confirmed the association of duodenal adenomas with colon neoplasia (Genta et al. 2014). In our database (Miraca Life Sciences database), there was a small but statistically significant inverse association of sporadic duodenal adenomas with concurrent *H. pylori* gastritis (Genta et al. 2014). However, we suspect that the likelihood that this represents a truly biologically relevant phenomenon is minimal, and we can assume that there is no association between *H. pylori* infection and duodenal adenomas.

16.14 Colonic Neoplasms

The relationship between *H. pylori* and colonic neoplasms has been investigated in several studies. Two meta-analyses of previous publications have suggested that infection with *H. pylori* confers a 1.4- to 1.6-fold increased risk for colon adenoma or colon cancer (Zhao et al. 2008; Zumkeller et al. 2006). The studies included in these meta-analyses relied on different variables to assess risk factors and the occurrence of colonic neoplasms. Although the majority of studies used positive serology as a marker for *H. pylori* infection, some also used the presence of gastritis or other abnormal gastric histopathologies (Bae et al. 2009; Inoue et al. 2010; Machida-Montani et al. 2007). The occurrence of colonic neoplasms was assessed by the endoscopic diagnosis of adenomatous polyps, villous adenoma, adenocarcinoma, or recurrence of adenomatous polyps after previous polypectomy (Fujimori et al. 2005; Mizuno et al. 2005; Siddheshwar et al. 2001). All studies included relatively small case populations, with the largest ones having evaluated fewer than 200 patients with adenomatous polyps.

In an attempt to provide evidence based on the analysis of large numbers of patients, we used a national database of 156,000 patients who had undergone both a colonoscopy and an EGD (Sonnenberg and Genta 2013). The database contained information about the histopathology of the colonoscopy, as well as the EGD, with detailed information about polyp size, number, and location. Our hypothesis was that *H. pylori* gastritis would be a risk factor for all types of colonic neoplasms, and that such risk would also affect the characteristics of the neoplasms with respect to histology, number, size, and location. Our results showed that *H. pylori* gastritis confers a significantly increased risk for colonic neoplasms. The risk applied to all types of colonic neoplasms: non-advanced adenomas (OR 1.80, 95 % CI 1.69–1.92), villous adenomas, adenomas with high-grade dysplasia or adenomas >1 cm (OR 1.97, 95 % CI 1.82–2.14), and adenocarcinomas (OR 2.35, 95 % CI 1.98–2.80). The risk also increased with advancing stage of the neoplasm from hyperplastic and adenomatous polyps to tubulovillous adenoma, adenoma with high-grade dysplasia, and adenocarcinoma. Such risk was not limited to *H. pylori* gastritis but was found similarly in all other types of gastric histopathology related to *H. pylori* infection, such as intestinal metaplasia, gastric adenoma, gastric lymphoma, and gastric cancer, even in the absence of active *H. pylori* infection at the time of the evaluation. In summary, all types of epithelial colon polyps are strongly associated with *H. pylori* gastritis. Although the mechanisms of this association remain in the realm of speculation, elevated gastrin levels in patients with *H. pylori* gastritis have been frequently invoked as the most likely cause for this association, (Singh et al. 2012), but this view is not supported by recent evidence (Lahner et al. 2012; Selgrad et al. 2014).

16.15 Conclusions and Outlook

In the gastrointestinal tract, polyps are mucosal elevations that owe their name to the imagination of ancient observers, who were reminded of the head of an octopus ("polypus" in Latin) lying on a flat surface. This purely morphologic term has no histopathologic inferences and encompasses lesions of mechanical, developmental, inflammatory, and neoplastic origin. Therefore, it is not surprising that there is no univocal relationship between gastrointestinal polyps and *H. pylori* infection. In the stomach, inflammatory and proliferative responses, irrespective of their etiology, seem to favor the development of inflammatory polyps. Intact oxyntic glands, unaffected by inflammatory changes, seem to be more vulnerable to focal dilatations induced by proton pump inhibitors, hence the strong negative association of these nonneoplastic, noninflammatory polyps with *H. pylori* gastritis. Gastric adenomas, precursors of adenocarcinoma, are likely related to *H. pylori* infection by the same mechanisms that link this infection to gastric cancer. In the colon, the indisputable association of *H. pylori* gastritis with both hyperplastic and neoplastic polyps remains largely unexplained.

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Chapter 17
Helicobacter pylori Infection and Gastric Cancer

Richard M. Peek Jr. and Lydia E. Wroblewski

Abstract Gastric cancer is widespread and remains a leading cause of cancer-related death worldwide. Infection with *Helicobacter pylori* is one of the strongest known risk factors for this malignancy. There is a high level of genetic diversity among *H. pylori* strains, and bacterial virulence factors play an important role in determining the risk of developing gastric adenocarcinoma following colonization with *H. pylori*. Infection with strains that contain a *cag* pathogenicity island or type s1 *vacA* alleles confers increased risk compared to infection with other strain types. Additional risk factors for gastric cancer include dietary factors, such as a high-salt diet and iron deficiency. *H. pylori* can interact with stem cell populations and in this way may promote gastric carcinogenesis. Recent studies suggest that components of the microbiome may also influence *H. pylori*-induced carcinogenesis. In this review article, we discuss mechanisms by which *H. pylori* infection can lead to gastric cancer.

Keywords *Helicobacter pylori* • Gastric adenocarcinoma • Type IV bacterial secretion system

17.1 Introduction

17.1.1 Gastric Cancer

Gastric adenocarcinoma is the fourth most common cancer and the second leading cause of cancer-related death worldwide, resulting in approximately 738,000 deaths in 2008 (de Martel et al. 2012; Fuchs and Mayer 1995; Parkin et al. 2005). The incidence rates of gastric adenocarcinoma can vary substantially in different regions of the world, with the highest rates found in East Asia, Central America, parts of South America, and Eastern Europe (de Martel et al. 2012; Forman and Pisani 2008; Fuchs and Mayer 1995; Soerjomataram et al. 2012).

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Adenocarcinoma is the most common type of cancer that affects the stomach, but other types of cancer can also arise, including lymphoma and leiomyosarcoma. Two distinct variants of gastric adenocarcinoma can be differentiated histologically: diffuse-type gastric cancer, which consists of individually infiltrating neoplastic cells that do not form glandular structures, and intestinal-type adenocarcinoma, which progresses through a series of well-defined histological steps, first described in 1975 (Correa 1992) (Fig. 17.1). Intestinal-type adenocarcinoma is initiated by the transition from normal mucosa to chronic superficial gastritis; this is followed by the development of atrophic gastritis and intestinal metaplasia, finally leading to dysplasia and adenocarcinoma (Correa 1996; Sipponen and Marshall 2000) (Fig. 17.1). Intestinal-type adenocarcinoma affects men twice as frequently as women, and it is thought that estrogen may confer some protection in women (Correa and Houghton 2007; Hatakeyama 2004). Typically, the diagnosis of gastric cancer is not established until late in the course of the disease and is often not detected until invasion of the muscularis propria has occurred. This delay in diagnosis likely contributes to poor survival rates; indeed, 5-year survival rates for gastric cancer in the USA are less than 15 % (Correa 2004). It is therefore of great importance to gain a comprehensive understanding of the factors that contribute to this malignancy and identify persons who are at the greatest risk of developing gastric cancer with a goal of developing strategies to prevent this devastating disease.

Over the past century, the incidence rates of gastric adenocarcinoma in developed countries have significantly decreased. This decline can primarily be attributed to a decline in intestinal-type adenocarcinomas in the distal stomach (Fuchs and Mayer 1995; Howson et al. 1986). Currently, in the USA, distal gastric adenocarcinoma is diagnosed most commonly in elderly persons and occurs more frequently in African-Americans, Hispanic-Americans, and Native Americans than among other ethnicities (Haile et al. 2012; Wu et al. 2007). While the incidence of gastric adenocarcinomas of the distal stomach has been declining, the incidence rates of proximal gastric adenocarcinomas as well as those originating in the gastroesophageal junction have been increasing in both the USA and Europe (Blot et al. 1991; Pera et al. 1993).

17.1.2 *Helicobacter pylori*

Histology studies performed decades ago indicated that intestinal-type gastric adenocarcinoma of the distal stomach was preceded by gastric inflammation (termed superficial gastritis) as well as several other histologic alterations, including intestinal metaplasia (presence of intestinal-type epithelium in the stomach), gastric atrophy (loss of specialized cell types such as parietal cells and chief cells), and dysplasia (Correa 1992; Correa et al. 1976) (Fig. 17.1). In the early 1980s, Robin Warren and Barry Marshall definitively identified *Helicobacter pylori* as a cause of gastritis by culturing an organism that had been visualized for almost a

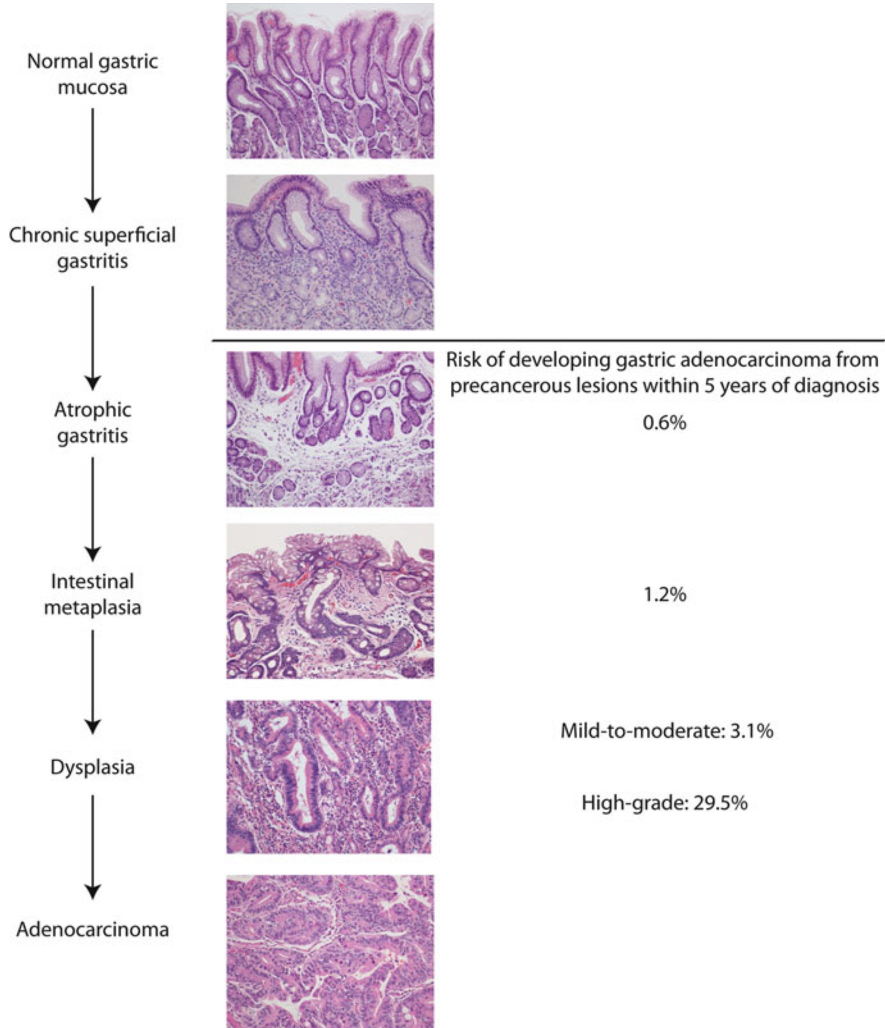

Fig. 17.1 Schematic representation showing the sequential steps of the precancerous cascade initiated by infection with *H. pylori*. The risk of developing gastric cancer within 5 years of diagnosis is indicated at each stage (de Vries et al. 2008) (Images provided courtesy of M. Blanca Piazuelo)

century in gastric biopsies (Marshall and Warren 1984). In 1994, *H. pylori* was recognized as a type I carcinogen by the WHO and is now considered to be the strongest known risk factor for distal gastric adenocarcinoma (Fox and Wang 2007; Polk and Peek 2010) and the most common etiologic agent of infection-related cancers, which represent 5.5 % of the global cancer burden (Parkin et al. 2005).

H. pylori infection is usually acquired in childhood, and without antimicrobial treatment, persists for the lifetime of the host, despite the harsh gastric environment

that it colonizes. Genetic studies indicate that *H. pylori* has colonized humans for at least 58,000 years (Linz et al. 2007), and currently, approximately half of the world's population is infected with *H. pylori*. Persons colonized with *H. pylori* reside in all regions of the world; however, the highest rates of colonization are found in developing countries with lower rates in developed nations (Everhart 2000).

Among *H. pylori*-infected individuals, only 1–3 % develop gastric adenocarcinoma (Peek and Crabtree 2006). Factors that influence pathologic outcomes of *H. pylori* infection include strain-specific bacterial constituents, host genetic factors, alterations of the stem niche and host microbiota, and environmental influences including diet (Blaser and Berg 2001). In this chapter, we consider the microbial factors, microbiome and stem cell alterations, as well as the dietary risk factors that specifically modify microbial factors that are known to influence the risk of *H. pylori*-associated gastric cancer. Host effectors are covered by El-Omar and coworkers in Chap. 14.

17.2 Epidemiology Linking *H. pylori* Infection to Gastric Cancer

A possible link between *H. pylori* and gastric cancer was debated for a number of years; however, numerous studies throughout the world have shown that colonization with *H. pylori* is associated with a significantly increased risk for developing gastric adenocarcinoma. A prospective study of 1526 Japanese patients reported that gastric cancer (both intestinal and diffuse types) developed in approximately 3 % of *H. pylori*-colonized persons compared with 0 % in uninfected persons over 8 years (Uemura et al. 2001).

Studies using stored blood samples to detect *H. pylori* by serology have also supported the link between *H. pylori* infection and development of gastric cancer (Nomura et al. 1991; Parsonnet et al. 1991). Results pooled from 12 geographically diverse studies comprised of 1228 gastric cancer cases and 3406 controls estimate that infection with *H. pylori* increased the risk for developing adenocarcinoma of the distal stomach almost sixfold over baseline (Helicobacter and Cancer Collaborative 2001).

Recent evidence indicates that Western blot analysis is a more sensitive method for detecting anti-*H. pylori* antibodies than enzyme-linked immunosorbent assay (ELISA) (Plummer et al. 2014). Analysis of studies that employed Western blot data only indicates that the worldwide attributable risk for *H. pylori* in non-cardia gastric cancer is actually 89 % versus 75 % when ELISA assays were used and that *H. pylori* infection accounts for 6.2 % of all cancers (Plummer et al. 2014). Depending on the study, the degree to which *H. pylori* increases the risk for gastric adenocarcinoma can vary and likely depends on numerous factors including, patient age, selection of controls, and the site and stage of gastric cancer.

17.3 *H. pylori* Virulence Factors That Influence Gastric Cancer Risk

There is a high level of genetic diversity among isolates of *H. pylori* harvested from unrelated persons (Blaser and Berg 2001). Bacterial virulence factors play an important role in determining the risk of developing gastric adenocarcinoma following colonization with *H. pylori* and will be discussed in the following sections.

17.3.1 The H. pylori cag *Pathogenicity Island*

One of the *H. pylori* determinants that clearly influences the risk of gastric cancer is the *cag* pathogenicity island (*cag*PAI), a 40-kB DNA insertion element which contains 27–31 genes, including *cagA* and genes which encode proteins that form a type IV bacterial secretion system (T4SS). The *cag* T4SS exports CagA from adherent *H. pylori* across the bacterial and epithelial membrane and into host cells (Fischer et al. 2001; Kwok et al. 2007; Odenbreit et al. 2000; Shaffer et al. 2011).

Persons that are seropositive for both *H. pylori* and CagA harbor a 5.8-fold increased risk of developing intestinal and diffuse gastric adenocarcinoma compared with uninfected persons, whereas persons infected with CagA-negative *H. pylori* are only at a 2.2-fold higher risk of developing distal gastric adenocarcinoma compared to uninfected persons (Parsonnet et al. 1997). A meta-analysis of studies examining cancer risk suggests that *H. pylori* strains harboring CagA increase the risk of developing distal gastric adenocarcinoma twofold over the risk incurred by CagA-negative strains of *H. pylori* (Huang et al. 2003).

Within the host cell, CagA can be tyrosine phosphorylated at glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs. Four different EPIYA motifs (EPIYA-A, EPIYA-B, EPIYA-C, or EPIYA-D) have been identified within CagA and can be used as indicators of disease severity (Hatakeyama 2004; Higashi et al. 2005; Naito et al. 2006). An increased number of CagA EPIYA-C sites are linked to an increased risk of developing gastric cancer (Basso et al. 2008), and strains containing the EPIYA-D motif induce more intense cellular morphologic aberrations and higher levels of IL-8, an indicator of increased pathogenesis, in cell culture than strains harboring C-type CagA (Argent et al. 2008; Hatakeyama 2004).

In rodent models of gastric cancer, *cagA*-isogenic mutant strains of *H. pylori*, or strains defective in *cag* T4SS function, fail to induce gastric cancer (Franco et al. 2008; Gaddy et al. 2013). Furthermore, transgenic mice expressing CagA demonstrate increased gastric epithelial cell proliferation rates and carcinoma in the absence of inflammation (Ohnishi et al. 2008). Thus CagA appears to function as a microbial oncoprotein. Further details on CagA function can be obtained in Chap. 4.

17.3.2 VacA

VacA is a toxin secreted by *H. pylori* and is another microbial constituent linked to the development of gastric cancer (Boquet and Ricci 2012; Cover and Blanke 2005). All *H. pylori* strains possess *vacA*, but there are considerable differences in *vacA* sequences among strains. The regions of greatest diversity are localized to the 5′ region of the gene, which encodes the signal sequence and amino-terminus of the secreted toxin (allele types s1a, s1b, s1c, or s2), an intermediate region (allele types i1 or i2), and a mid-region (allele types m1 or m2) (Atherton et al. 1995; Rhead et al. 2007). Strains containing type s1 and m1 alleles are strongly associated with gastric cancer (Atherton et al. 1995, 1997; Miehlke 2000 #197). Likewise, strains containing a *vacA* i1 versus an i2 allele are also strongly associated with gastric cancer. This association between type i1 alleles and gastric cancer may even be stronger than the risk incurred by *vacA* s or m types or even *cag* status (Rhead et al. 2007). In a recent study of a population in Belgium, *vacA* s1 and i1 genotypes were determined to be better markers of gastric cancer than *cagA* genotypes (Memon et al. 2014). Similar findings were reported in a British population where the presence of the *vacA* i1 genotype was strongly associated with intestinal metaplasia (Winter et al. 2014). Regardless of *cagA* positivity or *vacA* allele type, the presence of a *vacA* i2 genotype is almost exclusively linked to the absence of intestinal metaplasia (Winter et al. 2014).

17.3.3 BabA

Blood group antigen-binding adhesin (BabA) is another *H. pylori* constituent that has been linked to the development of gastric cancer. BabA is an outer membrane protein and is encoded by the *babA2* gene which binds to fucosylated Lewis^{b} antigen (Le^{b}) on the surface of gastric epithelial cells (Gerhard et al. 1999; Ilver et al. 1998; Oliveira et al. 2003; Yu et al. 2002). BabA is more frequently expressed by *cag* PAI-positive strains compared to *cag* PAI-negative strains, and the presence of *babA2* is associated with gastric cancer (Gerhard et al. 1999). When BabA is found in conjunction with *cagA* and *vacA* s1 alleles, it is associated with an even greater risk of developing more severe disease (Gerhard et al. 1999; Hennig et al. 2004). In a recent study of a Swedish population, BabA was found to be associated with adenocarcinoma of the gastric cardia (Song et al. 2014). *H. pylori* BabA expression can be lost in *H. pylori* isolates retrieved from human clinical samples as well as in macaque, mice, and gerbil animal models that are infected with *H. pylori* (Solnick et al. 2004; Styer et al. 2010). Loss of BabA prevents binding to Lewis B and may provide a mechanism through which *H. pylori* can modify its outer membrane in order to adapt to changing conditions that occur within an inflamed milieu (Solnick et al. 2004; Styer et al. 2010). Further details on VacA function can be obtained in Chap. 5.

17.3.4 SabA

Sialic acid-binding adhesin (SabA) is another *H. pylori* adhesin which binds to the carbohydrate structure sialyl-Lewis^{x}, antigen expressed on gastric epithelium, and is associated with increased gastric cancer risk (Yamaoka et al. 2006). Sialyl-Lewis^{x} expression is induced during chronic gastric inflammation, suggesting that *H. pylori* modulates host cell glycosylation patterns to enhance attachment and colonization (Mahdavi et al. 2002). SabA expression can rapidly be switched "on" or "off" through phase variation to adapt to changes exerted by inflammation in the gastric niche (Yamaoka et al. 2006), or SabA promoter activity can be fine-tuned by altering the length of the thymine nucleotide repeat tract (Aberg et al. 2014). Further details on BabA and SabA functions can be obtained in Chap. 6.

17.4 Dietary Risk Factors

The risk of gastric carcinoma is not only influenced by *H. pylori* strain constituents but also by environmental factors such as diet. Diets that are high in salted, pickled, smoked, or poorly preserved foods, those with a high meat content, and those with low fruit and vegetable content are most commonly associated with an increased risk for developing gastric cancer (Epplein et al. 2008; Gonzalez et al. 2006, 2012; Kim et al. 2004, 2010; Ren et al. 2012; Tsugane and Sasazuki 2007). Within the context of *H. pylori* infection, high dietary salt intake and low iron levels are most highly associated with increased gastric cancer risk and will be discussed in further detail in Sects. 4.1 and 4.2, respectively.

17.4.1 Salt

High dietary salt intake is associated with increased gastric cancer risk and has been reported in numerous studies (Lee et al. 2003; Tsugane 2005; Tsugane and Sasazuki 2007). *H. pylori* infection in combination with a high-salt diet further increases gastric cancer risk. Two independent investigations reported that *H. pylori*-infected subjects consuming a high-salt diet exhibited an increased risk of gastric cancer when compared to *H. pylori*-infected subjects who consumed lower levels of salt (Lee et al. 2003; Shikata et al. 2006). A third study also identified a positive association between *H. pylori* infection and levels of dietary salt (Beevers et al. 2004).

In Mongolian gerbils, the combination of *H. pylori* infection and a high-salt diet has been reported to exert synergistic effects on the development of premalignant lesions or gastric cancer (Gamboa-Dominguez et al. 2007; Kato et al. 2006; Sun et al. 2006). Gerbils maintained on a high-salt diet that are also infected with

H. pylori developed more severe gastric inflammation and exhibited increased gastric epithelial proliferation compared to *H. pylori*-infected gerbils consuming a regular diet (Gamboa-Dominguez et al. 2007). A high-salt diet in combination with *H. pylori* infection has also been found to induce gastric tumors in an IL-10-deficient mouse model. Of interest, *H. pylori*-induced pathogenesis in the presence of a high-salt diet was prevented by administration of licorice extracts, possibly due to antioxidant, anti-inflammatory, and antimutagenic effects of the extract (Park et al. 2014).

The mechanisms by which salt increases the risk for developing gastric cancer in humans are incompletely understood; however, one possibility is that salt may modulate virulence gene expression in *H. pylori*. Recent work has demonstrated that expression of *H. pylori* CagA, VacA, and UreA is significantly upregulated when certain strains of *H. pylori* are cultured in a medium containing high salt concentrations (Gancz et al. 2008; Loh et al. 2007) It is not entirely understood why some strains respond to high salt and others do not; however, salt-responsive strains of *H. pylori* more frequently harbor two copies of a 5′-TAATGA motif located within the *cagA* promoter, while strains containing only a single copy of this motif are less likely to upregulate CagA in response to salt (Loh et al. 2012).

17.4.2 Iron

In addition to salt, host iron levels have also been found to modulate the virulence potential of *H. pylori*, and iron deficiency is associated with an increased risk for gastric cancer. Iron deficiency is the most common nutritional disorder in the world and can be a result of a diet deficient in iron, blood loss, or alternatively colonization by certain *H. pylori* strains which has been associated with hemorrhagic gastritis (Yip et al. 1997). Long-term colonization with *H. pylori* can further exacerbate iron deficiency through the development of gastric atrophy which leads to decreased acid secretion, reduced ascorbic acid levels, and decreased iron absorption (Yip et al. 1997). Low iron levels in persons with *H. pylori* infection are not reversed by iron supplementation but can be normalized following eradication of *H. pylori* (DuBois and Kearney 2005). A recent study suggests that iron deficiency in *H. pylori*-infected persons accelerates the development of carcinogenesis by increasing the virulence potential of *H. pylori* (Noto et al. 2013a).

Rodent models of *H. pylori*-induced gastric cancer have been used to investigate potential relationships between iron, *H. pylori*, and gastric cancer risk. A frequently used rodent model of *H. pylori*-induced carcinogenesis is the transgenic INS-GAS mouse model. INS-GAS mice overexpress human gastrin, which results in hypergastrinemia and after 20 months of age develop gastric cancer (Thomson et al. 2012; Wang et al. 2000a). Infection with *H. felis* or *H. pylori* accelerates this process, suggesting that persistently elevated gastrin levels synergize with *Helicobacter* to augment cancer progression (Wang et al. 2000b). Using this model, it has recently been demonstrated that infection of INS-GAS mice with

H. felis results in decreased serum iron concentrations, decreased transferrin saturation and hypoferritinemia, and increased total iron binding capacity. Chronic infection with *H. felis* also altered expression of host genes important in iron metabolism and absorption such as hepcidin and transferrin receptor 1 (Thomson et al. 2012). One limitation of studies utilizing *H. felis* is that virulence factors present in *H. pylori* per se that are important in pathogenesis, such as the *cag*PAI, cannot be studied

A recent study determined the effects of dietary iron depletion on the development of *H. pylori*-induced carcinogenesis in gerbils (Noto et al. 2013). Infection of gerbils with CagA-positive *H. pylori* induced more severe gastritis in iron-depleted gerbils than in iron-replete gerbils. Furthermore, gastritis developed earlier in *H. pylori*-infected iron-depleted gerbils compared to infected iron-replete gerbils (Noto et al. 2013). *H. pylori* infection significantly increased the occurrence of dysplasia and gastric adenocarcinoma in iron-depleted gerbils compared to iron-replete gerbils (Noto et al. 2013). These effects were only present following infection with CagA-positive *H. pylori* and were not seen in gerbils on a low iron diet infected with an isogenic *cagA*-mutant strain. To investigate the molecular mechanisms underlying these changes, the proteomes of *H. pylori* strains cultured from gerbils fed an iron-depleted diet or an iron-replete diet were compared using two-dimensional (2D) DIGE/mass spectrometry. Proteins involved in survival, microbial adherence, and function of the *cag* T4SS were differentially regulated when comparing *H. pylori* strains isolated from iron-depleted versus iron-replete gerbils (Noto et al. 2013). CagA expression was significantly higher in *H. pylori* cultured from iron-depleted gerbils, and *H. pylori* FlaA and FlaB, the major flagellin subunits, were significantly upregulated in *H. pylori* isolated from the iron-depleted gerbils (Noto et al. 2013). To further investigate differences between these two types of *H. pylori* strains, gastric epithelial cells were cocultured with *H. pylori* strains isolated from iron-depleted gerbils or iron-replete gerbils. Levels of phosphorylated CagA (reflecting translocated CagA), the number of *cag* T4SS pili, and IL-8 induction were significantly higher following coculture with strains isolated from iron-depleted gerbils compared with strains isolated from iron-replete gerbils (Noto et al. 2013). Collectively these data demonstrate that dietary iron depletion significantly increases the severity of gastric inflammation and accelerates the development of *H. pylori*-induced disease via augmentation of the *cag* secretion system.

17.5 Gastric Stem Cells and *H. pylori*-Induced Gastric Cancer

Stem cells are critical for regulating the self-renewing gastric epithelium and maintaining homeostasis, and evidence from mouse models has indicated that *H. pylori* can interact with stem cell populations. *H. pylori* directly attach to gastric

epithelial cells in transgenic mice that overexpress Le^b (Falk et al. 1995; Guruge et al. 1998), and genetic ablation of parietal cells in Le^b-expressing transgenic mice permits stem cell populations to expand, which is accompanied by a corresponding expansion of *H. pylori* colonization and increased inflammation (Syder et al. 1999, 2003).

In vivo lineage tracing of the gastric epithelium has demonstrated that Lgr5 (leucine-rich repeat-containing G protein-coupled receptor 5)-positive cells are self-renewing, multipotent stem cells capable of generating an entire antral gastric gland (Barker et al. 2010). In the human stomach, *H. pylori* infection in patients with gastric cancer leads to an increased population of Lgr5-positive epithelial cells in the antrum compared to uninfected persons with cancer (Uehara et al. 2013). Furthermore, in *H. pylori*-infected persons with cancer, Lgr5-positive cells are more susceptible to DNA damage than Lgr5-negative cells (Uehara et al. 2013).

Lrig1 (Leucine-rich repeats and immunoglobulin-like domains 1) is a transmembrane protein that marks a distinct population of quiescent stem cells and functions as a tumor suppressor (Powell et al. 2012). Lrig1 is expressed in the gastric epithelium, and expression of Lrig1 is increased in the gastric epithelium of *H. pylori*-infected versus uninfected mice suggesting that infection with *H. pylori* increases this stem cell population as well (Noto et al. 2013).

Bone marrow-derived cells (BMDCs) are a heterogeneous population of cells with the ability to differentiate into cells of diverse lineages. Studies in mice infected with *Helicobacter* have demonstrated that BMDCs home to and engraft in sites of chronic gastric inflammation and repopulate the endogenous tissue stem cells (Houghton et al. 2004; Varon et al. 2012). Within an inflamed stomach, BMDCs degenerate into adenocarcinoma, suggesting that gastric epithelial carcinomas can originate from marrow-derived sources (Houghton et al. 2004; Varon et al. 2012).

A lack of molecular markers has hindered research into the possible interaction of *H. pylori* with corpus stem cells; however, many of the factors that *H. pylori* is known to modulate, such as the transcription factor NF-κB and reactive oxygen species, have been found to be involved in maintaining stem cells and cancer stem cells. It would therefore appear likely that the interaction of *H. pylori* with gastric stem cells may potentiate gastric carcinogenesis (Cabarcas et al. 2011).

17.6 Microbiome

The stomach harbors a bacterial community with colonization densities ranging from 10^1 to 10^3 colony-forming units/g which influence gastric homeostasis (Sheh and Fox 2013). Recent progress in molecular techniques has provided evidence that bacteria colonizing the gastric epithelium may influence *H. pylori*-associated pathogenesis. In transgenic INS-GAS mice, those harboring a complex microbiota spontaneously develop gastric cancer (Thomson et al. 2012; Wang et al. 2000a). However, in germfree INS-GAS mice, it takes over a year longer for gastric cancer

to develop (Lofgren et al. 2011). *H. pylori*-infected germfree INS-GAS mice develop less severe lesions and are slower to progress to gastrointestinal intraepithelial neoplasia than *H. pylori*-infected INS-GAS mice with a complex microbiota (Lofgren et al. 2011). Furthermore, antimicrobial therapies have been found to delay the progression to gastric cancer in both uninfected and *H. pylori*-infected INS-GAS mice (Lee et al. 2009). Taken together these findings suggest that components of the microbiome may exacerbate *H. pylori*-induced carcinogenesis. In support of this, a restricted microbiota containing only three species (ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus*, and ASF519 *Bacteroides* species) of commensal bacteria is sufficient to promote gastric cancer in *H. pylori*-infected INS-GAS mice to the same extent as seen in *H. pylori*-infected INS-GAS mice with a complex microbiota (Lertpiriyapong et al. 2014).

Extragastric constituents of the microbiota may also influence outcomes of *H. pylori*-induced gastric cancer. Coinfection of C57BL/6 mice with *H. bilis* or *H. muridarum* attenuated *H. pylori*-induced gastric pathology (Ge et al. 2011; Lemke et al. 2009). In contrast, preexisting infection with *H. hepaticus* increases *H. pylori*-induced gastric injury (Ge et al. 2011). Interestingly, new evidence suggests that helminth infections prevent *H. pylori*-induced changes in the microbiota of INS-GAS mice and may decrease the severity of *H. pylori*-induced disease (Whary et al. 2014). While progress is being made in understanding the complex interplay between the microbiota and *H. pylori* in the development of gastric cancer in animal models, detailed molecular studies are still needed in well-defined human populations to examine differences in the microbiota of *H. pylori*-infected persons with and without gastric cancer (Sheh and Fox 2013).

17.7 Conclusions and Outlook

Globally, gastric cancer leads to a high number of cancer-related deaths and understanding the risk factors for this disease is of utmost importance in identifying individuals that are most at risk of developing gastric cancer. Infection with *H. pylori* is extremely common, and in some areas of the world, infection prevalence rates approach 100 %; however, 97–99 % of colonized persons will never develop gastric cancer. The risk of developing gastric cancer is dependent on numerous factors including *H. pylori* strain-specific virulence factors, the host genotype, environmental factors such as diet, as well as alternations in stem cell populations and the microbiome. Interactions among these factors affect the outcome of long-term colonization of *H. pylori*; therefore, it is important to be able to utilize results from mechanistic studies to identify people who are most at risk of developing gastric cancer.

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Chapter 18
Helicobacter pylori Infection and MALT Lymphoma

Xavier Sagaert

Abstract Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT), also known as MALT lymphoma, is an indolent B-cell non-Hodgkin lymphoma, arising in lymphoid infiltrates that are induced by chronic inflammation in extranodal sites. The stomach is the most commonly affected organ, where MALT lymphomagenesis is clearly associated with *Helicobacter pylori* gastroduodenitis. Outside the stomach, the role of infectious agents is less clearly defined. In recent years, gastric MALT lymphoma became the focus of attention because of the involvement of its genetic aberrations in the nuclear factor kappa B (NF-κB) pathway, currently one of the most investigated pathways in the fields of immunology and oncology. This chapter presents gastric MALT lymphoma as an outstanding example of the close pathogenic link between *Helicobacter pylori*-induced chronic inflammation and tumour development. It also presents gastric MALT lymphoma as one of the best models of how genetic events initiate oncogenesis, determine tumour biology, dictate clinical behaviour and represent viable therapeutic targets. Moreover, in view of the association of gastric MALT lymphoma with deregulation of the NF-κB pathway, the latter signalling pathway is also discussed in depth in both physiological and pathological conditions.

Keywords Helicobacter pylori • MALT lymphoma • NF-κB pathway • Carcinogenesis

18.1 Introduction

The marginal zone (MZ) represents a microanatomic compartment of the B follicle and is especially well developed in lymphoid organs that are continuously exposed to antigenic stimulation (e.g. spleen, mesenteric lymph nodes and mucosa-associated lymphoid tissue or MALT) (Martin and Kearney 2002). The role of

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the splenic MZ has been well investigated. Splenic MZ B cells play a crucial role in the immune response to T-cell-independent antigens, like polysaccharides of encapsulated bacteria (*Haemophilus influenza*, *Neisseria meningitides* and *Streptococcus pneumoniae*) (Pillai et al. 2005). As such, a suboptimal function of the marginal zone, as seen in children under the age of 2 years or in splenectomised individuals, dramatically increases vulnerability to infections with encapsulated bacteria, and therefore vaccination in these groups is advised (Brigden and Pattullo 1999; Kruschinski et al. 2004). Marginal zone lymphomas (MZL) are believed to be the neoplastic counterpart of the MZ. They account for approximately 8 % of B-cell non-Hodgkin lymphomas, making it the third most frequent subtype after diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma. Based on their anatomic location, the World Health Organisation (WHO) makes a distinction between MALT lymphoma, splenic MZL and nodal MZL subtypes (Jaffe et al. 2008).

MALT lymphoma differs from its splenic and nodal counterpart as it arises in organs that normally lack lymphoid tissue (like stomach, lung, salivary and lachrymal glands) but that have accumulated B cells in response to either chronic infections or autoimmune processes (De Re et al. 2000; Ferreri et al. 2004; Hyjek and Isaacson 1988; Hyjek et al. 1988; Lecuit et al. 2004; Roggero et al. 2000; Stefanovic and Lossos 2009; Wotherspoon et al. 1991; Zucca et al. 2000). Sustained (auto) antigenic stimulation not only triggers a polyclonal B-cell proliferation but also attracts neutrophils to the site of inflammation, with the subsequent release of reactive oxygen species (ROS). The latter are genotoxic and cause a wide range of genetic abnormalities (Coussens and Werb 2002). Moreover, prolonged proliferation of B cells induced by chronic inflammation may also increase the risk of DNA damage, like double-strand DNA breaks, due to the intrinsic genetic instability of B cells during somatic hypermutation and class switch recombination (Goossens et al. 1998). Several of these genotoxic events in MALT lymphomas have been identified as either aneuploidy (trisomy 3, 7, 12 and 18) or disease-specific chromosomal translocations, being t(1;14)(p22;q32), t(14;18)(q32;q21), t(11;18)(q21;q21) and t(3;14)(p13;q32). Remarkably, the genes targeted by at least two of these aneuploidies and at least three of these translocations participate in one and the same pathway resulting in the activation of nuclear factor kappa B (NF-κB), which is a key transcription factor in the immune response and has been the focus of intense investigation over the past two decades (Bonizzi and Karin 2004). NF-κB regulates the expression of a number of survival- and proliferation-related genes in B cells (Ruefli-Brasse et al. 2003; Siebenlist et al. 2005), and as such, its constitutive activation may result in uncontrolled B-cell proliferation and thus lymphomagenesis (Packham 2008).

Here, we concentrate on MALT lymphomas at the most common site, the stomach. Gastric MALT lymphoma accounts for 5 % of all gastric cancers and at least 50 % of all gastric lymphomas, making it the most frequent lymphoma of the gastrointestinal tract. Because of its association with *Helicobacter pylori*, gastric MALT lymphoma represents a fascinating model of the close pathogenic link between chronic inflammation and lymphoma development. In this review, we will discuss the current insights into the pathogenesis of gastric MALT lymphomas

and integrate this information into daily clinical practice as well as provide strategies for new therapeutic targets.

18.2 Clinicopathological Features

Endoscopic biopsies remain the gold standard for the diagnosis of gastric MALT lymphomas. Histologically, the tumour appears as a diffuse spread of neoplastic lymphoma cells, surrounding reactive B follicles and invading epithelial structures resulting in so-called lymphoepithelial lesions (Fig. 18.1). Diagnosis can be difficult, especially in cases where lymphoepithelial lesions are not prominent and/or reactive B follicles cannot be recognised because of neoplastic colonisation. Molecular techniques such as polymerase chain reaction (PCR) can further support the diagnosis of a MALT lymphoma by identifying clonality of B cells, based on the fact that all lymphoma cells have the same immunoglobulin gene rearrangement. Most MALT lymphomas present as Ann Arbor stage IE disease (extranodal disease limited to the site of origin). In 10–20 % of cases, regional lymph nodes are involved (stage IIE), while bone marrow involvement occurs in 5–10 % (Montalban et al. 1995; Thieblemont et al. 2000). Multiple extranodal sites are involved in 10 % of cases at the time of presentation (e.g. small intestine, colon, the salivary gland and the splenic MZ). Consequently, extensive tumour staging should be performed at diagnosis, especially at the above mentioned sites. The disease is remarkably indolent and tends to remain localised for a long period of time. Transformation into an aggressive DLBCL may occur. The 10-year survival rate for gastric MALT lymphomas is approximately 90 %, with a disease-free survival of approximately 70 % (Cogliatti et al. 1991). However, once transformation to DLBCL occurs, the 10-year survival rate falls to approximately 45 % (Cogliatti et al. 1991). It is nowadays generally accepted that *H. pylori* eradication with antibiotics is the first choice of therapy for localised gastric MALT lymphoma (Zullo et al. 2009). The use of anti-infectious treatment in non-gastric locations is still under investigation, although two studies discourage the use of antibiotics in non-gastric MALT lymphoma (Grunberger et al. 2006a, b). Lack of response to *H. pylori* eradication, stable disease at 1 year or disseminated disease at diagnosis is usually considered an indication for chemo- or radiotherapy (Aviles et al. 2005; Martinelli et al. 2005; Schmelz et al. 2005; Tsang et al. 2003). Surgery only has a role in the treatment of gastric MALT lymphomas if local complications (e.g. perforation) occur.

18.3 Gastric MALT Lymphoma Pathogenesis

Gastric MALT lymphoma development is a multistep progression from a reactive, polyclonal to a neoplastic, monoclonal lymphoproliferation (Fig. 18.2).

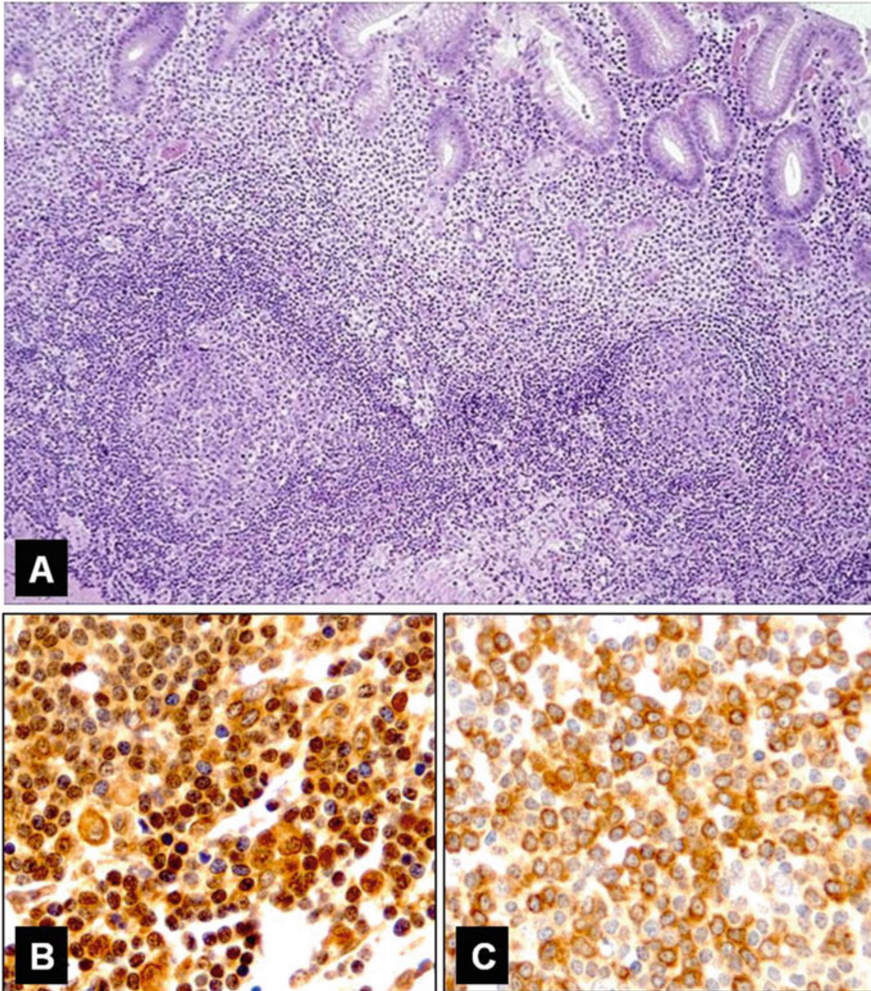

Fig. 18.1 Morphologic and immunohistochemical features of a MALT lymphoma. (**a**) Histology of a gastric MALT lymphoma (magnification 50×): the tumour cells surround reactive B follicles and invade gastric glandular epithelium, resulting in so-called lymphoepithelial lesions. (**b**) Nuclear BCL10 expression is a typical feature of t(11;18)(q21;q21)- and (1;14)(p22;q32)-positive MALT lymphomas (magnification 200×). (**c**) t(14;18)(q32;q21)-positive MALT lymphomas are characterised by perinuclear BCL10 expression (magnification 200×)

18.3.1 Step 1: Acquisition of MALT

MALT lymphomas define a distinct category of infection-related lymphomas, in which the infectious agent induces neoplastic lymphoid transformation by chronically triggering the immune system and, as such, maintaining a protracted proliferative status of lymphocytes. This is in contrast to the lymphotropic oncogenic

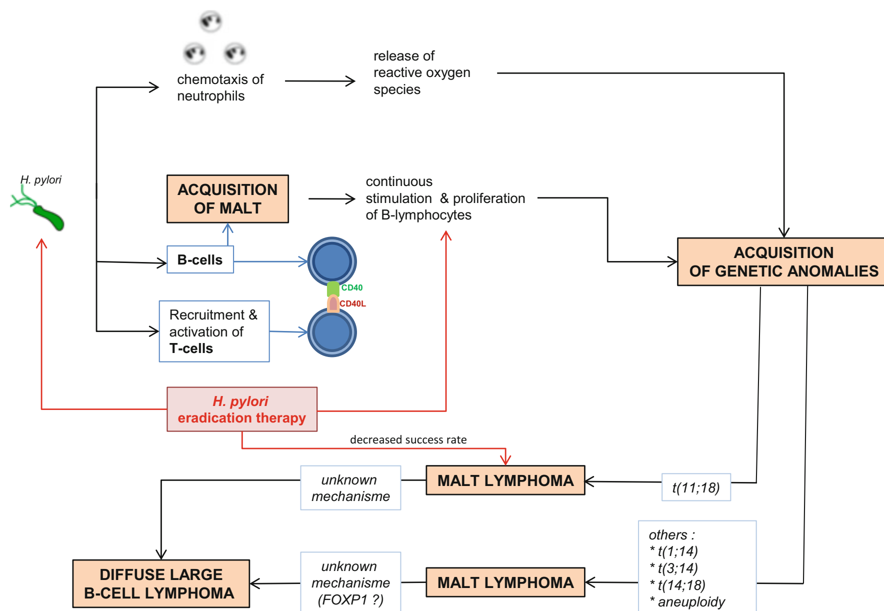

Fig. 18.2 Hypothetical model for the pathogenesis of gastric MALT lymphoma. *H. pylori* infection attracts B lymphocytes, T lymphocytes and neutrophils to the gastric mucosa. B-cell proliferation is driven by CD40-CD40L interaction with *H. pylori* activated, reactive T cells as well as by cytokines. The prolonged proliferative state of B cells as well as the release of reactive oxygen species by neutrophils present in the area of chronic inflammation, induce additional oncogenetic events which make the lymphoproliferation independent of antigenic stimulation. t(11;18)(q21;q21)-positive MALT lymphomas are resistant to *H. pylori* therapy and do not evolve to DLBCLs. Additional genetic alterations in t(11;18)(q21;q21)-negative MALT lymphomas may ultimately result in transformation to a clinically aggressive DLBCL

viruses human herpesvirus 8 (HHV-8), human T-lymphotropic virus 1 (HTLV-1), and Epstein-Barr virus (EBV), which directly infect and transform lymphoid cells into tumour cells. Increasing evidence suggests that MZ lymphomas, and MALT lymphomas in particular, are indeed proceeded by chronic antigenic stimulation. So far, five microbial species are associated with MZ lymphoproliferations: *H. pylori*, *Campylobacter jejuni (C. jejuni)*, *Chlamydia psittaci (C. psittaci)*, *Borrelia burgdorferi (B. burgdorferi)* and hepatitis C virus (HCV); they are linked to gastric MALT lymphoma, immunoproliferative small intestinal disease (IPSID), orbital MALT lymphoma, cutaneous MALT lymphoma and splenic MZ lymphoma, respectively (De Re et al. 2000; Ferreri et al. 2004; Lecuit et al. 2004; Roggero et al. 2000; Wotherspoon et al. 1991; Zucca et al. 2000).

Of all MZ lymphomas, the infectious aetiology of gastric MALT lymphoma has been documented the best. In healthy individuals, a thick layer of viscous mucus as well as gastric acid limit bacterial colonisation of the stomach. However, *H. pylori*, a Gram-negative bacterium also associated with peptic ulceration and gastric carcinoma (Parsonnet et al. 1994), survives in the acid environment by secreting

a pH buffering urease and triggers lymphoid infiltration. There is now compelling evidence that gastric MALT lymphoma is caused by *H. pylori* infection. First, the prevalence of *H. pylori* in both the gastric mucosa and serum of gastric MALT lymphoma patients is well above the infection frequency in other populations (Wotherspoon et al. 1991; Parsonnet et al. 1994). Second, gastric MALT lymphoma has the highest incidence in regions with endemic *H. pylori* infection (Doglioni et al. 1992). Third, *H. pylori* triggers T-cell-mediated B-cell growth in vitro by activating the CD40 pathway (Hussell et al. 1993a). Also, *H.* pylori eradication therapy leads to complete lymphoma regression in about 80 % of the cases with early stage disease (Bayerdorffer et al. 1995; Wotherspoon et al. 1993, 1994). Finally, gastric MALT lymphomas can be induced *in vivo* in mice models by prolonged *H. pylori* infection (Enno et al. 1995).

Remarkably, despite proliferation of tumour cells after *H. pylori* stimulation, the gastric MALT lymphoma-derived immunoglobulin recognises various autoantigens instead of *H. pylori* (Hussell et al. 1993b). As such, it may be hypothesised that gastric MALT lymphoma arises from *H. pylori*-stimulated, autoreactive B cells. Evidence for the role of an (auto)antigen in MALT lymphomagenesis is also supported by sequence analysis studies of the immunoglobulin heavy chain (*IGH*) gene in MALT lymphomas, which revealed the occurrence of somatic hypermutation with a pattern indicative of antigen selection (Bahler et al. 1997; Du et al. 1996a; Hara et al. 2001; Qin et al. 1995). Also, MALT lymphoma *IGH* genes were frequently derived from germline *IGH* genes that are commonly involved in autoantibody production (Bahler et al. 1997; Du et al. 1996b; Hara et al. 2001; Qin et al. 1995). In addition, ongoing mutation (i.e. intraclonal variation) of the *IGH* gene was observed in MALT lymphoma, emphasising the importance of continuing antigenic stimulation in the clonal B-cell expansion (Qin et al. 1995; Du et al. 1996a). However, the rate of ongoing mutation gradually declines during tumour progression, indicating that the role of direct antigenic stimulation in MALT lymphomagenesis decreases during tumour evolution. This is probably due to the occurrence of ROS-induced genetic anomalies, which make the lymphoproliferation progressively independent of antigenic stimulation.

Conversely, 5–10 % of gastric MALT lymphomas are *H. pylori* negative. In some of these cases, *H. pylori* infection may be undiagnosed. This is particularly the case if *H. pylori* testing was only performed on the biopsy, as lymphomas may arise in atrophic mucosa where *H. pylori* bacterial load is low. Therefore, a negative *H. pylori* test in gastric MALT lymphoma should prompt reinvestigation by means of the urea breath test, serological antibody test, biopsy immunostaining and/or stool culture. It has been suggested that some *H. pylori*-negative gastric MALT lymphomas are associated with *H. heilmannii*, and, interestingly, these lymphomas have been shown to respond to antibiotic therapy (Morgner et al. 2000).

Outside the stomach, the role of infectious agents is less clearly defined. Associations of previously mentioned bacteria (*C. jejuni*, *B. burgdorferi*, *C. psittaci)* with MALT lymphoma have been described, based on epidemiological studies and the detection of these bacteria in the affected tissue (Ferreri et al. 2004; Lecuit

et al. 2004; Roggero et al. 2000). Contrary to *H. pylori*, none of these infections fulfil the criteria postulated by Koch (i.e. is the bacteria detectable in the host's tissue in early disease stage? can the bacteria be cultivated from the affected tissue? can the bacteria induce the disease in animal models? can the bacteria be isolated from sick animals?). However, new criteria have been established by recent molecular advances that take into account the host specificity as well as putative uncultivability of certain microbial organisms (Frederickx and Relman 1996; Franco et al. 2004). Nevertheless, whether the observed association between *C. jejuni*, *B. burgdorferi* and *C. psittaci* and MALT lymphoma is a proof of causation remains to be investigated. Furthermore, it is well established that autoimmune diseases increase the risk of developing non-gastric MALT lymphomas. Autoreactive B cells infiltrate the thyroid gland in Hashimoto thyroiditis and the salivary glands in Sjögren syndrome and progressively organise into a MALT-mimicking lymphoproliferation. Patients with Sjögren syndrome have a 44-fold increased risk of developing a lymphoma, and MALT lymphomas account for approximately 85 % of lymphomas in patients with Sjögren syndrome (Royer et al. 1997). Patients with Hashimoto thyroiditis have a 70-fold increased risk of thyroid lymphoma, and thyroiditis in the adjacent gland is present in 94 % of thyroid lymphomas (Derringer et al. 2000).

18.3.2 Step 2: Acquisition of Genetic Abnormalities

Besides aneuploidy (trisomy 3, 7, 12 and 18), the chromosomal translocations t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21) and t(3;14)(p13;q32) all occur with variable frequencies in gastric MALT lymphomas, resulting in *API2-MALT1*, *IGH-BCL10*, *IGH-MALT1* and *IGH-FOXP1* rearrangements, respectively (Baens et al. 2000; Streubel et al. 2003; Willis et al. 1999; Wlodarska et al. 2005).

T(11;18)(q21;q21) is the most common structural chromosomal abnormality in MALT lymphomas. Remarkably, t(11;18)(q21;q21) is not found in other lymphoma types and its presence in MALT lymphoma correlates with the absence of any further genetic aberrations (Muller-Hermelink 2003; Starostik et al. 2002). Depending upon the study performed, it is present in 10–50 % of gastric MALT lymphomas, whereas this translocation rarely occurs in non-gastric MALT lymphomas, with the exception of pulmonary MALT lymphomas (Baens et al. 2000; Ye et al. 2003; Streubel et al. 2004). T(11;18)(q21;q21) fuses the amino-terminus of the *API2* gene (located at 11q21) to the carboxyl-terminus of the *MALT1* gene (located at 18q21), hereby creating the fusion protein API2-MALT1 (Dierlamm et al. 1999; Akagi et al. 1999) (Fig. 18.3). The apoptosis inhibitor protein API2 is a member of the inhibitor of apoptosis protein (IAP) family and inhibits the biological activity of certain caspases (Roy et al. 1997). It contains three BIR (baculovirus IAP repeat) domains, a CARD (caspase recruitment domain) motif and one RING (really interesting new gene) finger motif. MALT1, classified as a paracaspase, is an essential protein in the antigen receptor-mediated pathway that leads to NF-κB

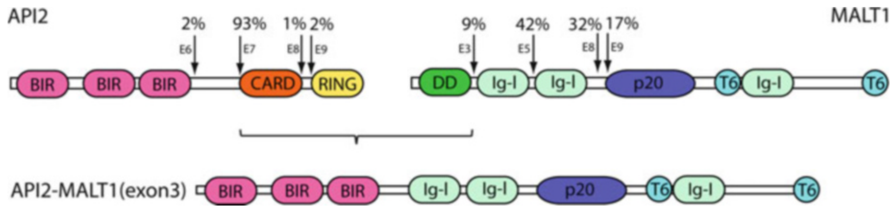

Fig. 18.3 Structure of the *API2* and *MALT1* gene structure. *Arrowheads* indicate known breakpoints and their frequencies. The breakpoints within the *API2* gene almost consistently occur in intron 7 (annotated now as intron 6 according to Ensembl gene ENSG00000023445), whereas the breakpoints within the *MALT1* coding DNA are more variable and are located in introns 2, 4, 7 and 8, resulting, respectively, in an *API2*/exon7-*MALT1*/exon3, *API2*/exon7-*MALT1*/exon5, *API2*/exon7-*MALT1*/exon8 and *API2*/exon7-*MALT1*/exon9 fusion products. *Abbreviations: BIR* baculovirus inhibitor of apoptosis repeat, *CARD* caspase recruitment domain, *RING* really interesting new gene, *DD* death domain, *Ig-l* immunoglobulin-like, *p20* caspase-like p20 domain, *T6* TRAF6 binding site

activation and comprises an N-terminal death domain (DD), two immunoglobulin-like domains and a C-terminal caspase-like domain (Uren et al. 2000). The breakpoints in both *API2* and *MALT1* are well characterised (Baens et al. 2000; Ye et al. 2003; Dierlamm et al. 1999; Remstein et al. 2000; Motegi et al. 2000; Kalla et al. 2000; Liu et al. 2001a). All breakpoints are fused in-frame and constitute a total of eight variant forms of the fusion transcripts, all of which comprise the three intact BIR domains of the N-terminal API2 portion and the intact caspase-like domain of the C-terminal MALT1 portion (Morgan et al. 1999). The selection of specific domains of *API2* and *MALT1* to form a functional fusion protein emphasises their synergetic importance in lymphomagenesis.

Presence of t(11;18)(q21;q21) in tumour tissue, either fresh frozen or paraffin embedded, can be detected by reverse transcription-PCR (RT-PCR) and fluorescence in situ hybridisation (FISH). One or both of these techniques are now routinely performed in most labs as the presence of t(11;18)(q21;q21) has direct clinical compact : not only does it confirm the diagnosis of a MALT lymphoma, moreover, t(11;18)(q21;q21)-positive gastric MALT lymphomas are more often resistant to *H. pylori* eradication treatment and only rarely if ever evolve to a DLBCL (Ye et al. 2003; Alpen et al. 2000; Liu et al. 2001b; Sugiyama et al. 2001). Also, the presence of t(11;18)(q21;q21) in gastric MALT lymphomas is significantly associated with infection by the CagA-positive *H. pylori* strains. The latter generates a strong inflammatory response with release of interleukin-8 (IL-8), a powerful chemokine for neutrophil activation and subsequent ROS secretion (Ye et al. 2003). Therefore, it is tempting to hypothesise that t(11;18)(q21;q21) is related to genotoxic oxidative stress caused by inflammatory responses in premalignant MALT-like lesions, specifically in the mucosa of organs where an abundant influx of exogenous environmental antigens is known to occur (e.g. stomach and lung).

T(1;14)(p22;q32) and its variant **t(1;2)(p22;p12)** are present in approximately 5 % of MALT lymphomas, which tend to present with advanced stage of disease

and typically reveal additional genetic aberrations (Willis et al. 1999; Achuthan et al. 2000; Wotherspoon et al. 1992). As a consequence of this translocation, the *BCL10* gene (located at 1p22) is brought under the control of the *IGH* gene enhancer located at 14q32 (or the immunoglobulin light chain κ gene enhancer at 2p12 in the case of the variant translocation), resulting in overexpression of the *BCL10* transcript (Ye et al. 2000). *BCL10* encodes a CARD-containing protein that plays a key role in the antigen receptor signalling to NF-κB (Thome 2004).

Depending upon the study performed, **t(14;18)(q32;q21)** is demonstrated in 2–20 % of MALT lymphomas (Sagaert et al. 2006a; Streubel et al. 2005). Similar to t(1;14)(q21;q21), this translocation is mediated by *IGH* and leads to MALT1 overexpression. In contrast to t(11;18)(q21;q21)-positive cases, t(14;18)(q32;q21)-positive MALT lymphomas mainly occur outside the gastrointestinal or pulmonary tract, presenting as tumours of the skin, breast, lachrymal glands, liver or salivatory glands (Streubel et al. 2005; Remstein et al. 2004). It is hypothesised that this polarisation reflects a distinct pathogenesis; that is that MALT lymphomas of the stomach and lung are associated with an (un)known infectious agent while lymphomas arising in the salivary glands and ocular adnexa are often linked to autoimmune disease. As such, genetic differences might reflect different exposures to various inflammatory agents associated with MALT lymphomas in different locations.

More recently, *FOXP1* (forkhead-box protein P1 gene ; located at 3p13) was identified as a new translocation partner of *IGH*, not only in MALT lymphomas but also in DLBCL with mainly extranodal location (Wlodarska et al. 2005; Streubel et al. 2005). In both lymphoma subtypes, the overall frequency of **t(3;14)(p13;q32)** is relatively low (Wlodarska et al. 2005; Streubel et al. 2005; Sagaert et al. 2006b; Haralambieva et al. 2006). Remarkably, a significant number of t(3;14)(p13;q32)-negative MALT lymphomas and DLBCLs harbour strong nuclear FOXP1 expression, suggesting that mechanisms other than underlying *FOXP1* rearrangements can upregulate FOXP1 expression (e.g. trisomy 3). The significance of this nuclear FOXP1 overexpression is still debated: two studies found FOXP1 to be associated with inferior survival in DLBCLs (Banham et al. 2005; Barrans et al. 2004), whereas this could not be confirmed in a third study (Hans et al. 2004). Also, two studies found strong nuclear FOXP1 expression to be confined to (gastric) MALT lymphomas that are at risk of transforming into a DLBCL with poor clinical outcome (Sagaert et al. 2006a; Han et al. 2009). The *FOXP1* gene encodes a member of the FOX family of transcription factors, which is characterised by a common DNA-binding winged helix or forkhead domain (Banham et al. 2001). It was shown that FOXP1 is essential for B-cell maturation in the bone marrow, since FOXP1-deficient mice showed an arrest in the transition from pro-B cell to pre-B cell, probably due to diminished expression of the recombination-activating genes *RAG1* and *RAG2* (Hu et al. 2006). However, so far, it is not clear yet how FOXP1 mediates signalling in the mature, peripheral B-cell pool and how FOXP1 could contribute to MALT lymphomagenesis.

18.3.3 Step 3: Deregulation of the NF-κB Pathway

Mounting evidence links the oncogenic activity of t(11;18)(q12;q21), t(1;14)(p22; q32) and t(14;18)(q32;q21) to aberrant NF-κB activation by API2-MALT1, BCL10 and MALT1, respectively. NF-κB was first described in B lymphocytes as a transcription factor binding to the κB site of the immunoglobulin κ-light chain enhancer. Soon after, it was found that NF-κB activity could be induced in all cell types. To date, five members of the NF-κB transcription factor family have been identified: RelA (p65), RelB, c-REL, NF-κB1 and NF-κB (Siebenlist et al. 2005). After post-translation modification, the NF-κB1 and NF-κB2 precursor molecules p105 and p100 are processed to p50 and p52, respectively. All 5 NF-κB family members share a conserved REL homology domain for DNA binding, and they are all essential for lymphocyte survival and activation. Two distinct NF-κB signalling pathways have been identified: the canonical and non-canonical pathway (Li and Verma 2002; Pomerantz and Baltimore 2002). The canonical pathway engages RelA/p50 dimers and is triggered by viruses, bacterial lipopolysaccharides and pro-inflammatory cytokines such as TNF-α (tumour-necrosis factor-α) and IL-1 (interleukin-1). In unstimulated B cells, inhibitory κB (IκB) proteins bind to the NF-κB molecules RelA and p50, forming latent complexes that are present in the cytoplasm. Activation of the canonical pathway induces polyubiquitination and activation of Iκ-B kinase-γ (IKK-γ), also known as NF-κB essential modulator (NEMO). This event results in the phosphorylation and subsequent proteasomal degradation of IκB by the IKK catalytic subunit IKK-α. This allows the RelA/p50 dimers to translocate to the nucleus and mediate transcription of several antiapoptotic genes, including *BCL-X*$_L$ and *BCL-2*, as well as pro-proliferative genes, such as *cyclin D2*. Conversely, the non-canonical pathway engages RelB/ p52 dimers and is induced by a limited number of stimuli including BAFF (B-cell activating factor), lymphotoxin-β and CD40L (CD40 ligand). In a NEMO-independent manner, it leads to the phosphorylation of p100 by IKK-α and the subsequent degradation of its carboxyl-terminal half, resulting in the nuclear translocation of the p52/RelB dimers. Both pathways switch on a different set of genes and therefore mediate distinct regulatory functions, one that is mostly involved in innate immunity (canonical pathway) and the other in adaptive immunity (non-canonical pathway) (Bonizzi and Karin 2004).

Although both pathways modulate MALT lymphomagenesis, most of our current knowledge relates to the canonical pathway. The last decade has witnessed a significant advance in our understanding of this pathway through the identification of two key molecules, BCL10 and MALT1, which function downstream of the antigen receptor and upstream of the IKK complex. Therefore, their overexpression induced by fusion with the *IGH* enhancer in t(1;14)(p22;q32) and t(14;18)(q32; q32), respectively, hints at a role for NF-κB deregulation in MALT lymphomagenesis. Studies of knockout mice established that both BCL10 and MALT1 are essential in transducing antigen receptor signals to activate the NF-κB pathway (Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003). In vitro

experiments showed that overexpressed BCL10 activates NF-κB, whereas overexpressed MALT1 can only do so after BCL10-induced oligomerisation (Lucas et al. 2001). Also, BCL10-induced NF-κB activation is reduced in MALT1-deficient cells, an effect that can only be reversed after reintroduction of MALT1 expression (Ruefli-Brasse et al. 2003). Taken together, these data suggest that physical and functional BCL10-MALT1 interaction is essential for optimal NF-κB activation. The physical association between BCL10 and MALT1 involves a short region downstream of the CARD motif of BCL10 and the immunoglobulin-like domain of MALT1. This interaction is probably constitutive as endogenous BCL10 and MALT1 can be co-immunoprecipitated from lysates of non-stimulated B and T cells (Uren et al. 2000). In 2001, the upstream activator of the BCL10/MALT1 complex was identified as CARMA1 (CARD, membrane-associated guanylate kinase [MAGUK], protein 1), a 130 kDa CARD-containing protein that is constitutively associated with cholesterol- and glycosphingolipid-enriched membrane microdomains, termed lipid rafts (Gaide et al. 2001; Bertin et al. 2001; Allister-Lucas et al. 2001). The following model of BCR (B-cell antigen receptor)-induced activation of the canonical pathway fits the best with all currently available data (Fig. 18.4). BCR ligation by an antigen initiates a tyrosine phosphorylation signalling cascade, culminating in the generation of second messengers that activate PKC (protein kinase C) isoforms (Rawlings et al. 2006). PKC-β then phosphorylates and structurally reconfigurates CARMA1, hereby exposing the CARD motifs of CARMA1 and allowing interactions with its downstream components (Rawlings et al. 2006). As such, CARMA1 recruits BCL10 and MALT1 to the lipid rafts and binds BCL10 through CARD-CARD interaction (Gaide et al. 2002; Wang et al. 2004). Subsequently, the high-molecular-weight BCL10/MALT1 oligomers interact with and induce oligomerisation of the TNF receptor-associated factor 6 (TRAF6) via the carboxyl-terminus of MALT1 (Sun et al. 2004). As a result, TRAF6 elicits the E3 ubiquitin ligase activity of its RING-E3-domain to synthesise a Lys63-linked polyubiquitin chain on its target proteins, including IKK-γ (NEMO) and TRAF6 itself. In contrast to Ly48-linked polyubiquitin chains, this type of polyubiquitination does not induce the proteasomal degradation of IKK-γ and TRAF6 but facilitates their interaction with and the activation of TAK1 (TGF-β activating kinase). Activated TAK1 subsequently fully activates the IKK complex via phosphorylation of its β-subunit, which results in IkB phosphorylation/degradation and nuclear translocation of the p65/p50 dimers.

As NF-κB regulates the transcription of pro-proliferative and antiapoptotic genes in B cells, it is evident that its constitutive activation may result in lymphomagenesis. In t(1;14)(p22;q32)-positive MALT lymphomas, characterised by BCL10 overexpression, BCL10 is thought to oligomerise through its CARD motif without the need for upstream signalling, thus triggering MALT1 oligomerisation and aberrant NF-κB activation. In t(14;18)(q32;q21)-positive MALT lymphomas, in which MALT1 is overexpressed, the oligomerisation of MALT1 with subsequent NF-κB activation is believed to be dependent on BCL10, as MALT1 does not have a structural domain to mediate self-oligomerisation, nor does its overexpression alone activate NF-κB in vitro (Uren

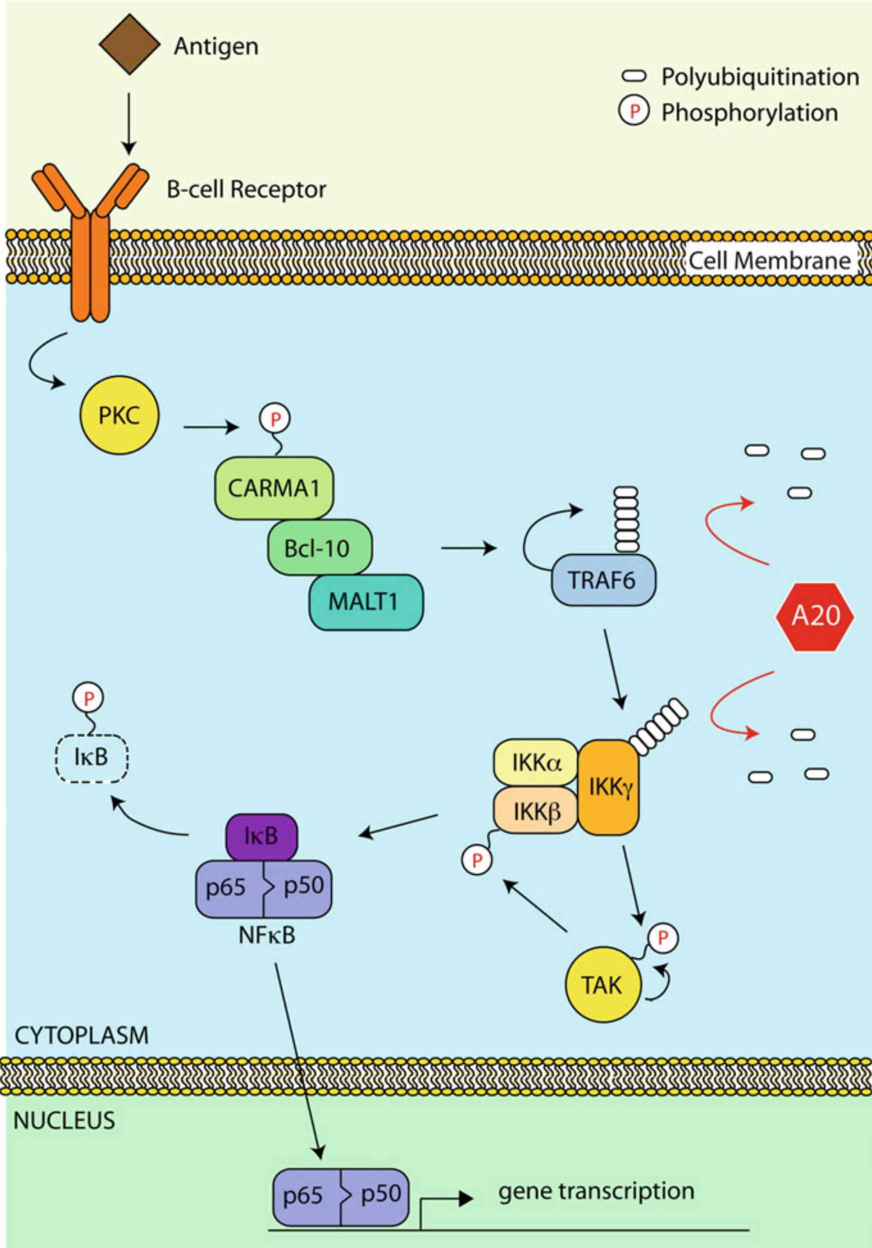

Fig. 18.4 Canonical NF-κB pathway. CARMA1 interacts with the antigen-activated B-cell receptor in the lipid rafts and induces the oligomerisation of its downstream components BCL10 and MALT1 with TRAF6. The latter elicits its ubiquitin ligase activity, resulting in polyubiquitination of IKK-γ (NEMO), which in turn phosphorylates IκB, hereby targeting IκB for phosphorylation and proteasomal degradation. This allows the REL-A/p50 dimers to enter the nucleus and mediate transcription of NF-κB-responsive genes

et al. 2000; Lucas et al. 2001). In t(11;18)(q21;q21)-positive MALT lymphomas, it is believed that the fusion protein API2-MALT1 activates NF-κB directly by increased IKK-γ polyubiquitination as demonstrated in cell lines and API2-MALT1 transgenic mice (Zhou et al. 2005; Baens et al. 2006). This process depends on the first BIR domain of API2 (Zhou et al. 2005; Baens et al. 2006). Also, the API2-MALT1 fusion protein was reported to reside in the lipid rafts of human B-cell lymphoma BJAB cells, which was associated with increased constitutive NF-κB activity and resistance to Fas-induced apoptosis (Ho et al. 2005). In fact, association of the MALT carboxyl-terminus with these lipid rafts, which is mediated by the API2 portion, is sufficient to trigger NF-κB activation via enhanced IKK-γ polyubiquitination activate (Baens et al. 2006). The oligomerisation of the API2-MALT1 fusion protein and/or the association of its MALT1 domains with downstream signalling molecules (e.g. TRAF6) might be eased by raft association, in this way bypassing the normal antigen-induced oligomerisation of MALT1-TRAF6 and thus ensuing constitutive NF-κB activation. In addition, it was shown that API2-MALT1 induces transactivation of the *API2* gene through NF-κB activation, hereby creating a positive feedback loop mechanism of self-activation by upregulating its own expression in t(11;18)(q21;q21)-positive MALT lymphomas (Hosokawa et al. 2005).

Finally, a remarkable feature of MALT lymphomas is the aberrant subcellular BCL10 location in the tumour cells (Fig. 18.1). In normal lymphoid tissue, BCL10 is weakly expressed in the cytoplasm of MZ B cells (Ye et al. 2000). Conversely, t(11;18)(q21;q21)- and (1;14)(p22;q32)-positive MALT lymphomas are marked by a, respectively, moderate to strong nuclear BCL10 expression, while t(14;18)(q32;q21)-positive MALT lymphomas are characterised by a perinuclear BCL10 location (Ye et al. 2000, 2005; Sagaert et al. 2006b). While the significance of this change in subcellular location is not yet known, it might be caused by disturbed nucleocytoplasmic shuttling of BCL10 which is regulated by MALT1 (Nakagawa et al. 2005). In the presence of t(1;14)(p22;q32), the overexpression of BCL10 results in nuclear BCL10 retention because of a shortage of MALT1 relative to BCL10. In t(14;18)(q32;q21)-positive MALT lymphomas, the location of BCL10 remains cytoplasmic because all nuclear BCL10 is exported by MALT1. The relative shortage of MALT1 due to the loss of one allele in t(11;18)(q21;q21)-positive MALT lymphomas might be responsible for the nuclear retention of BCL10 as the associated API2-MALT1 fusion is unable to export BCL10.

18.4 Conclusions and Outlook

Gastric MALT lymphomagenesis is a multistep process, provoked by *H. pylori* infection which induces genetic abnormalities and subsequent malignant transformation. As evident from the above discussion, the diagnosis of a gastric MALT lymphoma, made by endoscopic biopsy, should prompt investigation for the presence of *H. pylori* and t(11;18)(q21;q21) to clarify the therapeutic approach. An

important aim in the treatment of MALT lymphomas is to prevent transformation into an aggressive DLBCL, although the underlying mechanisms of that transformation are not known yet. Gastric MALT lymphoma-associated gene alterations, including *API2-MALT*, *IGH-BCL10* and *IGH-MALT*, result in constitutive activation of NF-κB. As such, pharmaceutical interference with the NF-κB pathway may represent an attractive treatment strategy in the future.

18.5 Key Points

- Diagnosis of a gastric MALT lymphoma is made by morphologic analysis of the endoscopic biopsy and supported by molecular techniques (PCR, FISH).
- Gastric MALT lymphoma is caused by *H. pylori* infection, and, therefore, every diagnosis of a gastric MALT lymphoma should prompt a thorough investigation for the presence of *H. pylori.*
- To date, gastric MALT lymphoma is the only malignancy in which antibiotics are the first choice of therapy.
- It is important to screen for the (gastric) MALT lymphoma-specific translocation t(11;18)(q21;q21) as its presence not only confirms the diagnosis of a MALT lymphoma but also predicts resistance to *H. pylori* eradication treatment and almost rules out evolution to a DLBCL.
- Gastric MALT lymphoma is characterised by a series of translocations, which affects molecules involved in one and the same pathway leading to the activation of NF-κB. Therefore, these molecules may represent attractive therapeutic targets.

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Chapter 19
Helicobacter pylori Infection in Children

Sibylle Koletzko and Francis Mégraud

Abstract *Helicobacter pylori* infection is acquired essentially in childhood and may persist lifelong. The prevalence varies widely in the world according to the socioeconomic status of the populations. It is very low in developed countries, while almost all children become infected in the developing world. While the exact routes of transmission are not fully determined, it appears that a gastro-oral (via vomitus) or oral-oral (via saliva after regurgitation) is the most important, and it occurs essentially, but not exclusively, from person to person inside the families. The pathology observed in children is usually milder compared to that in adults because of downregulation of the host immune response in the youngsters. Among clinical features, peptic ulcer disease may be found as a consequence of *H. pylori* infection but mostly in adolescents. Recurrent abdominal pain has multiple causes and is not an indication to test noninvasively and treat *H. pylori*. The other diseases caused by *H. pylori* are sideropenic anemia, chronic idiopathic thrombocytopenic purpura, and growth retardation. Some animal data also point the benefit of *H. pylori* infection as protector against atopic diseases in the youngest, especially asthma. Diagnostic tests in children are those used in adulthood. However, at age below 6 years, the urea breath test can lead to nonspecific results, and serology may not be sensitive enough. Preference must be given to invasive testing. Treatment should be tailored according to susceptibility testing.

Keywords Epidemiology • Pathogenesis • Clinical features • Diagnosis • Treatment

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19.1 Introduction

Childhood is an important period of life for *Helicobacter pylori* infection because it is the period of contamination. However, the consequences of this infection are limited in early life and occur essentially at an older age, so it can be said that it is "a pediatric infection with geriatric consequences." Epidemiological studies are lacking because of the difficulty in accessing the site of the infection for ethical reasons. More recently, the development of the stool antigen test brought us interesting data. Clinical studies were also carried out less frequently in children than in adults because of a limited prevalence of the infection in western countries, where technology is optimal. For these reasons, multicenter studies on diagnosis, treatment, and clinical outcome were implemented, especially in Europe, under the leadership of an *H. pylori* Pediatric Task Force resulting from the joint efforts of the European Society for Pediatric Gastroenterology Hepatology and Nutrition and the European Helicobacter Study Group. In this chapter, the particularities of *H. pylori* infection will be reviewed with regard to its epidemiology, pathogenesis, clinical manifestations, diagnosis, and treatment.

19.2 Epidemiology

19.2.1 Prevalence and Incidence

The first studies on the prevalence of *H. pylori* infection indicated a low prevalence in children which progressively increased during the life span (Megraud et al. 1989). However, it was quickly shown that *H. pylori* infection was not progressively acquired over the years but that this curve corresponded to a cohort phenomenon (Banatvala et al. 1993). Each age group (cohort) carries its own risk linked to the socioeconomic conditions existing at the time of their youth. However, in some developed countries like the Netherlands, it appears that the prevalence in children has stabilized given the similar prevalence in subsequent birth cohorts (den Hoed et al. 2011). Studies performed on cohorts of children and adults have confirmed that the incidence (new cases per year) is higher in children and that young children are more at risk than older ones.

The age of acquisition of *H. pylori* is not easy to determine because the infection is essentially asymptomatic or paucisymptomatic and can be confounded with a gastroenteritis. Incidence was monitored over 20-year period from ages 1–3 years to ages 22–24 years, in the Bogalusa cohort from Louisiana, USA, at the end of the twentieth century. The crude incidence rate decreased from 2.1 % at ages 4–5 years to 1.5 % at ages 7–9 years and 0.3 % at ages 21–23 years (Malaty et al. 2002). It was also shown in the Okinawan cohort followed for 10 years that the incidence in children (2.7 %/year) was higher than in adults (Banatvala et al. 1994). The same was observed in China (Mitchell et al. 1992) and in Chile (Russell et al. 1993).

Rowland and coworkers investigated 327 healthy children applying the ^{13}C urea breath test (UBT) for 4 years. At baseline, 28 children were already positive and 20 converted during follow-up. The highest incidence of 5.1 per 100 person years was observed between 2 and 3 years of age and continuously declined to 0.7 by ages 7–8 years (Rowland et al. 2006).

Although most of the infections remain for a long time, it also happens that the host can resolve them especially in the younger population. In the Bogalusa cohort, 2.2 % cleared the infection at ages 4–5 years vs. 0.2 % at ages 18–19 years (Malaty et al. 2002). Transient *H. pylori* infections were also documented in Japan by Okuda and coworkers using stool antigen test. Out of 16 children who became stool antigen test positive during a year, only four remained persistent positives (Okuda et al. 2007). These data confirmed those obtained by using serology in a cohort of Native American children (Perez-Perez et al. 2003).

The prevalence of the infection in children is extremely diverse throughout the world. In developing countries, it turns out to be high, while in developed countries it is rare. However, there are some exceptions and, for example, in Europe, *H. pylori* prevalence is still high in Portugal and some Eastern European countries. In a cohort of adolescents (age of 13 years) from schools in Porto, the prevalence was 66.2 % with an incidence of 1.1 per year (Bastos et al. 2013b).

The prevalence of *H. pylori* infection also remains high in children of immigrants to developed countries as was shown in Belgium for children 0–9 years: >30 % *H. pylori* positive vs. <5 % for those of European origin (Miendje Deyi et al. 2011). However, in some areas, a dramatic change in the prevalence of *H. pylori* infection is observed during childhood. For example, in St. Petersburg (Russia), the prevalence among children younger than 5 years decreased from 30 to 2 % between 1995 and 2005, linked to improvement in the standard of living and an increased use of antibiotics (Tkachenko et al. 2007).

19.2.2 Risk Factors

Risk factors for the acquisition of *H. pylori* are linked to the socioeconomic status in a given country. It is likely that almost all European children were indeed infected at the turn of the twentieth century when European countries still had a very low hygiene level. The risk of infection then progressively decreased, and it was amplified by the use of antibiotics which began after the Second World War.

Low socioeconomic status in terms of risk factors for families means low income, small homes, large households, low education, and lack of facilities for hygiene, all of which favor transmission of the bacterium.

In contrast, breastfeeding appears to be protective. IgG anti-*H. pylori* are present in breast milk when the mother is *H. pylori* positive. Human milk contains many anti-infectious factors such as lactoferrin, lysozyme, secretory IgA, and mucins which may act even in infants of noninfected mothers (Chak et al. 2009; Carreira et al. 2015).

The possibility of ethnic differences in susceptibility to the infection has been proposed since the incidence of the infection is very different, for instance, between Caucasian and African-Americans living in the same area (Malaty et al. 2002) or in young women in a multiethnic European city (den Hollander et al. 2013), but this difference is most likely linked to environmental conditions, especially the socio-economic status.

A study of monozygotic and dizygotic twins reared together or separately was the occasion to assess the importance of genetic effects on the acquisition of *H. pylori* infection. A correlation coefficient of 0.66 for monozygotic twins reared apart points to a positive, while moderate, effect of genetics (Malaty et al. 1994). However, when the environmental conditions of these twins were studied, it appeared that infected twins were raised in homes under poorer socioeconomic conditions than those of their noninfected co-twins.

Looking at genome wide associations, a Toll-like receptor 1 single nucleotide polymorphism was associated with *H. pylori*-positive serologic status (Mayerle et al. 2013); these data must be confirmed in ethnic groups other than Caucasians. For more details on the role of gene polymorphisms, we refer to Chap. 14.

19.2.3 Transmission

Transmission of *H. pylori* is essentially from person to person and mostly intrafamilial and vertical through transmission, particularly in low prevalence countries, while in high-prevalence settings, a horizontal transmission from outside the family with infections of multiple strains within one person is common (Schwarz et al. 2008). We can anticipate that about two-thirds of the transmission occurs between family members and one-third outside. Among the parents, the role of the mother appeared to be more important (Dominici et al. 1999). It was confirmed in a German study (Weyermann et al. 2009).

But according to others, the transmission may also involve older siblings. The risk of infection increases according to birth order in large families and is higher when the age interval with the older siblings is low (Goodman and Correa 2000). In another study, the number of siblings in a family rather than the birth order was associated with *H. pylori* infection (Ford et al. 2007).

There is also the possibility of transmission from grandmothers. In Japan, where the family structure is still traditional, they carried the third highest risk after siblings and mothers (Urita et al. 2013).

Considering acquisition from outside home, a systemic review and meta-analysis was carried out to evaluate childcare attendance as a risk factor for acquiring infections especially in settings with a high prevalence of the infection (Bastos et al. 2013a). In Portugal, for instance, when prevalence of the infection at ages 4–5 years was 30.6 %, it significantly increased with cumulative time of attendance in daycare centers/homes, from 13.2 % among those never attendees to 40.2 % among those attending for >36 months ($p < 0.001$) (Lunet et al. 2014).

In addition to epidemiological studies, molecular fingerprinting has also been performed to prove the transmission of the same strain in families. The first studies using ribotyping showed that the same strain was present in at least two family members in three out of four families (Bamford et al. 1993). In Japan, using random amplified polymorphic DNA (RAPD) fingerprinting, 76 % of 42 children showed DNA fingerprint patterns identical to those of at least one of the respective family members, essentially to the mother (69 %) and much less to the father (16 %) (Konno et al. 2008).

The schema of intrafamilial transmission may be challenged in developing countries. In Peru, also using RAPD fingerprinting, 30 % of the child-mother strain pairs did match, as well as 18 % child-father and 32 % from siblings, suggesting also transmission from unrelated individuals or via environmental sources (Herrera et al. 2008). The corresponding figures in Bangladesh were 46 % for mothers and 15 % for fathers (Nahar et al. 2009). Raymond and coworkers studied *H. pylori* in two French families with DNA microarrays: different matches of strains were found, while in a Moroccan family of seven, five different strains were identified (Raymond et al. 2008).

Our knowledge is also limited concerning the vehicle of transmission given the absence of detectable environmental reservoirs and outbreak scenarios. To go from one stomach to another stomach, the most probable route taken is the gastro-oral transmission. Indeed, all vomitus samples from infected subjects grow *H. pylori*, often in high quantities and even air samples obtained during vomiting (Parsonnet et al. 1999).

This vehicle may therefore be responsible for *H. pylori* transmission among siblings and in daycare centers if proper disinfection is not carried out.

In the USA, among 1752 noninfected household members, 30 new infections occurred over a 3-month period, and 75 % were attributable to exposure to an infected person with gastroenteritis especially because of vomiting (OR, 6) (Perry et al. 2006).

H. pylori may also be present in the oral cavity after emesis (Parsonnet et al. 1999) and following regurgitation in patients with gastroesophageal reflux disease (GERD) (Young et al. 2000).

Its presence is more likely transient than permanent but may allow transmission via saliva. It was shown that pre-mastication of food by mothers or wetting the mammilla before breastfeeding with saliva was a risk factor of transmission to their children (Albenque et al. 1990).

Stools do not seem to play a major role in the transmission of *H. pylori*, at least in developed countries. Indeed, all elements from the stomach will be eliminated in stools, but isolation of live *H. pylori* from stool samples commonly failed because the bacterium rarely survives the bowel passage. After induced diarrhea in *H. pylori*-positive subjects, growth of live *H. pylori* was obtained in only 22 % of the stools (Parsonnet et al. 1999).

This result indicates that *H. pylori* can only be transmitted by stools in the context of diarrheal diseases. Furthermore, it implies that fecal hygiene must not be adequate in order to have a direct contact or to contaminate water. A number of

studies claimed that water can be positive for *H. pylori*. However, these results are doubtful because most often, only one polymerase chain reaction (PCR) was performed detecting DNA and the lack of direct growth of live *H. pylori*. In water, as in the oral cavity and all specimens except the stomach, it is necessary to use several PCRs based on different targets and perform growth to get the same positive answer before making a conclusion.

The hypothesis that access to treated water and a sanitary sewage system that reduces the incidence of *H. pylori* infection was tested in a cohort of children at the US-Mexican border but did not provide a firm support for potential waterborne transmission of *H. pylori* (Travis et al. 2010). Acquisition of *H. pylori* infection in rhesus macaques is also most consistent with oral-oral transmission (Solnick et al. 2006). The seroepidemiology of *H. pylori* infection and of hepatitis A argues also against a common mode of transmission (Luzza et al. 1997).

In conclusion, *H. pylori* infection occurs essentially in childhood, mostly in the family, the mother being the main source. Once established, *H. pylori* infection may remain for life. Poor socioeconomic conditions are the main risk factor, and their improvement leads to a decrease in the prevalence of the infection in successive cohorts. A gastro-oral transmission via vomiting is the most likely.

19.3 Pathogenesis

The characteristics of *H. pylori* infection are dependent on the infecting strain and on the host response and can be influenced by other environmental factors.

There are very few differences between the strains infecting children or adults. The main known colonizing and pathogenic factors are the same, i.e., BabA, SabA, *cag*PAI, VacA, and others (for more details, we refer to Chaps. 3, 4, 5, 6, and 7). However, conversely, the host response can be different in children and in adults as they evolve over time. Furthermore, the changing gastric environment in response to infection may lead to changes in the expression of *H. pylori* virulence factors. Finally, most of the environmental conditions which can influence the evolution of the infection such as smoking, alcohol consumption, and drug intake are mostly absent in children, leading to more "pure" pathologies compared to adults, making it easier to study the relationship between putative diseases and virulence factors.

Harris and coworkers compared the differences in histology and T cell response between 36 children younger than 12 years (50 % infected) and 79 adults (65 % infected) in Chile (Harris et al. 2008). They observed a limited number of polymorphs and mononuclear cell infiltration, as well as an intact epithelium, in children vs. adults, independently of the type of the infecting strains and the bacterial load. They were able to demonstrate that the level of T regulatory cells (Treg) and Treg cytokines (the transforming growth factor beta (TGF-β) signaling pathway and interleukin-10 (IL-10)) is markedly increased in both infected and noninfected children compared to adults, while interferon alpha (IFN-α) expression was increased in adults. Then in children, it appears that Treg activity

downregulates the inflammatory response to *H. pylori* resulting in lower gastritis scores compared to adults (Harris et al. 2008).

Similar results were obtained by Freire de Melo and coworkers. Treg-associated cytokines were more predominant in children than in adults where Th17 cytokines are mainly present. They concluded that the lower inflammatory infiltrates of the gastric mucosa in children could be responsible for the children's increased susceptibility to infection (Freire de Melo et al. 2012) and persistence of the bacteria (Gil et al. 2014).

Freire de Melo and colleagues also studied the gastric concentrations of cytokines representative of the innate and Th1 response. IL-1α and TNF-α concentrations were significantly higher, while those of IL-2, IL12-p70, and IFN-γ were lower in the infected children than in the infected adults, but the gastric concentrations of these three latter Th1-associated cytokines increased with age in children and correlated with an increased degree of gastritis (Freire de Melo et al. 2014). Such a result, regarding IFN-γ secretion in the stomach of infected children, had already been highlighted in the past (Bontems et al. 2003). The same authors also showed that nuclear factor kappa-B (NF-κB) activation occurred essentially in adults, possibly a consequence of a lower CD3+ and CD8+ T cell recruitment in children (Bontems et al. 2014).

In China, Li and coworkers found that peptic ulcer disease (PUD) in children was essentially due to the most virulent *cag*A+ EPIYA-ABD, while in adults, four types of EPIYA motifs were identified (Li et al. 2009). Detailed explanation of EPIYA and ABD type in *cag*A are discussed in Chap. 4.

Oleastro and coworkers studying children's strains could also identify two markers for PUD, jhp0562 involved in lipopolysaccharide biosynthesis and jhp0870 an outer membrane protein (Oleastro et al. 2006).

In conclusion, the differences in pathophysiology between children and adults are essentially due to the host innate and adaptive immune response rather than the *H. pylori* strains. The lack of other environmental conditions which may influence the outcome allows the observation of "pure" pathologies.

19.4 Clinical Manifestations

19.4.1 Peptic Ulcer Disease

Drumm and coworkers described in the *New England Journal of Medicine* in 1987, for the first time convincingly, the association of primary gastritis and peptic ulceration and *H. pylori* (at that time still called *Campylobacter pylori*) in children (Drumm et al. 1987). In children with a normal histology, the bacteria could not be identified. Since then, many studies in children and adults have identified *H. pylori* as an important human pathogen that is responsible for the majority of cases with PUD. With the decreasing prevalence of infected children, other pathogenic factors

than *H. pylori* are becoming predominant as causes for gastric and duodenal ulcerations. A prospective, European multicenter, case-control study in 244 pediatric patients with gastric or duodenal erosions ($n = 153$) or ulcers ($n = 91$) and two age-matched controls for each from the same center was performed recently (Bontems et al. 2013). Children receiving antimicrobials or acid-suppressive drugs before endoscopy were excluded. *H. pylori* infection was detected more frequently in cases than in controls (32.0 % versus 20.1 %) ($p = 0.001$), but in two-thirds of the patients, erosions and ulcerations were due to other causes than *H. pylori*.

H. pylori-related ulcers are rare in symptomatic children undergoing endoscopy. In a large European study including 1322 *H. pylori*-infected children, gastric or duodenal ulcers were found in only 3.5 % of children younger than 6 years of age, in 4.6 % between the ages of 6 and 11 years, and in 10.4 % of those older than 11 years (Koletzko et al. 2006). In another multicenter study, only 64/454 (12.3 %) infected children presented with gastric or duodenal ulcer and/or erosions (Oderda et al. 2007). The low rate of PUD, particularly in children below 12 years of age, may be related to a shorter disease duration, but it is more likely due to the absence of additional risk factors for ulcerations such as smoking, alcohol consumption, and regular intake of ulcerogenic drugs (e.g., nonsteroidal anti-inflammatory drugs (NSAID)).

Duodenal ulcer patients have antral predominant, body-sparing, non-atrophic gastritis with a highly stimulated acid production. Without clearance of the infection, the relapse rate of the ulceration is high even after healing with acid-suppressive drug treatment. Meta-analyses of studies on adults consistently demonstrate a reduced risk for bleeding ulcers after eradication of the *H. pylori* infection (Leodolter et al. 2001). Although no such data are available for infected children, there is a clear recommendation that, in the presence of gastric or duodenal ulceration, the organism should be eradicated independently of age (Koletzko et al. 2011; Malfertheiner et al. 2012).

19.4.2 Recurrent Abdominal Pain

Recurrent abdominal pain as defined by Apley and Naish (1958) or weekly pain occurs in 10–17 % of school-aged children (Schwille et al. 2009). Abdominal pain and other complaints such as dyspepsia or nausea are nonspecific symptoms and can be caused by different organic diseases related and unrelated to the gastrointestinal tract. Most affected children suffer from functional pain. Even 30 years after the first publication by Marshall and Warren in the *Lancet* (Marshall and Warren 1984), there is still controversy regarding whether, in the absence of PUD, the infection causes abdominal symptoms in children. A few controlled studies on a limited number of children with different study designs showed no difference regarding improvement of dyspeptic symptoms after either successful or failed triple therapy (Wewer et al. 2001) or placebo treatment (Ashorn et al. 2004). A

causal relationship between recurrent abdominal pain and non-ulcer *H. pylori* infection in children can only be proved or disproved by randomized placebo-controlled intervention studies. So far, only one such study has been performed on children (Ashorn et al. 2004). No difference was found regarding dyspeptic symptoms after either successful triple therapy or proton pump inhibitor (PPI) treatment only, even 12 months after treatment. As functional pain is highly responsive to reassuring but also to placebo effect, the common improvement of symptoms in *H. pylori*-treated patients may be unrelated to bacterial clearance. A meta-analysis on 38 studies (23 case controls, 14 cross-sectional, and 1 prospective cohort study) did not find an association between recurrent abdominal pain and *H. pylori* infection: pooled OR 1.21 [95 % CI, 0.82–1.78] in the case-control studies and pooled OR 1.0 [0.761.31] in the cross-sectional studies (Spee et al. 2010). The absence of a significant relationship between abdominal pain and the infection is consistent with a previous meta-analysis (Macarthur 1999). The only significant association was found in patients referred to a pediatric gastroenterologist. However, the wide availability of noninvasive tests makes a referral bias of those with a positive test result very likely. Since the meta-analysis of Spee and coworkers, there have been further reports on the lack of relationship between *H. pylori* infection and abdominal pain from industrialized and developing countries (Guariso et al. 2010; Senbanjo et al. 2010; Cherian et al. 2010; Buonavolonta et al. 2011; Dore et al. 2012).

In conclusion, the reported absence of an association between abdominal pain and *H. pylori* infection supports the recommendations given by the European and North American Societies of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN and NASPGHAN) that abdominal pain in children is not an indication to test for *H. pylori* infection with a noninvasive test (Koletzko et al. 2011).

19.4.3 Extragastric Diseases

Extragastroduodenal disorders as a manifestation of *H. pylori* infection have been looked for in many epidemiological cross-sectional studies, some case-control studies, and only very few prospective interventional studies (Pacifico et al. 2014). Since *H. pylori* infections are not evenly distributed within a population or a birth cohort but are highly related to socioeconomic factors or emigrant status, it is very difficult to interpret positive findings from association studies. Factors such as poor growth or iron deficiency (ID) are themselves highly related to socioeconomic conditions. Only randomized prospective intervention trials can prove a causal relationship between *H. pylori* infection and these health outcomes. This also applies to potential beneficial effects of an *H. pylori* infection which have an inverse relationship to childhood asthma and allergies, obesity, and inflammatory bowel disease (Blaser et al. 2008; Amberbir et al. 2014; Chen and Blaser 2008; Sonnenberg and Genta 2012; Francois et al. 2011). Trials by randomized infection of infants or young children to evaluate the beneficial effect are not possible for ethical reasons since the bacterium has been declared as a carcinogen. However, the

underlying mechanisms can be studied in animal models to elucidate whether factors or pathogenic strains can be identified and transferred to children without producing a lifelong infection and an increased risk for PUD and gastric cancer.

19.4.3.1 Sideropenic Anemia

Of all the extragastroduodenal manifestations, ID and iron deficiency anemia (IDA) are those with the most available evidence to support a causal relationship. Pacifico and coworkers recently reviewed all published studies performed in children regarding ID and IDA and the possible biological mechanism (Pacifico et al. 2014). Several pathways seem to be involved alone or in combination. Most children with *H. pylori* gastritis only, in the absence of erosions or ulcerations, have no evidence of occult blood loss. Hypothesized mechanisms are related to a reduced acid output with a change to a higher gastric pH. This affects the reduction of the ferric form (Fe^{+++}) of the nonheme iron in the diet to the ferrous form (Fe^{++}) which is more easily absorbed. Ascorbic acid is an important promoter of the reduction process. At a pH of >5, an insoluble molecular complex between iron and ascorbic acid is formed, making the iron unavailable for absorption. In Chilean children undergoing upper endoscopy, *H. pylori* infection was more commonly found in those with hypochlorhydria (pH > 4) compared with those with a lower gastric pH (Harris et al. 2013). The link may be a higher gastric concentration of IL-1β which is related to a higher pH and a higher frequency of ID and IDA. Queiroz and colleagues demonstrated in Brazilian *H. pylori*-infected children that those with a certain polymorphism in the IL1β receptor have higher IL-1β concentrations leading to more severe hypochlorhydria in the acute phase of the infection (Queiroz et al. 2013b). Another possible mechanism may the absorption of lactoferrin bound iron in the stomach by the bacteria and therefore a competition with the availability for the host.

Epidemiological observational trials suffer from the coexistence of several risk factors for ID and IDA which are also closely related to *H. pylori* infection in childhood such as low hygiene and low socioeconomic status, crowding, helminth infections and poor nutrition, and particularly low intake of fruit (vitamin C), meat, and fish. Puberty normally accompanied by a growth spurt is a particular period in life with a high demand in iron, especially in menstruating girls. For this reason, age is a strong confounding factor for iron status which explains that *H. pylori* infection was found to be a risk factor only in teenagers and not in infants and young children (Muhsen et al. 2010; Choi 2003; Baggett et al. 2006). A recent multicenter study from Brazil, Chile, and the UK investigated risk factors including *H. pylori* infection for the iron status (hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), hematocrit (Hct,) transferrin saturation, and ferritin) in children undergoing upper endoscopy (Queiroz et al. 2013a). Children with mucosal lesions or intake of iron or other potentially interfering drug intake were excluded. Higher age, female gender, being born in Brazil, and *H. pylori* infection were independent variables for low ferritin and hemoglobin in a multiple linear regression model.

Several intervention trials have been performed in children with and without ID or IDA. In the absence of a depleted iron status, iron supplementation results in a larger increase in iron stores in children without the infection or who are treated successfully with eradication of the bacteria (Cardenas et al. 2011; Mahalanabis et al. 2005; Xia et al. 2012). In another randomized clinical trial in Bangladesh, iron plus eradication therapy was not better to improve iron status compared to iron supplementation and placebo. No effect was seen in children receiving anti-*H. pylori* therapy regarding the success of bacterial clearance or not (Sarker et al. 2008). Another large intervention trial in Western Alaska included *H. pylori*-infected children between 7 and 11 years of age ($n = 219$) with ID. They received either a 2-week anti-*H. pylori* therapy with 6 weeks of iron sulfate or iron supplementation only. No difference was observed regarding iron status up to 14 months after treatment. After 40 months, 176 children were available for reevaluation. Reinfection was very common (52 %). ID and IDA had improved only in those who stayed uninfected during the observation period.

In conclusion, *H. pylori* infection may be considered as a risk factor for IDA in a subgroup of children, particularly those living in developing countries. More studies are required from developed countries where other interfering factors are not or less prevalent. Considering the importance of iron for immunological, cognitive, and other important functions in the growing child, it is recommended that children with refractory IDA in which other causes have been ruled out or treated may be tested for *H. pylori* infection and treated in case of a positive result (Koletzko et al. 2011).

19.4.3.2 Chronic Immune Thrombocytopenic Purpura

Chronic Immune Thrombocytopenic Purpura (cITP) is an autoimmune disease with platelet autoantibody formation resulting in platelet destruction and thrombocytopenia for at least 6 months. As in many other autoimmune diseases, infections including *H. pylori* have been considered to trigger the autoimmune process. These triggering factors may differ in pediatric patients vs. adult patients, and spontaneous recovery occurs more commonly in children (~ one-third) compared to only 5 % in adults. In low prevalence countries for *H. pylori* infection, most children with cITP are not infected. For example, only 3 of 33 children in the Netherlands tested positive for *H. pylori* (Neefjes et al. 2007) and none of the children in a Finnish study (Rajantie and Klemola 2003). Therefore, most intervention trials have been performed in high-prevalence countries with conflicting results. A weakness of the studies is the lack of treatment in the *H. pylori*-negative control group. It is possible that a 2-week therapy with PPI and two or three different antibiotics may have an effect even in *H. pylori*-uninfected children by changing the luminal milieu or the gut microbiome, and the transient or persistent positive effects on the platelet counts may be unrelated to the clearance of the *H. pylori* infection.

In conclusion, there is no evidence that *H. pylori* infection plays a role in cITP in developed countries and very little evidence in high-prevalence countries. Further larger-scale randomized clinical trials including children with cITP from different countries are required before a general screening for *H. pylori* infection can be recommended.

19.4.3.3 Growth Retardation

The effect of *H. pylori* infection on growth has been assessed in many studies. While most previous studies were not or inadequately controlled for potential confounders affecting growth such as parental height, socioeconomic status, dietary factors, and others, more recent studies have tried to account for this. If *H. pylori* has a negative effect on growth, this can most likely be seen more easily when the infection takes place within the first 6–12 months of life when the growth velocity is highest. However, the type of feeding, breastfeeding or formula feeding, has a strong influence on the growth pattern in the first year of life, but it also differs markedly depending on the socioeconomic status.

The biological plausibility of early *H. pylori* infection affecting growth may be related to the described transient hypochlorhydria during the acute infection which increases the risk for intestinal infection with vomiting and diarrhea. *H. pylori* gastritis may also change the ghrelin secretion from the gastric mucosa, consequently affecting appetite and satiety. Most studies on the effect of *H. pylori* infection on ghrelin and changes after spontaneous or treatment-related clearance of the infection have been performed in children (Plonka et al. 2006; Pacifico et al. 2008; Yang et al. 2012; Deng et al. 2012) and none on infants and toddlers. Most but not all studies showed an increase in plasma ghrelin levels after successful treatment. Most studies on the effect of *H. pylori* infection on growth have been performed in developing countries on populations under poor socioeconomic conditions with a high prevalence of both *H. pylori* infection and malnutrition during early childhood, such as the Gambia (Thomas et al. 2004), Colombia (Bravo et al. 2003; Mera et al. 2012; Goodman et al. 2011), Ecuador (Egorov et al. 2010), and Peru (Jaganath et al. 2014). Results are controversial with some showing a transient decrease in growth velocity but catch up growth not affecting the height at the last follow-up (Thomas et al. 2004; Mera et al. 2012), while other reported a persistently lower height in *H. pylori* infected compared to noninfected children (Goodman et al. 2011; Egorov et al. 2010). Jaganath and coworkers followed 183 infants from a peri-urban shanty town outside Lima from birth to 24 months of age. UBT was performed from 6 months of age. Almost all (>97 %) became infected with *H. pylori* within the first 2 years and 77 % within the first 12 months of life (Jaganath et al. 2014). A low socioeconomic status increased the risk of early infection until 12 months (HR 1.59, 95 % CI 1.1.6–2.19), while breastfeeding had a preventive effect (HR 0.63, 95 % CI 0.40–0.96). The infection was not independently associated with decreased growth ($p = 0.58$). However, early infection in conjunction with several diarrheal episodes negatively affected

height at 24 months. It remains to be determined whether *H. pylori* infection itself has an effect on growth either directly or indirectly or whether early infection is only a marker for poor socioeconomic circumstances with a negative impact on health and nutritional status.

19.4.4 Possible Beneficial Effects of H. pylori Infection

Many studies focused on the adverse health effects such as peptic ulcer disease or gastric cancer and also on the effect of having an *H. pylori*-positive gastritis on unspecific pain, dyspepsia, or gastric emptying. During the first 20 years of *H. pylori* enthusiasm, hardly anybody asked the question whether the infection may have any benefits for the host. Research by Linz and coworkers disclosed that *H. pylori* migrated with the first humans about 60,000 years ago from East Africa and spread over the world (Linz et al. 2007). Thus, for mankind, chronic *H. pylor*i infection was the normal situation with adaptation for thousands of years while not being infected was the exception. The disappearance of one species from the stomach may change the environment and the conditions for the remaining bacteria. The immunological response may differ in relation to the *H. pylori* infectious status. As pointed out above, a Treg response with high IL-10 and low IL-17 expression predominates in children compared to adults (Freire de Melo et al. 2012). In a mouse model using sensitization with ovalbumin (OVA), a protective effect has been shown against asthma airway hyperresponsiveness and tissue inflammation with eosinophils, Th2 cells, and Th17 cells through the induction of regulatory T cell during early *H. pylori* infection (Arnold et al. 2011). The protective effect was most robust when mice were infected as neonates compared to later in life and was abrogated by antibiotic eradication of *H. pylori*. Systemic Treg depletion abolished asthma protection; conversely, the adoptive transfer of purified Treg populations was sufficient to transfer protection from infected donor mice to uninfected recipients. These results gave experimental evidence for a beneficial effect of *H. pylori* infection on the development of allergen-induced asthma.

In humans, only epidemiological data are available. A meta-analysis including nine cross-sectional, seven case-control, and three cohort studies showed a risk reduction of the infection on children but not adult onset asthma (OR, 0.81 (95 % CI 0.72–0.91) versus 0.88 (0.71–1.08)) (Wang et al. 2013). A recent meta-analysis including 16 studies confirmed the reduced odds of atopy by *H. pylori* infection, particularly in those with raise allergen-specific IgE (OR = 0.75; 95 % CI 0.62–0.92, $p < 0.01$, seven studies) (Taye et al. 2015). In a recent study from a low-income birth cohort in Ethiopia, 863 children were followed for up to 5 years for incidence and prevalence of allergic disease and sensitization (Amberbir et al. 2014). *H. pylori* infection was assessed by the stool antigen test and was found to be positive in 25 % at both time points, in 21 % at age 5 years only, and in 17 % at age 3, but not at age 5. After adjustment for possible confounders, *H. pylori* infection at age 3 years reduced the risk of incident eczema between ages 3 and

5 years (adjusted OR, 0.25; 0.007–0.92). Sensitization was inversely related to *H. pylori* infection. A similar negative association between *H. pylori* infection could be shown with other so-called immune-mediated disorders such as inflammatory bowel disease (IBD) (Sonnenberg and Genta 2012; Roka et al. 2014). Although these association studies are good for generating hypothesis and are biologically plausible and/or supported by experiments in animals, they are not sufficient to prove a causal relationship. However, as long as there is the plausibility that an early infection may be beneficial for the long-term immune response, the indication to treat a child for the indication should be based on solid grounds to balance the risk-benefit ratio.

19.5 Diagnosis

The numerous tests used to diagnose *H. pylori* infection in adults may also be used in children. However, some noninvasive tests like UBT and serology have limitations.

19.5.1 Invasive Tests

The endoscopic aspect of gastric mucosa can orientate itself toward the presence of *H. pylori*. Antral nodularity is a specific feature of the infection but with a poor sensitivity and positive predictive value (42 %) (Prasad et al. 2008). Gastric biopsies can be obtained for histology, culture, molecular tests, and rapid urease test (RUT). According to the guidelines, two biopsies from the antrum and two from the corpus are necessary for histology (for the Sydney system classification) and one of each for culture. In a series of children in Brazil, histological features indicate a moderate to severe chronic active gastritis, more frequent and of higher grade in the antrum than in the corpus with a topographic distribution of mostly pangastritis (62 %), followed by antral gastritis (32 %) and corpus only gastritis (6 %). No significant atrophy or intestinal metaplasia was present (Carvalho et al. 2012). In contrast to what occurs in adults, in children, *H. pylori* may be present without inflammatory stigmata in the case of an early stage of infection.

Molecular tests correspond essentially to real-time PCR. This test allows detection of *H. pylori* with an excellent sensitivity and specificity and also detection of the mutations associated with *H. pylori* resistance to macrolides if the target is the 23S rDNA.

To render the procedure less invasive, especially in asymptomatic children, an alternative in getting biopsies is to use the string test to obtain gastric juice. The material is suitable for culture and molecular tests (Goncalves et al. 2013).

19.5.2 Noninvasive Tests

19.5.2.1 Urea Breath Test (UBT)

The UBT has its limitations in young children. A special device is needed to obtain breath air (mask with unidirectional valve with a breath bag), the 75 mg dose of ^{13}C urea in adults can be decreased to 50 mg (Bazzoli et al. 2000), and the test meal consisting of citric acid can be replaced by orange juice to be acceptable to children. More importantly, a problem of specificity has been highlighted in infants and children under 6 years of age (Imrie et al. 2001; Leal et al. 2011b). Kindermann and coworkers proposed to define a gray zone rather than a cutoff for children less than 6 years of age (Kindermann et al. 2000).

Some authors proposed to normalize the UBT results for CO_2 production in children by calculating the "urea hydrolysis rate" to improve the performances (Elitsur et al. 2009). The accuracy was not improved in children under 6 years of age suggesting that factors other than endogenous CO_2 production could be responsible (Yang et al. 2008).

The question of UBT specificity was addressed essentially in developed countries and on small numbers of cases. More recently, Queiroz and coworkers performed a large study in South America (Brazil and Peru) where they evaluated the agreement between stool antigen test and UBT and indeed found UBT reliable in infants and toddlers. Discrepant results did occur in 5.1 % of the samples. They also found that delta over baseline (DOB) values of UBT increased with age between birth and 2 years (Queiroz et al. 2013c).

19.5.2.2 Antibody-Based Tests

Serology

Serology in adults using ELISA was considered as a diagnostic test with limited specificity. The main reason was because all of the results were aggregated in the systematic reviews, independently of the kit used. Indeed, there is a great heterogeneity between kits available as pointed out by Leal and coworkers (Leal et al. 2008); some have poor performances, but a few have excellent sensitivity and specificity (Burucoa et al. 2013).

When serology is used in young children, it is also supposed to lack sensitivity (Leal et al. 2008). Indeed, the serological response is mounted progressively, and the amount of specific IgG may be below the usual threshold of detection. It may be worth having special cutoffs for young children. Furthermore, at this age given that we may be faced with a primo-infection, it is important to look for specific IgM. In contrast to ELISA, Western blots showed high overall performances and heterogeneity in a meta-analysis of ten pediatric studies (Leal et al. 2008).

The measurement of specific IgG in urine and saliva has been carried out. Unfortunately the lack of sensitivity does not allow to recommend them (Leal et al. 2008; Okuda et al. 2013).

19.5.2.3 Stool Antigen Test

The stool antigen test appears to be a test well suited to children, especially infants and young children. Current kits using monoclonal antigens have shown good sensitivity and specificity, while tests based on polyclonal antibodies were inferior (Leal et al. 2011a; Zhou et al. 2014). Indeed, sensitivity could theoretically be altered in the case of diarrhea, leading to lower concentration of antigens in stools than usual and specificity from the eventual presence of helicobacters other than *H. pylori*. However, this does not appear to be common.

In contrast, the rapid immunochromatographic tests currently on the market show a lower accuracy, and there are problems with interpretation (Prell et al. 2009).

19.5.2.4 PCR from Stool Samples

The real-time PCR developed for detecting *H. pylori* in gastric biopsies and clarithromycin resistance can also be applied to stool specimens. The limitation is the difficulty in obtaining DNA without Taq polymerase inhibitors which correspond to polysaccharides from vegetable origin and follow DNA during the extraction (Monteiro et al. 2001). So while this method has an excellent specificity, the sensitivity is unsatisfactory (Lottspeich et al. 2007; Vecsei et al. 2010).

A multicenter study was carried out in Europe to compare the accuracy of noninvasive tests using a combination of invasive tests as the reference. Concerning the 316 children recruited (133 *H. pylori* positive including children below 6 years), the best results were obtained for UBT (96.8 % accuracy) followed by serology (91.5 %) and stool antigen test (87 %). The performance of antibody detection in urine was not good (Megraud 2005). In contrast, a literature review concluded that the best noninvasive methods in children were immunoblot and stool antigen test (Guarner et al. 2010).

The current recommendation is not to test and treat using a noninvasive test for the diagnosis, because in children, gut symptoms are very unspecific, and causes other than *H. pylori* infection are more likely than *H. pylori* infection itself. In cases with significant complaints and suggestion of organic disease such as GERD, it is important to perform an upper digestive endoscopy to carry out a global exploration (Koletzko et al. 2011). For *H. pylori* diagnosis, it is recommended to perform a histological examination and another test. Because of the high antibiotic resistance of strains infecting children and the lack of treatment options overcoming this problem such as the bismuth-based regimen, it is also highly recommended to perform a culture and susceptibility testing or at least a molecular-based test to determine resistance to clarithromycin in order to tailor the therapy accordingly.

19.6 Treatment

To treat *H. pylori* infection, the regimens have followed those successively proposed for adults with some particularities. The PPI-based triple therapies have been applied, especially omeprazole-amoxicillin-nitroimidazole for a week.

With the PPI-clarithromycin-amoxicillin combination for a week, the added value of PPI was essential. The results per protocol (PP) were 80 % with PPI but only 10 % without PPI (Gottrand et al. 2001).

Bismuth-based triple therapies have also been used in children. They included essentially amoxicillin-nitroimidazole as antibiotics. In a recent review, the PP result was 86 % (Pacifico et al. 2012). The addition of a PPI did not improve the results. The pediatric European registry for treatment of *H. pylori* (PERTH) showed a better efficacy of bismuth-based triple therapies (77 % eradication) compared to PPI-based triple therapies (64 % eradication) (Oderda et al. 2007).

These last years, 10-day sequential therapy emerged as an alternative to previous regimens, also in children (Francavilla et al. 2005). In a meta-analysis, Horvath et al. found a higher eradication rate when compared to standard clarithromycin-based triple therapy (78 % vs. 71 %) (Horvath et al. 2012). All of these studies were faced with the problems of diversity in the posology and length of treatment, a small number of children in each arm, and no clear randomization procedure, and therefore, they are subject to criticism. Furthermore, data on susceptibility testing are missing in most studies, even though this is by far the main risk factor for treatment failure. With the current knowledge, a 10-day sequential therapy cannot be recommended as first-line treatment without prior antibiotic susceptibility testing.

A large prospective multicenter study, carried out in Europe from 1999 to 2002, pointed out an overall resistance rate to clarithromycin of 20 %, higher in boys, in children less than 6 years old, and in patients from Southern Europe. The resistance rate to metronidazole, which has less impact on the treatment outcome, was indeed 23 % (Koletzko et al. 2006). Ten years later, a survey was conducted on adults but also included children. The clarithromycin resistance rate was 31.8 %, and for metronidazole it was 25.7 % (Megraud et al. 2013). A similar trend was also found in other countries like South Korea where over a 20-year period, clarithromycin resistance increased from 6.9 to 18.2 % and metronidazole resistance decreased from 32.8 to 27.3 % (Seo et al. 2013).

The other aspect to consider is safety, and all current regimens lead to adverse events, e.g., diarrhea, bad taste, etc. Fortunately, it is seldom that the patients must stop the treatment. However, these factors may contribute to poor compliance with drug intake, which is after antibiotic resistance, the major risk factor for low eradication rates of *H. pylori*.

Another potential negative effect not explored is the impact on the intestinal microbiome. The intestinal microbiome is very important for various diseases, and the resilience capacity most likely varies between individuals and according to the different antibiotics used.

Current guidelines (Koletzko et al. 2011) recommend as first-line therapy either (1) the standard triple therapy (PPI-amoxicillin-clarithromycin or nitroimidazole) tailored to results from prior antibiotic susceptibility testing or (2) the bismuth-based triple therapy with amoxicillin. Given the importance of antibiotic resistance as a first factor for failure and the fact that bismuth-based drugs are not licensed for use in pediatric patients in many countries, the logical consequence is to prescribe a tailored treatment based on antimicrobial susceptibility testing. In children with a fully susceptible strain or resistance to metronidazole, a 10- or 14-day triple therapy using PPI, amoxicillin, and clarithromycin is recommended. In case of clarithromycin resistance, metronidazole should be used instead. Dosage should be calculated on body weight with PPI 1–1,5 mg/kg (max. 60 mg), amoxicillin 60–70 mg/kg (max. 3 g), clarithromycin 20–25 mg/kg (max. 1 g), and metronidazole 20–25 mg/kg (max 1 g) per day divided in two doses. In case of double resistance against both antibiotics, a 14-day high-dose regimen with PPI, amoxicillin, and metronidazole was successful in 73 % of those who complied to the regimen (Schwarzer et al. 2011).

19.7 Conclusion

H. pylori infection in children remains a challenge in many aspects. The long-term impact of early infection on the immune response with possible reduction of immune-mediated disorders is still unclear. This needs to be balanced against an increased risk for later development of PUD and gastric cancer. Animal experiments indicate that not only the infection itself but also the time point of infection, infancy versus childhood, may play an important role for long-term health. The chronic infection itself is rarely cause of symptoms; the risk for PUD is low before puberty and for *H. pylori*-related gastric malignancy nonexistent. In contrast, treatment options are less compared to adults, and the same treatment regimens seem less effective during childhood. For these reasons, testing for *H. pylori* in children should be restricted to those who will benefit from eradication therapy and should include biopsies for antibiotic susceptibility to guide treatment.

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Part IV
Treatment of *Helicobacter pylori*

Chapter 20
Helicobacter pylori Therapy

Javier Molina-Infante and David Y. Graham

Abstract Eradication of *Helicobacter pylori* infection provides symptomatic benefit for many non-ulcer dyspepsia patients, heals and prevents recurrence of peptic ulcer disease, reduces the risk of development of gastric cancer, and can be a curative therapy for gastric mucosa-associated lymphoid tissue lymphoma. An effective therapy is defined as one achieving at least a 90 % eradication rate with the first attempt. Cure rates of *H. pylori* with triple therapy have declined to unacceptable levels worldwide, mostly due to increasing clarithromycin resistance rates. As such, first-line treatment should be chosen upon local prevalence of *H. pylori* antimicrobial resistance or, if unavailable, empirically based on a combination of using only regimens that have proven to be reliably excellent locally and knowledge of prior use of antibiotics by the patient. The preferred empirical choices are currently 14-day concomitant therapy and 14-day bismuth quadruple therapy, using high-dose proton pump inhibitor therapy. Rescue therapy after failure eradication regimens should be tailored based on antimicrobial susceptibility testing and, if not available, chosen upon which treatments were used initially (e.g., bismuth or non-bismuth quadruple therapy) and the prevalence of fluoroquinolone resistance locally. Where available, furazolidone- and rifabutin-containing therapies are likely to be successful as rescue therapy.

Keywords *Helicobacter pylori* • Eradication • Drug resistance • Triple • Non-bismuth quadruple • Concomitant • Sequential • Bismuth quadruple • Rescue therapy

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20.1 Introduction

Helicobacter pylori (*H. pylori*) is a worldwide infection that affects millions of people. This microorganism, discovered ~30 years ago, is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer. Over the last 20 years, the most recommended treatment for eradication of *H. pylori* by all international guidelines has been the so-called standard triple therapy, consisting of a proton pump inhibitor (PPI) plus the combination of two antibiotics (clarithromycin plus amoxicillin or metronidazole) (Chey and Wong 2007; Fock et al. 2009; Malfertheiner et al. 2012). However, the effectiveness of triple therapy dramatically declined a decade ago to unacceptably low levels, largely related to the development of resistance to clarithromycin (Graham and Fischbach 2010). The general lack of antibiotic susceptibility testing has limited the adoption of susceptibility test-guided therapy, and, therefore, the scientific community has just recently taken on the task of exploring novel empiric therapeutic schemes to overcome antibiotic resistance. However, an ideal universal regimen to treatment has not been identified yet, largely because of large geographical variations in antibiotic resistance. Effectiveness of treatment is determined by the details of the regimen (the drugs used, their doses, formulations, the frequency of administration, the duration of therapy, etc.), host-related parameters, such as compliance with therapy and genetic differences in metabolism of the drugs, especially the PPI used, and the local pattern of antimicrobial resistance. Some general rules have been established, the most important of which is that one can expect similar, if not identical, results of a regimen anywhere if the pattern of resistance is the same (Graham et al. 2014).

20.2 Definition of a Successful Eradication Regimen

For an infectious disease where one can expect to cure essentially 100 % of cases with susceptible organisms (such as *H. pylori*), an optimal regimen is defined as a regimen that reliably cures at least 90–95 % of infections with susceptible strains (Graham et al. 2014). As with most bacterial infectious diseases, an appropriate therapy should be devised based on antimicrobial susceptibility testing. However, susceptibility testing is seldom available for *H. pylori* therapy and therapies are mostly prescribed empirically. Regardless of the lack of antimicrobial data, the therapeutic goal should be the same, so eradication therapies can be currently classified according to their cure rates on a per-protocol analysis: excellent (>95 % success), good (>90 % success), borderline acceptable (85–89 % success), or unacceptable (<85 % success) (Graham et al. 2014) Using less stringent therapeutic thresholds will only lead to implementation of suboptimal therapies in clinical practice and subsequent selection of *H. pylori* resistant strains (Graham 2009, 2010). In addition, rescue drugs after *H. pylori* therapy failure might be unavailable (bismuth, tetracycline, furazolidone) or may potentially lead to serious

side effects (rifabutin), so the clinician should be sure of using the most effective first "hit" to eradicate the bacteria (Graham et al. 2014).

20.3 Difficulties to Eradicate *H. pylori* Infection

Unlike the bulk of bacterial infections, *H. pylori* resides in the stomach, which is an acid antinatural environment for microorganisms. Acute *H. pylori* infection may result in hypochlorhydria, which is thought to facilitate survival of the organism and colonization of the stomach (Schubert and Peura 2008). Moreover, several bacteria-, environmental-, and drug-related factors may also account for difficulties associated with the cure of the infection. Upon this unique picture, *H. pylori* infection should be distinctly treated by means of a combination of acid-suppressive agents and several antibiotics. By far, the most important factor impairing the efficacy of eradication regimens is the presence or development of *H. pylori* genetic mutations conferring antimicrobial resistance (Graham et al. 2014).

20.3.1 Bacteria-Related Factors

20.3.1.1 *H. pylori* Genotypical Resistance to Antimicrobial Drugs

Resistance to bismuth is not thought to occur in *H. pylori*, and acquired resistance to either amoxicillin or tetracycline (after previous exposure to these antibiotics) is rare in most regions. Accordingly, they can be reused despite failure of an eradication regimen where they were prescribed, as we do with amoxicillin and bismuth in clinical practice (Graham and Fischbach 2010). Clarithromycin is a typical macrolide, and one can expect cross resistance to occur such that prior or frequent use of other macrolides, such as erythromycin or azithromycin, which results in a high prevalence of clarithromycin resistance (Megraud et al. 2013). Resistance to clarithromycin has increased in most regions over time and empiric triple therapy is strongly discouraged when clarithromycin resistance rate is prevalent (Chey and Wong 2007; Fock et al. 2009; Malfertheiner et al. 2012). Treatment success falls below 90 % at 7 days with about 5 % clarithromycin resistance and with about 8 % with 14-day therapy. At 15–20 % resistance population, results of 70–75 % are expected. The highest frequency of clarithromycin resistance in adults (40 %) has been reported in southern and central European countries (Megraud et al. 2013), although it is also fairly common in other settings such as the USA, Mexico, Japan, Russia, Turkey, and Iran, usually over 15 % (Graham et al. 2014). In general, one should consider that a high rate of clarithromycin resistance is present, unless proven otherwise. Metronidazole resistance remains around 20–40 % in Western countries but is often as high as 60–80 % in countries such as China, India, Iran, and

Central and South America (Graham and Fischbach 2010). Resistance to fluoroquinolones (e.g., levofloxacin, moxifloxacin, sitafloxacin) is rapidly increasing worldwide (e.g., Europe 14 % in 2013) (Megraud et al. 2013). Of note, resistance to clarithromycin, fluoroquinolones, or rifabutin cannot be overcome by increasing the dose or duration (Graham et al. 2014). As such, previous exposure to macrolides or fluoroquinolones (due to previous eradication therapies or ear-nose-throat, respiratory, or urinary tract infections) in patients carrying *H. pylori* infection increases the likelihood of harboring antibiotic-resistant strains, and alternative antibiotic schemes should be sought. On the contrary, metronidazole resistance can be at least partially overcome by means of higher doses and longer duration of therapies, especially when used as a part of a bismuth-containing quadruple therapy (Graham et al. 2014).

20.3.1.2 *H. pylori* Phenotypic Resistance to Antimicrobial Drugs

Eradication treatment may fail even when the organism genetically remains susceptible to the antibiotic (Graham and Fischbach 2010). This is most commonly seen with amoxicillin. This form of reversible resistance is termed *phenotypic antibiotic resistance*, and it is thought to be due to the presence of nonreplicating population of organisms. Bacteria usually oscillate between a nonreplicating (phenotypically resistant) and replicating state (phenotypically susceptible), during which they cannot and can be eradicated, respectively· (Graham and Fischbach 2010). As such, short doses, short duration, or extended dosing interval of antibiotic drugs may limit the presence of antibiotics during these susceptibility periods.

20.3.1.3 High Bacterial Load (Inoculum Effect)

The total number of *H. pylori* in the stomach is very high, resulting in an inoculum effect, which stands for the decreasing efficacy of an antibiotic with increasing bacterial density (Graham and Fischbach 2010). For example, if the spontaneous mutation rate for a particular resistance was 1 in 10 million and there were 50 million organisms present, it would be statistically likely that a small population of resistant organisms would always be present. In fact, resistance usually develops because of the outgrowth of a small existing population of resistant organisms (Graham and Fischbach 2010). Strategies aiming to overcome *H. pylori* high bacterial load include increasing the dose and duration of antibiotic therapy, combining several antibiotics (one of which will probably kill the resistant organisms) and pretreatment with PPI and bismuth, reducing the bacterial load (which would make survival of the minor populations less likely).

20.3.2 Environmental-Related Factors

The natural pH in the acid gastric environment remains between 1 and 2, especially during fasting and starting meal ingestion (Schubert and Peura 2008). *H. pylori* becomes phenotypically resistant with a pH range between 3 and 6, and this is thought to be the main reason why acid-suppressive agents, along with antibiotics, are often indispensable in *H. pylori* therapy. Increasing gastric pH to 6 or 7, by means of PPI therapy, allows the bacteria to enter the replicative state, where they become susceptible to specific antibiotics such as amoxicillin and clarithromycin (Graham and Fischbach 2010). Therefore, insufficient acid suppression, due to either low doses or rapid/extensive PPI metabolism, may predispose microorganisms to a phenotypically resistant state, and eradication regimens may fail in spite of genetic susceptibility to prescribed antibiotics.

20.3.3 Drug-Related Factors

Amoxicillin and clarithromycin are antibiotics that require microbial replication to kill the organisms. As such, powerful acid suppression (high-dose PPI) and adequate antibiotic doses, dosing intervals, and durations are key to avoid *H. pylori* entering in a non-replicative state, where antibiotics are less effective (Graham and Fischbach 2010). Clarithromycin must bind to ribosomes in order to kill *H. pylori*. Acquired resistance is associated with failure to bind to ribosomes, such that resistance cannot be overcome by increasing the dose or duration. Likewise, resistance to fluoroquinolones (i.e., levofloxacin, moxifloxacin) is not responsive to changes in dose or duration. Metronidazole resistance can be partially overcome by increasing the dose and duration, especially with bismuth-containing therapies (Graham et al. 2014). In contrast, bismuth resistance does not occur, and resistance to either amoxicillin or tetracycline is rare in most regions.

20.4 Pivotal Considerations for an Optimal Therapeutic Decision-Making

20.4.1 Choice of Antibiotic Therapy

The strongest predictor of *H. pylori* treatment failure using a regimen proven to be effective elsewhere is antimicrobial resistance. From a microbiological standpoint, treatment results are best when regimens are used to treat patients with organisms susceptible to the antimicrobials chosen, but this approach is currently limited by the unavailability of *H. pylori* culture (invasive procedure) or of molecular testing

in stools or gastric biopsies (expensive, time consuming, and limited largely to clarithromycin) in most of the cases (Graham et al. 2014; Molina-Infante and Gisbert 2014). Another choice would be using bismuth quadruple therapy; owing to tetracycline resistance is negligible and metronidazole resistance can be partially overcome by increasing doses and duration. Nonetheless, this approach is limited to regions where bismuth salts and tetracycline are both available (Megraud 2012; Graham et al. 2014). The launch of Pylera®, the three-in-one capsule containing bismuth, tetracycline, and metronidazole, which decreases the pill burden and theoretically might improve compliance, is one option where it is available (see below under specific therapies). Therefore, one often must choose antibiotic therapy, empirically making the best approach being to use regimens that have proven to be reliably excellent locally. That choice should take advantage of knowledge of local resistance patterns, clinical experience, and especially patient history. The history of the patient's prior antibiotic use and any prior therapies will help identify which antibiotics are likely to be successful and those where resistance is probable (Graham et al. 2014). All of these crucial variables in the decision-making process for an optimal first-line eradication therapy are summarized in Fig. 20.1.

20.4.2 Optimization of Therapies

In this era of antibiotic resistance, all therapies should be optimized (Graham et al. 2014; Molina-Infante and Gisbert 2014). We recommend high-dose PPI (i.e., 40 mg of omeprazole or equivalent b.i.d. (*bis in die* or twice a day)) and a 14-day duration in order to ensure the greatest effectiveness. A 14-day regimen has proven to achieve higher eradication rates for triple, bismuth quadruple, and non-bismuth quadruple sequential and concomitant therapy (Salazar et al. 2012; Yuan et al. 2013; Liou et al. 2013; Graham et al. 2014; Molina-Infante and Gisbert 2014). PPI therapy should be given at doses guaranteeing effective and prolonged gastric acid suppression, since *H. pylori* becomes phenotypically resistant and less susceptible to amoxicillin and clarithromycin when the pH in their microenvironment is lower than 6 and higher than 3 (Graham and Fischbach 2010). All PPIs are metabolized by cytochrome P450 (*CYP*) *2C19*. Four different genotypes have been described: ultrarapid metabolizer, rapid metabolizer, intermediate metabolizer, and poor metabolizer. Plasma PPI levels and intragastric pHs during PPI treatment are inversely related to *CYP2C19* genotype, so they are lowest in the more extensive or rapid metabolizer group and highest among the poor metabolizers (Graham and Fischbach 2010). Subsequently, several meta-analyses have shown eradication rates that are inversely related to the ability to metabolize the PPIs (e.g., the ultrarapid and rapid metabolizer groups have lower eradication rate compared to other groups) (Zhao et al. 2008; Tang et al. 2013). The prevalence of *CYP2C19* rapid metabolizers has been shown to be highest in Europe and North America (56–81 %), while the proportion is lower (27–38 %) in the Asian population. As

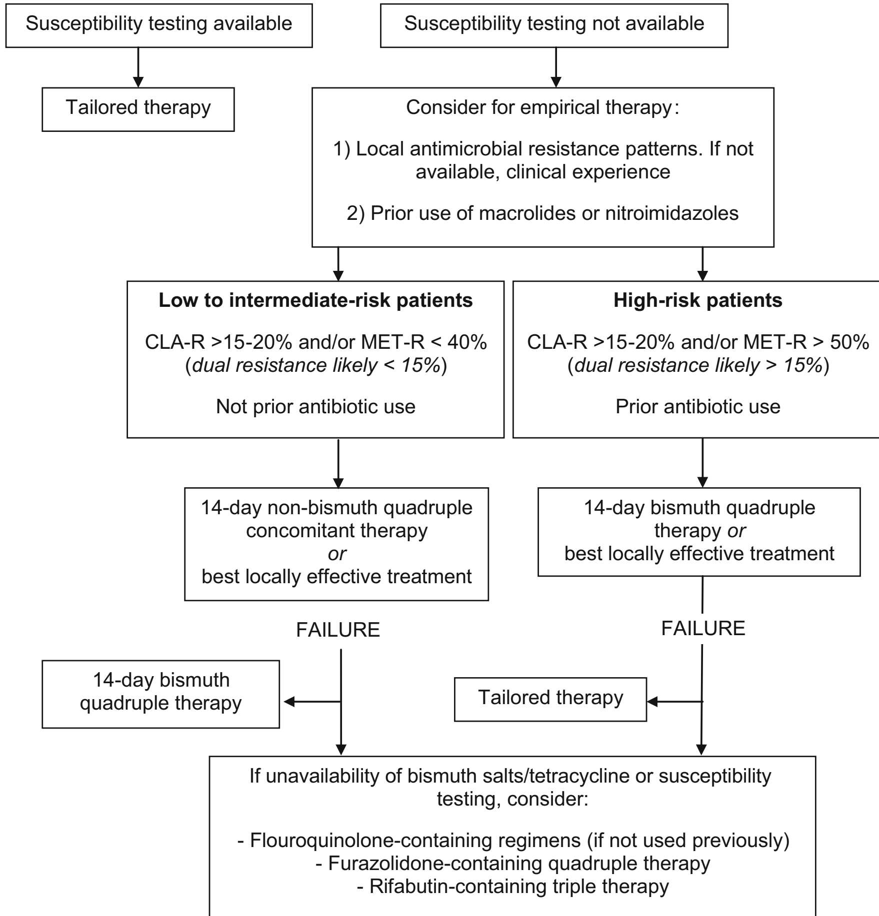

Fig. 20.1 Recommended approach for *Helicobacter pylori* therapy

such, it is conceivable that all patients, especially in Europe and North America, should routinely receive higher-dose PPI twice a day (dosing interval) therapy in order to achieve similar effects in either rapid and poor metabolizers (Graham et al. 2014; Molina-Infante and Gisbert 2014). In this regard, two other recent meta-analyses have demonstrated that, at standard doses, esomeprazole and rabeprazole provide better overall *H. pylori* eradication rates, especially in *CYP2C19* rapid metabolizers (McNicholl et al. 2012; Tang et al. 2013). Available first-line regimens with preferred drug doses and dosing intervals are summarized in Table 20.1.

Table 20.1 Current first-line therapeutic recommendations in the era of increasing clarithromycin and metronidazole resistance

	Preferred doses and dosing intervals	Caveat
14-day bismuth-containing classical quadruple therapy	Bismuth salts q.i.d.	Availability
	PPI (double doses) b.i.d.	Complexity
	Tetracycline 500 mg q.i.d.	Side effects
	Nitroimidazole 500 mg t.i.d.	Compliance
14-day bismuth-containing quadruple therapy using Pylera®	PPI (double doses) bid	Availability
	Pylera 3 pills q.i.d.	Cost
		Relatively low tetracycline doses
14-day non-bismuth quadruple concomitant therapy	PPI (double doses) b.i.d.	Cure rates ≤90 %
	Amoxicillin 1 g b.i.d.	if dual resistance rate ≥15 %
	Clarithromycin 500 mg b.i.d.	
	Nitroimidazole 500 mg b.i.d.	
14-day non-bismuth quadruple hybrid therapy	*7 days*	Cure rates <90 %
	PPI (double doses) b.i.d.	if dual resistance rate >9 %
	Amoxicillin 1 g b.i.d.	
	7 days	
	PPI (double doses) b.i.d.	
	Amoxicillin 1 g b.i.d.	
	Clarithromycin 500 mg b.i.d.	
	Nitroimidazole 500 mg b.i.d.	
14-day non-bismuth quadruple sequential therapy	*7 days*	Cure rates <90 %
	PPI (double doses) b.i.d.	if dual resistance rate >5 %
	Amoxicillin 1 g b.i.d.	Not recommended as an empirical therapy
	7 days	
	PPI (double doses) b.i.d.	
	Clarithromycin 500 mg b.i.d.	
	Nitroimidazole 500 mg b.i.d.	
14-day triple therapy	PPI (double doses) b.i.d.	Cure rates <90 % if clarithromycin resistance >15 %
	Amoxicillin 1 g b.i.d.	
	Clarithromycin 500 mg b.i.d.	Not recommended as an empirical therapy

Dual resistance rate: H. pylori microorganism resistant to both clarithromycin and metronidazole

20.5 First-Line Therapy

The preferred empirical choices should be currently 14-day bismuth quadruple therapy or 14-day concomitant therapy, depending on local conditions and patient history of antibiotic use and possible drug allergies (Fig. 20.1) (Graham et al. 2014). Sequential therapy is no longer recommended as it contains the same drugs as concomitant therapy and is more complicated, and concomitant therapy will always

be equal or superior to it. Of note, none of the non-bismuth quadruple regimens is infallible, and sequential, hybrid, and concomitant therapy have all been shown to fail (<90 % eradication) if the rate of dual clarithromycin- and metronidazole-resistant strains is >5 %, >9 %, or >15 %, respectively (Graham et al. 2014). As such, empirical non-bismuth quadruple therapies would likely be poor choices in settings with documented high clarithromycin and metronidazole resistance or with documented high resistance (e.g., <50 % to clarithromycin or metronidazole) in some specific high-risk patients (e.g., women in whom metronidazole has been used for *Trichomonas* infections, immigrants from developing countries, patients who previously failed sequential or PPI-clarithromycin-metronidazole triple therapy, and those in whom the risk of acquired metronidazole resistance is high) (Graham et al. 2014).

20.5.1 Triple Therapy (Currently Considered as Obsolete as an Empiric Therapy)

Despite many still current recommendations, triple therapy should be no longer prescribed on an empirical basis. Its empiric use should be strictly restricted to a minority of settings where clarithromycin resistance is established by culture known to be low (e.g., Northern Europe or Thailand (Megraud et al. 2013; Vilaichone et al. 2013) or where cure rates >90 % have been documented in clinical practice (Prasertpetmanee et al. 2013)). When used, it should be given for 14 days with double-dose PPI twice a day, since this can increase eradication rates by 10 % (Malfertheiner et al. 2012).

20.5.2 Non-bismuth Quadruple Therapies

20.5.2.1 Sequential Therapy (Currently Considered Obsolete as an Empiric Therapy)

Sequential therapy was developed in Italy in 2000 as a replacement for triple therapy. It initially consisted of 5 days of PPI therapy plus amoxicillin, followed by a further 5 days of PPI with two other antibiotics, usually clarithromycin and a nitroimidazole (Zullo et al. 2000; De Francesco et al. 2001). Meta-analyses conducted between 2007 and 2009, pooling mostly Italian evidence, confirmed the advantage of 10-day sequential (cure rates >90 %) over 7- or 10-day triple therapy (Zullo et al. 2007; Jafri et al. 2008; Tong et al. 2009; Gatta et al. 2009). In 2012 and 2013, two updated meta-analyses (Horvath et al. 2012; Yoon et al. 2013) and two systematic reviews (Zullo et al. 2013a; Kate et al. 2013), including studies on sequential therapy from Asia, Europe, and Latin America, showed that the mean eradication rates were dramatically lower (79–84 %) than those reported in early

Italian trials. These poor results were confirmed in a global meta-analyses in 2013 with overall cure rates of 84 % (95 % CI 82.1–86.4 %) (Gatta et al. 2013). Further analysis showed that the Achilles heels for sequential therapy was metronidazole resistance and dual clarithromycin resistance (Liou et al. 2013; Graham et al. 2014). Because concomitant therapy is effective in isolated metronidazole or clarithromycin resistance, it will thus always be equal or superior to sequential therapy in regions where sequential therapy is effective. We conclude that sequential therapy is an obsolete regimen.

20.5.2.2 Concomitant Therapy

The concept of a “non-bismuth quadruple regimen” or “concomitant” regimen consists of converting standard triple therapy to a quadruple therapy by the addition of 500 mg of metronidazole or tinidazole twice daily. This therapeutic regimen resurfaced in 2010 as an alternative therapy to triple and sequential therapy. Meta-analyses have consistently shown its advantage over triple therapy (Essa et al. 2009; Gisbert and Calvet 2012b). Indeed, several recent studies (2010–2013) have evaluated its effectiveness in Latin America, Asia (Thailand, Japan, Taiwan, China, and Korea), and Europe (Spain, Greece, Italy, and Turkey). Regardless of the duration of the therapy, all studies showed intention-to-treat cure rates from 85 to 94 %, with the exceptions of four studies conducted in regions where dual resistance was high (Latin America, Korea, and Turkey) or where the duration of therapy (Latin America, Italy) tested was too short (Molina-Infante and Gisbert 2014).

The efficacy of 14-day concomitant therapy is not impaired by neither clarithromycin- nor metronidazole-isolated resistance, but it is expected to fall below 90 % in regions where the prevalence of dual clarithromycin and metronidazole-resistant strains is >15 % (Graham et al. 2014). Currently, 14-day concomitant therapy should be the preferred non-bismuth quadruple therapy as it has shown to be the most effective to overcome antibiotic resistance. When dealing with dual clarithromycin- and metronidazole-resistant strains, concomitant therapy has shown a remarkable advantage over sequential therapy (Georgopoulos 2013b; Molina-Infante and Gisbert 2014). However, it is important to stress that it should not be recommended in settings with high rates of metronidazole resistance (>50–60 %) along with high clarithromycin resistance (i.e., Latin America, Turkey, and Korea) or in populations at high risk of dual resistance (i.e., following clarithromycin or metronidazole treatment failures) (Graham et al. 2014; Molina-Infante and Gisbert 2014). These recommendations are in agreement with acceptable to excellent results (86–95 % eradication rates) in Greece, Spain, and Italy in Southern Europe (Georgopoulos et al. 2012, 2013a; Molina-Infante et al. 2012; Zullo et al. 2013b; Molina-Infante et al. 2013; McNicholl et al. 2014; De Francesco et al. 2014) and Thailand, Taiwan, China, and Japan in Asia (Wu et al. 2010; Kongchayanun et al. 2012; Huang et al. 2012; Yanai et al. 2012; Hsu et al. 2014), where clarithromycin ranges from low (9 %) to high (40 %), but metronidazole

resistance remains relatively low (<30–40 %). As a matter of fact, if metronidazole resistance remains stable in Southern Europe, clarithromycin resistance would need to exceed 50 % to undermine 14-day concomitant therapy. These successful eradication results for concomitant therapy have not been replicated in settings with high rates of dual clarithromycin- and metronidazole-resistant *H. pylori* strains, such as Turkey (Toros et al. 2011; Sharara et al. 2014), Korea (Lim et al. 2013; Heo et al. 2014) and possibly Latin America (Greenberg et al. 2011), where the duration was also too short.

20.5.2.3 Hybrid (Sequential-Concomitant) Therapy

Hybrid sequential-concomitant regimen is a therapeutic innovation which includes a PPI plus amoxicillin for 14 days, adding clarithromycin and a nitroimidazole for the final 7 days (Hsu et al. 2011). In other words, it is a 7-day first dual phase (PPI + amoxicillin), followed by a 7-day quadruple phase (PPI + amoxicillin + clarithromycin + nitroimidazole). Several recent studies conducted between 2011 and 2014 have consistently shown cure rates ≥86 % in Taiwan (Hsu et al. 2011; Wu et al. 2014), Iran (Sardarian et al. 2013), Spain, and Italy (Molina-Infante et al. 2013; Zullo et al. 2013b), but less satisfactory results have been reported in other studies conducted in Italy (De Francesco et al. 2014) and Korea (Oh et al. 2014). Hybrid therapy has been shown to be non-inferior to concomitant therapy, besides improved safety, convenience, or better compliance. It could be considered in the same populations where concomitant therapy is recommended; however, 14-day hybrid therapy is expected to fall below 90 % when clarithromycin-metronidazole resistance exceeds 9 % (Graham et al. 2014). A recent first meta-analysis on hybrid therapy has not shown relevant differences in terms of efficacy with the sequential and concomitant therapy (Wang et al. 2014). Therefore, further studies are required to validate this therapy in settings with different patterns of resistance.

20.5.3 Bismuth-Quadruple Therapy

This is the oldest effective therapy and a resurfacing one on account of increasing failure of clarithromycin-containing therapies. Using this regimen at full doses and for 14 days, one can expect 95 % or greater treatment success irrespective the level of metronidazole resistance (Graham et al. 2014; Salazar et al. 2012). The main disadvantages of this therapy are its complexity and frequent side effects, both of which may hamper compliance with therapy. In addition, there are often issues with the availability of bismuth salts or tetracycline. Generally, doxycycline has proven not to be an adequate substitute for tetracycline (Graham et al. 2014). This is likely the main reason why we lack more evidence on this therapy over the last decade. Because of the relative high rate of side effects, optimization is needed in terms of

formulations, forms of bismuth, doses, and dosing intervals. Recent studies conducted in Italy (Dore et al. 2011) and China (Liang et al. 2013) have shown similar success rates using twice a day bismuth and full q.i.d. (*quarter in die* or four times a day) antibiotic doses. In this context, bismuth-based quadruple therapy in its most recent galenic formulation, bismuth subcitrate potassium, metronidazole, and tetracycline (BMT, sold under license as Pylera®) has been suggested as a possible first-line therapeutic option (Malfertheiner et al. 2011). However, its use is currently limited by high cost and the fact that only a 10-day regimen is available in a prepackaged form. The dose of tetracycline is 1500 mg, which is lower than usually recommended. Head to head comparisons with standard bismuth therapy and with b.i.d. dosing are needed, especially in population with high rates of metronidazole resistance.

20.6 Rescue Therapy

Rescue therapy is defined as therapy after two treatment failures with two different regimens. Even with the current most effective treatment regimens, a variable proportion of patients will fail to eradicate *H. pylori* infection at the first attempt (Marin et al. 2013; Graham et al. 2014). Most will be cured by using the alternate regimen among the two different preferred quadruple regimens (Marin et al. 2013; Graham et al. 2014). Despite the number of studies, the optimal retreatment regimen has not yet been defined. If susceptibility testing can be obtained, one can almost always identify a regimen that will prove effective. Our therapeutic target, similarly to first-line regimens, should be at least 90 % cure rates. The empiric choice of a rescue treatment primarily depends on which treatment was used initially (e.g., bismuth or non-bismuth quadruple therapy) and the local rate of fluoroquinolone resistance. The available regimens, with preferred doses and interval dosing, are summarized in Table 20.2.

After failure of a clarithromycin- or metronidazole-containing treatment, clinicians should assume that *H. pylori* is likely resistant to the antibiotic previously used, so it is not appropriate to repeat the same antibiotics. An exception to this rule is amoxicillin, which rarely induces acquired resistance. In the absence of pretreatment antibiotic resistance testing, the most commonly used empirical strategies for a second-line therapy are bismuth quadruple therapy and, where not available, a fluoroquinolone-containing therapy. This latter should only be considered if no fluoroquinolone, including ciprofloxacin, was used before (Gisbert and Morena 2006; Marin et al. 2013; Graham et al. 2014). Furazolidone quadruple therapy (where available) and rifabutin triple therapy are typically reserved as salvage therapies of last resort. A recommended therapeutic algorithm for *H. pylori* rescue therapy, after failure, is displayed in Fig. 20.1.

Table 20.2 Current rescue therapeutic recommendations, in the era of increasing fluoroquinolone resistance, in patients with high risk of acquired clarithromycin and/or metronidazole resistance after failure of first eradication regimen

	Preferred doses and dosing intervals	Caveat
14-day fluoroquinolone triple therapy	PPI (double doses) b.i.d.	Cure rates ≤90 %
	Amoxicillin 1000 mg b.i.d.	if levofloxacin resistance ≥12 %
	Levofloxacin 500 mg	
14-day bismuth-containing fluoroquinolone quadruple therapy	Bismuth salts 240 mg b.i.d.	Cure rates ≤90 %
	PPI (double doses) b.i.d.	if levofloxacin resistance ≥25 %
	Amoxicillin 1000 mg b.i.d.	
	Levofloxacin 250 mg b.i.d.	
14-day bismuth quadruple concomitant therapy	PPI (double doses) b.i.d.	Availability
	Bismuth salts 240 mg b.i.d.	Side effects
	Plus a combination of 2 antibiotics among	Complexity
	Amoxicillin 1 g t.i.d./b.i.d.	Compliance
	Nitroimidazole 500 mg t.i.d.	Potential genotoxic and carcinogenetic effects of furazolidone
	Tetracycline 500 mg q.i.d.	
	Furazolidone 100 mg t.i.d.	
14-day rifabutin-containing therapy	PPI (double doses) b.i.d.	Cost
	Amoxicillin 1000 mg t.i.d.	Potential severe side effects (*myelotoxicity and hepatotoxicity*)
	Rifabutin 300 mg	Risk of development of resistance in *M. Tuberculosis*

20.6.1 Fluoroquinolone-Containing Therapies

Levofloxacin is a fluoroquinolone with a broad spectrum of activity against *H. pylori*. Fluoroquinolone-based therapy has been proposed in several international guidelines as a rescue therapy (Chey and Wong 2007; Fock et al. 2009; Malfertheiner et al. 2012). However, prevalence of fluoroquinolone resistance has increased rapidly in recent years, mostly due to the widespread use of levofloxacin for ear, nose and throat, respiratory tract, and urinary infections. Recent studies have reported high levofloxacin resistance rates, ranging from 63 % in China to 14 % in Europe (Graham et al. 2014). In line with these data, a recent review revealed a weighted efficacy of 76 % (Marin et al. 2013), whereas several meta-analyses have shown that 7-day fluoroquinolone triple therapy (including PPI, amoxicillin, and levofloxacin) provides cure rates of typically <80 % and extending the duration to 10 days for improved outcome, but the treatment success typically remained typically below 90 % (Gisbert and Morena 2006; Marin et al. 2013; Gisbert et al. 2013). Treatment success >90 % requires 14-day fluoroquinolone triple therapy. However, treatment success will fall below 90 % with 14-day

fluoroquinolone triple therapy when fluoroquinolone resistance rates exceed approximately 12 % (Chuah et al. 2012) and with 14-day bismuth-containing fluoroquinolone quadruple therapy in areas where fluoroquinolone resistance exceeds 25 % (Liao et al. 2013). As such, awareness of local resistance rates or close monitoring of cure rates are mandatory in order to promptly detect inefficacy of these therapies. The role of newer fluoroquinolones, such as sitafloxacin and gemifloxacin, to overcome fluoroquinolone resistance, needs to be validated in further studies.

20.6.2 Bismuth-Quadruple Therapy, Including Furazolidone-Containing Regimens

Classical bismuth quadruple regimen has been also proposed in several international guidelines as a rescue therapy (Chey and Wong 2007; Fock et al. 2009; Malfertheiner et al. 2012). In a recent review, its weighted efficacy was 77 % (including different durations, drug doses, and interval dosing) (Marin et al. 2013). Concerns with this therapy are complexity, patient adherence, and availability. Meta-analyses comparing fluoroquinolone-based and bismuth quadruple therapies for rescue therapy did not find significant differences between both therapies regarding efficacy, albeit fluoroquinolone therapy was significantly better tolerated and induced significantly fewer side effects. Importantly, the results with both regimens were unacceptably low due to poor choices of doses, duration, and the presence of resistance (Saad et al. 2006; Marin et al. 2013).

Bismuth quadruple therapy, however, is the regimen with the most unanswered questions regarding what are the optimal doses and frequencies of drug administration. A recent study from China has evaluated the efficacy of bismuth quadruple therapies (PPI, bismuth salts, and two antibiotics) with different antibiotic combinations in patients with previous eradication treatment failure (Liang et al. 2013). They used for all four evaluated regimens twice a day bismuth (220 mg b.i.d.) and full doses and adequate dosing intervals for the antibiotics (amoxicillin 1000 t.i.d. (*tres in die* or three times a day), tetracycline 500 mg q.i.d., metronidazole 400 mg q.i.d., and furazolidone 100 mg t.i.d.). Cure rates were excellent (>90 %), regardless of the presence of clarithromycin, levofloxacin, or metronidazole resistance, with the best results for the furazolidone-containing regimens. Of note, these bismuth quadruple regimens were equally effective for patients allergic to penicillin, combining tetracycline with either metronidazole or furazolidone. However, furazolidone is seldom available in developed countries, and although it has been declared a class C carcinogen (meaning there is no evidence that it is a carcinogen), concern about possible genotoxic and carcinogenetic effects is often voiced. This study, however, importantly highlights that bismuth quadruple therapy compliance and efficacy can be improved through optimization of therapy.

20.6.3 Rifabutin-Containing Therapy

Rifabutin is a rifamycin-S derivative, which shares many of the properties of rifampin (rifampicin). Rifabutin is commonly used to treat *Mycobacterium avium* and *Mycobacterium intracellulare*, and it has shown utility against *H. pylori*, seeing as the in vitro sensitivity is high and prevalence rate of rifabutin resistance is very low, only about 1 %. A recent systematic review disclosed that mean *H. pylori* eradication rate with rifabutin-containing rescue regimens was 73 % (Gisbert and Calvet 2012a). Respective cure rates for second-line, third-line, and fourth/fifth-line rifabutin therapies were 79, 66, and 70 %. The most prescribed regimen has been a triple therapy combining PPI, amoxicillin, and rifabutin. The most effective dose is 300 mg/day and the ideal length remains unclear, although 10–14-day regimens are generally recommended. The most successful cure rates ($\geq$90 %) have been reported using high-dose PPI (pantoprazole 80 mg t.i.d.), high-dose amoxicillin (1 or 1.5 g t.i.d.), and rifabutin (150 mg b.i.d.) (Borody et al. 2006). The main disadvantages of this drug are its high cost, uncommon but relevant adverse effects (mainly myelotoxicity and hepatotoxicity), and the potential development of resistance to *M. tuberculosis* in populations with a high prevalence of tuberculosis. Owing to all these reasons, most consider it as one of the therapeutic options of last resort.

20.7 Conclusions and Outlook

H. pylori infection has proven challenging to eradicate due to bacteria-, environmental-, and drug-associated factors. Antibiotic resistance and patient adherence are the critical factors responsible for eradication treatment failure. Due to lack of updated reliable data regarding antimicrobial susceptibility, most eradication therapies are empirically prescribed. An optimal *H. pylori* regimen is defined as one that reliably achieves at least 90–95 % of infections at first attempt. First-line empiric triple therapy for *H. pylori* infection has become ineffective in most settings worldwide because of clarithromycin resistance rates. Therefore, the choice of therapy may depend on the patient's previous antibiotic treatment, local patterns of antibiotic resistance, and drug availability. Optimization of all eradication regimens (including duration, PPI/antibiotic doses, and dosing intervals) is key to maximize efficacy. Currently, the most effective first-line eradication regimens are 14-day bismuth and non-bismuth concomitant quadruple therapies. No trial has compared the efficacy of both regimens yet. Pylera®, the three-in-one capsule containing bismuth, tetracycline, and metronidazole, requires taking 14 capsules daily, whereas the Chinese quadruple therapy with b.i.d. bismuth/PPI and 500 mg capsules of tetracycline and metronidazole requires only 10 daily. Research to improve tolerability and adherence with bismuth quadruple therapy is warranted. The golden rule for choice of treatment is only to use what works locally

(>90–95 % success) and to closely monitor its effectiveness over time. Rescue regimens should also target at least 90 % cure rates. Whenever possible, rescue therapy should be based on antimicrobial susceptibility test data. The empiric choice of rescue therapy primarily depends on which treatment was used initially (e.g., bismuth or non-bismuth quadruple therapy) and the local rate of fluoroquinolone resistance. Furazolidone- and rifabutin-containing regimens might be also effective as rescue treatments.

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Chapter 21
Management of *H. pylori* Infection in Europe

Peter Malfertheiner and Michael Selgrad

Abstract Over the last decades, *Helicobacter pylori* (*H. pylori*) has been recognized as the main risk factor for various gastroduodenal diseases. This knowledge has dramatically changed the clinical management of *H. pylori* infection and related gastroduodenal diseases. Nowadays, *H. pylori*-related diseases, such as peptic ulcer disease and MALT lymphoma, can be cured by *H. pylori* eradication therapy. Furthermore, *H. pylori* eradication has the potential to prevent gastric cancer. In this chapter, we have selected the most significant aspects of *H. pylori* infection. We reviewed indications for *H. pylori* eradication therapy and data about diagnosis and therapy of *H. pylori* infection. New treatment regimens or modifications of established therapies have been developed with the goal to overcome increasing antibiotic resistance rates that can be regarded as the main reason for treatment failure. Finally, more and more evidence has been gathered that prevention of gastric cancer is feasible by eradication of *H. pylori*. This approach is likely to be highly effective in reducing the incidence of gastric cancer.

Keywords *H. pylori* • Diagnostic strategies • Treatment options

21.1 Introduction

Management of *H. pylori* in Europe needs to consider specific regional/national differences concerning prevalence of the infection and related diseases, environmental, lifestyle, and health-care systems factors. The prevalence of *H. pylori* infection varies among European countries and even among different regions in the same country (Bastos et al. 2013; Mayerle et al. 2013; Wex et al. 2011). Over the last decades, there is a significant decrease in prevalence of *H. pylori* infection, and the prevalence is lowest in childhood (Bureš et al. 2012). The prevalence of the infection in the adult population ranges from 20 to 60 % and is age dependent, with the highest prevalence in patients above 50 years.

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In spite of these differences, there are common general aspects relevant for the management of *H. pylori* infection, which have been updated in European consensus reports in 4–5 year intervals over the last 18 years. The first European consensus meeting was held in Maastricht in 1996 (Malfertheiner et al. 1997) and provided a general frame intending to guide and facilitate the management of *H. pylori* infection. The European consensus meetings were since held to four different occasions. Some national societies have adapted and fine-tuned the guidelines to their specific local needs.

The most current European consensus of *H. pylori* management is based on the Maastricht-Florence four consensus report (Malfertheiner et al. 2012), which addresses the issues of:

(a) *Whom to treat*
(b) *How to test*
(c) *How to treat*

The challenges in Europe are similar to those as in other parts of the world:

(a) *Emergent resistance of* H. pylori *to antibiotics used in conventional eradication regimen*
(b) *The gastric cancer burden which is highly variable in different European countries*

21.2 Indications

Indications for treatment of *H. pylori*-related gastroduodenal pathologies have been broadened over the past two decades and are summarized in Table 21.1. The clinical evidence for *H. pylori* as a pathogen has grown immensely over the last 30 years (Malfertheiner et al. 2014) and public awareness has been spread. Therefore among different clinical indications, the wish of patient even in the absence of symptoms or specific risk conditions has been recommended as an indication for testing and treating the infection. The third edition of the European Maastricht-Florence consensus report (Malfertheiner et al. 2007) has been the first to recommend *H. pylori* eradication in some extra-alimentary conditions, which are potentially related to the infection (Malfertheiner et al. 2012) (Table 21.1).

There has been an immense progress in the management of mucosa-associated lymphoid tissue (MALT)-lymphoma with the recommendation that *H. pylori* should always be the first step of treatment, including patients with advanced gastric MALT lymphoma as well. Eradication is worth trying also in *H. pylori*-negative patients with MALT lymphoma (Ruskoné-Fourmestraux et al. 2011).

In young patients with dyspepsia and no alarm symptoms below the age of 45–50 years, a "test and treat" strategy is considered appropriate because of the low prevalence of gastric malignancies in these age groups in most European countries (Wee 2013). However, the adoption of this strategy continues to be debated although clinical trials confirmed the value of this strategy. In countries with easily

Table 21.1 Indications for *H. pylori* treatment

Who should be treated for *H. pylori*-related diseases?
Gastrointestinal diseases
Peptic ulcer (duodenal – gastric), complicated and non-complicated
Functional dyspepsia
Dyspepsia (noninvasive test based)
Beneficial before starting NSAID treatment
Advisable in NSAID and aspirin users, because of reduced ulcer risk
If patients with ulcers on NSAID, but PPI needs to be continued
In aspirin users with previous history of gastroduodenal ulcer
Chronic atrophic gastritis
Following gastric cancer resection
MALT lymphoma
Extradigestive diseases
Idiopathic thrombocytopenic purpura
Iron deficiency anemia
Vitamin B12 deficiency
Improvement in the bioavailability of certain drugs (i.e., l-thyroxine, l-dopa)

accessible endoscopy, the “scope and test” strategy remains the recommended strategy for management of all patients with dyspeptic symptoms, which has been emphasized in the German national guidelines (Fischbach et al. 2009). Arguments in favor of the “scope and treat” strategy are the definitive diagnosis by direct endoscopic inspection and by including histological assessment. A further argument is the low cost of endoscopy in several European countries. Arguments against the “scope and test” strategy in the young age group are a normal endoscopic finding in most cases. Complications (i.e., ulcer, neoplasia) associated to the *H. pylori* infection are extremely rare in the young age group. Furthermore, *H. pylori* eradication would be the treatment of choice even in case of peptic ulcers. In Europe, treatment of non-complicated duodenal ulcer can be restricted to the duration of eradication therapy without further proton-pump inhibitor (PPI) therapy for additional weeks. In gastric ulcer, PPI needs to be continued until healing of the gastric ulcer is confirmed and malignancy is excluded via a follow-up endoscopy.

In patients with dyspeptic symptoms undergoing upper gastrointestinal endoscopy either age dependent (older than 45–50 years) or because of the need for histological assessment, testing for *H. pylori* infection should routinely be performed independent of presence or absence of gross morphologic changes of the gastric mucosa. *H. pylori* eradication should routinely be offered to patients with dyspeptic symptoms (functional dyspepsia) with a positive *H. pylori* test result. A positive urease test is considered sufficient to start with the eradication therapy even if additional management would eventually be necessary on the basis of the histological findings. The histological assessment is mostly performed according to the Sidney system. In cases of suspected atrophic gastritis, the updated Sidney-Houston criteria are preferred and gastritis staging systems with special

reference to gastric atrophy (OLGA) (Rugge et al. 2008) or intestinal metaplasia (OLGIM) (Capelle et al. 2010) are recommended.

The evidence that *H. pylori* eradication prevents/reduces gastroduodenal ulcers in patients before long-term nonsteroidal anti-inflammatory drug (NSAID) exposure and prevents the recurrence of ulcer bleeding in patients on low dose aspirin has led to the implementation of a series of considerate recommendations in the European consensus (Table 21.1). However, persisting high rates of NSAID and aspirin-induced upper gastrointestinal bleeding display an indirect evidence for room of improvement in the translation of these recommendations. There is great demand for creating more awareness of the potential to prevent ulcers and complications by eradicating *H. pylori* in patients exposed to gastrolesive medication.

Secondary prophylaxis by *H. pylori* eradication in aspirin users with *H. pylori* infection and previous ulcer bleeding is recommended based on a positive study conducted in Asia (Chan et al. 2013).

A similar study is missing in Europe and no study on primary prophylaxis of aspirin-induced gastroduodenal lesions by *H. pylori* eradication is available at present. The ongoing claim *H. pylori* may be protective for the esophagus and beneficial for gastroesophageal reflux disease (GERD) and GERD-related complications have been rejected in the European consensus on the basis of available evidence (Malfertheiner et al. 2012), but the issue remains debated on a global level; however there is no objection to *H. pylori*.

The role of *H. pylori* in the pathogenesis of selected systemic diseases has been corroborated by clinical studies in the most recent European consensus (Banić et al. 2012; Franceschi et al. 2014). For extragastric diseases causally related with *H. pylori* (Table 21.1), it is important to emphasize that other possible causes need to be ruled out before starting the eradication therapy.

It is also clearly stated in the European consensus that for several other extragastric diseases associated with *H. pylori* but with insufficient evidence for causality, more research is required and eradication is not recommended at present. There are several autoimmune, neuroinflammatory, and metabolic associations with *H. pylori* that deserve further investigations. The hygiene theory has been revisited and *H. pylori* has been proposed as an ideal "surrogate marker" for poor hygiene in childhood and to be beneficial in the prevention of atopic diseases. Clinical evidence is poorly supporting this relationship but experimental studies provided positive findings and stimulate further clinical research in this area (Arnold et al. 2012).

21.3 Prevention Strategies of Gastric Cancer

"Search and treat" and "screen and treat" are strategies with great potential in the prevention of gastric cancer and other *H. pylori*-related pathologies (Table 21.2). However at present in Europe, the recommendation of "search and treat" strategy for prevention is recommended only in first-degree family members of patients with

Table 21.2 European strategies

Strategies adopted in Europe
Variable among countries
Test (noninvasive) and treat – in young patients (<50) with no alarm symptoms
Scope (endoscopy based) and treat – in all conditions requiring precise diagnosis of gastroduodenal pathologies
Search and treat – in selected populations at risk, i.e., family members of first-degree relatives with gastric cancer
Screen and treat – in study protocols, proposed in high to moderate risk areas for gastric cancer! Currently not adopted in any European country

Table 21.3 *H. pylori* eradication for gastric cancer prevention

H. pylori eradication to prevent gastric cancer in individuals with increased risk
To be considered in:
First-degree relatives of family members with gastric cancer
Patients with:
Previous gastric neoplasia already treated by endoscopy or partial gastrectomy
Severe pan-gastritis, corpus-predominant gastritis, severe atrophy
[a]Long-term (>1 year) PPI therapy
Strong environmental risk factors for gastric cancer
Fear of gastric cancer

[a]PPI so far only associated with the accelerated development of gastric neoplastic condition

gastric cancer. A recent study from Portugal strengthens this recommendation as 20 % of asymptomatic subjects from these families presented with preneoplastic conditions (i.e., atrophy, intestinal metaplasia) of their stomach (Marcos-Pinto et al. 2013). Those patients are at an increased risk for the development of gastric cancer. These subjects require eradication and regular follow-up after successful eradication. Patients with preneoplastic conditions of the gastric mucosa should in any case undergo regular endoscopy controls at scheduled intervals (Dinis-Ribeiro et al. 2012).

A "search and treat" strategy is also recommended in patients on long-term PPI. In those patients, the long-term PPI use may have a negative effect by accelerating the development of preneoplastic conditions (i.e., gastric atrophic changes) in the presence of *H. pylori* infection.

In the European consensus report, commitment to implement prevention strategies in areas/countries also in Europe with moderate to high prevalence of gastric cancer in the population has been recommended (Table 21.3) and some initiatives in this respect have been started. The positive association of *H. pylori* with colon neoplasms (Selgrad et al. 2012, 2014a) and the established clinical benefits obtained with colonoscopy screening for colorectal cancer (De Angelis et al. 2014) prompted a European-wide initiative to screen for gastric preneopastic conditions in the context of screening colonoscopy ("Healthy Stomach Initiative – study proposals" n.d.).

21.4 Diagnostic Tests

The full spectrum of noninvasive and invasive tests is in use in Europe with different indications and preferences according to the clinical conditions. Among noninvasive tests, both the 13C-UBT and acid stool antigen test are considered equivalent. Both tests are used as primary diagnostic tools in the "test and treat" strategy of dyspeptic patients and they have the primary role for confirmation of the *H. pylori* eradication success.

All patients undergoing eradication therapy with non-complicated peptic ulcer, dyspepsia, and other nonmalignant pathologies should be tested 4 weeks after eradication by a noninvasive test. Endoscopy-based testing after therapy is demanded in patients with complicated duodenal ulcer and in all cases of gastric ulcer, preneoplastic conditions (severe atrophy), and MALT lymphoma and after surgical subtotal gastric resection or endoscopic resection of gastric neoplasia. If endoscopy is carried out for clinical reasons after first-line failure of *H. pylori* eradication, culture with susceptibility testing of antibiotics used in eradication regimen should be performed. After second-line failure, therapy should generally be chosen in the basis of antibiotic susceptibility testing.

Serology is useful in conditions of recent use of antimicrobials and PPI and in ulcer bleeding. Serology is also appropriate for testing in patients with dyspeptic symptoms if other noninvasive tests are not available. The use of rapid in office tests is discouraged because of insufficient accuracy. *H. pylori* serology combined with serum pepsinogen I/II and gastrin 17 offers the possibility to identify patients with advanced preneoplastic conditions (i.e., gastric atrophy) (Agréus et al. 2012). This test is widely accepted in Asia as a screening tool for patients at risk for gastric cancer development.

Novel technologies including in situ hybridization methods for clarithromycin and fluoroquinolone resistance on gastric biopsies are new options if standard testing on culture is not possible; however they are not generally available yet.

21.5 Treatment

As in other parts of the world, the efficacy of standard triple PPI (clarithromycin, amoxicillin, and metronidazole) for *H. pylori* eradication has significantly dropped in many European countries with eradication rates below 80 %. The increasing resistance of *H. pylori* to clarithromycin has been identified as the principal reason and this is not surprising as the most effective individual antibiotic in eradication regimen is clarithromycin.

In contrast, resistance to metronidazole although generally higher than for clarithromycin has much less impact on *H. pylori* eradication failures. The main reason for antibiotic resistance represents the occurrence of point mutations of *H. pylori* DNA, which can be explained by inappropriate antibiotic use. In Europe

Table 21.4 Antibiotic resistance in Europe

Country	CLA-R	MZ-R	LEV-R	TC-R	RIF-R	AC-R	References
Europe overall	17.5 %	34.9 %	14.1 %	–	–	–	Megraud et al. (2013)
Poland	23.3 %	66.7 %	6.7 %	–	–	–	Gośiniak et al. (2014)
Belgium	13.3 %	26.1 %	–	–	–	0.8 %	Vekens et al. (2013)
Ireland	–	–	11.7 %	0 %	0 %	–	O'Connor et al. (2013)
Italy	35.2 %	59.3 %	22.1 %	–	–	–	Saracino et al. (2012)
Germany	6.7 %	29.4 %	14 %	–	–	–	Wüppenhorst et al. (2014)
Germany	7.5 %	32.7 %	11.7 %	0 %	0.8 %	0 %	Selgrad et al. (2013)

CLA-R clarithromycin resistance, *MZ-R* metronidazole resistance, *LEV-R* levofloxacin resistance, *TC* tetracycline resistance, *RIF-R* rifabutin resistance, *AC-R* amoxicillin resistance

clarithromycin resistance rates show a huge difference between Northern and Southern European countries. Low rates of resistance have been described for the Scandinavian countries. In Germany, clarithromycin resistance remained comparably low (<10 %), but it has to be noted that there has been a significant increase over the last decade (Selgrad et al. 2013; Wolle et al. 2002; Wüppenhorst et al. 2014). In contrast, in Southern Europe (e.g., Italy, Spain, Portugal, and Greece), clarithromycin resistance is very high and therefore failure of clarithromycin-based therapies is high. Table 21.4 gives a general overview about the antibiotic resistance rates in Europe in the years 2012–2014.

Based on the prevalence of clarithromycin resistance, recommendations for first-line therapy have been revised and no clarithromycin-containing regimen is recommended for use in areas where clarithromycin resistance of >15 % is reported. The prevalence of clarithromycin resistance is very different in European countries and also within countries (Megraud et al. 2013; Selgrad et al. 2014b). Accordingly different solutions for improving treatment success have been offered in various European regions. In areas with clarithromycin resistance >15 (20 %), bismuth-based quadruple is recommended as first line. In a European multicenter study, bismuth quadruple (O-BMT= omeprazole, bismuth, metronidazole, tetracycline) has been significantly superior to standard triple PPI containing clarithromycin (Malfertheiner et al. 2011). In some European countries, bismuth is not available and therefore several non-bismuth-based quadruple, sequential (ST), concomitant (CT), or even hybrid therapies have been used successfully (Gatta et al. 2013; Molina-Infante et al. 2013) (Table 21.5).

In clinical studies the eradication rates of complex non-bismuth quadruple regimens are above 90 % (Molina-Infante et al. 2013), but in routine clinical practice, the eradication rate drops below 90 % (McNicholl et al. 2014). Antibiotic resistance testing in the individual patient with the selection of the regimen based on antibiotic susceptibility is not recommended as first-line treatment. However it

Table 21.5 Current (proposed) treatment regimens and dosages

Standard triple therapy	**PPI, clarithromycin, amoxicillin, or metronidazole**	7–10 days
	Standard dose b.i.d., 500 mg b.i.d., 1,000 mg b.i.d. (or 500 mg b.i.d.)	
Bismuth-containing quadruple therapy	**PPI, tetracycline, metronidazole, bismuth (Pylera®)**	10 days
	Standard dose b.i.d., 500 mg q.i.d., 125 mg q.i.d., standard dose q.i.d.	
Sequential therapy	**Days 1–5, PPI, amoxicillin**	10 days
	Standard dose b.i.d., 1,000 mg b.i.d.	
	Days 6–10, PPI, clarithromycin (levofloxacin), metronidazole	
	Standard dose b.i.d., 500 mg b.i.d., (250 mg b.i.d.) 500 mg b.i.d.miku	
Concomitant therapy	**PPI, clarithromycin, amoxicillin, or metronidazole**	7–10 days
	Standard dose b.i.d., 500 mg b.i.d., 1,000 mg b.i.d., 500 mg b.i.d	
Hybrid therapy	**Days 1–7, PPI, amoxicillin**	14 days
	Standard dose b.i.d., 1,000 mg b.i.d	
	Days 8–14, PPI, amoxicillin clarithromycin, metronidazole	
	Standard dose b.i.d., 1,000 mg b.i.d., 500 mg b.i.d. 500 mg b.i.d.	

should be considered in patients undergoing upper gastrointestinal endoscopy for diagnostic purpose. Second-line regimen should be chosen in consideration of the first line and can either be O-BMT or based on levofloxacin-containing regimen (Table 21.6). Levofloxacin has emerged as the antibiotic of choice for second line, either in the absence of bismuth quadruple or after O-BMT failure. It is worrisome that also levofloxacin resistance is increasing in many European countries (Megraud et al. 2013) and therefore in countries with high levofloxacin resistance, it is advisable to test for levofloxacin resistance before its use. In spite of some excellent results of levofloxacin in first line (Federico et al. 2012), it should never be used in first-line therapy without susceptibility testing. There is a general recommendation to perform antibiotic susceptibility tests after second-line failure (Table 21.6). Rifabutin for third-line combination is a valid option (Gisbert and Calvet 2012). In this context, it is worth mentioning that for amoxicillin, tetracycline, and rifabutin, resistance rates are none or neglectable in Europe. Several studies have been performed in Europe to test for the add-on value of probiotics and for some of them a positive effect has been shown (Szajewska et al. 2010). The beneficial effect is considered to be obtained by lowering side effects with better adherence of patients to the eradication therapy (6). The decision of simultaneous administration of a "valid" probiotic with eradication therapy should be on a case

Table 21.6 *H. pylori* – therapy algorithm

Regions with a ***low*** clarithromycin resistance	Regions with a ***high*** clarithromycin resistance
1st line	
PPI-clarithromycin-amoxicillin/ metronidazole	**Bismuth quadruple therapy**
Or	If not available:
Bismuth quadruple therapy	**Quadruple therapy without bismuth** (**SQT or CCT**)
2nd line	
Bismuth quadruple therapy	**PPI-levofloxacin/amoxicillin**
Or	
PPI-levofloxacin/amoxicillin	
3rd line	
Only based on susceptibility testing	Only based on susceptibility testing

by case basis taking into account the susceptibility for side effects reported by patients from the experience with prior antibiotic therapy.

21.6 Conclusions and Outlook

Current recommendations for *H. pylori* eradication in Europe are based on good to excellent evidence regarding gastroduodenal pathologies and related symptoms. Some extragastric diseases are potentially caused by *H. pylori* infection and deserve individual consideration. This is an area of important future research activities. Commitment for gastric cancer prevention strategies is increasingly recognized in Europe, especially in countries that continue to carry a significant gastric cancer burden. Bismuth-based quadruple therapy and various non-bismuth-based quadruples are effective alternatives to standard triple PPI therapies in areas of high antibiotic (i.e., clarithromycin) resistance. Following failure of first-line therapy, an algorithm of successive therapies is advised.

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Chapter 22
Population-Based Strategies for *Helicobacter pylori*-Associated Disease Management: Latin American Perspective

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Abstract In 2012 almost 65 % of all cancer deaths occurred in less developed regions of the world, including Latin America, and this frequency is predicted to increase in the coming years. We aim to offer an outlook of the burden that gastric cancer represents for the region, the challenges faced, actions taken by some countries, and regional efforts to deal with the problem. Latin America is a region with contrasting gastric cancer mortality rates, with areas presenting among the highest in the world (Central America and the Andean countries) and areas with the lowest mortality rates (Paraguay and Argentina). In Colombia, a chemoprevention trial was carried out in a high-risk region to investigate whether anti-*Helicobacter pylori* therapy, vitamin supplements, or both prevented the progression of gastric premalignant lesions. All three interventions resulted in significant regression of precancerous lesions. A clinical trial in seven Latin American regions proved the feasibility of *H. pylori* eradication in community-based programs using less expensive and locally available generic drugs. A multidisciplinary Latin American team worked on a consensus to deal with the problem, and they stated that "the potential benefit of eradicating *H. pylori* in primary prevention of gastric cancer is highly suggested. However, there is insufficient evidence to justify large-scale implementation in the general population." In 2013 IARC gathered worldwide experts to make recommendations on *H. pylori* treatment for gastric cancer prevention. The group recommended the inclusion of gastric cancer prevention in national cancer

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programs, including controlled interventions for *H. pylori* eradication, particularly in high-incidence areas like Latin America.

Keywords Latin America • Gastric cancer • *Helicobacter pylori* • Primary prevention • Eradication treatment • Population based

22.1 Introduction

22.1.1 Global Burden of Cancer and the Current Situation in Latin America

The International Agency for Research on Cancer (IARC) from the World Health Organization released in December 2013 its last world report on cancer epidemiology, where they inform that the most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0 % of the total), breast (1.7 million, 11.9 %), and colorectum (1.4 million, 9.7 %) (Ferlay et al. 2013). They also reported that the most common causes of cancer death were cancers of the lung (1.6 million, 19.4 % of the total), liver (0.8 million, 9.1 %), and stomach (0.7 million, 8.8 %). The global burden of cancer is projected to rise, and IARC estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and aging of the global population (Ferlay et al. 2013). Of relevance for this chapter is the observation that in 2012 more than half of all cancers (56.8 %) and cancer deaths (64.9 %) occurred in less developed regions of the world, and these proportions will increase further in the coming years. Latin America is among these developing regions where cancer burden is projected to increase significantly and utmost attention should be given by countries in the area. In this chapter we aimed to offer a general outlook of the burden that gastric cancer represents for the region, the challenges faced to address the issue, and examples of actions taken by some of the countries as well as regional efforts to deal with the problem.

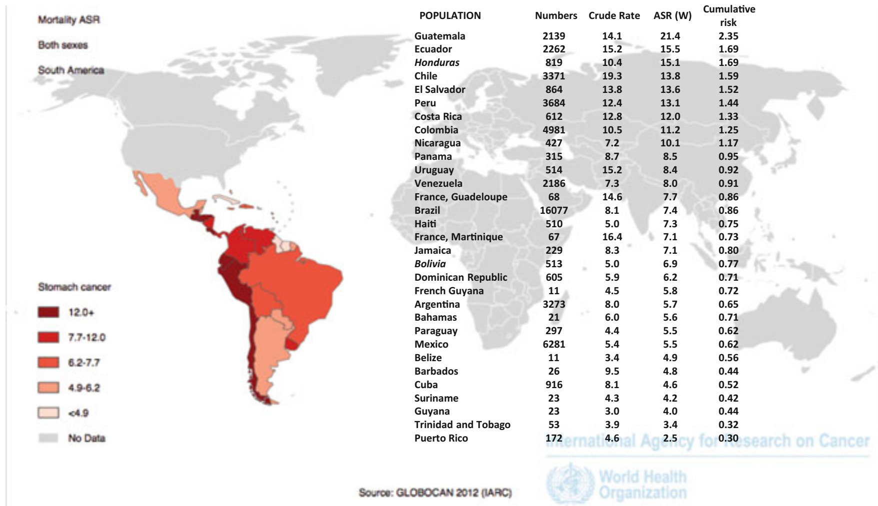
Projections for Latin America and the Caribbean estimate that in 2030 1.7 million cases of cancer will be diagnosed, and more than a million deaths will occur annually. As indicated above, aging of the population is among the factors responsible for this expected rise, and this is exemplified by the estimate that by 2020 more than 100 million people older than 60 years will be living in Latin America and the Caribbean (WHO 2012). A recent report did a critical and thorough analyses on the causes associated with increasing burden and mortality due to cancer in the region (Goss et al. 2013), from which selected points are summarized next. In Latin America, low screening rates, delayed referrals, and failure to seek medical help when symptoms develop contribute to advanced disease at presentation and hence to increased mortality for breast, cervical, and gastric cancer. For example, in the USA 60 % of breast cancers are diagnosed in the earliest stages, whereas in Brazil only 20 % and in Mexico only 10 % are diagnosed at an early stage. The problem is further complicated if we consider that of

590 million inhabitants in Latin America, an estimated 54 % (almost 320 million) do not have health-care coverage. The causes for this include language barriers, unemployment, underemployment, geographic isolation, low education levels, and health illiteracy. In addition, it is estimated that some 400 different indigenous groups live in Latin America, representing 10 % of the population or about 60 million people (Chomitz et al. 2005). This is relevant because rural and remote populations are especially vulnerable to adverse cancer outcomes, since they often reside in areas where oncologists and experts in cancer care are not available and local health centers cannot provide specialized cancer prevention, screening services, treatment, or survivor care. Furthermore, the transition to a lifestyle that mirrors developed countries is increasing obesity, and concomitant cancer risk is becoming a greater disease burden than infectious diseases in Latin America (Goss et al. 2013). The above information summarizes the complexity of the situation in Latin America that challenges health authorities, politicians, and scientists to work on the search for solutions. Some of the actions taken mostly by the scientific and medical community are described in the following sections.

22.2 *Helicobacter pylori* Infection and Gastric Cancer in the Region

Helicobacter pylori infection is highly prevalent in Latin America where in most countries prevalence in adults is around 80 %, (Porras et al. 2013; Pest et al. 1999) although lower frequencies are reported in a few countries like Paraguay and Argentina (around 60 % in adults) (Flores-Luna et al. 2013). In spite of the high prevalence of *H. pylori* in most countries, Latin America is a region with contrasting gastric cancer mortality rates between countries, with areas presenting among the highest mortality rates in the world (particularly in Central America and the Andean countries), but also other areas among the lowest in mortality rates (like Paraguay and Argentina) (Fig. 22.1). It has been noted that the highest burden of gastric cancer mortality in the region is concentrated along the Pacific Rim, following the Andes Cordillera, from Venezuela to Chile, and the Sierra Madre and Cordillera in Central America (Torres et al. 2013).

What is relevant is that even within these countries, there are marked contrasts in mortality, with the highest mortality rates occurring in communities in the mountains and the lowest in the coastal regions. These differences are illustrated by studies in Colombia, where the Andean Pasto region presents age-standardized incidence rate of 150, whereas the coastal Tumaco area, only 100 miles away, has an age-standardized incidence rate of 6, representing a 25-fold difference in incidence rates (Correa et al. 1976). Maps comparing the distribution of the Andean cordillera and the distribution of gastric cancer mortality rates in Colombia clearly illustrate the consistent association of higher mortality rates in districts in the mountainous regions (Fig. 22.2).



POPULATION	Numbers	Crude Rate	ASR (W)	Cumulative risk
Guatemala	2139	14.1	21.4	2.35
Ecuador	2262	15.2	15.5	1.69
Honduras	819	10.4	15.1	1.69
Chile	3371	19.3	13.8	1.59
El Salvador	864	13.8	13.6	1.52
Peru	3684	12.4	13.1	1.44
Costa Rica	612	12.8	12.0	1.33
Colombia	4981	10.5	11.2	1.25
Nicaragua	427	7.2	10.1	1.17
Panama	315	8.7	8.5	0.95
Uruguay	514	15.2	8.4	0.92
Venezuela	2186	7.3	8.0	0.91
France, Guadeloupe	68	14.6	7.7	0.86
Brazil	16077	8.1	7.4	0.86
Haiti	510	5.0	7.3	0.75
France, Martinique	67	16.4	7.1	0.73
Jamaica	229	8.3	7.1	0.80
Bolivia	513	5.0	6.9	0.77
Dominican Republic	605	5.9	6.2	0.71
French Guyana	11	4.5	5.8	0.72
Argentina	3273	8.0	5.7	0.65
Bahamas	21	6.0	5.6	0.71
Paraguay	297	4.4	5.5	0.62
Mexico	6281	5.4	5.5	0.62
Belize	11	3.4	4.9	0.56
Barbados	26	9.5	4.8	0.44
Cuba	916	8.1	4.6	0.52
Suriname	23	4.3	4.2	0.42
Guyana	23	3.0	4.0	0.44
Trinidad and Tobago	53	3.9	3.4	0.32
Puerto Rico	172	4.6	2.5	0.30

Fig. 22.1 Gastric cancer mortality (ASR) in Latin America as reported by Globocan 2012 (Ferlay et al. 2013). Central America and the Andean countries in the Pacific Rim present among the highest mortality rates in the world

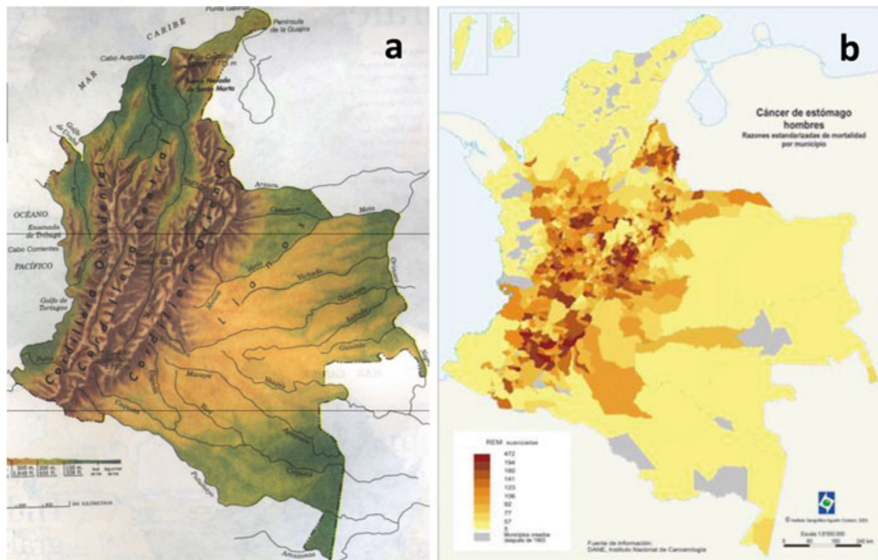


Fig. 22.2 The distribution of gastric cancer mortality in Colombia follows the location of the Andes Cordillera. (**a**) Map showing the distribution of the Andes Cordillera; (**b**) colormap showing gastric cancer mortality per districts in Colombia (Torres et al. 2013)

A clear correlation with altitude has been documented in Central America, and in Costa Rica age-standardized incidence rates of 50 were observed in males of communities located in the "Cordillera Volcánica Central," whereas in males of communities in coastal regions, age-standardized incidence rates of 10 have been reported (Torres et al. 2013); similar observations were found in Honduras and Venezuela. The epidemiology of gastric cancer in Chile presents important differences with the other Andean countries, whereas age-standardized incidence rates in the country are also among the highest in the world, the rates per counties do not follow altitude, and highest rates are observed in districts in the center and south of the country, in regions where prevalence of *H. pylori* infection in young people is higher (Torres et al. 2013). We need to better understand this rather complex geographic diversity of gastric cancer burden in the area for a more rational design of regional programs for the control of gastric cancer.

22.3 Lesson from Studies in Colombia

The creation of the Cali Cancer Registry in southeastern Colombia in 1962 led to the identification of very high rates of gastric cancer in the Nariño region of the Andes Mountains (Correa et al. 1970, 1975a, b). In a study conducted from 1972 to 1974, the incidence rate for the high-altitude area of Nariño was estimated to be 150 per 100,000 persons, the rate for the coastal and low-altitude valley areas of Nariño was 40 per 100,000 persons, while the incidence rate in Cartagena, on the Atlantic coast, was 6 per 100,000 (Correa et al. 1976). Alongside with these observations, multiple studies were carried out in the 1960s and 1970s that involved systematic evaluation of numerous gastric specimens from necropsies and gastric biopsies from Colombian subjects (Cuello et al. 1976; Correa et al. 1990a, b). These studies led Correa and colleagues to propose the sequence of histologic lesions that currently define the gastric precancerous process: non-atrophic gastritis, multifocal non-metaplastic atrophic gastritis, intestinal metaplasia, and dysplasia (Correa 1992; Correa et al. 1975b). After *H. pylori* was first documented as a causal agent of gastritis in 1983 (Marshall and Warren 1983), it was recognized that the precancerous process starts with the colonization of the gastric mucosa by this bacterium.

A double-blind randomized chemoprevention trial was carried out in the high-risk region of Nariño between 1991 and 1996 (Correa et al. 2000a, b). The purpose was to investigate the role of anti-*H. pylori* therapy and vitamin supplements in preventing the progression of gastric premalignant lesions. Adult volunteers with gastric precancerous lesions documented by histology (multifocal atrophic gastritis with or without intestinal metaplasia or dysplasia) were randomly assigned to receive anti-*H. pylori* therapy for 2 weeks (amoxicillin, metronidazole, and bismuth subsalicylate) and/or supplementation for 6 years with beta-carotene and/or ascorbic acid or their corresponding placebos in a three-way factorial design. Subjects assigned to the anti-*H. pylori* treatment arms, who tested positive for

H. pylori at 3 years of follow-up, were retreated for 14 days (amoxicillin, clarithromycin, and either omeprazole or lansoprazole). A total of 630 subjects completed the 6-year intervention and underwent new endoscopic procedure with gastric biopsy sampling. All three basic interventions resulted in statistically significant increases in the rates of regression of precancerous lesions. Relative risks of regression in subjects with non-metaplastic atrophic gastritis at enrollment were 4.8 (95 % CI = 1.6–14.2) for anti-*H. pylori* treatment, 5.1 (95 % CI = 1.7–15) for beta-carotene treatment, and 5.0 (95 % CI = 1.6–14.2) for ascorbic acid. Corresponding relative risks of regression in subjects with intestinal metaplasia were 3.1 (95 % CI = 1.0–9.3), 3.4 (95 % CI = 1.1–9.8), and 3.3 (95 % CI = 1.1–9.8). No additive effect for any of the combinations was detected. At the end of the 6-year trial, eradication of the infection was achieved in 75 % of treated subjects. Anti-*H. pylori* therapy was then offered to the infected subjects that had not received it. Results from the 12 years ($n = 456$) (Mera et al. 2005) and 16 years ($n = 456$; unpublished data) of follow-up showed that significant regression of lesions was observed among subjects free of *H. pylori* infection during 3–6 years, but the effect of vitamin intervention was lost. Furthermore, the highest beneficial effect of anti-*H. pylori* therapy was observed in subjects that started with the least advanced lesions, mainly regressing from non-metaplastic atrophic gastritis to non-atrophic gastritis. The rate of *H. pylori* reinfection/recrudescence in this cohort has hovered around 5 new infections per 100 person-years of exposure, while the rate of spontaneous clearance has been significantly increasing from 2 per 100 person-years at the beginning of the trial to 7 per 100 person-years in the 12–16 years of follow-up (our unpublished data).

Characterization of *H. pylori cagA* and *vacA* genotypes in a subset of subjects ($n = 165$) of this cohort showed that failure to completely eradicate *H. pylori* from the stomach resulted in some patients being colonized by less virulent strains (Correa et al. 2000b). This finding suggests that treatment may be more effective in eliminating more virulent strains and less virulent strains are less responsive to treatment.

The reasons for the variation in gastric cancer incidence rates in Colombia were unknown for a long time, but recent studies have offered possible explanations. Among the possible explanations are differences in dietary habits, host genetic susceptibility, and host-bacterium interactions. Inhabitants of the high-altitude Andes Mountains are predominantly "mestizos," mixture of aboriginal Amerindians and European migrants. In contrast, residents of the Pacific coast, only about 100 miles apart, are predominantly of African ancestry. This phenomenon in Colombia is similar to the fact described by the African enigma. Similarly high prevalence rates (>90 %) of *H. pylori* infection are observed in the adult population in both high- and low-risk gastric cancer areas, but differences in virulence of the *H. pylori*-infecting strains might partially account for differences in cancer risk. A study showed higher prevalence of infection with more virulent *H. pylori* strains in subjects living in the high-risk area for gastric cancer than in those from the low-risk coastal area (Bravo et al. 2002). The corresponding prevalence rates of *H. pylori* genotypes from high- and low-risk areas were *cagA*-positive, 90.4 %

vs. 81.1 %; *vacA* s1 genotype, 93.2 % vs. 83.7 %; and *vacA* m1 genotype, 83.3 % vs. 70.2 %.

In addition to the mentioned virulence determinants, the phylogeographic origin of the *H. pylori* strains seems to play a role in the presence and severity of gastric precancerous lesions in Colombia. Multilocus sequence typing of seven housekeeping genes was used to identify the ancestral origin of 64 *cagA*-positive *H. pylori* isolates from these two populations (de Sablet et al. 2011). All *H. pylori* isolates from the habitants of the mountains showed a predominantly European phylogeographic origin. In contrast, two thirds of the *H. pylori* isolates from African descendants living on the coast displayed predominantly African origin and one third displayed European origin. Overall, subjects carrying *H. pylori* strains of European origin showed more advanced gastric precancerous lesions and greater oxidative damage in the gastric mucosa than subjects infected with strains of African origin.

A recent study involving subjects ($n = 126$, 40 years or older) from both Colombian regions (mountain and coast of Nariño) showed that interactions between the host and *H. pylori* ancestries completely accounted for the difference in the severity of gastric lesions (Kodaman et al. 2014). In particular, African *H. pylori* ancestry was relatively benign in humans of African ancestry but was deleterious in individuals with substantial Amerindian ancestry. Thus, coevolution likely modulated disease risk, and the disruption of coevolved human and *H. pylori* genomes may explain the high incidence of gastric disease in the mountain population.

Another factor potentially related with carcinogenesis is oxidative stress related to polyamines, which are generated by the rate-limiting enzyme ornithine decarboxylase (ODC). During *H. pylori* infection, the enzyme spermine oxidase (SMOX) is induced, which generates hydrogen peroxide from the catabolism of the polyamine spermine. When cocultured with gastric epithelial cells, *H. pylori* clinical strains from the high-risk region induced more SMOX expression and oxidative DNA damage and less apoptosis than low-risk strains. In Mongolian gerbils, a *H. pylori* strain from the high-risk region induced more SMOX, DNA damage, dysplasia, and adenocarcinoma than a strain from the low-risk region. Treatment of gerbils, with either an inhibitor of ODC or an inhibitor of SMOX, reduced gastric dysplasia and carcinoma, as well as apoptosis-resistant cells with DNA damage. These data indicate that aberrant activation of polyamine-driven oxidative stress is a marker of gastric cancer risk and a target for chemoprevention (Chaturvedi et al. 2015).

22.4 Actions Taken in Chile

As described above, Chile is a high-risk area for gastric cancer in Latin America, with gastric cancer risk increasing from north to south of the country (Ferreccio et al. 2007). This population experienced a downward trend of 43 % in the gastric cancer mortality rates from 1960 to 1980 (from 32 to 20 per 100,000 population);

nevertheless after the 1980s, the rates leveled off and remained around 20 per 100,000 in 2009 (http://www.deis.cl/defunciones-y-mortalidad-por-causas/), still representing the first cancer killer in the country. Unfortunately, nothing has been done in the area of primary prevention to reverse gastric cancer mortality in Chile. However, with respect to secondary prevention of gastric cancer, Chile is the only Latin American country with a national program, the AUGE program. This program guarantees endoscopic examination, including *H. pylori* detection, biopsy, and treatment for symptomatic adults aged 40 years and above. A pilot study of this program was performed from 1996 to 2006 in a county of Santiago, the capital city, with a population of 223,708. In this 10-year period, 10,284 individuals were screened, and 190 gastric cancer cases were identified, of which only 32.1 % were at early stages. The specific 5-year survival rate in this group was 40 % (Galleguillos 2006), which is significantly higher than the previously reported population-based survival of 8 % in an unscreened population of Chile (Heise et al. 2009). Based on these promising results, in 2006 the Ministry of Health initiated a nationwide gastric cancer detection program, which was "opportunistic," focused only on symptomatic individuals (including only patients on demand); it guaranteed endoscopic examination and *H. pylori* treatment, if indicated (http://www.supersalud.gob.cl/difusion/572/articles-651_guia_clinica.pdf). Unfortunately, until now, this program has presented no real impact on reducing national gastric cancer mortality, most probably due to its very low population coverage (18 % in 2009) (http://epi.minsal.cl/wp-content/uploads/2012/07/Informe-ENS-2009-2010.-CAP-5_FINALv1julioccepi.pdf); because of these results, the program is under review and other more feasible strategies are being considered. It is currently agreed that the most cost-effective intervention would be *H. pylori* eradication in high-risk populations, accompanied with a more efficient early detection scheme, which may include biomarker-based screening before stomach endoscopy, and this approach is one of the strategies under consideration.

22.5 Regional Efforts to Analyze Evidences and Reach Consensus

Under the sponsorship of the Chilean Society of Gastroenterology (http://sociedadgastro.cl), the consensus organizing committee derived from the V American Workshop for the study of *H. pylori* assembled a multidisciplinary group of gastroenterologists, epidemiologists, and basic scientists with expertise in various aspects of *H. pylori* infection and associated diseases. A modified three-round Delphi method was used to reach consensus on relevant topics. Consensus statements to deal with the high burden of gastric cancer in the region included (Rollan et al. 2014):

I. The potential benefit of eradicating *H. pylori* in primary prevention of gastric cancer is highly suggested. However, there is insufficient evidence to justify

large-scale implementation in the general population. Further studies should be performed on high-risk populations in Latin America to confirm the expected benefit and to evaluate potential adverse effects.

II. The eradication of *H. pylori* infection is recommended as a routine measure to prevent recurrence in gastric cancer patients receiving either subtotal surgical gastrectomy or endoscopic resection.
III. In patients with gastric premalignant lesions, the eradication of *H. pylori* infection halts the progression of chronic atrophic gastritis and probably that of intestinal metaplasia. Although the evidence is still limited, current data favors the eradication of *H. pylori* infection in these patients.
IV. High-risk gastric premalignant conditions, such as severe or extensive chronic active gastritis, intestinal metaplasia, or dysplasia, require periodic follow-up. Endoscopic examination is recommended every 2–3 years for patients with moderate to severe chronic active gastritis or intestinal metaplasia, annually for those with low-grade dysplasia, and every 3–6 months for those with high-grade dysplasia and no focal lesion on endoscopy.

Recommendations for the region included the following: (a) further investigate implementation issues and health outcomes of *H. pylori* eradication for primary prevention of gastric cancer in high-risk populations, and (b) in order to improve the population health, more information and demonstration projects are urgently needed to identify effective and safe primary prevention strategies targeted to the general population of high-risk areas.

In 1994 Brazil experts formed the *Brazilian Helicobacter Study Nucleus*, now part of the Brazilian Federation of Gastroenterology. The group has held three consensus conferences on *H. pylori* infection, with the third held in April 2012 (Coelho et al. 2013). Delegates included gastroenterologists, pathologists, epidemiologists, and pediatricians, and the meeting sought to reexamine the role of *H. pylori* in dyspepsia, gastric cancer, and extra-digestive diseases, as well as address diagnosis and therapeutic options for treating and retreating infection. Results were detailed in 30 statements and a section of proposals for actions to reduce *H. pylori* prevalence, as well as suggestion for action to be implemented by government agencies to reduce infection in Brazil. Two important statements for gastric cancer were the following: (1) The optimal timing of *H. pylori* eradication to prevent gastric cancer is before the appearance of preneoplastic conditions (atrophic gastritis and intestinal metaplasia). (2) In Brazil, surveying and treating the population as a measure to prevent gastric cancer is not indicated. This last statement might be due to the fact that Brazil with a reported age-standardized rate of 7.4 (see Fig. 22.1) is not among the Latin American countries with the highest age-standardized rates (Ferlay et al. 2013).

22.6 Is Eradication of *H. pylori* Feasible in Communities of Latin America?

H. pylori is considered as the main risk for distal gastric cancer, and studies have suggested that eradication of the infection in regions where this cancer is burdensome may reduce incidence and mortality rate (Ma et al. 2012; Wong et al. 2004). Few studies have addressed the question of feasibility of *H. pylori* eradication at community level, and most of them have been performed in Asia and Europe. We performed a randomized clinical trial to compare effectiveness of empiric 14-day triple, 5-day concomitant, and 10-day sequential therapies for *H. pylori* eradication in six Latin American countries, Chile, Colombia, Costa Rica, Honduras, Mexico, and Nicaragua (Greenberg et al. 2011). The study also aimed to provide insight into the feasibility of community-based programs of *H. pylori* eradication in the region. The work included a random sample of 1,469 individuals recruited from the general population in urban and rural communities. Based on the assumption that large programs for *H. pylori* eradication should require inexpensive antibiotic regimens that are effective when used in the communities, we chose to work with generic drugs locally available. The rate of eradication at 6 weeks with the 14-day standard triple therapy was 82.2 %, which was 8.6 % (95 % CI = 2.6–14.5) higher than concomitant therapy and 5.6 % (95 % CI = 0.04–11.6) higher than sequential therapy. At 1 year the rates of eradication were 80.4 %, 79.8 %, and 77.8 % for the standard triple, the concomitant, and the sequential therapies. These results showed that in contrast to studies in other parts of the world, in Latin America standard triple therapy reaches eradication rates similar to sequential and a little higher than concomitant therapies. The study also proves the feasibility of *H. pylori* eradication in community-based programs of countries where gastric cancer is burdensome, using less expensive and locally available generic drugs. The long-term effectiveness of *H. pylori* eradication programs for reducing gastric cancer will depend on the coverage, the acceptability by the population, the compliance with the treatment schedule, and recurrence rate, whether due to treatment failure or to reinfection. This latter aspect has been particularly worrisome in high-prevalence countries like in Latin America (Leal-Herrera et al. 2003; Soto et al. 2003). In our study, the risk of *H. pylori* recurrence 1 year after treatment was 11.5 %, resulting in a 1-year effectiveness among all enrolled participants (independently of their compliance) of 72.7 % (Morgan et al. 2013). Recurrence was significantly associated with study site, nonadherence to initial therapy, and children in the household.

A recent report described a longitudinal study conducted in two remote rural villages of Bolivia (Sivapalasingam et al. 2014) to evaluate annual recurrence rates after mass eradication treatment with a triple antibiotic therapy (lansoprazole, amoxicillin, and clarithromycin). The study included 1,153 individuals, children, and adults, who were tested for *H. pylori* infection using the ^{13}C urea breath test (UBT), and overall cure rates were similar in all age groups (92–98 %). Importantly, annual recurrence rate was as high as 20 % in children <5 years of age, but 8 % in adults. The study concluded that one-time population-based *H. pylori* screen

and treat, as strategy to reduce gastric cancer, might be feasible in adults but not in children in regions with high prevalence of *H. pylori* infection.

A recent systematic review estimated region and country-specific prevalence of *H. pylori* antibiotic resistance in Latin America and found a high resistance to first-line anti-*H. pylori* antibiotics in the area (Camargo et al. 2014). The report stresses the need for appropriate surveillance programs, improved antimicrobial regulation, and increased public awareness in the region. The above studies are relevant in the design of public health programs for primary prevention of gastric cancer in high-incidence countries of Latin America.

22.7 Recommendations for Latin America by the IARC Expert's Group

In December of 2013, a working group organized by IARC gathered scientific experts from around the world to discuss the evidence and controversies and to make recommendations on *H. pylori* treatment for gastric cancer prevention (IARC 2014). The high number of cases occurring worldwide, particularly in Asia, Latin America, and Eastern Europe, in addition to its high lethality, makes it the third cause of cancer death, with an incidence of nearly one million cases per year. The demographic transition, resulting in aging populations, will result in stable or increasing burden of disease, despite general trends of declining incidence rates for the next several decades. The main etiologic agent of the disease, mainly non-cardia gastric cancer, has been identified, with approximately 90 % of the cases caused by chronic infection with *H. pylori* in conjunction with poorly identified cofactors. The precancerous process has been characterized in detail for the epithelial subtype, although many questions remain about the specific carcinogenic mechanisms. The working group considered the fact that in most high-incidence areas, infection with *H. pylori* is very common in adults, with a prevalence of about 60–80 %, and noted the extreme regional differences in gastric cancer incidence between and within countries. Treatment of *H. pylori* infection is feasible with properly tailored combinations of antibiotics and antacids, and several trials, mainly in China, have demonstrated, with some limitations, that treatment of the infection can reduce the incidence of gastric cancer approximately 30–40 %. In addition, cost-benefit analyses mainly in developed countries indicate that population-based treatment programs would be cost-effective. However, the working group noted that, with very few exceptions, population-based *H. pylori* treatment programs are not been established as public health interventions (IARC 2014). The reasons for this inaction have to do with the false notion that gastric cancer is disappearing in most places, that mass eradication of large fractions of the population may not be feasible or effective, and that the absence of *H. pylori* infection could have negative effects, including antibiotic resistance, increases in adenocarcinoma of the esophagus, and obesity, emphasizing the uncertainty and limitations

of the available data to guide evidence-based interventions. Ongoing research is likely to provide additional valuable information, but the working group considered that inaction is no longer acceptable in view of the large future burden of disease and enormous cost to society represented by gastric cancer. The group recommended the inclusion of gastric cancer prevention in national cancer control programs, particularly in high-incidence areas, including interventions for *H. pylori* treatment in the context of demonstration projects or trials generating the necessary data on effectiveness, acceptability, and potential adverse effects.

It was also discussed the role of biomarkers in the strategies to reduce gastric cancer in populations of Latin America. In contrast to extensive studies in Asia probing the utility of pepsinogen test to identify individuals with chronic atrophic gastritis and increased risk to develop gastric cancer (Miki 2006), in Latin America the limited number of studies has shown poor results with this test (Ley et al. 2001). There are a number of studies on the correlation of polymorphisms in genes associated with inflammation and risk for gastric cancer, but they are still far from being representative of the Latin American region and should be extended before further consideration. It should be emphasized that Latin America is a region with limited resources and any test suggested for massive screening should be noninvasive, simple, and cheap. In this context, we should consider that (a) *H. pylori* infection is the most important risk factor to develop gastric cancer, and longitudinal studies have shown that gastric cancer developed only in those with a basal infection; (b) precancerous gastric lesions associated with *H. pylori* are usually presented in adults (most commonly after the 40 years of age); (c) Latin America, and in particular Central America, and the Andean countries present among the highest age-standardized incidence rates for gastric cancer worldwide; and (d) within these Latin American countries with high mortality rates, there are districts or countries with even higher mortality rates. These arguments could be used to propose a strategy to screen and treat individuals infected with *H. pylori* in high-risk communities as follows (Fig. 22.3).

22.8 Conclusions and Outlook

In summary, the main issues addressed in this chapter include the following: (I) Central America and the Andean countries in the Pacific Rim present among the highest gastric cancer mortality rates in the world. Still, in Latin America low screening rates, delayed referrals, and failure to seek medical help when symptoms develop contribute to advanced disease at presentation and hence to increased mortality for gastric cancer. (II) In Colombia, a chemoprevention trial in a high-risk region for gastric cancer demonstrated that anti-*H. pylori* therapy, vitamin supplements, or both prevented the progression of gastric premalignant lesions. Test and treat population-based studies in the region have documented an annual *H. pylori* recurrence rate of around 10 % in adults. (III) Although the potential benefit of eradicating *H. pylori* in primary prevention of gastric cancer is highly

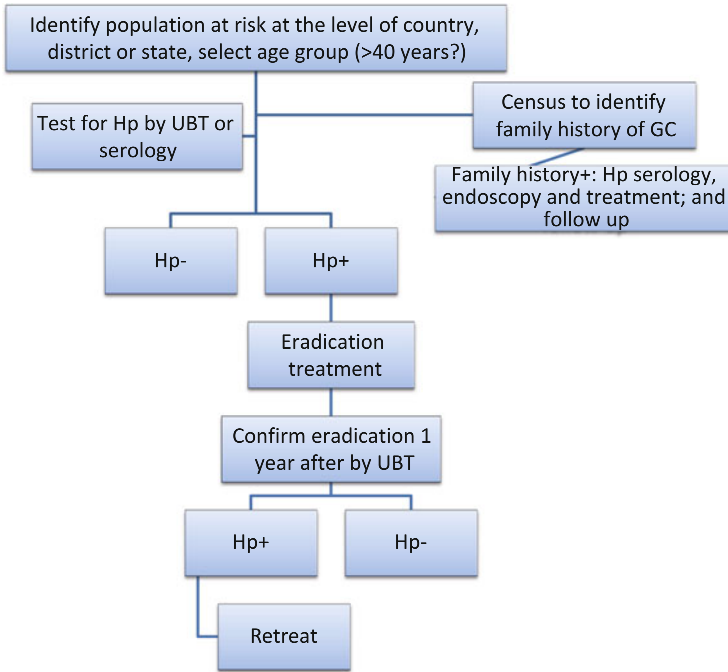


Fig. 22.3 Suggested minimal screening for identification of patients at risk for gastric cancer in the Latin American region and follow-up strategies. *Hp H. pylori*, *UBT* urea breath test, *GC* gastric cancer

suggested, there is insufficient evidence to justify large-scale implementation in the general population. (IV) In countries with low age-standardized incidence rates like Brazil, Paraguay, Argentina, or Mexico, mass eradication of *H. pylori* is not recommended. (V) And recently IARC gathered world experts to discuss the evidence and controversies and to make recommendations on *H. pylori* treatment for gastric cancer prevention. The group recommended the inclusion of gastric cancer prevention in national cancer control programs, particularly in high-incidence areas, including interventions for *H. pylori* treatment in the context of demonstration projects or trials generating the necessary data on effectiveness, acceptability, and potential adverse effects.

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Chapter 23
Population-Based Strategies for *Helicobacter pylori*-Associated Disease Management: Asian Perspective

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Abstract Asia has the largest population of any continent and the highest incidence of gastric cancer in the world, making it very important in the context of *Helicobacter pylori* infection. Several new guidelines in East Asian countries include expanded indications for *H. pylori* eradication. Importantly, the Japanese national health insurance system now covers expenses for all *H. pylori*-infected subjects up to second-line treatment. According to current guidelines, standard triple therapy containing a proton pump inhibitor (PPI) and two antibiotics, clarithromycin and amoxicillin/metronidazole, is still the preferred first-line regimen for treatment of *H. pylori* infection. However, in following years, the efficacy of legacy triple regimens has been seriously challenged, and they are becoming ineffective. Moreover, some regions in Asia show patterns of emerging antimicrobial resistance. Therefore, clarithromycin-containing triple therapy should be abandoned, as it is no longer effective unless local clarithromycin resistance is low or culture confirms susceptibility to clarithromycin. More effective clarithromycin-based regimens are now replacing standard triple therapies as empirical first-line treatments on the basis of the understanding of the local prevalence of *H. pylori* antimicrobial resistance. These include the bismuth and non-bismuth quadruple, sequential, and dual-concomitant (hybrid) regimens.

Keywords *Helicobacter pylori* • Asia • Antibiotic resistance • Proton pump inhibitor (PPI) • Clarithromycin • Amoxicillin • Metronidazole

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Abbreviations

cagA	Cytotoxin-associated gene
DU	Duodenal ulcer
IM	Intestinal metaplasia
MALT	Mucosa-associated lymphoid tissue
PPI	Proton pump inhibitor
PUD	Peptic ulcer disease

23.1 Introduction

Helicobacter pylori infection is regarded as a high risk factor for severe gastritis-associated diseases, including peptic ulcers and gastric cancer (Suerbaum and Michetti 2002). Asia is a very important continent in the context of *H. pylori* infection. It has the largest population of any continent (4.4 billion people) and the highest incidence of gastric cancer in the world, with an age-standardized incidence rate of 15.8/100,000 (available from the International Agency for Research on Cancer; GLOBOCAN2012, http://globocan.iarc.fr/). The population of India is approximately 1.2 billion people; if *H. pylori* prevalence was 60 %, then more than 726 million individuals in India would be infected with *H. pylori*. Furthermore, the estimated prevalence of duodenal ulcer (DU) in India is 3 %, meaning that at least 18 million people could need anti-*H. pylori* therapy (approximately 50 000 per day if treated over 1 year) (Thirumurthi and Graham 2012). Asia is subdivided into high-risk, intermediate-risk, and low-risk regions by age-standardized incidence rate for gastric cancer (Ferlay et al. 2010). High-risk areas include East Asian countries such as China, Japan, and Korea, where the age-standardized incidence rate is greater than 20 per 100,000. Intermediate-risk countries (age-standardized incidence rate 11–20/100,000) include Malaysia, Singapore, and Taiwan, while low-risk areas (age-standardized incidence rate <10/100,000) include India, Thailand, and Indonesia.

In Asia, there is geographic variation in the prevalence rates of *H. pylori* infection. Generally, developing countries have a higher prevalence than developed countries (Fock and Ang 2010). Interestingly, the incidence rate of gastric adenocarcinoma in Asia tends to mirror the prevalence rate of *H. pylori* infection. Among East Asian countries, China has the highest seroprevalence rate (58.1 %) (Wang and Wang 2003) and Japan the lowest (39.3 %) (Fujisawa et al. 1999), while seroprevalence rates in South Korea and Taiwan are similar (59.6 % and 54.5 %, respectively). In Southeast Asian countries, reported seroprevalence rates are 35.9 %, 31.0 %, and 57.0 % in Malaysia, Singapore, and Thailand, respectively (Fock and Ang 2010). High *H. pylori* seroprevalence rates have also been reported in Bhutan (70.2 %) (Vilaichone et al. 2013b) and Vietnam (74.6 %) (Hoang et al. 2005). Interestingly, Indonesia has a low prevalence rate of *H. pylori* infection (Miftahussurur et al. 2014). Using five different methods, the prevalence rate in

Indonesia was measured as only 11.5 % (Miftahussurur et al. 2015), similar to that of Australia, which has an overall seroprevalence rate of 15.1 % (Moujaber et al. 2008). In Western Asia, prevalence rates among different countries are similar. Almost 90 % of the adult population is infected with *H. pylori* in Iran (Malekzadeh et al. 2004; Massarrat et al. 1995). Prevalence has been reported to be about 82 % in Jordan (Bani-Hani and Hammouri 2001) and 78.4 % among industrial workers and 64.3 % among referent workers in the United Arab Emirates (Bener et al. 2002). In Turkey and Kuwait (Novis et al. 1998), the overall prevalence of *H. pylori* infection is 81 % and 84 %, respectively. In Southern Central Asia, the prevalence of *H. pylori* infection was reported to be almost identical among two ethnic groups (79 % and 80 % in Russians and Kazakhs, respectively) in Kazakhstan (Nurgalieva et al. 2002). However, a high prevalence of *H. pylori* infection is not always associated with a high incidence of gastric cancer. For example, despite the high infection rate in South Asian countries (79 %, 45 %, and 60.2 % in India, Pakistan, and Bangladesh, respectively), the incidence of gastric cancer in the region is low, which is known as an "Asian enigma" (Miwa et al. 2002). Eradicating *H. pylori* not only heals peptic ulcers but also prevents their recurrence and reduces the risk of development of gastric cancer (Malfertheiner et al. 2012). Furthermore, other *H. pylori*-associated disorders such as mucosa-associated lymphoid tissue (MALT) lymphoma, chronic atrophic gastritis, and intestinal metaplasia (IM) regress after treatment with antibiotics (Sugiyama et al. 2002; Vannella et al. 2011). It is therefore suggested that *H. pylori* should be eradicated in Asia.

23.2 Indications for the Treatment of *H. pylori* Infection in Asia

While European and US guidelines for the management of *H. pylori* infection have been available for many years, there were no such guidelines for the Asia-Pacific region until 1997, when guidelines were developed in Singapore (Lam and Talley 1998). These guidelines recommended that all gastric ulcer (GU) and DU patients infected with *H. pylori* should be treated for *H. pylori*, regardless of whether the ulcer is active or in remission. Patients requiring long-term nonsteroidal anti-inflammatory drug therapy who have a current or recent history of dyspepsia, patients with early gastric cancer or low-grade gastric MALT lymphoma, and patients with a family history of gastric cancer should be treated (Lam and Talley 1998). The second guidelines in 2008 expanded the indications for treatment of *H. pylori* infection (Fock et al. 2009). In addition to the previous indications, *H. pylori* eradication was also indicated for *H. pylori*-infected patients with functional dyspepsia, in those receiving long-term maintenance proton pump inhibitors (PPIs) for gastroesophageal reflux disease, and in cases of unexplained iron-deficiency anemia or idiopathic thrombocytopenic purpura (Table 23.1). In

Table 23.1 Comparison indication of treatment of *H. pylori* infection by four guidelines in Asia

Second Asia-Pacific Consensus 2009	Japan 2013	China 2013	South Korea 2013
Indications (grade of recommendation)	*Approved by the Japanese national health insurance system*	*Strongly recommended*	*Strongly recommended*
Peptic ulcer disease (A)	PUD	Peptic ulcer (regardless of activeness or complications)	Peptic ulcer disease
MALT lymphoma (A)	After resection of early gastric cancer	Gastric MALT lymphoma	Low-grade gastric MALT lymphoma
Atrophic gastritis (B)	Gastric MALT lymphoma	*Recommended*	After resection of early gastric cancer
After gastric cancer resection (B)	Idiopathic thrombocytopenic purpura	Chronic gastritis with dyspepsia	*Recommended*
Patients who have first-degree relatives of patients with gastric cancer (B)	*H. pylori*-related gastritis	Chronic gastritis with mucosal atrophy/erosion	Chronic atrophic gastritis or IM
Patients' wishes (after full consultation with their physician) (A)		Early gastric cancer resected endoscopically or by subtotal gastrectomy	Family history of gastric cancer
Nonulcer dyspepsia (A)		Long-term use of PPI	Functional dyspepsia
To reduce the risk of peptic ulcer and upper gastrointestinal bleeding in NSAID-naive users (A)		Family history of gastric cancer	Long-term aspirin/NSAID medication with history of peptic ulcer disease
Before starting long-term aspirin therapy for patients at high risk for ulcers and ulcer-related complications (B)		Planning to take long-term NSAID (including low-dose aspirin)	Idiopathic thrombocytopenic purpura
Patients receiving long-term low-dose aspirin therapy and who have a past history of upper gastrointestinal bleeding and perforation (B)		Iron-deficiency anemia of unknown causes	
GERD patients requiring long-term proton pump inhibitor (B)		Idiopathic thrombocytopenic purpura	
As a strategy for gastric cancer prevention in communities with high incidence of gastric cancer (A)		Other *H. pylori*-related diseases (lymphocytic gastritis, gastric hyperplastic polyps, Ménétrier disease, etc.)	

(continued)

Table 23.1 (continued)

Second Asia-Pacific Consensus 2009	Japan 2013	China 2013	South Korea 2013
Unexplained iron-deficiency anemia or idiopathic thrombocytopenic purpura (C)		Requested by individual patient	

(Fock et al. 2009; Kim et al. 2013a; Asaka 2013; Chinese Society of Gastroenterology et al. 2013)

addition, a population "test-and-treat" strategy for *H. pylori* infection in communities with high incidence of gastric cancer was considered to be an effective strategy for gastric cancer prevention. It was recommended that *H. pylori* infection should be tested for and eradicated prior to long-term aspirin or nonsteroidal anti-inflammatory drug therapy in patients at high risk for ulcers and ulcer-related complications (Fock et al. 2009).

The new revised 2013 guidelines from China, Japan, and South Korea include expanded indications for *H. pylori* eradication (Table 23.1). In Japan, the first guidelines for management of *H. pylori* infection were developed in 2000. GU and DU were the only diseases for which *H. pylori* eradication therapy was recommended by these guidelines. The second guidelines in 2003 advanced the field greatly. Patients with MALT lymphoma were recommended to receive eradication therapy in addition to those with active GU and/or DU. In addition, the following three clinical outcomes were upgraded to advisable indications: early gastric cancer after endoscopic mucosal resection, atrophic gastritis, and gastric hyperplastic polyp. The guidelines were revised substantially again in January 2009. Most importantly, in the new guidelines all "*H. pylori* infection" subjects were put into the "level A" group for eradication therapy (strongly recommended based on strong evidence). Therefore, all *H. pylori* infections should be considered as an indication for eradication irrespective of the background diseases (Shiota et al. 2010). In 2013, the Ministry of Health, Labour and Welfare of Japan announced that from February 2013, the Japanese national health insurance system would begin covering expenses from *H. pylori* eradication in all infected subjects. The current indications consist of five categories: (1) peptic ulcer disease (PUD), (2) after resection of early gastric cancer, (3) gastric MALT lymphoma, (4) idiopathic thrombocytopenic purpura, and (5) *H. pylori*-related gastritis. These expanded indications are intended not only for the prevention of *H. pylori*-related diseases such as gastric cancer but also for the prevention of dissemination. Since infection seems to disseminate from parents to a child during the childhood period, dissemination of *H. pylori* can be prevented by eradicating all infected adults (Asaka 2013).

The Fourth National Consensus Conference on the management of *H. pylori* infection in China expanded the indications for *H. pylori* eradication to include two strongly recommended diseases (PUD and gastric MALT lymphoma) and ten

recommended diseases. Most notably, the *H. pylori*-related diseases lymphocytic gastritis, Ménétrier disease, and gastric hyperplastic polyps were recommended for eradication. Chronic gastritis with mucosal atrophy/erosion was also indicated for eradication but not IM. The reason for this is based on the concept that dysplasia is frequently accompanied by atrophy and/or IM. Atrophy and metaplasia can occur after repetitive erosions. Although the optimal period for preventing gastric cancer by *H. pylori* eradication is prior to the development of atrophy and IM, eradication during this period can still ameliorate the inflammatory response, slow or stop the progression of atrophy, and it is even possible to partially reverse the atrophy. It is difficult, however, to reverse IM (Wang et al. 2011). Two existing areas of controversy were mentioned. Although a *H. pylori* "test-and-treat" strategy has been recommended by several other consensus guidelines, in China, the cost of endoscopic examination is low, and the prevalence of upper gastric cancer is high. Therefore, the introduction of a "test-and-treat" strategy has a high risk of missing the detection of gastric cancer (Li et al. 2005). The consensus panel also believes that *H. pylori* eradication might increase the risk of developing gastroesophageal reflux disease in Eastern countries, due to the presumed higher incidence of gastric body dominant gastritis in this region than in the Western countries (Chinese Society of Gastroenterology et al. 2013).

The latest version of the South Korean guideline consists of 11 statements for the indication of *H. pylori* eradication, four statements for diagnosis, and four statements for treatment (Kim et al. 2013a). Highly recommended indications for *H. pylori* eradication are (1) PUD including scar, (2) low-grade gastric MALT lymphoma, and (3) after resection of early gastric cancer. Although the level of evidence is lower than for these indications, *H. pylori* eradication may also be considered for the prevention of gastric cancer in subjects with (4) chronic atrophic gastritis or IM and (5) a family history of gastric cancer. South Korean studies have demonstrated the importance of a family history of gastric cancer in determining when *H. pylori* eradication is indicated, especially in patients aged 40 years or younger (Kim et al. 2013a; Lee 2014). Compared to the second Asia-Pacific and World Gastroenterology Organisation global guidelines, indications in three guidelines from East Asian countries are more expansive and aggressive, including younger populations with acute gastric lesions, who will show markedly greater improvements than older populations with chronic gastric lesions. As East Asia is a high-risk region, *H. pylori* infection would be better eradicated at a reversible stage before the development of a precancerous condition and prevent many infected East Asians from going untreated. Such differences in the indications for *H. pylori* treatment among countries might be due to differences in the approvals granted by governments and/or national health insurance systems in each country (Lee 2014).

23.3 Antibiotic Resistance in Asia

Triple therapy regimens that include one PPI and two antimicrobial agents such as clarithromycin, metronidazole, amoxicillin, levofloxacin, ciprofloxacin, and tetracycline have been widely used to eradicate *H. pylori* (Malfertheiner et al. 2007; Fock et al. 2009; Chey et al. 2007). However, prevalence of antibiotic resistance is now increasing worldwide and varies by the geographic area; it is generally higher in developing countries than in developed regions (Megraud 2004). Antibiotic resistance is the most common factor causing treatment failure, the likelihood of which is further increase by poor compliance or if the patient is a smoker (Jenks 2002; Qasim and O'Morain 2002; Suzuki et al. 2006a). In addition, the antibiotic resistance rate often parallels the antibiotic consumption rate in the population (Megraud 1998; Broutet et al. 2003). Table 23.2 summarizes antibiotic resistance rates from 16 countries and four regions in Asia.

Clarithromycin resistance has been shown to be associated with any one of three well-known point mutations in the 23S rRNA gene of *H. pylori*; these three mutations are responsible for more than 90 % of clarithromycin resistance cases in developed countries (Megraud and Lehours 2007). Interestingly, the point mutations inducing clarithromycin resistance in Asian countries differ from those in Europe and North America (Ierardi et al. 2013). Additional mutations such as T2183C and A2223G have been frequently found to be the cause of observed clarithromycin resistance, while the A2143G mutation, which has a much stronger impact than A2142G, and A2142C (De Francesco et al. 2006), which are responsible for 90 % of cases of primary clarithromycin resistance in *H. pylori* strains isolated in Western countries (Oleastro et al. 2003), accounted only for 23 % of resistant strains in Asia (Oleastro et al. 2003). In East Asian countries, high levels of clarithromycin resistance have been recorded. For example, in Japan the most recent estimate of resistance rate is 32.4 % (Kato and Fujimura 2010), and annual surveillance for 5 years conducted between 2002 and 2006 showed that the mean nationwide clarithromycin resistance rates had increased from 18.9 % (2002) to 27.2 % (2006) (Kobayashi et al. 2007). In China, resistance rates ranged between 21.5 and 23.8 % from 2000 to 2009 (Su et al. 2013; Gao et al. 2010), and the overall resistance increased annually from 14.8 to 65.4 % (Gao et al. 2010). Increasing rates of clarithromycin primary resistance have also been reported in South Korea (17.2–23.7 %) from 2003 to 2012 (Lee et al. 2013). However, the primary resistance rate of clarithromycin in Taiwan is only 8.3 %. In South Asia, India (58.8 %) has a higher resistance rate than Pakistan (36 %) (Khan et al. 2012; Pandya et al. 2014; Poon et al. 2009). In Western Asia, resistance rates have been increasing over the last 20 years. In Iran, clarithromycin resistance has increased from 1.4 % in 1997 to 26.5 % in 2013 (Fakheri et al. 2014; Safaralizadeh et al. 2006). Turkey and Bahrain also have high rates of clarithromycin resistance (21.3 and 32.5 %) (Ozbey et al. 2013; Bindayna 2001). Interestingly, while resistance rates in Vietnam and Indonesia are considered high (33 % and 27.8 %, respectively) (Binh et al. 2013), in Thailand and Singapore the resistance rates are very low (3.7 % and 6 %,

Table 23.2 Antibiotic resistance rates from 16 countries and four regions in Asia

Reference	Country	Year	Patients	Methods	Clarithromycin (%)	Metronidazole (%)	Levofloxacin (%)	Tetracycline (%)	Amoxicillin (%)	Others
Eastern Asia										
Kobayashi et al. (2007)	Japan	2002–2003	1069	Agar dilution method	18.9	4.9	–	–	15.2	–
		2003–2004	1381		21.1	5.3	–	–	21.4	–
		2004–2005	1257		27.7	3.3	–	–	16.3	
Gao et al. (2010)	China-Beijing	2000–2009	290	Epsilometer test	23.8	56.6	36.9	1.0	0.3	Moxifloxacin (41.2 %)
Su et al. (2013)	Southeast China	2010–2012	17,731	Agar dilution method	21.5	95.4	20.6	–	0.1	Furazolidone (0.1 %), gentamicin (0.1 %)
Wang et al. (2000)	China-Hong Kong	NM	83	Agar dilution method	10.8	49.4	–	–	–	
Poon et al. (2009)	Taiwan	1998–2004	218	Epsilometer test	8.3	31.7	–	–	0.0	1998–2004
Lee et al. (2013)	South Korea	2003–2005	70	Agar dilution method	22.9	34.3	5.7	18.6	7.1	Azithromycin (25.7 %), moxifloxacin (5.7 %)
		2006–2008	201		25.5	26.0	27.4	32.8	9.5	Azithromycin (27.4 %), moxifloxacin (27.9 %)
		2009–2012	162		37.0	35.8	34.6	35.2	18.5	Azithromycin (34.0 %), moxifloxacin (34.6 %)

Western Asia										
Abadi et al. (2011)	Iran	2009	197	Disk diffusion method	45.2	65.5	37.1	–	23.9	Ciprofloxacin (34.5 %), furazolidone (61.4 %)
Ozbey et al. (2013)	Turkey	2009–2010	61	Disk diffusion method	21.3	42.6	3.3	0.0	0.0	
Eltahawy (2002)	Saudi Arabia	2002	223	Disk diffusion method	4.0	80.0	–	0.4	1.3	
Bindayna (2001)	Bahrain	1998–1999	83	Epsilometer test	32.5	57.0	–	0.0	0.0	
Southern Asia										
Pandya et al. (2014)	India	2008–2011	80	Disk diffusion method	58.8	83.8	72.5	53.8	72.5	Ciprofloxacin (50 %)
Khan et al. (2012)	Pakistan	2005–2008	178	Not mentioned	36.0	89.0	–	12.0	37.0	Ofloxacin (18.5 %)
South Eastern Asia										
Kumala and Rani (2006)	Indonesia	2006	72	Disk diffusion method	27.8	100.0	1.4	–	19.4	Ciprofloxacin (6.9 %), moxifloxacin (1.4 %), ofloxacin (6.9 %)
Vilaichone et al. (2013a)	Thailand	2004–2012	400	Epsilometer test	3.7	36.0	7.2	1.7	5.2	Ciprofloxacin (7.7 %)
Hua et al. (2000)	Singapore	1995–1998	282	Disk diffusion method	6.0	46.0	–	–	–	
Ahmad et al. (2011)	Malaysia	2004–2007	187	Epsilometer test	2.1	36.4	1.0	0.0	0.0	Ciprofloxacin (0.0 %)
Vilaichone et al. (2013c)	Bhutan	2010	111	Epsilometer test	0.0	82.9	2.7	0.0	0.0	Ciprofloxacin (2.7 %)
Binh et al. (2013)	Vietnam	2008	103	Epsilometer test	33.0	69.9	18.4	5.8	0.0	

respectively) (Vilaichone et al. 2013a; Hua et al. 2000). Moreover, in Bhutan and Malaysia, no *H. pylori* strains showed resistance to clarithromycin (Vilaichone et al. 2013c; Goh and Navaratnam 2011). This suggested that Southeast Asia is the region of Asia with the lowest clarithromycin resistance rates.

Metronidazole is another agent frequently included in regimens to eradicate *H. pylori*. Therefore, the presence of metronidazole resistance may also affect therapeutic outcomes. The mechanisms of metronidazole resistance are complex but are largely associated with inactivating mutations of the *rdxA* and *frxA* genes, which encode reductases required for the activation of metronidazole (Gerrits et al. 2004). However, development of metronidazole resistance can occur independently of these mutations, suggesting alternative, as yet unknown, resistance mechanisms exist (Bereswill et al. 2003). In East Asian countries, China has the highest prevalence of metronidazole resistance (56.6–95.4 %) (Su et al. 2013; Gao et al. 2010). Prevalence of resistance in Hong Kong is 49.4 % (Wang et al. 2000), while the 10-year prevalence of resistance in South Korea is 34.3–35.8 % (Lee et al. 2013). However, contrary to the general phenomenon whereby prevalence of clarithromycin resistance tends to be much lower than that of metronidazole, the metronidazole resistance rate in Japan is only 3.3–4.9 %, as recorded by annual surveillance for 5 years (Kobayashi et al. 2007). In Southeast Asia, only Thailand and Malaysia (Vilaichone et al. 2013a; Ahmad et al. 2011) have metronidazole resistance rates below 40 %. Rates of resistance to metronidazole were found to be in 46 % in Singapore and 69.9 % in Vietnam (Hua et al. 2000; Binh et al. 2013). Bhutan (82.9 %) and Indonesia (100 %) have the highest prevalence of metronidazole resistance in this region (Vilaichone et al. 2013c; Kumala and Rani 2006). High prevalence of metronidazole resistance was also reported in Western and Southern Asia. Resistance rates in Iran, Turkey, Bahrain, and Saudi Arabia have been reported as 65.5 %, 42.6 %, 57.0 %, and 50.0 %, respectively (Abadi et al. 2011; Ozbey et al. 2013; Eltahawy 2002; Bindayna 2001). High resistance rates are also reported in Pakistan and India (89 % and 83.8 %, respectively) (Khan et al. 2012; Pandya et al. 2014).

Loss of penicillin-binding protein is known to be associated with amoxicillin resistance (De Francesco et al. 2006). However, research into rates of amoxicillin resistance is limited. Although most studies estimate rates of resistance to amoxicillin as <1 % in China, Taiwan, Turkey, Bahrain, Malaysia, Bhutan, and Vietnam, the resistance rate in Japan is >10 % (Kobayashi et al. 2007). Increasing amoxicillin primary resistance rates have also been reported in South Korea (7.1–18.5 %) (Lee et al. 2013). Both countries in Southern Asia also have high resistance rates to amoxicillin (72.5 % and 37.0 % for India and Pakistan) (Pandya et al. 2014; Khan et al. 2012). In Southeast and Western Asia, only Indonesia and Iran have reported high resistance rates of 19.4 % and 23.9 %, respectively (Abadi et al. 2011; Kumala and Rani 2006).

Fluoroquinolones, especially levofloxacin-based triple therapy, achieve good *H. pylori* eradication rates. As with other bacteria, resistance of *H. pylori* to fluoroquinolones is due to point mutations in the quinolone resistance determining regions of gyrA (Megraud 1998). Rates of resistance to fluoroquinolones also

mirror the level of use of these kinds of drugs. In Asia, fluoroquinolone resistance rates differ among countries. The resistance is higher in the Southeast coastal region of China (20.6 %) and Beijing (36.9 %). A high rate (41.2 %) of primary moxifloxacin resistance was also reported in Beijing (Su et al. 2013; Gao et al. 2010). Moreover, the primary levofloxacin and moxifloxacin resistance rate in South Korea rose from 5.7 % in 2003–2005 to 34.6 % in 2009–2012 (Lee et al. 2013). In Western and Southern Asia, Turkey was found to have a low levofloxacin resistance rate (3.3 %) (Ozbey et al. 2013), whereas high levofloxacin and ciprofloxacin resistance rates (37.1 % and 34.5 %, respectively) were reported in Iran and India (72.5 % and 50.0 %, respectively) (Abadi et al. 2011; Pandya et al. 2014). Although resistance rates of 18.4 % have been reported in Vietnam (Binh et al. 2013), levofloxacin resistance rates in Southeast Asia are otherwise low. Results of a nationwide survey in Thailand found rates of ciprofloxacin and levofloxacin resistance to be 7.7 % and 7.2 %, respectively (Vilaichone et al. 2013a). Levofloxacin and ciprofloxacin resistance rates are reported to be 1 % and 0 % in Malaysia (Ahmad et al. 2011), 1.4 and 6.9 % in Indonesia (Kumala and Rani 2006), and both of 2.7 % in Bhutan (Vilaichone et al. 2013c), respectively.

The tetracycline resistance mechanism has been characterized as a change in three contiguous nucleotides in the 16S rRNA gene (AGA 926-928RTTC). Resistance to tetracycline is very low, or even absent, in most countries (Megraud 1998). Indeed, resistance to tetracycline has been shown to be infrequent in Beijing, occurring in 1 of 49 (2.0 %) cases in 2006–2007, 0 of 63 cases in 2008, and 1 of 52 (1.9 %) cases in 2009 (Gao et al. 2010). In contrast, higher values were found in South Korea: these increased from 18.6 % in 2003–2005 to 35.2 % in 2009–2012 (Lee et al. 2013). Resistance rates in Saudi Arabia, Thailand, and Vietnam have been reported to be 0.4 %, 1.7 %, and 5.8 %, respectively. However, in South Asia (India and Pakistan), tetracycline resistance rates are high (Eltahawy 2002; Vilaichone et al. 2013a; Binh et al. 2013). Tetracycline resistance is absent in Turkey, Bahrain, Malaysia, and Bhutan (Vilaichone et al. 2013c; Ozbey et al. 2013; Bindayna 2001; Ahmad et al. 2011).

23.4 The Relationship Between Virulence Factors and *H. pylori* Antibiotic Resistance in Asia

In addition to host factors and diet, virulence factors of *H. pylori* have been demonstrated to be predictors of gastric atrophy, IM, and severe clinical outcomes (Yamaoka 2010). Several studies have reported a relationship between *H. pylori* antibiotic resistance patterns and virulence factor genotypes in Asia. In 184 patients in Turkey treated with clarithromycin-based triple therapy for *H. pylori* eradication, the eradication rate was higher in cytotoxin-associated gene (*cagA*)-positive patients (87.4 %) than in those who were *cagA*-negative (71.9 %). The same study found that TNF-α levels were higher in the *cagA*-positive than in the *cagA*-

negative group. This result is concordant with a meta-analysis by Suzuki et al. (2006b), which reported pooled successful *H. pylori* eradication rates of 84 % for *cagA*-positive and 73 % for *cagA*-negative cases. The summary risk ratio for eradication failure in *cagA*-negative relative to *cagA*-positive cases was 2.0 (95 % CI, 1.6–2.4; $P < 0.01$). The relationship of *cagA* with the success and failure of *H. pylori* eradication therapy is clarified by enhanced gastric mucosal inflammation. A close correlation between *cagA* positivity and severe gastric inflammation has been confirmed (van der Hulst et al. 1997). Patients with severe inflammatory cell infiltration in the antral mucosa have significantly higher cure rates than those with milder inflammation. As gastric inflammation increases mucosal blood flow, the increased blood flow may facilitate the diffusion of antibiotics (Maeda et al. 1999). Another study found that vacuolating cytotoxin gene (*vacA*) m1 allele was disproportionately represented among patients with eradication failures (68 %) than in those with successful eradication (39 %) but found no significant association of *vacA* s1 with eradication failure. However, there was no relationship between clarithromycin resistance and *cagA* or *vacA* status in a Japanese population where all cases were *cagA*-positive and *vacA* s1m1 (Shiota et al. 2012).

Lu et al. (2005) were first to describe a novel virulence factor, the duodenal ulcer-promoting gene (*dupA*), which is located in the plasticity region of the *H. pylori* genome. Furthermore, they reported that infections with *dupA*-positive strains increased the risk for DU but were protective against gastric atrophy, IM, and gastric cancer in Japanese, Korean, and Colombian subjects. A more recent study showed that gastric acid output is significantly higher in *dupA*-positive than in *dupA*-negative patients (Imagawa et al. 2010); serum gastrin levels were also higher in the *dupA*-positive group than in the *dupA*-negative group, suggesting that gastric acid secretion might be higher in the *dupA*-positive group than in the *dupA*-negative group (Shiota et al. 2012). In an in vitro study, Lu et al. reported that the presence of *dupA* was associated with increased resistance to low pH (Lu et al. 2005). Strains that are *dupA*-positive may induce a high level of gastrin and high gastric acid secretion. The high gastric acid secretion in the *dupA*-positive group might have been related to the lower rate of eradication, although the prevalence of *dupA* was not different between DU and gastritis cases in this study (Shiota et al. 2012). If *dupA* positivity is associated with high acid output, its prevalence should be higher in DU than in gastritis. Other mechanisms may correlate with the low rate of eradication in patients with *dupA*-positive *H. pylori* infection. Therefore, further study is necessary to identify the mechanism of the lower rate of eradication in *dupA*-positive groups.

23.5 Treatment Regimens for *H. pylori* Eradication in Asia

Various combinations of PPIs and antimicrobial agents have been designed to treat *H. pylori* infection. These regimens include triple therapy, bismuth- and non-bismuth-containing quadruple therapy, sequential therapy, and hybrid therapy. As a general rule, clinicians should prescribe therapeutic regimens that have a ≥90 % or preferably ≥95 % eradication rate locally (Rimbara et al. 2011). If no available regimen can achieve ≥90 % eradication, clinicians should use the most effective regimens available locally.

Guidelines for the management of *H. pylori* infection are still evolving and vary according to the geographic area. First-line, alternative first-line, second-line, or even third-line therapies have been proposed. Recent guidelines proposed for the Asia-Pacific region, World Gastroenterology Organisation global guidelines for developing countries, and guidelines for three countries in East Asia are summarized in Table 23.3 (Fock et al. 2009; Kim et al. 2013a; Asaka 2013; Chinese Society of Gastroenterology et al. 2013). However, some regimens are confined to very small geographic districts, therefore not encouraging the development of therapeutic guidelines that could be valid worldwide. Therefore, geographic patterns of antibiotic resistance must be considered. According to current guidelines, standard triple therapy containing a PPI and two antibiotics, clarithromycin and amoxicillin/metronidazole, is still the first-line regimen for treatment of *H. pylori* infection (Fock et al. 2009; Kim et al. 2013a; Asaka 2013; Chinese Society of Gastroenterology et al. 2013). However, in recent years, the efficacy of legacy triple regimens has been seriously challenged, and eradication rates lower than 70 % are now reported in many countries (Papastergiou et al. 2014). In East Asia, the revised 2013 version of the Japanese guideline recommends a lower dose of antibiotics for a shorter duration (7 days) than guidelines from China or South Korea. No 14-day treatments or bismuth-based regimens are recommended as first- or second-line treatments in Japan. The first-line therapy approved by the Japanese health insurance system is clarithromycin-containing triple therapy. Therefore, many Japanese physicians currently prescribe clarithromycin-containing triple therapy according to the national health insurance system, even with the knowledge that this regimen is not effective in areas with a high prevalence of clarithromycin-resistant strains. Although the Japanese health insurance system has not approved a metronidazole-containing regimen as a first-line eradication regimen yet, it would be a better future first-line therapy in Japan than clarithromycin-containing triple therapy. When *H. pylori* eradication fails in patients undergoing clarithromycin-based triple therapy, metronidazole-based triple therapy can be used as a second-line eradication regimen. This second-line therapy was reported to be highly successful, with an eradication rate of more than 90 % in Japan (Murakami et al. 2008; Shimoyama

Table 23.3 Treatment regimens for *H. pylori* eradication in Asia

Guidelines	First-line treatment	Second-line treatment
Second Asia-Pacific Consensus 2009	*Standard PPI-based triple therapy: 7–14 days*	*Quadruple therapy: 7–14 days*
	PPI, amoxicillin 1 g, clarithromycin 500 mg twice daily	PPI twice daily, bismuth 240 mg twice daily, metronidazole 400 mg twice daily or three times daily, tetracycline 500 mg four times daily
	PPI, metronidazole 400 mg, clarithromycin 500 mg twice daily	Levofloxacin-based triple therapy: 10-day PPI, levofloxacin 250 mg (or 500 mg), amoxicillin 1 g twice daily
	PPI, amoxicillin 1 g, metronidazole 400 mg twice daily	
	Quadruple therapy: 7–14 days	Rifabutin-based triple therapy: 7–10-day PPI, rifabutin 150 mg, amoxicillin 1 g twice daily
	PPI twice daily, bismuth 240 mg twice daily, metronidazole 400 mg twice daily or three times daily, tetracycline 500 mg four times daily	
Global guidelines for developing countries	*Triple therapy: 7 days*	*Quadruple therapy*
	PPI + amoxicillin + clarithromycin /furazolidone, twice daily	PPI + bismuth + tetracycline + metronidazole for 10–14 days
	Quadruple therapy (clarithromycin resistance > 20 %): 7–10 days	PPI + furazolidone + tetracycline + bismuth for 10 days
	PPI twice daily + bismuth + tetracycline + metronidazole all four times daily	*Triple therapy*
	Quadruple therapy (no known clarithromycin resistance, bismuth unavailable): 14 days	PPI + furazolidone + levofloxacin for 10 days
	PPI + clarithromycin + metronidazole + amoxicillin	PPI + amoxicillin + clarithromycin for 7 days
	Sequential therapy: 10 days	PPI + amoxicillin + levofloxacin for 10 days
	PPI + amoxicillin for 5 days followed by PPI + clarithromycin and a nitroimidazole (tinidazole) for 5 days	
Japan 2013	*Triple therapy: 7 days*	*Triple therapy: 7 days*
	Amoxicillin 750 mg, clarithromycin 200 mg (or 400 mg), and PPI twice daily	Amoxicillin 750 mg, metronidazole 250 mg, and PPI twice daily
China 2013	*Triple therapy: 7–14 days*	*Quadruple therapy: 10–14 days*
	Amoxicillin 1 g (or metronidazole 400 mg), clarithromycin 500 mg, and PPI twice daily	Bismuth 220 mg, tetracycline 750 mg, metronidazole 400 mg twice, and PPI twice daily for 10 or 14 days
Korea 2013	*Triple therapy: 7–14 days*	*Quadruple therapy: 7–14 days*
	Amoxicillin 1 g, clarithromycin 500 mg, and PPI twice daily	Bismuth 120 mg four times, tetracycline 500 mg four times, metronidazole 500 mg thrice, and PPI twice daily for 7–14 days

et al. 2004). Using the metronidazole breakpoint of 8 μg/ml established by the European Study Group, resistance rates did not change from 2002–2003 to 2004–2005 (4.9 %and 3.3 %, respectively) (Kobayashi et al. 2007). In Asia, only Japan, Thailand, and Malaysia have populations with <40 % metronidazole resistance (Table 23.2), and the Maastricht III Consensus Report stated that the use of the PPI + clarithromycin + metronidazole regimen is preferable for these countries. On the contrary, a regimen including metronidazole is not suitable and should not be chosen as first-line treatment therapy in most other Asian countries. Amoxicillin-containing combinations are recommended over those containing metronidazole, due to metronidazole resistance rates. Alternatively, PPI + clarithromycin + amoxicillin treatment is the recommended first-choice treatment in populations with less than 15–20 % clarithromycin resistance (Malfertheiner et al. 2007). Therefore, this treatment combination is preferred in almost all Southeast Asian countries (Thailand, Singapore, Malaysia, and Bhutan).

Bismuth quadruple therapy is not completely novel but rather represents an enhanced evolution of the older regimen comprising a bismuth salt, tetracycline, and metronidazole (Papastergiou et al. 2014). This regimen is now designated as a preferred first-line empirical treatment, achieving >90 % eradication in the presence of clarithromycin resistance and >85 % in regions with a high rate of metronidazole resistance (Fischbach et al. 2004). Increased efficacy against metronidazole-resistant strains, which offsets the ability of standard therapies to overcome clarithromycin resistance, is likely the key for the improved performance with bismuth quadruple therapy. It is also a valid, and cost-effective, rescue option after failure of clarithromycin-based regimens. Second Asia-Pacific consensus and global guidelines for developing countries recommend bismuth quadruple therapy as an alternative first-line treatment or as a second-line treatment. As second-line treatment, bismuth quadruple therapy combined with high-dose metronidazole (2000 mg/day) resulted in 90.8 % per-protocol efficacy rates in a Taiwanese study (Kuo et al. 2013). Additionally, efficacy rates of 93.1 % were obtained using standard-dose metronidazole (1600 mg/day) in Shanghai, China, in a setting of high metronidazole resistance (Liang et al. 2013). Interestingly, re-treatment with bismuth quadruple therapy was also acceptable as a third-line option (66.7 % by intention-to-treat analysis and 75.0 % by per-protocol analysis) after second-line eradication failures with the same regimen in Korea (Lee et al. 2011). Although bismuth quadruple therapy has been officially substituted for standard triple therapy in high clarithromycin resistance areas, due to its side effects, bismuth is no longer available in many countries, including Japan, Malaysia, Indonesia, and Australia. Therefore, sequential treatment or a non-bismuth quadruple therapy (concomitant) treatment is recommended as the alternative first-line treatment in areas of high clarithromycin resistance (Yang et al. 2014; Yanai et al. 2012). Unfortunately, out of a total of nine bismuth quadruple therapy regimens tested in Iran, with varying (7-, 10-, and 14-day) durations, none had acceptable eradication rates. Similarly,

only one of nine studies conducted in Turkey has reported a 90.1 % per-protocol eradication rate (Fakheri et al. 2014).

A novel non-bismuth quadruple therapy combination has also been developed. This was originally developed in an attempt to decrease the duration of treatment for *H. pylori* infection. In studies performed in the late 1990s, data from Europe and Japan suggested that a short course of 3–5 days using three antibiotics and a PPI could achieve reasonable eradication rates. A meta-analysis of 2070 patients including 14 studies from Asia revealed a mean *H. pylori* intention-to-treat cure rate of 88 % for non-bismuth quadruple therapy (PPI + clarithromycin + amoxicillin + nitroimidazole) (Gisbert and Calvet 2012). Two studies in Japan using PPI + clarithromycin + amoxicillin + metronidazole as quadruple therapy reported intention-to-treat cure rates of 92.5 and 94.5 % (Nagahara et al. 2000, 2001). On the another hand, two studies in South Korea reported intention-to-treat cure rates of only 81.1 % for the PPI + clarithromycin + amoxicillin + metronidazole regimen. The rate was increased by using levofloxacin-based non-bismuth quadruple therapy (intention-to-treat cure rate 87.5–91.4 %) for 5 and 7 days (Kim et al. 2013b; Kwon et al. 2000). However, we must remember the importance of levofloxacin-based therapy as a rescue treatment after failure with other regimens.

Although it consists two dosing periods, sequential therapy is a quadruple therapy consisting of one PPI and three antibiotics. Hypothetically, amoxicillin during the first 5 days of therapy would weaken the bacterial cell wall, which prevents the formation of the channels that prevent clarithromycin from entering the bacterium and hence confer resistance to the antibiotic. Then, in the second phase of therapy, clarithromycin and a nitroimidazole are added for a further 5 days. PPI is continued throughout the treatment. Global guidelines recommend sequential therapy as an alternative first-line treatment. A meta-analysis of 46 randomized controlled trials, including several countries in Asia (nine in China, seven in South Korea, and three in Taiwan), found that sequential therapy was superior to 7 days of triple therapy and marginally superior to 10 days of triple therapy, but not superior to 14 days of triple therapy, bismuth quadruple therapy, or non-bismuth quadruple therapy (Gatta et al. 2013). Although several studies have demonstrated the efficacy of sequential therapy, most of them did not perform susceptibility testing. A study conducted a multicenter randomized controlled trial to compare the efficacy of sequential therapy for 10 and 14 days with that of 14 days of triple therapy as first-line treatment (Liou et al. 2011). In addition to antibiotic resistance, they examined host cytochrome P 2C19 polymorphisms and bacterial virulence factors such as *cagA* and *vacA*. They found that the successful eradication rate was significantly higher following 14 days of sequential therapy than 14 days of triple therapy. In addition, 14 days of sequential therapy was more effective against either clarithromycin-sensitive or clarithromycin-resistant strains of *H. pylori* than 10 days of sequential therapy. Cytochrome P 2C19 polymorphisms and bacterial virulence factors did not influence the rate of successful eradication, but the eradication rates of each treatment were affected by clarithromycin resistance.

Even after 14 days of sequential therapy, successful eradication was achieved in only 71 % of patients with clarithromycin-resistant *H. pylori* strains. This finding suggests that antibiotics other than clarithromycin, rather than sequential therapy, are better for eradication in this population.

Dual-concomitant (hybrid) therapy is another novel regimen, consisting of dual therapy with a PPI and amoxicillin over the first 7 days, followed by a concomitant quadruple therapy containing a PPI plus amoxicillin, clarithromycin, and metronidazole over the second 7 days. This regimen seems to be effective in areas with dual resistance to metronidazole and clarithromycin. A study by Hsu et al. reported a 99 % per-protocol eradication rate with hybrid therapy (Hsu et al. 2011). In an Iranian study, 420 patients were randomized to receive either hybrid or sequential therapy. The eradication rates with hybrid therapy were 92.9 % and 89.5 % on per-protocol and intention-to-treat analysis, respectively, while sequential regimen had 79.9 % per-protocol eradication and 76.7 % intention-to-treat eradication rates (Fakheri et al. 2014).

23.6 Conclusions and Outlook

In conclusion, eradicating *H. pylori* not only heals peptic ulcers but also prevents their recurrence, reduces the risk of development of gastric cancer, may prevent the spread of infection, and reduces future costs arising from treatment of subsequent *H. pylori*-associated diseases. Several new guidelines in East Asian countries contain expanded indications for *H. pylori* eradication. According to current guidelines, standard triple therapy containing a PPI and two antibiotics, clarithromycin and amoxicillin/metronidazole, is still the preferred first-line regimen for treatment of *H. pylori* infection. However, in recent years, the efficacy of legacy triple regimens has been seriously challenged, and their rates of effectiveness have fallen. Moreover, some regions in Asia are exhibiting emerging patterns of antimicrobial resistance. Clarithromycin-containing triple therapy should be abandoned, as it is no longer effective unless local clarithromycin resistance is low or culture confirms susceptibility to clarithromycin. More effective clarithromycin-based regimens are now replacing standard triple therapies as empirical first-line treatments, on the basis of local rates of *H. pylori* antimicrobial resistance (Table 23.4). These regimens include bismuth and non-bismuth quadruple, sequential, and dual-concomitant (hybrid) regimens.

Table 23.4 Resistance region and possibility regimens for *H. pylori* eradication in Asia

		First- and second-line therapy						Rescue therapy	
Resistance region type	Country	Clarithromycin-based triple therapy	Metronidazole-based triple therapy	Bismuth-based quadruple therapy	Non-bismuth quadruple “concomitant” therapy	Sequential therapy	Hybrid therapy	Levofloxacin-based triple therapy	Rifabutin-based triple therapy
Low four antibiotic resistance	Taiwan, Thailand, Malaysia	√	√	√	√	√	√	√	√
High clarithromycin resistance (>20 %)	Japan		√	√	√	√	√	√	√
High metronidazole resistance (>40 %)	China-Hong Kong, Saudi Arabia, Singapore, Bhutan	√		√	√	√	√	√	√
High clarithromycin and metronidazole resistance	Turkey, Bahrain, Vietnam			√	√		√	√	√

High clarithromycin and levofloxacin resistance	South Korea		√	√	√	√	√	√	√
High clarithromycin, metronidazole, and levofloxacin resistance	China-Bei-jing and Southeast China			√	√			√	√
High clarithromycin, metronidazole, and amoxicillin resistance	Indonesia			√			√	√	√
High clarithromycin, metronidazole, amoxicillin, and levofloxacin (ciprofloxacin) resistance	Iran, India, Pakistan			√					√

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Chapter 24
Probiotics as an Alternative Therapy for *Helicobacter pylori*-Associated Diseases

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Abstract Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host. Probiotics are not part of the current treatment therapies prescribed for *Helicobacter pylori* eradication, but there are numerous studies, most of them using probiotics as adjuvants to therapy, showing a reduction of side effects in the greater majority of cases. The probiotics administered vary hugely in the composition of microorganisms used, as well as the duration and mode of administration, which renders the comparison difficult. However, the most used probiotics for *H. pylori* infection are composed of *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, and *Streptococcus*. The mode of action of these probiotics relies on competition for nutrients and for adhesion to cell receptors, antimicrobial activity, and modulation of the immune system and microbiota.

Keywords *Helicobacter pylori* • Probiotics • Microbiota • *Lactobacillus* • *Bifidobacterium* • *Saccharomyces* • *Streptococcus*

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24.1 Introduction

Helicobacter pylori, a curved, microaerophilic, Gram-negative bacterium which colonizes the mucus layer of the gastric epithelium, is the causative agent of chronic active gastritis and ulcer disease and it is associated with an increased risk for gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Basso et al. 2010).

Eradication of *H. pylori* is recommended in patients with (a) peptic ulcer disease and low-grade MALT lymphoma, (b) gastric precancerous lesions, (c) first-degree relatives of patients with gastric cancer, and (d) patients with unexplained iron deficiency anemia (Malfertheiner et al. 2012). Gastric cancer is a major public health issue and the global burden of gastric cancer is increasing, particularly in developing countries (Crew and Neugut 2006). Several studies highlight that *H. pylori* eradication could reduce the risk of gastric cancer, thus playing a central role in the prevention of this malignancy (Wong et al. 2004).

H. pylori eradication is currently a challenge in many parts of the world, due to enormous raise in antibiotic-resistant strains. Therefore, alternative therapeutic regimens are urgently needed.

Probiotics are living organisms that have a long history of use to promote human health. In relation to disease, their beneficial effects have been explored as well, especially in the prevention and treatment of gastrointestinal disorders such as diarrhea, inflammatory bowel disease, irritable bowel syndrome, and liver disease.

In the context of *H. pylori* infection, the effect of probiotics is not so straightforward, due to specific niche of *H. pylori*, the human stomach.

In theory, probiotics could be used as a single therapy against *H. pylori*. Indeed, lactic acid bacteria and bifidobacteria produce several substances, such as organic acids, hydrogen peroxide, carbon dioxide, and other bactericidal compounds with the potential to inhibit *H. pylori* (Servin 2004). In addition, probiotics could compete with *H. pylori* for adhesion receptors and nutrients and strengthen the mucin barrier of the epithelium, inhibiting the bacteria's adherence to epithelial cells (Mattar et al. 2002; Nam et al. 2002). However, to date, no study has demonstrated complete eradication of *H. pylori* infection by probiotic treatment and therefore, their use as a single therapy is currently unviable.

Nevertheless, the use of probiotics as an adjuvant of antibiotic-based therapies, in order to reduce side effects or even to increase therapy efficacy, is an interesting approach. Probiotics can modify the immunologic response of the host by interacting with epithelial cells and modulating the secretion of anti- and pro-inflammatory cytokines, what could result in a reduction of gastric activity and inflammation associated with *H. pylori* infection, as experimentally demonstrated with animal models (reviewed in (Patel et al. 2014)). These effects could increase the efficacy of antibiotic therapies and also help the injured gastric epithelium to restore itself more rapidly. Accordingly, the use of probiotics with concomitant antibiotic therapy against *H. pylori* would serve a role in restoring the homeostasis of microbiota and therefore in reducing side effects. However, the

clinical trials using probiotics as a complement to *H. pylori* eradication treatment are very diverse in their design and probiotic content, which may explain the diversity of the effect observed (Canducci et al. 2000; Goldman et al. 2006).

24.2 The Human Gut Microbiota

The definition of gut microbiota corresponds to the collective set of microorganisms that colonize the human gastrointestinal tract. A typical human may carry over 40,000 different bacterial species in the intestinal microbiome (McFarland 2010). Until recently, newborn babies were considered to be born sterile, but current evidence points otherwise (Jimenez et al. 2008), showing that *Bacilli* and other *Firmicutes* are the main bacteria groups detected in the meconium while *Proteobacteria* dominate in the fecal samples (Moles et al. 2013). Indeed, most phylotypes belong to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* (Gotteland et al. 2006). At the microbiome phylum level, humans are similar to one another and to other mammals, whereas at the genus, species, and strain population levels, the diversity is highly specific for each individual (Blaser 2010; Costello et al. 2009). Several pathologies are associated with alterations in host-associated microbial communities, including obesity, malnutrition, and a variety of inflammatory diseases of the skin, mouth, and intestinal tract. The human body today is viewed as an ecosystem, and human health can be construed as a product of ecosystem services delivered in part by the microbiota (Costello et al. 2012).

A recent study showed that the host diet is of paramount importance on gut microbiota composition and activity, strengthening the ecosystem view. Schnorr and co-workers (2014) studied the phylogenetic diversity and metabolite production of the gut microbiota from 27 hunter-gatherers, the Hadza of Tanzania, and compared them with 16 urban Italian adults from Bologna, Italy. They observed that their gut microbiome profiles are quite distinct even at the phylum level. *Firmicutes* and *Bacteroidetes* are the dominant phyla in both Hadza and Italian gut microbiome; Hadza are characterized by a relatively higher abundance of *Bacteroidetes* and a lower abundance of *Firmicutes*. The two gut microbiome ecosystems are remarkably different with respect to subdominant phyla (<10 % relative abundance): Hadza are largely enriched in *Proteobacteria* and *Spirochetes*, which are extremely rare in the Italian, while *Actinobacteria*, an important subdominant component of the Italian microbiome, are almost completely absent from the Hadza microbiome. They also conclude that the microbiome, as a diverse and responsive ecosystem, is adapting continuously as a commensal component of the host supraorganism; the functional redundancy found in bacterial communities indicates that microbial activity is the important aspect, rather than species composition.

The gut microbiome is so complex and diverse that one may have the impression that it will take a long time before some of this information can be used to treat

certain human pathologies. The normal microbiome remains to be defined but when known may be useful for its reposition in patients (Ling et al. 2013). Indeed, the administration of exogenous bacteria has been shown to improve some conditions, such as *Bacteroides fragilis* which in a mouse model corrected gut permeability, altered microbial composition, and improved defects in communicative, stereotypic, anxiety-like, and sensorimotor behavior (Hsiao et al. 2013), or the fecal transplants in the treatment of the *Clostridium difficile* infection, so far the most efficient way to remove the pathogen from the gut (van Nood et al. 2013).

The gut microbiota may serve as a virtual endocrine organ, which is corroborated by their direct role in metabolic pathways which produce and regulate multiple compounds that reach the circulation and influence the function of distal organs and systems. For example, the probiotic *Lactobacillus rhamnosus* PL60 produces conjugated linoleic acid which has been shown to reduce body weight gain and white adipose tissue without effects on food intake (Clarke et al. 2014).

H. pylori colonize a peculiar ecological niche, the stomach mucin layer, probably being the principal bacteria present in the stomach of infected patients, although the presence of a transient gastric flora, to which *Lactobacillus* strains belong, cannot be ignored (Gotteland et al. 2006). Moreover, a recent study reports that *Bifidobacteriaceae* species present in the oral cavity can colonize the omeprazole-treated hypochlorhydria stomach to quite a large degree (Mattarelli et al. 2014). There are conflicting findings regarding the influence of *H. pylori* in the stomach microbiota (Wang et al. 2014). The microbiota of the human stomach and its influence on *H. pylori* colonization has been characterized showing that *H. pylori* does not affect the composition of the gastric community (Bik et al. 2006). However, a positive *H. pylori* status has been associated to an increased relative abundance of non-*Helicobacter* bacteria from the *Proteobacteria*, *Spirochetes*, and *Acidobacteria* and with decreased abundance of *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* (Wang et al. 2014).

The gastric microbiota is highly controlled by pH and may play a role in the development of gastric cancer. In the presence of lower acid secretion due to gastric atrophy, bacteria overgrowth is favored in the gastric fluid, enhancing the production of carcinogenic N-nitrosamine compounds (Wang et al. 2014). The gastric microbiota in gastric cancer patients is comprised predominately of *Veillonella*, *Haemophilus*, *Streptococci*, *Lactobacillus*, *Prevotella*, and *Neisseria* (Dicksved et al. 2009).

24.3 The Omics Era in Probiotics

The first genome to be completely sequenced was published in Nature in 1976. The sequencing was carried out by Walter Fiers and his group in Gent, Belgium. It concerned the bacteriophage MS2, whose genome is a small linear single-stranded RNA molecule with 3569 bases, which codes for four genes (Fiers et al. 1976). Twenty-one years later, the first complete microbial genome of *Haemophilus*

influenzae Rd, with 1,830,137 base pairs, was published (Fleischmann et al. 1995), and soon after a large number of other bacteria were sequenced, as well as the fruit fly, baker's yeast, and the human genome.

All of this sequencing interest stimulated the improvement of the DNA sequencing method developed by Fred Sanger as well as the search for novel sequencing technologies, and today, there are several companies selling large and small sequencing machines, based on several different technologies. They are faster, less expensive, and more informative than the pioneer Sanger method (Brown 2013; Sanger et al. 1977). These new technologies, known as "next-generation sequencing technologies" (NGS), are able to surpass the major limitation of microbiology: the inability to cultivate most of the earth's microorganisms (Brown 2013).

The first attempts to understand the diversity of microorganisms in the human body were carried out using polymerase chain reaction (PCR)-based technologies, targeting 16S RNA sequences, and soon it was clear that the number of species present in the human gut was very large (Hayashi et al. 2002; Suau et al. 1999). Using NGS will probably clarify the (1) identity of the microbes that populate each host, (2) the role of the microbiota, (3) the response of the host to these microbes, (4) the forces that maintain equilibrium among the populations, and (5) the unique characteristics of each individual (Blaser 2010). After a full comprehension of the microbiota, it will be possible to study how probiotics interfere and module it. The National Institute of Health (NIH) decided to start a program to finance the study of the microbiota by examining at least four body sites: the gastrointestinal tract, the mouth, the vagina, and the skin. The Human Microbiome Project was designed and its major goal was to demonstrate the feasibility of characterizing the human microbiome well enough to enable study of its variation and its influence on disease. Fifteen projects were funded, and more than half were gut related (Peterson et al. 2009).

24.3.1 Probiotics

The term probiotics means "for life" and was most probably first addressed by Ilya Mechnikov (1908 Nobel Prize winner, natural scientist/microbiologist at Pasteur Institute), who proposed the use of diet containing milk fermented by bacilli which produce large amounts of lactic acid to prevent the multiplication of certain bacteria that poison the body (reviewed in the Nobel Lectures, (Nobel Foundation 1967)). Also in France, the pediatrician Henry Tissier suggested the administration of *Bifidobacterium* (designated as Y-shaped bacteria abundant in healthy children) to patients with diarrhea to restore gut flora (Tissier 1906).

Presently, probiotics are defined as "live organisms which administered in adequate amounts confer a health benefit to the host" (Pineiro and Stanton 2007). Although probiotics may exert their function by products of theirs metabolism, such as enzymes or bacteriocins (proteins lethal to bacteria other than the producing strain) that do not require live cells, dead microorganisms are not probiotics.

Moreover, microbiota microorganisms are not probiotics. Microbiota may be the source of probiotics, but the term should only be used after strain isolation and characterization, namely, considering their health effects, dose, and stability (Sanders 2008). Specificity of probiotic action and the ability to remain viable at the target site is the most important point rather than the source of the microorganism (Pineiro and Stanton 2007). In fact, the guidelines established by Food and Drug Administration (FAO) and World Health Organization (WHO) recommend (1) the identification to the level of strain of all probiotics in the product, (2) the depositing of all strains in an international culture collection, (3) naming the strain according to the International Code of Nomenclature, (4) characterization of strain safety and function (probiotic effects are strain specific), and (5) validation of health benefits in human studies, including the dose required to provide the benefit (FAO and WHO 2001, 2002). The mode of action of probiotics is discussed below, but their action may prevent pathogenic bacteria infection through stimulation of the immune response, competition with pathogenic bacteria, prevention of antibiotic side effects, and improvement of eradication rates (Lesbros-Pantoflickova et al. 2007; Lionetti et al. 2010; Miki et al. 2007; Vitor and Vale 2011).

The increasing difficulty in eradicating *H. pylori* when therapy is recommended has incited a search of new treatments and an attractive field within *H. pylori* research. Probiotics are probably at the top of the list for alternative therapies against *H. pylori*, although frequently administrated in conjugation with antibiotics (Vitor and Vale 2011). According to the Web of Science, the number of publications addressing probiotics and *H. pylori* increased especially in the ten last years (Fig. 24.1). The total number of publications in Thomson Reuters Web of Science addressing *H. pylori* and probiotics is 394 (December 2014). The analysis of Fig. 24.1 shows that the most studied probiotic microorganism is *Lactobacillus* followed by *Bifidobacterium*, *Saccharomyces*, and *Streptococcus*. The two

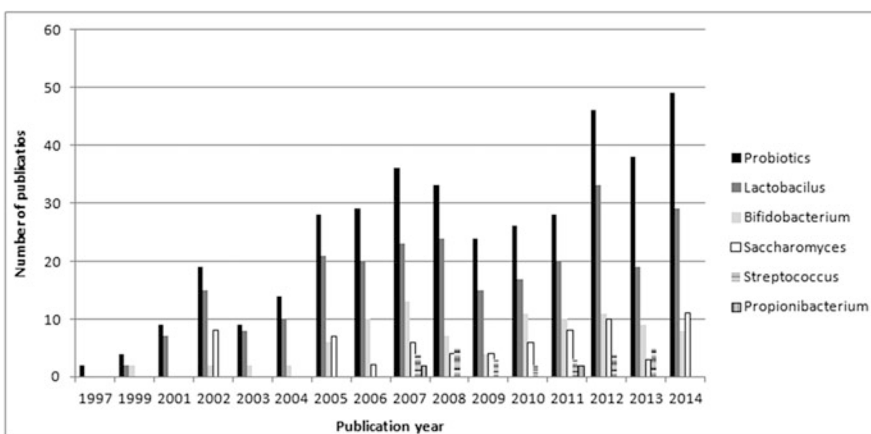

Fig. 24.1 Number of Thomson Reuters Web of Science publications addressing *H. pylori* and probiotics from 1997 to 2014. The number of publications is only displayed if ≥ 2

microorganisms used the most for studying their impact on *H. pylori* infection, *Lactobacillus* and *Bifidobacterium*, belong to the genera most present as probiotics in food (Pineiro and Stanton 2007). The number of publications addressing the use of probiotics reflects their increasing importance in *H. pylori* infection, as scientific evidence continues to accumulate on the properties, functionality, and benefits of probiotics for the promotion of human health, namely, improving immunological and digestive functions and alleviating infectious disease (Pineiro and Stanton 2007).

Probiotic studies include not only a wide range of microbial species, administered alone or in combination with each other, which complicates the comparison of studies. However, an observation of the most recent meta-analysis shows that the majority of the probiotics reveal a consistent, statistically significant increase in eradication rates and reduced side effects from antibiotic therapy (Molina-Infante and Gisbert 2013).

The clinical trials included in the meta-analysis of Tong and co-workers (2007) applying multiple strains from different species (Cao et al. 2005; Cremonini et al. 2002; Myllyluoma et al. 2005; Sheu et al. 2002) presented an odds ratio (OR) favoring probiotics, except for the study of Goldman et al. (2006) which only included children. However, only one of the trials (Sheu et al. 2002) applying a combination of *Lactobacillus* and *Bifidobacterium* was statistically significant. Furthermore, when the Goldman study was compared with other clinical trials performed on children (Pacifico et al. 2014), it favored probiotic administration even though the results were not statistically significant. Pacifico and colleagues reported only one study applying a mixture of probiotics to children (Ahmad et al. 2013) was statistically significant (Pacifico et al. 2014).

The effect of probiotics is known to be species specific and the next topics present work developed specifically according to the bacterial genus.

24.3.2 Lactobacillus

Over 100 species belong to the genus *Lactobacillus*, which is the largest group among the *Lactobacteriacea*. *Lactobacilli* are nutritionally fastidious and are associated with a large variety of plants and animals, including the human gut microbiota. These lactic acid bacteria, whose primary fermentation end product is lactic acid, are used extensively for the fermentation of dairy products (Canchaya et al. 2006). The species used the most frequently in clinical trials are *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, and *Lactobacillus sporogenes* (Pacifico et al. 2014; Tong et al. 2007; Wang et al. 2013).

Lactobacillus species is not only the most studied probiotic but also the first tested against *H. pylori*. Shortly after the discovery of *H. pylori*, *L. acidophilus* was described in the in vitro inhibition of *H. pylori*, as was the inhibition by both

L. acidophilus culture supernatant and 1 and 3 % lactic acid (Bhatia et al. 1989). At this time, the inhibitory effect was related to the extracellular secretory products of *Lactobacillus*, since *H. pylori* was also inhibited by solutions of lactic acid (Bhatia et al. 1989; Lambert and Hull 1996). Other *Lactobacillus* species, especially *L. casei*, were then also associated with an inhibitory effect on the growth of *H. pylori*. Strain specificity concerning an inhibitory effect on *H. pylori* was also described, since not all strains tested presented the same effect (Midolo et al. 1995). There are numerous in vitro studies demonstrating the capacity of inhibition of *H. pylori* growth by *Lactobacillus* spp. (reviewed in (Gotteland et al. 2006; Vitor and Vale 2011)). *Lactobacillus* spp. belong to the group of microbiota gut bacteria which being acid resistant can persist in the stomach longer than other bacteria (Gotteland et al. 2006). An important additional characteristic of *Lactobacillus* spp. is their ability to adhere to gastric cells in vitro (Conway et al. 1987) and in vivo, both in animals (Declich et al. 2000) and in humans (Valeur et al. 2004), and to inhibit *H. pylori* adherence to gastric epithelial cells, which is fundamental for the establishment of the infection, as demonstrated in vitro using cultured standard laboratory cell lines such as AGS (Lin et al. 2009; Rokka et al. 2008) and Caco-2 (Myllyluoma et al. 2008).

Valeur and co-workers demonstrated by fluorescence in situ hybridization (FISH) the colonization of the human stomach, duodenum, and ileum by *L. reuteri* after the administration to 19 volunteers of a dietary supplementation of strain ATCC 55730, a heterofermentative autochthonous bacterium of the gastrointestinal tract of humans and animals (Valeur et al. 2004). The presence of probiotics was also highlighted by strain-specific real-time PCR, showing that the concentrations of probiotics were significantly higher in the probiotic group than in the placebo group during the probiotic intervention (Karjalainnen et al. 2012; Myllyluoma et al. 2005), reinforcing the capacity of colonization of the gut by probiotics.

Recent meta-analyses reinforce the increased eradication rate and reduction of antibiotic side effects during the (antibiotic) treatment of *H. pylori* supplemented by probiotics containing *Lactobacillus*, as well as *Bifidobacterium* (Tong et al. 2007; Wang et al. 2013). Moreover, the most recent meta-analysis considering ten clinical trials presented an odds ratio (OR) of 0.31 (95 % confidence interval (CI), 0.12–0.79) for the incidence of total side effects in the probiotic supplementation (*Lactobacillus* and *Bifidobacterium*) versus treatment without probiotics, while eradication rates by intention-to-treat (ITT) analysis presented an OR of 2.07 (95 % CI 1.40–3.06), favoring probiotics. However, current clinical trials, besides being very diverse in their design, have not included all human populations, namely, North Americans and black Africans (Wang et al. 2013).

Clinical trials using *Lactobacillus* or placebo supplementation to antibiotic therapy present eradication rates favoring probiotics (Tong et al. 2007), especially in the study by Canducci and colleagues (2000), which showed a statistical significant result in comparison to other clinical trials (Armuzzi et al. 2001a, b; Sykora et al. 2005). The comparison of seven clinical trials targeting children (Pacifico et al. 2014) with one group receiving antibiotic therapy and another receiving the

same therapy plus a probiotic showed a globally beneficial effect of probiotics concerning the probiotic group, although of the three studies that used only *Lactobacillus* strains (Lionetti et al. 2006; Sykora et al. 2005; Szajewska et al. 2009), only one (Sykora et al. 2005) presented a statistically significant result. In a clinical trial, patients who received a lyophilized and inactivated culture of *L. acidophilus* showed an improved eradication rate in comparison to patients just receiving antibiotic therapy (Canducci et al. 2000), but according to the definition of probiotic, this is not a probiotic trial.

Several clinical trials rely on the administration of dairy products. The storage conditions to preserve the required number of probiotics are extremely important. For instance, the initial dose of colony-forming units (CFU) ($1–1.4 \times 10^7$) of *L. gasseri* (LG 21) present in a yogurt decreased by approximately 50 % following 1 week of storage (Sakamoto et al. 2001), which may introduce a bias in the clinical trials since the probiotic load appears to be very important for a successful eradication.

24.3.3 Bifidobacterium

The genus *Bifidobacterium* belongs to the phylum *Actinobacteria* and is characterized by high G+C content Gram-positive bacteria, which are common inhabitants of the gastrointestinal tract of mammals, birds, and certain cold-blooded animals (Turroni et al. 2011). Bifidobacteria were first isolated from feces of a breast-fed infant in 1899 and were quickly associated with a healthy infant gut because of their predominance in breast-fed infants in comparison to formula-fed ones. The human milk seems to be a source of living bifidobacteria for the infant gut (Martin et al. 2009). Due to their morphological and physiological features, similar to lactobacilli, they were classified as members of the genus *Lactobacillus* during a large part of the twentieth century and have been recently recognized as a different genus. Currently, the genus *Bifidobacterium* is comprising over 30 species. Bifidobacteria are important members of the human gut microbiota and play a beneficial role in maintaining the health of the host (Martin et al. 2009; Turroni et al. 2011). Together with lactic acid bacteria, bifidobacteria are used in yogurt production and are known to have probiotic effects (Wang et al. 2004).

Bifidobacteria are known to adhere to epithelial cells and inhibit enteropathogenic cell interactions (Bernet et al. 1993). The inhibition of *H. pylori* by *Bifidobacterium* appears to occur via immunomodulatory effects (Shirasawa et al. 2010; Wang et al. 2013; Zhang et al. 2008). In fact, *H. pylori* upregulates the expression of the inflammatory chemokine interleukin (IL)-8 in the gastric mucosa. A microarray study demonstrated that the expression of IL-8 diminished in the human scirrhus-type gastric cancer epithelial cell line, GCIY, which had been preincubated with the probiotic *Bifidobacterium bifidum* BF-1 and then infected with *H. pylori*. Moreover, the action of *Bifidobacterium* appears to affect the regulatory mechanism of the nuclear factor kappa-B (NF-κB) signaling pathway,

since *H. pylori*-induced and *B. bifidum*-suppressed genes overlap with the genes regulated by NF-κB (Shirasawa et al. 2010).

The majority of the clinical trials that include Bifidobacteria also include other probiotic microorganisms. For example, in one trial, *Bifidobacterium* BF1 and *Streptococcus thermophilus* YIT 2021 fermented milk were administrated to a group of 79 randomized healthy individuals and appeared important for the maintenance of stomach health (Miki et al. 2007); others are described in Table 24.1. Nevertheless, in vitro and in vivo animal studies using only *Bifidobacterium* strains (*B. bifidum* CECT 7366) showed anti-*H. pylori* effects and partially repaired damages in the gastric tissue of the BALB/c mouse model (Chenoll et al. 2011).

24.3.4 Saccharomyces

The third most used probiotic is a eukaryotic microorganism. The yeast *Saccharomyces* comprises eight species (Hittinger 2013). Most of the yeast strains used for alcoholic fermentation are now recognized as *Saccharomyces cerevisiae*, which is used for baking, brewing, winemaking, and distillation industries, and it was first used for the production of fermented beverages in China about 9000 years ago (Dequin and Casaregola 2011). The probiotic yeast is *Saccharomyces boulardii*, which is used worldwide to combat antibiotic side effects. This probiotic yeast was originally isolated from litchi fruit in Indonesia and described as a separate species but is now considered to be conspecific with *S. cerevisiae*. However, the probiotic strains of *S. boulardii* present tight clustering both genetically and metabolically (MacKenzie et al. 2008).

A recent meta-analysis focusing on the efficacy and safety of *S. boulardii* in treating several diseases recommends the use of 1000 mg/day for 2 weeks in supplementation to standard triple therapy to prevent *H. pylori* symptoms (McFarland 2010).

S. boulardii is considered to be safe but may cause fungemia, especially in patients with central venous lines, and this issue should be considered while the number of antimycotics available is reduced (Vandenplas et al. 2009).

Most clinical trials are consensual in demonstrating that the administration of *S. boulardii* diminishes the incidence of side effects (Cindoruk et al. 2007; Duman et al. 2005; Hurduc et al. 2009; Song et al. 2010) but rarely points to an improvement in the eradication rate (Song et al. 2010).

S. boulardii survives in gastric acid and bile only short time (Vandenplas et al. 2009), as less than 1 % of the *S. boulardii* cells are viable 2 h under gastric conditions (Vanhee et al. 2010). A stable concentration of this probiotic is reached after three consecutive days of administration and *S. boulardii* is undetectable 1 week after the end of the treatment (Vandenplas et al. 2009). *S. boulardii* appears to alter the structure of *H. pylori*, as demonstrated in an ultrastructure study in which the closest bacteria to the yeast showed altered morphology (Vandenplas et al. 2009). *S. boulardii* inhibits the NF-κB inflammation pathway via the secretion

Table 24.1 Probiotics tested in blind, placebo-controlled, randomized trials

Probiotic	Patients enrolled	Improved eradication	Improved side effects	Other comments	Reference	Meta-analysis results A – Tong et al. (2007) B – Wang et al. (2013) C – Li et al. (2014)
Lactobacillus casei, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *and Streptococcus thermophilus*	180	No	Yes/no	1×10^8 CFU, twice daily for 14 days Less frequent diarrhea/more frequent abdominal pain	Shavakhi et al. (2013)	
	66 (children)	Yes	Yes	1×10^9 CFU once daily for 4 weeks	Ahmad et al. (2013)	C – Improved eradication and diminished total side effects and nausea/vomiting
Lactobacillus plantarum, *Lactobacillus reuteri*, *Lactobacillus casei subsp. rhamnosus*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus salivarius*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *and Lactobacillus sporogenes* (Probinul – CaDiGroup)	68 (children)	No	Yes	1×10^9 CFU to 5×10^9 CFU daily for 7 days	Tolone et al. (2012)	C – Diminished total side effects and diarrhea
Saccharomyces boulardii	991	Yes	Yes	1.8×10^9 CFU (Bioflor250, Kuhnil Pharmacy), three times a day for 4 weeks. Helped completing therapy	Song et al. (2010)	
	90 (children)	No	Yes	1.8×10^9 CFU (Enterol, Biocodex) once daily for 4 weeks	Hurduc et al. (2009)	C – Diminished total side effects

(continued)

Table 24.1 (continued)

Probiotic	Patients enrolled	Improved eradication	Improved side effects	Other comments	Reference	Meta-analysis results A – Tong et al. (2007) B – Wang et al. (2013) C – Li et al. (2014)
	124	No	Yes	500 mg twice daily for 2 weeks (Reflor, Sanofi–Synthelabo Ilac A.S.)	Cindoruk et al. (2007)	
	389	No	Yes	Prevented antibiotic-associated diarrhea 500 mg twice daily for 2 weeks	Duman et al. (2005)	A – Diminished side effects (diarrhea)
Lactobacillus GG	83 (children)	No	No	10^9 CFU for 7 days	Szajewska et al. (2009)	C – Did not improved eradication or side effects
	60	No	Yes	6×10^9 (Giflorex®, Errekappa Euroterapici) twice daily for 14 days	Armuzzi et al. (2001a)	A – Did not improved eradication Diminished side effects (diarrhea, taste disturbance, and nausea)
	120	No	Yes	6×10^9 twice daily for 14 days	Armuzzi et al. (2001b)	A – Did not improved eradication Diminished side effects (diarrhea and taste disturbance)
Lactobacillus reuteri	40	No	Yes	10^8 CFU (Reuterin, Nóos) once daily for 4 weeks. Decreased the occurrence of dyspeptic symptoms	Francavilla et al. (2008)	
	40 (children)	No	Yes	10^8 CFU (Reuterin, Nóos) once daily for 20 days	Lionetti et al. (2006)	
Lactobacillus casei DN-114 001	86 (children)	Yes	Infrequent side effects	10^{10} CFU – fermented milk (Actimel) for 2 weeks	Sykora et al. (2005)	A – Improved eradication C – Improved eradication
Lactobacillus casei subsp. *casei* DG	70	Yes	Yes	10^9 CFU twice daily for 10 days	Tursi et al. (2004)	A – Diminished total side effects

Lactobacillus johnsonii La1	326 (children)	Yes	–[a]	$>10^7$/ml (80 ml Chamyto, Nestlé-Chile SA) for 4 weeks	Cruchet et al. (2003)	
Lactobacillus gasseri OLL2716	29	Yes	–[a]	1–1.4 × 10^7 CFU/g (90 g yogurt) twice daily for 16 weeks	Sakamoto et al. (2001)	
Bacillus subtilis and *Streptococcus faecium*	352	Yes	Yes	8 weeks	Park et al. (2007)	
Bacillus clausii (*Enterogermina*, Sanofi–Synthelabo)	120	No	Yes	2 × 10^9 spores	Nista et al. (2004)	A – Eradication not significant
				Once daily for 3 weeks		Diminished side effects (diarrhea, epigastric pain, and nausea)
Bifidobacterium animalis and *Lactobacillus casei*	65 (children)	No	No	10^7/ml (200 ml yogurt) once daily for 3 months	Goldman et al. (2006)	A – Did not improve eradication
						B – Did not improve eradication
						C – Did not improve eradication
Lactobacillus and *Bifidobacterium*	138	Yes	Yes	5 × 10^9/ml (200 ml AB-Yogurt, President Corp) for 4 weeks	Sheu et al. (2006)	A – Improved eradication
						B – Improved eradication
	160	Yes	Yes	Restored the depletion of *Bifidobacterium* in stools after triple therapy. 5 × 10^9/ml (200 ml AB-Yogurt, President Corp) twice daily for 5 weeks	Sheu et al. (2002)	A – Improved eradication
						B – Improved eradication

(continued)

Table 24.1 (continued)

Probiotic	Patients enrolled	Improved eradication	Improved side effects	Other comments	Reference	Meta-analysis results A – Tong et al. (2007) B – Wang et al. (2013) C – Li et al. (2014)
Lactobacillus acidophilus LB and *Saccharomyces boulardii*	254 (children)	Yes	–[a]	LA 10^9 heat killed (Lacteol Forte, Laboratory of Dr. Boucard), SB (Perenteryl, Merck Química Chilena) 250 mg for 8 weeks	Gotteland et al. (2005)	
				S. boulardii-eradicated *H. pylori* in 12 % of the colonized children, and *L. acidophilus* in 6 %		
Lactobacillus plantarum, *Lactobacillus reuteri*, *L. casei*, *Lactobacillus salivarius*, *Lactobacillus acidophilus*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Streptococcus thermophilus*	206	Yes	Yes	1–2 × 10^9 (Lf100, Dicofarm®; and Probinul, CaDiGroup®) twice daily for 7 days	de Bortoli et al. (2007)	B – Improved eradication, diminished side effects
	31	No	No	LR 10^8 CFU (Reuflor), others 1–5 × 109 CFU (Probinul) twice daily for 14 weeks	Scaccianoce et al. (2008)	B – Eradication and side effects did not improve
Lactobacillus acidophilus La5 or *Bifidobacterium lactis* Bb12	70	Yes	–[a]	10^7 CFU (AB-Yogurt) twice daily for 6 weeks	Wang et al. (2004)	
Lactobacillus acidophilus HY2177, *Lactobacillus casei* HY2743, *Bifidobacterium longum* HY8001, and *Streptococcus thermophilus* B-1	337	No	No	LA, LC >10^5 CFU/ml, BL >10^6 CFU/ml, ST >10^8 CFU/ml (150 ml Will yogurt) once daily for 4 weeks	Yoon et al. (2011)	B – Eradication and side effects did not improve
	347	Yes	No	Will yogurt once daily for 3 weeks	Kim et al. (2008)	
Lactobacillus rhamnosus GG, *Lactobacillus rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99	58	–	Yes	10^9 CFU/ml twice daily for 1 week and once daily for 3 weeks. Probiotic combination resulted in only minor changes in the microbiota	Myllyluoma et al. (2007)	

L. rhamnosus GG, *L. rhamnosus LC705*, *Bifidobacterium breve Bb99*, *and Propionibacterium freudenreichii ssp. shermanii JS*	47	No	Yes	1×10^9 CFU/ml Twice daily during 7 days and once daily for 3 weeks	Myllyluoma et al. (2005)	A – Eradication and side effects not significant
Lactobacillus GG, *S. boulardii*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis*	85	No	Yes	$5–6 \times 10^9$ twice daily 14 days (Giflorex, Errekappa Euroterapici; Codex, SmithKline Beecham; Ferzym, Specchiasol)	Cremonini et al. (2002)	A – Eradication not significant Diminished side effects (diarrhea and taste disturbance)

[a]Probiotics administered alone (without antibiotic therapy)

of a soluble, heat-stable, small (<1 kDa) anti-inflammatory factor. This anti-inflammatory factor blocks NF-κB activation and NF-κB-mediated IL-8 gene expression, which may be responsible for the anti-inflammatory action of *S. boulardii* (Sougioultzis et al. 2006).

24.3.5 *Streptococcus*

Streptococcus spp. are Gram-positive anaerobic aerotolerant bacteria and the genera *Streptococcus* and *Lactobacillus* belong to the same order, *Lactobacillales*. The most used *Streptococcus* species is *Streptococcus thermophilus*, which is also considered as a lactic acid bacterium, since it is found in milk-derived products. The clinical trials that included *S. thermophilus* used combined probiotics, together with *Lactobacillus* and *Bifidobacterium* (Shavakhi et al. 2013; Ahmad et al. 2013; de Bortoli et al. 2007; Scaccianoce et al. 2008; Kim et al. 2008; Yoon et al. 2011). The results obtained with these clinical trials are rather diverse, although the majority point to a decrease in side effects (Table 24.1). Streptococcal virulence-related genes (associated with pathogenic streptococci) are either inactivated or absent in sequenced genomes of *S. thermophilus* (Prajapati et al. 2013). *S. thermophilus* isolates produce bacteriocins, which are excreted antimicrobial peptides with the ability to inhibit the growth of other bacteria (Gul et al. 2012). Sterilized and neutralized fluid supernatants of *S. thermophilus* (and *Lactobacillus* spp.) were shown to inhibit *H. pylori* in vitro, which suggests the presence of bacteriocin-like substances (Aslim et al. 2011). The other tested probiotic *Streptococcus* is *Streptococcus faecalis*, administrated in combination with *Bacillus subtilis* (Park et al. 2007). *S. faecalis* is a microbiota bacterium of the human gastrointestinal tract. This isolated study showed an increased eradication rate and decrease in side effects (Table 24.1).

24.3.6 *Propionibacterium*

The *Propionibacterium* genus, as well as the *Bifidobacterium* genus, belongs to the *Actinobacteria* class. *Propionibacterium* are Gram-positive, high G+C%, mesophilic, and aerotolerant bacteria, with pleomorphic rods. The main end product of fermentation is named propionic acid. The *Propionibacterium* genus is currently comprised of 14 species, divided in two groups, dairy and cutaneous propionibacteria. Four typical dairy species were described early: *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, and *Propionibacterium thoenii*. *P. freudenreichii*, a food grade bacterium consumed both in cheeses and in probiotic preparations, first described more than a century ago in Swiss Emmental cheese (Thierry et al. 2011), has been used in combination with other probiotics to adjuvant *H. pylori* therapy. *P. freudenreichii*

was in the past divided into two subspecies: *P. freudenreichii* subsp. *freudenreichii* which is unable to ferment lactose and shows nitrate reductase activity and *P. freudenreichii* subsp. *shermanii* which exhibits the opposite properties. *P. freudenreichii* consumption modulates the gut microbiota, enhancing the bifidobacterial population and decreasing the *Clostridium* and *Bacteroides* populations (Saraoui et al. 2013). In vitro studies showed that *P. freudenreichii* subsp. *shermanii* inhibits *H. pylori* adhesion to epithelial cells and, in combination with other probiotics, inhibits *H. pylori*-induced cell membrane leakage and *H. pylori*-induced IL-8 release (Myllyluoma et al. 2008). *P. freudenreichii* ssp. *shermanii* JS has been used in combination with other probiotics (Myllyluoma et al. 2005, 2007), showing an improvement of side effects, which were not confirmed by further meta-analysis studies (Tong et al. 2007).

24.3.7 Other Probiotic Microorganisms

Clinical trials presented in Table 24.1 also show two isolated studies that used *Bacillus* species. Park and co-workers used *Bacillus subtilis* in combination with *S. faecium* (see above) (Park et al. 2007) and Nista and colleagues used *Bacillus clausii* spores alone (Nista et al. 2004), both showing reduction of antibiotic side effects. *Bacillus* are Gram-positive bacteria capable of forming spores. *Bacillus clausii* spores can survive the gastric pH, activate and reach the intestinal tract where they germinate to vegetative forms (Urdaci et al. 2004). *B. clausii* is a probiotic used for viral diarrhea in children and for antibiotic-related side effects that can release antimicrobial substances. *B. clausii* spores and cells can adhere to the bowel wall and colonize the mucosa (Nista et al. 2004).

The list of other probiotics tested in vitro on *H. pylori* cultures is much more extensive. The in vitro test of the effect of 32 microorganisms against *H. pylori* clinical isolates was determined by a diffusion method, showing that several species presented an inhibitory action against *H. pylori*. These were both Gram-positive and Gram-negative microorganisms, such as *Staphylococcus* spp. (*Staphylococcus auricularis*, *Staphylococcus epidermis*, *Staphylococcus hominis*, and *Staphylococcus aureus*), *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Stenotrophomonas maltophilia* (Lopez-Brea et al. 2008). Excluding the pathogenic microbes, the other microorganisms could be a starting point to explore other probiotics to be used with *H. pylori* infection.

24.4 Characterization of Probiotics Studies

A probiotic study typically starts with in vitro tests, where a potential probiotic microorganism is tested by a diffusion method or something similar on an *H. pylori* culture. Microorganisms presenting an inhibitory effect against *H. pylori* are then

further characterized. To determine if the inhibitory action is dependent on a secreted antimicrobial metabolite, culture supernatants of the probiotic may be obtained, concentrated, and tested again by the agar diffusion method (Lopez-Brea et al. 2008). Inhibition liquid culture assay may alternatively be used to screen potential anti-*H. pylori* activity of probiotics (Hsieh et al. 2012). Also of importance is the study of adherence to mucus and/or human epithelial cells and cell lines. The use of gastric epithelial cells cocultured with *H. pylori* and the probiotic allows further characterization, for instance, of changes in the adhesion of *H. pylori* to these cells or alteration of cytokine secretion by the epithelial cells (Hsieh et al. 2012; Myllyluoma et al. 2008; Shirasawa et al. 2010). Of course, before proceeding to animal trials, certain tests should be performed, namely, the resistance to acidity, bile acid resistance, and the ability to reduce pathogen adhesion to surfaces (FAO and WHO 2002). Animal models permit further study of the gastric acid regulation, the histopathological analysis, the bacterial load and motility, and immunologic response, among others (Hsieh et al. 2012; Isobe et al. 2012). The own microbiota of the animal model is not currently considered but may interfere with the effect of the probiotics administered. The final step is the use of clinical trials (Table 24.1). The randomized, double-blind placebo-controlled human trials available are still few, some involving a small numbers of patients, and often difficult to compare because of differences in probiotic strains employed, doses, and formulation (Bergonzelli et al. 2005).

24.5 Probiotics and Immune System Stimulation

The beneficial effect of probiotics on human health was originally attributed to improvements in the intestinal microbial balance, but there is now growing evidence that probiotics can also provide benefits by modulating immune functions. A few examples of probiotic impact at different levels of the immune system will be given.

Probiotics can act at different levels as immunomodulators, by influencing specially the adaptive immunity, both the systemic and the mucosal types (reviewed in (Kemgang et al. 2014)).

The mucosal immunity is mediated by intraepithelial lymphocytes and other immunomodulatory cells of the mucosal lamina propria, such as cytokine-producing cells, phagocytic cells, goblet cells, and IgA-secreting cells.

Several studies support the effect of probiotic lactobacilli in IgA secretion in intestinal fluid and in the number of IgA-secreting cells. For example, two studies using mice models showed the ability of *L. casei* Zhang (Ya et al. 2008) and *Lactobacillus crispatus* KT-11 (Tobita et al. 2010) to increase the resistance of gastrointestinal mucosa to infections by inducing a significant increase in the concentration of secretory IgA in intestinal fluid, in a dose-dependent manner compared with the controls. Another study showed that yogurt supplemented with

L. acidophilus and *Bifidobacterium* spp. administered to mice stimulated enhanced mucosal and systemic anticholera toxin IgA (Sanders and Klaenhammer 2001).

The most extensively studied effect of immunomodulation by probiotics is cytokine secretion from intestinal epithelial cells, which is strongly dependent on the probiotic strain, with differences being observed even among strains of the same species.

Several probiotic strains can prevent degradation of the NF-κB inhibitor, therefore preventing the expression of pro-inflammatory cytokines like IL-8 by the intestinal epithelial cells (Thomas and Versalovic 2010). Zhang et al. (2005), using an epithelial cell model, demonstrated the effect of both viable and heat-killed *Lactobacillus* GG in the decrease in degradation of the NF-κB inhibitor and subsequent inhibition of NF-κB translocation into the nucleus, resulting in decreased IL-8 production (Zhang et al. 2005). Another study showed that pretreatment of epithelial cells with *L. casei* DN-114 001 decreased *Shigella flexneri*-induced NF-κB activation due to inhibition of NF-κB inhibitor degradation (Tien et al. 2006).

Toll-like receptors (TLR) are proteins involved in the prokaryotic macromolecular motifs recognition. TLR stimulation by probiotics has been extensively studied in vivo and in vitro with different types of immune cells, and this effect appears to be strain specific. An example of this effect is the probiotic *Lactobacillus paracasei* ssp. *paracasei* DC412 stimulation of TLR2- (peptidoglycan recognition) and TL4-mediated (lipopolyssaccharide recognition) signaling events that lead to secretion of several cytokines (Kourelis et al. 2010). These strong pro-inflammatory responses after TLR stimulation are important host-defense mechanisms against dangerous exogenous and endogenous factors. Another example of the immunomodulatory effect of probiotics at the mucosal level is their action on mucins and antimicrobial peptide production. Recently, Tobita and co-workers demonstrated that *L. crispatus* K-11 increased mucin 13 and defensin alpha genes expression in Peyer's patches by twofold compared with control, ensuring constant protection against the attack of digestive fluid, microorganisms, pollutants, and toxins (Tobita et al. 2010).

Via the lymph nodes, immune cells can migrate from the lamina propria into the circulation system, therefore influencing the systemic immune system involving the production of IgA, IgG antibodies, and a wide range of cytokines (Th1, Th2, and regulatory cytokines). It was recently demonstrated that *Lactobacillus plantarum*, *L. salivarius*, and *Lactococcus lactis* attenuate Th2 response and increase the production of regulatory cytokines in healthy mice in a strain-dependent manner (Smelt et al. 2012).

Another study, using seven probiotic strains from *Lactobacillus* and *Bifidobacterium* genera, administered individually in the form of two capsules/day for 21 days containing 1×10^{10} CFU/capsule to 83 healthy volunteers aged 18–62 years, also receiving an oral cholera vaccine, showed an effect of some of the tested strains on specific humoral responses. During early response (day 0–21), serum IgG significantly increased in subjects consuming *B. lactis* Bl-04 and *L. acidophilus* La-14 compared with controls. These results suggest that specific

strains of probiotics may thus act as adjuvants to the humoral immune response following oral vaccination (Paineau et al. 2008).

24.6 Mechanism of Action

The mode of action of probiotics is not completely understood, but they probably replace normal microflora following antibiotic therapy until recovery is achieved (McFarland 2010). The use of probiotics to directly inhibit *H. pylori* does not seem viable in the acidic stomach. However, probiotics can help the microbiome of the patients during and after antibiotic therapy.

The specificity of action rather than the source of the microorganism is the most important (Pineiro and Stanton 2007).

In a general way, probiotics' action occurs via competition for adhesion sites and nutrients. This impairs the colonization of the pathogen and increases the production of antimicrobial compounds like acids or bacteriocins which inhibit pathogen growth and reduce inflammation and tissue destruction, enhancing the host immune response and thus promoting indirectly pathogen elimination (Rastogi et al. 2011). Most of the described mechanisms are related to the immune system modulation described above.

There are a few examples in which the mechanism of action is more detailed and they are presented below. The active compounds identified in *Propionibacterium* are 1,4-dihydroxy-2-naphtoic acid (DHNA), which is the penultimate intermediate in the biosynthesis pathway of vitamin K2 (Isawa et al. 2002), and conjugated linoleic acids (CLAs), which have anticarcinogenic properties (Thierry et al. 2011).

The *L. casei* strain Shirota YIT9029 presented in vitro and in vivo antagonism of *H. pylori* and *Salmonella*, inhibiting the swimming motility of these bacteria. The probiotic action on *Salmonella* was reversible. However, this probiotic produced an irreversible inhibition of the swimming motility of *H. pylori* accompanied by the presence of coccoid morphologies and loss of FlaA and FlaB flagellin expression (Le et al. 2013). *L. gasseri* OLL2716 strain also induces the coccoid conversion of *H. pylori* (Fujimura et al. 2012).

24.7 Safety and Delivery Systems of Probiotics

Probiotics are living microorganisms and therefore may represent a risk for human health. They are all nonpathogenic; however, since probiotics interfere with commensal microflora, as well as have immunomodulatory effects, they can result in opportunistic outcomes in the host due to bacteremia and fungemia. The main observed adverse effects of probiotics are sepsis, fungemia, and gastrointestinal ischemia. Generally, critically ill patients in intensive care units, critically sick children, postoperative and hospitalized patients, and patients with immune-

compromised complexity are the most at-risk populations (reviewed in (Didari et al. 2014; Redman et al. 2014)).

Another important aspect of probiotic safety is the antibiotic resistance characteristics. Indeed, they should not carry transmissible antibiotic resistance genes that could potentially be transferred by horizontal gene transfer to pathogenic bacteria. For example, *Lactobacilli*, one of the most common probiotics, display a wide range of antibiotic resistances naturally (Charteris et al. 1998), but in most cases, antibiotic resistance is not of the transmissible type. In addition, plasmid-linked antibiotic resistances are not very common among lactobacilli, but they can occur (Rinckel and Savage 1990), and their safety implications should be taken into consideration. Resistance transfer from commensal microbiota to probiotics is also an issue of concern.

Many systems have been developed for the delivery of probiotics to the gastrointestinal system including both conventional pharmaceutical systems and nonconventional commercial products. Probiotic delivery systems vary greatly in efficacy to exert health benefits for a patient. The degree of health benefits provided by these probiotic formulations varies in their ability to deliver viable, functional bacteria in large enough numbers (effectiveness), to provide protection against the harsh effects of the gastric environment and intestinal bile (in vivo protection), and to survive formulation processes (viability) (reviewed in (Govender et al. 2014)).

Nonconventional probiotic formulations include mostly cheeses, yogurts, creams, chocolates, milk, and meat. Due to their easy availability and convenience, they are good delivery systems that, in addition, can be beneficial to the patient, when effective. Some of these products have the ability to deliver viable probiotic bacterial cells to the human intestine but this ability differs greatly. This difference is a result of various reasons ranging from formulation processes and viability of dosed bacteria, as well as variability in the ability of different species of bacteria to survive physiological conditions. For example, *Lactobacillus* spp. are more viable in gastric conditions compared to other probiotic species, making it the most ideal probiotic for delivering systems that do not provide gastric protection (Maragkoudakis et al. 2006). In the last decades, a considerable amount of research has been consecrated to improving commercial food-based probiotics delivery systems.

Conventional pharmaceutical products tend to be more effective regarding protection of the probiotic bacteria from the human gastrointestinal tract. Indeed, providing probiotic living cells with a physical barrier against adverse environmental conditions is an approach which is currently of considerable interest. In addition, these systems have been characterized much more compared to commercial food-based carrier systems. Examples of pharmaceutical formulations for the delivery of probiotics currently include beads, capsules, and tablets (Schrezenmeir and de 2001; Solanki et al. 2013).

24.8 Screening of New Probiotic Bacteria, Production, and Available Products

The classical procedures and cultured media that have been used so far for bacterially based drug discovery are unable to increase the number of bacteria available for to assay. These limitations and the frequent rediscovery of known compounds led the pharmaceutical companies to abandoned screening for new antibiotics. A novel approach to screening is needed. A good example of a new and efficient methodology for finding new antibiotics was recently described: sequencing soil microbiomes and using bioinformatics methods to identify gene clusters predicted to encode for metabolites that are evolutionarily related to families of natural products with known bioactivities (Charlop-Powers et al. 2014). A similar approach could be envisaged for probiotics selection. The Human Microbiome Project is financing several projects that hopefully will bring new probiotics.

The starting point in screening new probiotic bacteria are in vitro tests as mentioned above. Of course, there are characteristics which are necessary for the probiotics, namely, viability at the target site, acid (and bile) tolerance, antimicrobial production, and adherence ability to human intestinal cells (Pineiro and Stanton 2007). The mechanism of action should be studied. In vivo tests start with animal models which, if effective, are then translated into clinical trials. These include randomized double-blind placebo-controlled human trials which should be undertaken to establish the efficacy of the probiotic product. Eradication rate and secondary effects are the most used indicators to evaluate the efficacy of probiotics for which a statistically significant result is necessary.

Probiotics are often used in fermented foods (Table 24.2) and fermentation metabolic products appear in the food product, including acetic acid, lactic acid, and possibly bacteriocins, resulting in a decrease of the product's pH. Such changes may affect the stability of probiotic bacteria and may alter the probiotic's functional properties. Moreover, long-term industrial use of the starter culture for production purposes may influence viability and functional properties, as well as storage. Thus, it is important to constantly control the properties of the probiotics (Tuomola et al. 2001).

The European Medicines Agency does not coordinate the evaluation of probiotics; these are evaluated and approved at a national level by the regulatory agencies of each country. The Food and Drug Administration (FDA) approves the probiotics available in the USA. The complete list of currently available probiotics is very difficult to obtain, due to the constant changes, variability of compositions, and designations given in each country. Table 24.2 presents a nonexhaustive list of generally recognized as safe (GRAS) microorganisms added to food from the FDA. There is a high variation in the composition of microorganisms of each product.

Table 24.2 FDA generally recognized as safe (GRAS) microorganisms added to food

Request by	Species	Intended use	Date of closure
Avitop, Germany	*Bacteroides xylanisolvens* DSM 23964	Fermented low-fat and skim milk and milk products. Heat-inactivated cells	Dec 16, 2013
Cargill, Inc., USA	*Bifidobacterium animalis* subsp. *lactis* Bf-6	Foods	Sep 29, 2011
Danisco, Inc., USA	*Bifidobacterium animalis* subsp. *lactis* strains HN019, Bi-07, Bl-04, and B420	Ready-to-eat breakfast cereals, bars, cheeses, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit "ades" and fruit drinks, chewing gum, and confections	Apr 10, 2013
Morinaga Milk Industry Co., Ltd., Japan	*Bifidobacterium breve* M-16V	Baked goods, breakfast cereals, fruit juices and nectars, fruit ices, vegetable juices, milk-based drinks and powders, dairy product analogs, frozen dairy desserts, processed cheese, imitation cheese, cheese spreads, butter-type products, snack foods, gelatin, pudding, fillings, meal replacements, snack bars, nut and peanut spreads, hard and soft candies, cocoa-type powder, and condiment sauces	Sept 27, 2013
454 Morinaga Milk Industry Co., Ltd., Japan	*Bifidobacterium breve* M-16V	Nonexempt powdered term infant formulas (milk or soy based) and exempt powdered term infant formula containing partially hydrolyzed milk or soy proteins	Sept 27, 2013
Danone Trading B.V., the Netherlands	*Bifidobacterium breve* M-16V	Exempt term powdered amino acid-based formulas	Sept 30, 2013
Nestlé, USA	*Bifidobacterium lactis* Bb12 and *Streptococcus thermophilus* Th4	Milk-based infant formula that is intended for consumption by infants 4 months and older	Mar 19, 2002
Morinaga Milk Industry Co., Ltd., Japan	*Bifidobacterium longum* BB536	Breads/baked goods, cereals, dairy products/dairy-based foods and dairy substitutes, fruit products, candy, chewing gum, cocoa powder, condiment sauces, flavored beverage syrups, fruit-	July 8, 2009

(continued)

Table 24.2 (continued)

Request by	Species	Intended use	Date of closure
		flavored powder beverage mixes, gelatin desserts, gravies, margarine, peanut and other nut butter/spreads, snack foods, and in milk-based powdered infant formula	
Danisco, Inc., USA	*Lactobacillus acidophilus* NCFM	In certain dairy products, functional beverages, nutritional powders, juices, bars, ready-to-eat breakfast cereals, chewing gum, and confections	Apr 19, 2011
Nutrition Physiology Corp., USA	*Lactobacillus acidophilus*, *Lactobacillus lactis*, and *Pediococcus acidilactici*	Antimicrobial to control pathogenic bacteria in meat and poultry products	Dec 7, 2005
Yakult Honsha Co., Ltd., Japan	*Lactobacillus casei* Shirota	Ingredient in fermented dairy products	Dec 10, 2012
Mead Johnson & Company, USA	*Lactobacillus casei* subsp. *rhamnosus* GG	Ingredient in term infant formula	May 29, 2008
BioGaia AB, Sweden	*Lactobacillus reuteri* DSM 17938	Ingredient in processed cheeses, yogurt, ice cream, fruit juices, fruit drinks, processed vegetables, processed vegetable drinks, beverage bases, energy bars, energy drinks, and chewing gum	Nov 18, 2008
Nestlé Nutrition, USA	*Lactobacillus reuteri* DSM 17938	Ingredient in powdered whey-based term infant formula	Mar 26, 2012
Micropharma Ltd., Canada	*Lactobacillus reuteri* NCIMB 30242	Beverages and beverage bases, breakfast cereals, cheeses, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk products, processed fruits and fruit juices, and sugar substitutes	Feb 12, 2013
Fonterra Co-operative Group, New Zealand	*Lactobacillus rhamnosus* HN001	Various foods, including certain beverages and beverage bases (excluding soft drinks); cheeses; milk drinks; milk products; meal replacements; energy bars; ready-to-eat	Nov 1, 2009

(continued)

Table 24.2 (continued)

Request by	Species	Intended use	Date of closure
		cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and hard candies	
Fonterra Co-operative Group, New Zealand	*Lactobacillus rhamnosus* HN001 produced in a milk-based medium	Milk-based powdered term infant formula that is intended for consumption from the time of birth, as well as in milk-based powdered follow-on formula	Aug 31, 2009
PURAC, the Netherlands	Cultured dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources fermented by *Streptococcus thermophilus*, *Bacillus coagulans*, *Lactobacillus acidophilus*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus bulgaricus,* and *Propionibacterium freudenreichii* subsp. *shermanii* or mixtures of these strains	Antimicrobial agents in a variety of food categories	Mar 26, 2012
Meiji Co., Ltd., Japan	Heat-killed *Propionibacterium freudenreichii* ET-3 culture (powder)	Beverages and beverage bases, breakfast cereals, cheeses, coffee and tea, fats and oils, frozen dairy desserts and mixes, gelatins, puddings, and fillings, grain products and pastas, milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and soft candy	Dec 26, 2012

24.9 Conclusions and Outlook

Taking into consideration the increasing knowledge of the human gut microbiota and the fact that probiotics are generally regarded as safe, reducing greatly the secondary effects produced by current therapies applied, probiotics appear to be here to stay. The administration of probiotics alone does not eradicate *H. pylori* but consistently diminish the side effects when added as an adjuvant to the therapy. Indirectly, probiotics can contribute to the eradication of *H. pylori*. The mechanisms of action are not fully understood but appear to be based on three pillars: competition, antimicrobial activity, and modulation of the immune system and

microbiota. The new tools to study the human microbiome will probably give new probiotics. Quality assurance protocols should be constantly applied to guarantee the safety of probiotics.

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Chapter 25
Vaccination Against *Helicobacter pylori* Infection

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Abstract *Helicobacter pylori* is well adapted to colonize the human gastric mucosa and induces a relatively mild but persistent inflammation and activation of adaptive B- and T-cell responses. A subset of infected individuals experience symptoms or develop ulcer disease or gastric adenocarcinoma that might be treatable with antibiotics. At the same time, the resistance to antibiotics is rapidly increasing among *H. pylori* isolates, and access to an efficient vaccine would improve treatment options considerably. Still, the complex pathogenesis and many different virulence factors of the bacterium have made vaccine development a challenging task. In this review, we discuss the possibilities of constructing a future vaccine against *H. pylori* based on the choice of antigens and mucosal adjuvants and describe our knowledge of protective immune responses that may be necessary to generate for eradication of *H. pylori*. In addition, the preclinical and clinical testing of available vaccine candidates is reviewed. The immunological response to *H. pylori* infection is multifaceted, and inflammatory responses are mounted side by side with a prominent regulatory response. The potential problems caused by the immune response, comprising both tolerance induction by the infection and the risk of developing post-immunization gastritis, are also discussed.

Keywords *Helicobacter pylori* • Vaccine • Adjuvant • Cytokine • T cell • Gastritis • Regulatory T cell • Clinical trial

25.1 Introduction

Helicobacter pylori infection has a major impact from a public health-care perspective since an estimated half of the world's population chronically harbor this bacterium in the stomach. A birth cohort study in a rural area of Bangladesh showed that *H. pylori* infection was already acquired by 60 % of the children within the first 2 years of their life (Bhuiyan et al. 2009). The highest prevalence of the infection in

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children is in low- and middle-income countries and can be related back to the low socioeconomic status of parents (Malaty et al. 1996a, b). The infection is mainly acquired via close contact with family members, between mother and child (Yamaoka et al. 2000) or between siblings (Goodman and Correa 2000). Although the fecal-oral route of transmission has been suggested as for other enteric infections, *H. pylori* may also be spread via the oral-oral route during outbreak of *Vibrio cholerae* or enterotoxigenic *Escherichia coli* (ETEC) infections (Janzon et al. 2009).

Approximately 1 in 10 *H. pylori*-infected individuals will develop symptoms, including PUD, and, in the worst case, approximately 1 in 100, gastric cancer. Whether chronic infection will lead to symptoms (or not) is dependent both on the host and bacterial genetics (Ernst and Gold 2000; Lochhead and El-Omar 2007). As discussed in detail in Chap. 12, there is also a vast body of epidemiological and experimental evidence to suggest that chronic infection with *H. pylori* during early childhood can protect from asthma and allergy later in life (Kosunen et al. 2002; Blaser et al. 2008). In infected individuals, activation of innate and adaptive immune system leads to the recruitment to the stomach of a wide range of cell types, including dendritic cells, macrophages, neutrophils, mast cells, and T and B cells (Robinson et al. 2007). *H. pylori* colonization also induces a strong systemic antibody response with a rise in *H. pylori*-specific IgA and IgG which has been exploited for diagnostic purposes. The immune cells recruited to the stomach secrete a range of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), interferon-γ (IFN-γ), and IL-17. In spite of the robust immune response evident in the stomach, the chronic lifelong infection does not protect against a second encounter with the bacteria, and spontaneous eradication of the bacteria is rare. This has led to a high prevalence of the infection which can be up to 90 % of the adult population in low- and middle-income countries where *H. pylori* infection is endemic.

25.2 The Need for a Vaccine

In most individuals, the infection will persist for life unless they seek antibiotic treatment. The current *H. pylori* eradication treatment schemes with antibiotics combined with proton pump inhibitors have been improved in recent years and are discussed in detail in Chap. 20. Although the treatment leads to eradication of the infection, in many cases depending on the country, it still poses several difficulties related to side effects and development of antibiotic resistance. Epidemiological studies have reported a reinfection rate of 20–30 %/year after antibiotic treatment in low- and middle-income countries which is accompanied by the return of peptic ulcer disease (PUD) and the risk for gastric cancer (Parsonnet 2003). Thus, there is an urgent need to develop novel treatment strategies not dependent on the use of antibiotics and, ideally, a highly efficacious vaccine, based on our understanding of *H. pylori* bacteria and its interplay with the human host. Candidate prophylactic and

therapeutic vaccines are already in clinical trials and have been projected to be cost-effective in reducing the prevalence of the infection and incidence of gastric cancer in the USA and Japan (Rupnow et al. 2000, 2001). Since the prevalence of the infection in developed countries is decreasing naturally without intervention, a vaccination scheme running for 10 years has been predicted to eradicate the infection from the population (Rupnow et al. 2001). Furthermore, the vaccine need not be highly efficacious or have wide coverage. In low- and middle-income countries, however, the predicted scenario is different; the high prevalence rate means that it would require not only a longer time (>10 years of continuous vaccination) but also a wider vaccine reach for it to effectively reduce the prevalence of *H. pylori* infection in the population and symptoms related to chronic infection (Rupnow et al. 2001).

It is noteworthy that the oral Dukoral cholera vaccine was found to provide not only direct protection to vaccine recipients in Bangladesh but also conferred significant herd immunity to non-vaccinated individuals (Ali et al. 2005). Since the mode of transmission of *H. pylori* seems to be similar to that of *V. cholerae* as evidenced by the fact that new infections with *H. pylori* in young children occur during the same biannual peaks as diarrheal diseases (Janzon et al. 2009), it would be interesting to evaluate whether a *H. pylori* vaccine would also afford herd immunity which might reduce the need for the vaccine to have a wide reach in the population as discussed above. It is also important to consider the target group for vaccination against *H. pylori* infection in low- and middle-income countries. Considering the evidence that suggests that childhood *H. pylori* infection might be protective against allergy and asthma (Blaser et al. 2008), it might be worth vaccinating only symptomatic adults in conjunction with antibiotic therapy. Such a treatment may be considered as a prophylactic vaccine in the traditional sense because the vaccine can be given to adults that have cleared their infection with antibiotics and protection against reinfection can be followed.

25.3 Protective Immune Mechanisms for Eradication of *H. pylori*

The complex pathogenicity and large variability of *H. pylori* are well described in Chaps. 1 and 9 of this book and thus make vaccine development a major challenge. To design an effective vaccine, we need to identify the protective immune mechanisms that will eventually lead to bacterial eradication, keeping in mind that they are most probably different, quantitatively or qualitatively, from what the infection as such gives rise to. Most of this work will, by necessity, be performed in small rodent models, but will eventually have to be verified in humans or nonhuman primate models.

The definition of protection also deserves some clarification. Most vaccination regimens in mice lead to a significant one- or two-log decrease in bacterial burden

in the gastric tissue, but rarely to complete eradication of the bacterium. However, most studies have examined bacterial numbers a few weeks after challenge, and there are data suggesting that waiting for a longer time will actually lead to a complete vaccine-induced eradication of the infection (Garhart et al. 2002). In humans, the situation is even more complicated by the patchy colonization pattern in the stomach, which makes it hard to determine the overall bacterial density even when multiple sampling sites are examined. It is thus hard to evaluate the effect of model vaccines if complete eradication is not achieved. In this chapter, we use the word "protection" to indicate a significant reduction in the gastric colony-forming units.

25.3.1 T-Cell-Mediated Protection

When research on anti-*Helicobacter* vaccines was first initiated, it was generally believed that a secretory IgA response would suffice to prevent infection, based on the extracellular lifestyle of the bacterium and the success of other oral vaccines promoting the barrier function. However, early studies in mice clearly showed that $CD4^+$ T helper cells, but not $CD8^+$ cytotoxic T cells or antibodies, were crucial for vaccine-induced protection (Ermak et al. 1998; Blanchard et al. 1999; Pappo et al. 1999). In addition, there is a prominent gastric IgA response to the infection (Luzza et al. 1995; Mattsson et al. 1998b), without any apparent effect on bacterial colonization. Much attention has since been devoted to elucidating the function, especially cytokine production, of protective T helper cell subsets.

In summary, several studies using knockout animals or antibodies to neutralize cytokine signaling indicate that both Th1- and Th17-type responses are important for vaccine-induced protection against *H. pylori* in mice. The importance of Th1 cells for protection after oral immunization with *H. pylori* sonicate and cholera toxin (CT) adjuvant was shown by Akhiani and coworkers (Akhiani et al. 2002), in IFN-$\gamma^{-/-}$ mice, and later confirmed by Sayi and coworkers (Sayi et al. 2009). In other studies, however, no effect of IFN-γ on vaccine-induced protection could be detected (Sawai et al. 1999; Garhart et al. 2002; Flach et al. 2011a). However, it was consistently shown that IL-12 p40 subunit was important for protection (Akhiani et al. 2002; Garhart et al. 2003; Hitzler et al. 2011; Ding et al. 2013). This later raised the question whether Th17 responses supported by IL-23 (a cytokine sharing the p40 subunit with IL-12) were actually the ones mediating the documented protection. Studies using IL-12 p35$^{-/-}$ mice lacking IL-12 showed varying effects of the IL-12 defect on protection, which was dependent on the genetic background of the host (Panthel et al. 2003; Hitzler et al. 2011). When IL-23 p19-deficient animals lacking IL-23 became available, it was shown that also IL-23 was needed to mount a fully protective immune response following vaccination, as p19$^{-/-}$ mice display some but not as good protection as wild-type mice (Hitzler et al. 2011; Ding et al. 2013).

IL-23 is a potent driver of Th17 responses, and both Th1 and Th17 responses appear to contribute to the *H. pylori*-induced chronic gastritis, as outlined in Chap. 12. Thus, the results using IL-12- and IL-23-deficient animals raised the question about the relative importance of Th1 and Th17 responses also in protective immunity after vaccination. The first studies of IL-17 in protective immunity after immunization used neutralizing antibodies during the effector phase of the immune response and showed that IL-17 was necessary for protection (Velin et al. 2009; Flach et al. 2011a). However, when IL-17A- or IL-17 receptor A (IL-17RA)-deficient animals were prophylactically immunized, they were protected as well as the wild-type mice (DeLyria et al. 2011). As the different studies cited above have used partly different model systems and immunization schedules, it is difficult to make absolute statements about the need for either Th1 or Th17 cells to achieve protective immunity, but it seems reasonable to assume that both Th1 and Th17 responses arise following immunization and that they both contribute to bacterial elimination. In some experimental systems, they may both be needed for successful immunization, while in others they may compensate for each other and the effect may not be seen due to redundancy.

Furthermore, it was also shown in the *H. felis* model that expression of the mucosal homing receptor integrin α4β7 was necessary for vaccine-induced protection in mice following prophylactic immunization (Michetti et al. 2000). Integrin α4β7 binds to mucosal addressin cell adhesion molecule (MAdCAM-1) which is expressed at high levels by the vascular endothelium in gastrointestinal mucosae. These findings are also supported by studies showing local T-cell activation in the gastric mucosa following immunization with various vaccine preparations, but less in systemic lymphoid tissues (Sayi et al. 2009; Becher et al. 2010; Flach et al. 2011a; Hitzler et al. 2011). Thus, we can conclude that $CD4^{+}$ T helper cells with the ability to migrate to mucosal effector sites and secrete either Th1 or Th17 cytokines, or both, are crucial for vaccine-induced protection. In this work, we must also remember that the pathology created by *Helicobacter* infection also appears to be mediated by T cells.

Mice lacking conventional T cells harbor large bacterial numbers, but are free from inflammation and epithelial damage (Roth et al. 1999; Hitzler et al. 2012), and in virtually all immunization studies, gastritis is correlated to lower bacterial burden. Therefore, much work has been devoted to trying to separate inflammation driving and protective immune responses, to avoid the so-called post-immunization gastritis (discussed later). However, a thorough study following immunized and infected mice for a year showed that although a strong post-immunization gastritis developed during the first weeks after infection in the prophylactically immunized animals, this response was reduced with time and had virtually disappeared (as did the bacteria) 1 year after infection (Garhart et al. 2002).

25.3.2 Innate Immune Functions Mediating Protection

Presumably, T cells are not directly responsible for bacterial elimination, and some attempts have been made to define the final effector mechanisms mediating the clearance of bacteria. In this regard, it is interesting to note that protective immunity induced by vaccination leads to gastric production of chemokines, small chemotactic proteins, with the ability to recruit not only effector T cells but also mast cells and neutrophils (DeLyria et al. 2009; Flach et al. 2012). These cells are also increased in the gastric mucosa in protected animals (Velin et al. 2005; DeLyria et al. 2009; Flach et al. 2012), and several studies have been performed to investigate the effect of neutrophils and mast cells on *H. pylori* clearance. Neutrophils are capable to translocate across the gastric epithelium into the lumen, where they can contribute to *H. pylori* clearance by phagocytosis (Zu et al. 2000). Antibody-mediated depletion of neutrophils during the effector phase of a vaccine-induced immune response abrogated the ability to mount a protective response to *H. pylori* (DeLyria et al. 2009). Further support for the role of neutrophils during *H. pylori* elimination comes from experiments in IL-$10^{-/-}$ mice that usually clear *H. pylori* spontaneously. However, if neutrophils are depleted from the IL-10-deficient mice, the bacterial clearance is delayed (Ismail et al. 2003). Thus, neutrophils appear to contribute to vaccine-induced protection, but their effect may be dependent on the infection model (see below).

Mast cells can kill *H. pylori* directly, at least in vitro, and have also been shown to degranulate in the gastric mucosa of immunized mice (Velin et al. 2005). However, in models of systemic bacterial infection, mast cells promote bacterial clearance rather by enhancing the recruitment of neutrophils to the site of infection (Echtenacher et al. 1996; Malaviya et al. 1996). The potential importance of mast cells for protection against *H. pylori* infection has been investigated in two separate studies. It was shown that mast cell-deficient mice were only partly protected compared to wild-type mice following immunization and infection with *H. pylori*, and they also failed to recruit neutrophils to the gastric mucosa during the effector phase of the immune response (Ding et al. 2009). Furthermore, in another experimental system, mast cell-depleted mice were unable to clear *H. felis* infection after immunization (Velin et al. 2005). In this model, however, neutrophil depletion had no effect on protection. Instead, the mast cells needed $CD4^+$ T cells for their function in anti-*Helicobacter* immunity (Velin et al. 2005). Recent studies indicate that a possible mechanism for the effect of mast cells and neutrophils in *Helicobacter* vaccination may be via activation of protease-activated receptors (PARs) on innate immune cells (Wee et al. 2010; Velin et al. 2011; Chionh et al. 2015). PARs are activated by serine proteases, which can be derived from both *Helicobacter* bacteria and endogenously from degranulating mast cells and neutrophils (Ossovskaya and Bunnett 2004; Kajikawa et al. 2007). The effects of PAR1 and PAR2 have been studied in *Helicobacter* infection, and they appear to balance the host's pro-inflammatory and tissue-protective responses, thereby influencing the outcome of infection and vaccination. Activation of PAR2 promotes

pro-inflammatory responses, and PAR2$^{-/-}$ mice have a higher gastric *Helicobacter* burden than wild-type mice (Wee et al. 2010) and were not as well protected against *H. felis* infection following immunization as wild-type mice (Velin et al. 2011). It was suggested that activation of PAR2 on dendritic cells (DCs) leads to improved Th17 responses, and activation of PAR2 may thus be part of a positive feedback loop where neutrophil degranulation leads to improved Th17 responses and more neutrophil recruitment, altogether contributing to bacterial clearance (Velin et al. 2011). In contrast, PAR1$^{-/-}$ mice have lower colonization than wild-type mice and display better protection after immunization in a *H. pylori* challenge model (Wee et al. 2010; Chionh et al. 2015).

25.4 Mechanistic Studies in Experimental Animals for Optimization of Immunization Protocols

A large body of evidence in the literature show that protection against *H. pylori* infection can be achieved by vaccination either prophylactically or therapeutically in animal models although prophylactic studies dominate. Based on numerous vaccination studies in the mouse model of *H. pylori* infection (summarized in Tables 25.1 and 25.2), one can come to the conclusion that the choice of adjuvant and route of immunization plays a crucial role in the induction of the protective immune responses which will be important to consider when proceeding to clinical trials.

25.4.1 Choice of Antigen(s)

The feasibility of mucosal immunization was first demonstrated in mice immunized orally with bacterial lysates or formalin-inactivated whole-cell bacteria together with CT, enterotoxigenic *E. coli* heat-labile toxin (LT), or their nontoxic mutants. If included in a *H. pylori* vaccine, crude antigens like the lysate preparation of the bacteria will be difficult if not impossible to pass through regulatory authorities, due to difficulties in batch-to-batch variation and lack of thorough characterization of the preparation. The lysate preparation does have the advantage that it contains all the known immunodominant and protective antigens such as CagA, HpaA, Urease, VacA, etc. (*unpublished observations*). However, the lysate preparation also contains a high concentration of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), flagellin, and unmethylated cytosine guanine dinucleotides (CpG DNA), that could potentially trigger an inflammatory reaction when given together with a strong mucosal adjuvant.

A striking feature of *H. pylori* LPS is the expression of Lewis (Le) and blood group antigens in the O-antigen component of the LPS molecule (Moran 2008). The

Table 25.1 Prophylactic vaccination studies against *H. pylori* infection in the mouse model[a]

Antigen	Adjuvant	Route	Fold protection[b]	Reference
Formalin whole cell	M-cell targeting	IG	10-fold	Chionh et al. (2009)
	dmLT	IG	10–100-fold	Summerton et al. (2010)
Urease	LT	IG	50–100-fold	Kleanthous et al. (1998) and Weltzin et al. (2000)
		IN	10–100-fold	Ermak et al. (1998), Kleanthous et al. (1998), and Park et al. (2000)
	Alum	s.c.	5-fold	Ermak et al. (1998)
		IN	100-fold	Park et al. (2000)
	LT	Rectal	100-fold	Kleanthous et al. (1998)
	LT	s.c.	50-fold	Weltzin et al. (2000)
	LTB	IG	No protection	
	LTB	s.c.	50-fold	
	CT	SL	10-fold	Flach et al. (2011b)
Salmonella expressing UreB		IG	5-fold	Gomez-Duarte et al. (1998)
HpaA	CT	SL	10-fold	Flach et al. (2011b)
	CT	IG	10-fold in BALB/c mice. No protection in B6	Sutton et al. (2007)
Lysate	CT	IN	100-fold	Garhart et al. (2002)
	CT	IG	10-fold	Pappo et al. (1999) and Raghavan et al. (2010)
	CT	SL	200-fold	Raghavan et al. (2010)
	CTA1-DD	IN	10-fold	Akhiani et al. (2006)
	IFA	sc/ip	50-fold	Eisenberg et al. (2003)
	CFA	sc/ip	50-fold	Eisenberg et al. (2003)
	CpG	IG	3-fold	Taylor et al. (2008)
	dmLT	IG	10-fold	Sjokvist Ottsjo et al. (2013)
	dmLT	SL	200-fold	Sjokvist Ottsjo et al. (2013)
	CpG-CTB	IN	3-fold	Nystrom-Asklin et al. (2008)
HpaA and UreB	CT	SL	40-fold	Flach et al. (2011b)
		IG	5-fold	
	dmLT	SL	44-fold	Sjokvist Ottsjo et al. (2013)

[a]Only studies where *H. pylori* were used as a challenge strain for infection- and colony-forming units subsequently quantified were included

[b]Fold protection was roughly calculated as the decrease in the number of colony-forming units in the stomach compared to unvaccinated infected mice

Table 25.2 Therapeutic vaccination studies against *H. pylori* infection in the mouse model[a]

Antigen	Adjuvant	Route	Fold protection	Reference
Formalin whole cell	CT	IG	10–100-fold	Raghavan et al. (2002a)
Urease	LT	IG	100-fold	Guy et al. (1999)
	QS21	s.c. back	<1000-fold	
Salmonella expressing CagA, VacA, and UreB		IG	10-fold	Liu et al. (2011)
HpaA	CT	IG	10-fold	Nystrom and Svennerholm (2007) and Sutton et al. (2007)
Lysate	CT	IG	5-fold	Raghavan et al. (2002b)
	Alum	ip	2-fold	Nystrom et al. (2006)
HpaA and UreB	CT	IG	>100-fold	Nystrom and Svennerholm (2007)

[a]Only studies where *H. pylori* were used as a challenge strain for infection- and colony-forming units subsequently quantified were included

expression of Le^x and Le^y antigens is a common property of *H. pylori* strains since as many as 80–90 % of isolates from various geographical regions worldwide have been found to express these antigens when screened using anti-Le antibodies as probes (Simoons-Smit et al. 1996; Moran 2008). Although controversial, there could be the possibility that vaccination with *H. pylori* LPS containing lysate preparation or whole-cell bacteria together with an adjuvant could induce antibodies to the bacterial Le^x and Le^y that could cross-react and bind to the Le^x and Le^y structures expressed by the host epithelium and trigger autoimmunity (Negrini et al. 1996; Appelmelk et al. 1998). If whole-cell bacteria/lysates are to be used as vaccine in humans, it would be important to perhaps reduce the LPS content and follow closely the Le^x and Le^y antibody titers in the vaccinated individuals.

Bacterial vectors for the delivery of antigens to the host have also been exploited for the delivery of *H. pylori* antigens to the host particularly urease. For example, attenuated *Salmonella typhi*, *Lactobacillus* ssp., and polio virus have all been used as vectors to deliver urease antigen (Corthesy et al. 2005; Smythies et al. 2005; Aebischer et al. 2008). The advantage on this approach is that it avoids the need for a mucosal adjuvant. Attenuated *Salmonella* as a vector has been the most extensively studied both for oral and intranasal delivery. The *Salmonella* vector expressing urease was based on the licensed typhoid fever vaccine strain Ty21a which is a chemically induced avirulent mutant of *S. enterica* serovar Typhi with an ability to induce broad cellular and humoral immunity at mucosal sites. The results showed that although the vaccination with *Salmonella* expressing urease worked well in mice, when taken to clinical trials, the response to urease in the volunteers was weak and did not provide protection to infectious challenge (discussed below).

Finally, some of the most promising results on vaccine-induced protection have been obtained using recombinant antigens from *H. pylori* either alone or in

combination. Numerous studies have tested the efficacy of urease as a protective antigen using different adjuvants and routes of immunization (Tables 25.1 and 25.2). The efficacy of prophylactic or therapeutic protection against *H. pylori* infection in mice has also been demonstrated for a variety of other native and recombinant antigens such as shock proteins and native and recombinant VacA, CagA, NapA, catalase, and HpaA among others (Radcliff et al. 1997; Marchetti et al. 1998). Importantly, a synergistic effect on vaccine-induced protection was seen when two antigens, HpaA and UreB, were combined together with CT or double-mutant heat-labile toxin from *E. coli* (dmLT) as an adjuvant compared to immunization with either antigen alone (Nystrom and Svennerholm 2007; Flach et al. 2011b; Sjokvist Ottsjo et al. 2013). In the future, information derived from the knowledge of the *H. pylori* genome that is now easily available will probably lead to the identification of additional candidate antigens that could be safely included in a multicomponent vaccine against *H. pylori* infection.

25.4.2 Choice of Adjuvants

The mucosal route of immunization requires the use of a strong adjuvant because proteins are poor immunogens when given mucosally. The strongest mucosal adjuvants are bacterial toxins such as CT and LT; however, they both cause severe diarrhea limiting their use in humans. Both CT and the heat-labile toxin from *E. coli* (LT) belong to the class of AB5 toxins with a characteristic A1 subunit linked to a pentamer of B subunits via the A2 fragment (Rappuoli et al. 1999) (Fig. 25.1). The B subunit is responsible for the binding to monosialotetrahexosylganglioside (GM1) present on all nucleated cells, and the A1 subunit is a ribosyltransferase promoting adenosine diphosphate ribosylation of stimulatory guanine nucleotide-binding proteins (G proteins), resulting in increased intracellular levels of cAMP and enhanced fluid secretion and diarrhea. The ribosyltransferase activity of the A1 subunit has been shown to be responsible also for the adjuvanticity of CT and LT (Giuliani et al. 1998). Intense efforts in the last 20 years have focused on developing molecules with reduced toxicity but intact adjuvanticity. Site-directed mutagenesis was used to replace single or two amino acids within the enzymatic A1 subunit of LT leading to reduced or eliminated enzymatic activity (LTK63, LTR192G, and dmLT) (Giuliani et al. 1998; Norton et al. 2011) (Fig. 25.1). Another approach to decrease the toxicity of CT has been to link the enzymatically active CTA1 subunit to the cell-binding moiety of *Staphylococcus aureus* protein A (CTA1-DD) (Agren et al. 1997). These nontoxic mutant molecules have been tested as mucosal adjuvants in combination with *H. pylori* antigens in the mouse model, primates, and human clinical trials (described below).

Studies on the proof of principle that a *H. pylori* vaccine can promote immunity and reduce bacterial load in the stomach of mice were performed initially using CT as an adjuvant. Prophylactic or therapeutic mucosal vaccination with a range of *H. pylori* antigens was found to protect against *H. pylori* infection (Tables 25.1 and

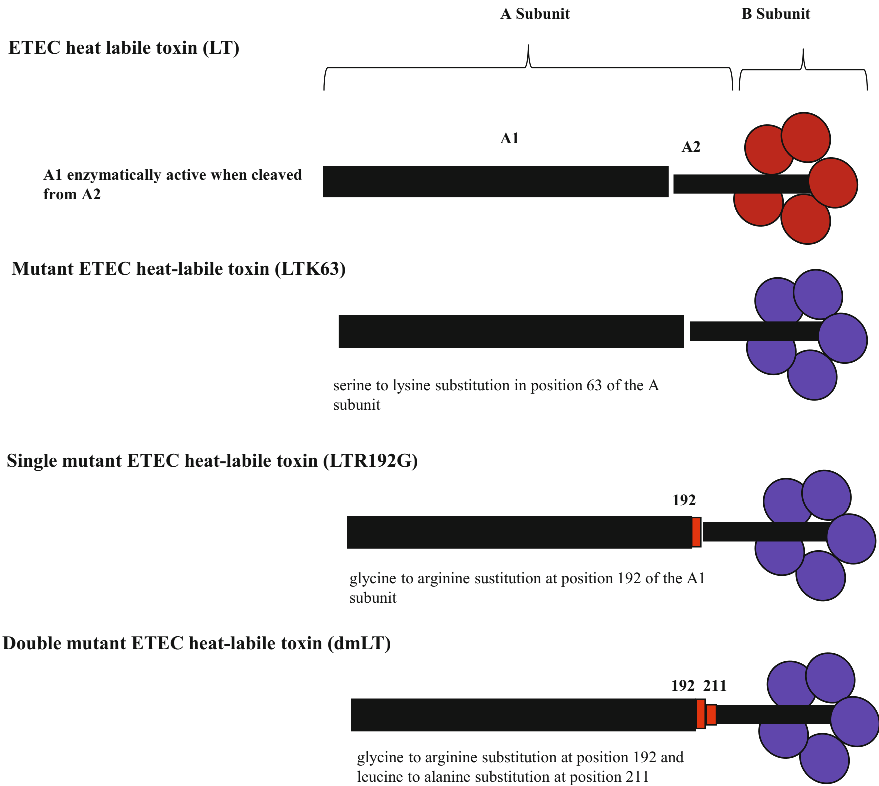

Fig. 25.1 Site-directed mutagenesis to reduce/remove the ADP ribosylase activity of the A1 subunit of the ETEC heat-labile toxin (LT). Several startegies have been employed to reduce the toxicity of LT while retaining the adjuvanticity of the molecule. The figure shows a schematic representation of native LT and the different LT variants with mutations in selected sites that have been evaluated as mucosal adjuvants together with candidate *H. pylori* antigens

25.2). Most of the animal studies on the protective immune responses after vaccination described earlier have also been carried out using CT. Thus, CT has served as a "golden standard" for evaluating other nontoxic mucosal adjuvants to be included in a *H. pylori* vaccine. Similar to CT, LT has been used as a mucosal adjuvant in animal studies (Tables 25.1 and 25.2). The nontoxic derivatives of LT, LTK63, LTR72, and dmLT have also been found to strongly potentiate immune response to various parenterally and mucosally administered *H. pylori* antigens making them promising adjuvants to include in future vaccines (Lu et al. 2010; Summerton et al. 2010; Norton et al. 2011; Sjokvist Ottsjo et al. 2013). Finally, CTA1-DD has been shown to function as an adjuvant with a *H. pylori* vaccine in mice when administered intranasally but not intragastrically or sublingually (Akhiani et al. 2006). The choice of the adjuvant and mucosal route of immunization will be crucial for the safety aspect and induction of appropriate and long-lasting immune responses against *H. pylori* antigens as discussed below.

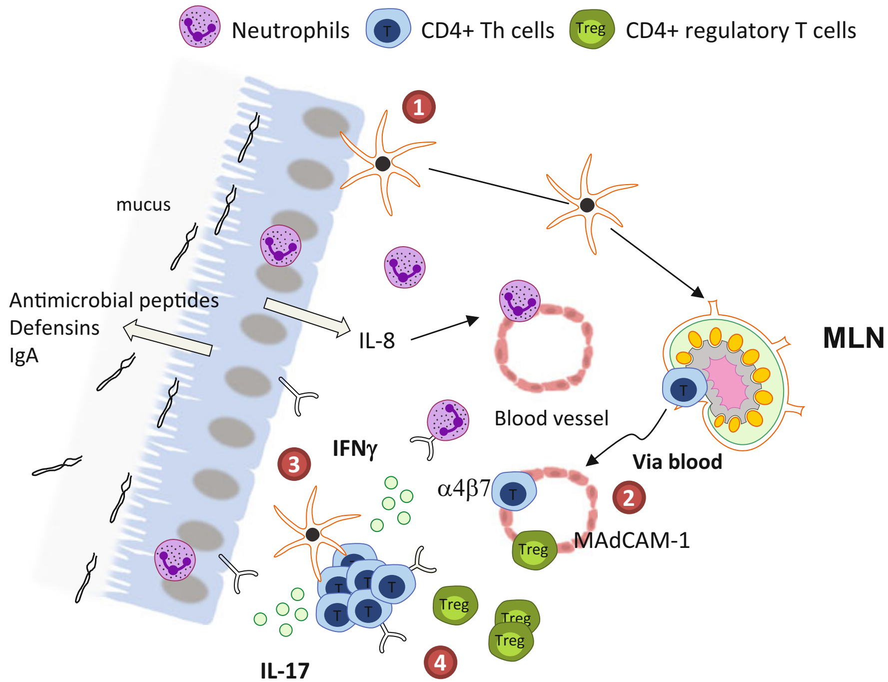

Fig. 25.2 Interactions between CD4+ T-cell subsets and the gastric epithelium and cytokines important for vaccine-induced protection. *H. pylori* reside in the mucous gel layer. (1) Antigens shed by *H. pylori* are picked up by dendritic cells which migrate to the draining lymph node to prime CD4+ T cells and induce regulatory T cells (Treg). IL-8 secretion by the epithelial cells in response to the infection can directly recruit neutrophils to the site of infection. (2) *H. pylori*-specific CD4+ T cells and Treg home to the gastric mucosa via α4β7 interaction with MAdCAM-1 and proliferate locally in the tissue. (3) CD4+ effector T cells secrete IFN-γ and IL-17A in response to *H. pylori* infection which may bind to their respective receptors on the epithelium. (4) Finally, Treg can suppress the activation of effector T cells in the stomach

25.4.3 Vaccination Routes

Since the bacteria are localized extracellularly in the stomach, particular emphasis has been given to immunization via the intragastric route although other mucosal routes of immunization as well as the parenteral route and prime-boost regimens have also been evaluated. Alternatives to the intragastric route of immunization have been sought since the stomach poses additional challenges on the stability of the antigen and adjuvants used. Indeed, the intranasal and the sublingual route of immunization has consistently proved to induce a stronger protection against *H. pylori* infection (in the range of 100–200-fold reduction in bacterial load) compared to the intragastric route which gives a reduction in bacterial load in order of five to tenfold (Tables 25.1 and 25.2) irrespective of the antigen and

adjuvant used. However, the intranasal route of immunization may not be the route of choice since studies in humans have indicated that the nasal route of immunization is ineffective in stimulating immune responses in the intestine or stomach (Johansson et al. 2004). In addition, intranasal immunization is associated with a risk of translocation of GM1-binding adjuvants like CT to the olfactory bulb of the brain, restricting its applicability in humans (van Ginkel et al. 2000).

The sublingual route of immunization on the other hand has been reported to be safe at least in mice and particularly ideal when using antigens or adjuvants that might be sensitive to the harsh environment of the stomach acid (Czerkinsky et al. 2011). $CD4^{+}$ T cells primed in the cervical lymph node after sublingual immunization have been shown to migrate to peripheral lymphoid organs and further to the mucosal tissues such as the lungs, stomach, intestine, and vagina and afford protection against H1N1 influenza, *H. pylori*, and genital papilloma virus infections (Song et al. 2009; Raghavan et al. 2010; Czerkinsky et al. 2011). The induction of rather poor immunity after intragastric administration of the *H. pylori* vaccine could be either due to the degradation of antigens or due to dilution of the vaccine in the contents of the gastrointestinal tract, an aspect that must be kept in mind when designing clinical trials in humans.

Another approach for the delivery of vaccines has been to target it to the M cells lining the payer patches of the small intestine. M cells selectively absorb the antigen by endocytosis or pinocytosis and direct it to antigen-presenting cells (macrophages, DCs, B lymphocytes). Whole-cell bacteria agglutinated with the Ulex Europaeus Lectin 1 (UAE1) and administered orally to mice could presumably bind to M-cell glycocalyx in the small intestine where they can be taken up for presentation to the immune system. Remarkably, vaccination with lectin-agglutinated bacteria was as effective as using formalin-inactivated whole-cell bacteria and CT (Chionh et al. 2009). An advantage of mucosal vaccination or M-cell targeting of the vaccine is that mucosal priming induces the lymphocytes to express the $\alpha4\beta7$ integrin. As mentioned previously, $\alpha4\beta7+$ cells have been shown to be essential for vaccine-induced protection against *Helicobacter* infection in mice indicating the importance of priming at a mucosal site (Michetti et al. 2000). By, contrast T cells that are primed peripherally typically display the $\alpha4\beta1$ integrin and CC chemokine receptor 4 (CCR4) and so do not migrate to or respond in mucosal sites. This selective expression of integrin on lymphocytes explains why mucosal vaccination might be required to protect against mucosal infections and why peripheral administration of vaccine antigens is often ineffective against mucosal infections which may be the case for a *H. pylori* vaccine.

Another approach that has been evaluated for *H. pylori* vaccines is the mucosal prime and systemic boost strategy particularly with purified antigens that might be weakly immunogenic. This strategy would require that the antigen and adjuvant are safe to administer via the mucosal and the systemic route. Mucosal immunization alone often requires two to three boosters for an effective immune response and reduction in the bacterial load in the stomach of mice (Sutton et al. 2000). With the mucosal prime-systemic boost strategy, on the other hand, mucosally induced T cells could be effectively expanded with a single systemic boost. The systemic

boost would also be dose sparing both for the antigen and adjuvant. In this regard, intranasal mucosal priming with *Salmonella* expressing urease and systemic boost with purified recombinant urease with alum was shown to be more effective in reducing the bacterial load in the stomach of mice post-challenge compared to single mucosal or parenteral immunizations alone (Londono-Arcila et al. 2002). In another study, using two separate adjuvants, Ermak and coworkers showed that mucosal intranasal prime with urease and LT and systemic boost with urease and alum was effective in reducing the bacterial load in the stomach of mice (Ermak et al. 1998). The mucosal prime and systemic boost strategy although promising might be difficult to implement in the field as it may increase the cost of the vaccine.

25.5 Studies in Nonhuman Primates and Clinical Trials

25.5.1 Immunization Studies in Nonhuman Primates

The use of nonhuman primates in medical research is inevitably connected with high costs and difficult ethical considerations. Still, in *Helicobacter* research, the model carries several advantages, such as the route of transmission, which is presumably the same in humans and socially housed Rhesus macaques (*Macaca mulatta*), the resulting chronic active gastritis (Dubois et al. 1994), and the ability of the bacterium to re-infect previously infected individuals treated with antibiotics (Dubois et al. 1999). A further advantage is the possibility to follow individual animals with repeated biopsy sampling after treatment or vaccinations attempts. Several immunization studies using Rhesus macaques to evaluate antigens and immunization routes were performed in the late 1990s, but in the last decade, there has been a few vaccination studies using this model that has made it all the way to publication.

Dubois and coworkers (1998) showed that prophylactic immunization of socially housed macaques with urease together with *E. coli* LT as adjuvant induced significant protection against infection acquired from other monkeys in the colony and also that the immunized but subsequently infected monkeys had significantly lower gastritis score than untreated and infected animals. In contrast, a prophylactic immunization with urease and LT given orally or intramuscularly failed to induce protection during subsequent experimental challenge with *H. pylori* (Solnick et al. 2000).

At the same time, attempts at therapeutic immunization with urease and LT adjuvant of already infected macaques had no effect on gastric bacterial load in a separate study (Lee et al. 1999b). Still, when the same animals were treated with antibiotics to eliminate the infection, vaccinated again, and then rechallenged with *H. pylori*, they had significantly lower bacterial carriage than previously unvaccinated but infected monkeys. In an attempt to define better immunization schedules, the same group also combined mucosal and parenteral immunizations in

antibiotic-treated animals with previous infection (Lee et al. 1999a). This treatment reduced bacterial numbers in the vaccinated animals, but did not achieve full eradication after challenge.

The factors differing between these studies suggest that in order to achieve successful vaccination actually preventing *H. pylori* infection, prophylactic immunizations, the natural infection route rather than challenge studies, and a reasonably large number of animals should be used. If full eradication is not achieved, the patchy distribution of *H. pylori* in the gastric mucosa will probably make it difficult to assess potential differences in bacterial load. Finally, it is encouraging to note that none of the studies cited above reports any serious post-immunization gastritis. If anything, gastritis scores appear to be slightly reduced in immunized and subsequently infected animals compared to animals who were immunologically naïve when they were infected.

25.5.2 *Clinical Trials of* H. pylori *Vaccines*

Urease was also used in the first trials with human volunteers receiving experimental *H. pylori* vaccines, and LT was again used as adjuvant. This carries obvious problems with toxin-induced diarrhea, and native LT will not be used as adjuvant in a human vaccine, when available. Oral immunization with urease and LT in asymptomatic but *H. pylori*-infected volunteers induced specific serum IgA responses and circulating IgA-secreting cells (Michetti et al. 1999) indicating the induction of a gastrointestinal immune responses (Brandtzaeg and Johansen 2005). There was also a modest but significant decrease in the gastric bacterial counts 1 month after immunization, but the volunteers were then treated with antibiotics, and no further effect on the bacterial counts could be followed. The same group also evaluated lower doses of LT in uninfected volunteers and found that these generally reduced the frequencies of responders to oral urease immunization (Banerjee et al. 2002). Still, a significant increase in $CD69^{+}$ activated lymphocytes within the gut-homing $\alpha 4\beta 7^{+}$ population could be documented following immunization. Another approach, using a mutated LT without toxic effect (LT_{R192G}) combined with a formalin-inactivated whole-cell vaccine, was also evaluated at the same time (Kotloff et al. 2001; Losonsky et al. 2003). This formulation induced mucosal IgA responses in both uninfected and *H. pylori*-infected volunteers, and a systemic T-cell response evident as secretion of IFN-γ, but interestingly enough only in the uninfected volunteers. Still, this vaccine formulation could not achieve bacterial eradication when given to asymptomatic *H. pylori*-infected volunteers. As the bacterial density was not examined, it could not be evaluated if the vaccination had any effect on bacterial density in the stomach.

In addition to the inactivated vaccine formulations, attempts to use live recombinant *S. typhi* Ty21a strains expressing urease have been partly successful. The *S. typhi* Ty21a is the same strain as in the Vivotif™ vaccine against typhoid fever and has an attractive safety profile. When engineered to express UreA and UreB

from *H. pylori*, it induces very weak B-cell responses, but significant T-cell responses to urease following oral delivery in a subset of volunteers, especially if the vaccinees had been exposed to the carrier *Salmonella* strain previously (Bumann et al. 2001; Metzger et al. 2004).

As *H. pylori* preferentially infects young children living in crowded households with poor hygiene (Kivi and Tindberg 2006), the use of natural infection to evaluate the efficacy of vaccine candidates is not a viable starting point, even though the initial primate studies suggested that this may be a possible way to record protective immune responses. In order to evaluate the protective effect of any vaccine candidate in healthy adults, the access to a safe and relevant human challenge model is therefore invaluable. One such model was developed some 10 years ago and made use of an antibiotic-sensitive *cag*PAI$^-$ *H. pylori* strain isolated from a patient with mild gastritis (Graham et al. 2004). Inoculation with this strain induced a typical active chronic gastritis, infiltration of CD4$^+$ and CD8$^+$ T cells in the gastric mucosa, and development of a serum IgM response (Graham et al. 2004; Nurgalieva et al. 2005). This challenge model was also used in subsequent attempts to correlate immune responses to vaccine-induced protection, again using the urease-expressing *S. typhi* Ty21a as vaccine (Aebischer et al. 2008). The live vaccine was delivered orally three times every second day, and the volunteers challenged with *H. pylori* 1–5 months later. This regimen resulted in the prevention of infection in some (three out of nine) volunteers in a first round of experiments, but none in a subsequent series of 12 vaccinees (Aebischer et al. 2008). The comprehensive analysis of immune responses made it possible to correlate low or absent *H. pylori* infection with detectable CD4$^+$ T-cell responses to the vaccinees antigens. It was shown that presence of circulating CD4$^+$ integrin β7$^+$ T cells producing IFN-γ or IL-2 following antigen-specific stimulation was much more common in protected individuals (defined as having no or few remaining *H. pylori*), while vaccine-specific B-cell responses could not be detected in any of the vaccinees (Aebischer et al. 2008). These data strongly indicate that CD4$^+$ gut-homing (α4β7$^+$) effector T cells may be important for protection in humans as well. However, the presence of such cells has not yet been evaluated in the gastric mucosa of vaccinated volunteers.

A second line of studies investigated the route antigen delivery that resulted in gastric immune responses using already available oral vaccines and the homing commitments of gastrically activated effector B and T cells. The immunogenic and well-tolerated oral Dukoral™ cholera vaccine was used for several of these studies. This is an inactivated whole-cell vaccine substituted with cholera toxin B subunit (CTB). Initial studies in *H. pylori*-infected and uninfected volunteers showed that although the two groups of volunteers displayed similar vaccine-specific B-cell responses in the duodenum, only the infected volunteers had detectable gastric B-cell responses to the cholera antigens (Mattsson et al. 1998a). The ability to induce gastric B-cell immunity in uninfected individuals was shown a few years later, when another study showed weak gastric reactivity to a higher dose of LT_{R192G} (25 μg versus 10 μg CTB in Dukoral) also in uninfected volunteers (Losonsky et al. 2003), but again, duodenal immune responses were considerably

higher. To investigate if gastric B-cell responses could be induced directly in the gastric mucosa or if they were the result of migration of cells activated at distant sites, such as Peyer's patches, another series of experiments with gastric or intestinal delivery of the Dukoral vaccine was performed. These experiments showed that both gastric and intestinal antigen delivery could induce gastric B-cell responses, but only in the *H. pylori*-infected individuals (Quiding-Jarbrink et al. 2001b). T-cell reactivity was not evaluated in the gastric cell suspensions, but another study showed that *H. pylori*-reactive T cells in the circulation of infected individuals carry the $\alpha 4\beta 7$ mucosal homing receptor, as do specific B cells induced by gastric antigen delivery (Quiding-Jarbrink et al. 2001a). The increased migration of effector B cells to the *H. pylori*-infected gastric mucosa could be caused by an increased production of the chemokine CCL28, which specifically recruits IgA^{+} plasmablasts to mucosal tissues (Kunkel et al. 2003), and was found to be increased in *H. pylori*-infected human stomach (Hansson et al. 2008). Taken together, these studies suggests that induction of adaptive gastric immune responses can be achieved by intestinal antigen administration and that a therapeutic vaccine given to already infected individuals should have the possibility to induce a strong gastric response. However, these studies were all performed with adult volunteers, and it may well be that young children or infants respond differently, and this possibility must be taken into account when designing future vaccine trials.

A different approach to *H. pylori* vaccination in humans is the use of three well-characterized recombinant *H. pylori* antigens (CagA, VacA, and NapA) given intramuscularly with alum adjuvant (Malfertheiner et al. 2008). As would be expected, this antigen regimen resulted in strong serum IgG response, and there was also a substantial IFN-γ production from restimulated circulating T cells. However, when volunteers receiving this vaccine were challenged with a *cag*PAI^{+} *H. pylori* strain, there was no difference in bacterial clearance in the vaccine and placebo groups (Malfertheiner et al. 2012).

When human and animal studies are considered together, it appears that an appropriate T-cell response is a key to protective immune responses against *H. pylori* infection. In this regard, it is interesting and encouraging that the human studies performed so far do not report on any overt vaccine-induced gastritis, even when a T-cell response to *H. pylori* has clearly been induced. In the previous primate studies, mucosal IgA responses were recorded, but at that time, unfortunately no attempts were made to correlate T-cell immunity to bacterial burden. Both in the human and primate studies performed so far, a long-time follow-up of gastric bacterial counts are lacking, to properly evaluate the effect of vaccine candidates. This would obviously be a time-consuming and a costly endeavor, but will probably be necessary to evaluate a future promising vaccine candidate.

25.6 Remaining Obstacles for Successful Vaccination Against *H. pylori*

25.6.1 Treg-Mediated Suppression of Effector Functions

In spite of a vigorous immune response generated in the stomach with infiltration of T and B cells, it is not clear exactly how *H. pylori* infection persists for many decades in the infected individuals. One reason that has been suggested is that the immune system has developed mechanisms to protect the host by ensuring the development of an immune response in the absence of harmful inflammation and damage to the host tissue. The mucosal immune system in particular is highly adapted toward tolerance, the breakdown of which can result in disease (Izcue et al. 2006). A specific subset of $CD4^{+}$ T cells co-expressing CD25 called regulatory T cells (Tregs) was first described in 1995 to be crucial in the active suppression of autoimmune inflammation and colitis in healthy individuals (Sakaguchi et al. 1995; Izcue et al. 2006; Ohkura et al. 2013). These Tregs could be identified, purified, and characterized by their high expression of CD25, CTLA-4, and later the transcription factor Foxp3 (FOXP3 in humans). The Tregs also specifically secreted TGFβ and IL-10 when activated through their T-cell receptor. In subsequent studies, Tregs have been shown to be readily induced by oral administration of antigen, but this induction must be avoided if mucosal vaccination is to be successful.

The role of Tregs in *H. pylori* infection was first described in the mouse model where it was shown to dampen the *H. pylori*-induced immunopathology by reducing the activation of $CD4^{+}$ IFN-γ-producing cells (Raghavan et al. 2003; Kaparakis et al. 2006; Rad et al. 2006; Stuller et al. 2008; Sayi et al. 2009). Indications that Treg can suppress *H. pylori*-induced inflammation can perhaps also be deduced from the fact that both B-cell knockout and NADPH phagocyte oxidase knockout mice that both have lower frequencies of Treg in vivo compared to wild-type controls also have much lower bacterial loads and exacerbated inflammation in the stomach (Blanchard et al. 2003; Akhiani et al. 2004).

Vaccination with *H. pylori* antigens and a strong mucosal adjuvant to overcome Treg activity could possibly be a mechanism to enhance immunity. However, the vaccine-specific response may itself be dampened by the presence of Treg as evidenced by the presence of Treg in the stomach of vaccinated mice (Becher et al. 2010) and lack of sterilizing immunity reported in several studies (Tables 25.1 and 25.2). Thus, vaccination combined with Treg depletion has been suggested as strategy to enhance the protective effect of the vaccine. Currently there is only one study reporting enhanced vaccine-induced response to *H. pylori* infection after depletion of Treg in mice (Hitzler et al. 2011). Surprisingly the bacteria were still not eradicated from the stomach of Treg-depleted mice indicating that $Foxp3^{neg}$ regulatory cells such as IL-10-producing T regulatory 1 cells might play a role in controlling effector T-cell responses in the stomach. Whether vaccination combined with short-term Treg depletion would be a viable option for the treatment of

H. pylori infection remains to be seen, as there are inherent risks of blocking Treg activity due to their role in preventing autoimmunity and inflammation.

25.6.2 Post-immunization Gastritis

Several studies have characterized the immune cell infiltration in the stomach of vaccinated mice compared to unimmunized infected mice reporting enhanced infiltration of CD4+ T cells, B cells, neutrophils, M1 macrophages, DCs, mast cells, and eosinophils and chemokine and cytokine response post-challenge (Ermak et al. 1998; Quiding-Jarbrink et al. 2010; Flach et al. 2011a, 2012; Hitzler et al. 2011) (Fig. 25.2) often referred to as post-immunization gastritis. In this complicated picture of the inflammatory infiltrate in the stomach of vaccinated mice, it is a daunting task to decipher the function of the individual cell types and cytokines in inflammation and protection. As mentioned previously, an increase in the IFN-γ and IL-17 response in the stomach of vaccinated mice has been shown to correlate with a decrease in bacterial load in the stomach of individual mice (Flach et al. 2011a). Recent studies have shown that vaccinated IL-12p35$^{-/-}$ and IFN-γ$^{-/-}$ mice are protected against *H. pylori* infection with minimal inflammation with a consequent increase in IL-17A levels in the stomach (Ding et al. 2013; Sjokvist Ottsjo et al. 2015), indicating that post-immunization gastritis might be promoted by IFN-γ, while IL-17A mainly affects the bacterial load in the stomach. These results were consistent with another study showing that depletion of macrophages using loaded liposomes reduced vaccination-induced gastritis presumably by blocking macrophage derived IFN-γ response (Kaparakis et al. 2008) but did not affect protection (Walduck et al., *personal communication*). Further studies are warranted and important because future vaccines should be designed based on the induction of appropriate immune response with minimal post-immunization gastritis.

25.6.3 Challenges in Vaccination Against **H. pylori** *in the Developing World*

The main target population for a vaccine against *H. pylori* infection are symptomatic individuals living in low- and middle-income countries and those with the risk for developing gastric cancer. Thus, one of the biggest challenges for the vaccine would be to make it available at an affordable price. Another important aspect to consider is that previous oral live vaccines have shown reduced immunogenicity when used in low- and middle-income countries compared to industrialized countries (Richie et al. 2000; Ogra et al. 2011). The reasons for the difference in immunogenicity although not completely defined have been suggested to be due

to nutrition-related factors including protein-calorie and micronutrient malnutrition. In addition, interference of vaccine take by maternal IgA antibodies during breastfeeding, presence of intestinal parasitic infection, and possibly also host genetic factors have been shown to contribute to the reduced efficacy of oral vaccines in low- and middle-income countries. Indeed zinc supplementation and temporary withdrawal of breast milk during vaccination in infants has given promising results when evaluated with the oral cholera vaccine (Ahmed et al. 2009).

Another aspect that needs to be taken into consideration is that due to high costs for production of the vaccine according to Good Manufacturing Practices, the involvement of a pharmaceutical company would be required. Previously several companies were actively pursuing the development of a *H. pylori* vaccine, but due to the poor results later in clinical trials, the interest has declined. With the development of new and promising mucosal adjuvants, hopefully there will be renewed interest in the production of an *H. pylori* vaccine for evaluation in clinical trials.

25.7 Conclusions and Outlook

As with other bacterial vaccines, either inactivated whole cells or a mixture of putative protective recombinant antigens should be included in the vaccine against *H. pylori* with a strong, safe, and effective mucosal adjuvant that can induce mucosal homing $CD4^+$ T-cell responses. Vaccination of symptomatic adults in conjunction with antibiotic therapy might be considered due to the recent data suggesting that *H. pylori* infection during childhood may actually protect against development of asthma and allergies. Indeed, it has been demonstrated in adult volunteers that it is possible to induce stronger immune responses by vaccination in the *H. pylori*-infected mucosa compared to uninfected mucosa; thus, a therapeutic vaccine would probably have a chance for success (Mattsson et al. 1998a). Furthermore, the evaluation of the safety of the *H. pylori* mucosal vaccine in future clinical trials will be of utmost priority. Two recently developed mucosal vaccines for human use against rotavirus diarrhea and influenza were unfortunately withdrawn after a short period due to reported adverse reactions, emphasizing the future challenging task in formulating a *H. pylori* vaccine which would be safe to use in children and adults primarily in developing countries.

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