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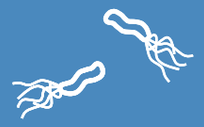

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PERSISTENT *HELICOBACTER PYLORI* INFECTION AND HOST RESPONSE

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ABSTRACT

Helicobacter pylori persistently colonizes the gastric mucosa of approximately one half of the world's population. Colonization always leads to chronic gastric inflammation, which may progress to peptic ulcer disease, gastric cancer or MALT lymphoma. The nature of *H. pylori* infection is determined by bacterial, host and environmental factors. Variability in strain virulence may be explained by the high genetic diversity of the species. The work presented in this thesis focuses on diverse bacterial factors and mechanisms that modulate persistent *H. pylori* infection.

In paper I, we characterized the effects of clinical *H. pylori* isolates with diverse inflammatory background on human gastric adenocarcinoma cells (AGS) and studied their capacity to cause maturation and activation of monocyte-derived dendritic cells (DCs). The inflammatory background of the bacteria was defined by the infiltration of lymphocytes and granulocytes at the site of infection. We found that the expression of the cytokines interleukin (IL)-6 IL-12, TNF-α and IL-1β was higher when DCs were infected with *H. pylori* of a higher inflammatory background. Such a correlation could not be described when analyzing IL-8 induction in AGS cells. We suggest, that DCs play a major role in *H. pylori* infection and that the bacterium can influence the outcome of the interaction.

In papers II and III, we investigated the prevalence of *H. pylori* virulence factors, the combination of virulence factors and their association with disease development in three major ethnicities in Malaysia and Singapore. The genes encoding an intact *cag* PAI, *babA*, *oipA* ON and *vacA* s1 and i1 were present in more than 85% of the isolates, irrespective of the disease state or ethnicity. This demonstrates that these factors are not reliable predictors of disease in these populations. In contrast, the prevalence of *dupA*, *hp0521* alleles and EPIYA motifs varied with ethnicity. We observe an association between EPIYA motifs and *hp0521* alleles and suggest a novel function for HP0521, probably correlated to CagA. The prevalence of *dupA* was further investigated in Swedish and Australian isolates. It varied between the geographic groups and could not be consistently associated with duodenal ulcer or gastric cancer across the investigated ethnic groups. The *dupA* gene was highly conserved in *H. pylori* strains with different geographic and ethnic background. A role in IL-8 induction in AGS cells could not be confirmed.

In paper IV, we examined the genetic diversity of type I restriction-modification (R-M) systems in clinical isolates of *H. pylori*. Due to the high sequence variability observed, we focused on the HsdS subunit, which is responsible for sequence specificity of the enzyme. A high number of novel allelic *hsdS* variants could be described. To investigate intra-strain diversity, we analyzed isolates obtained from six individuals at two different time points, four years apart. Most sequence variation could be observed for two isolates from patients with ongoing atrophy development. We therefore propose that *H. pylori* alters the specificity of type I R-M systems in response to a changing gastric environment, which may lead to adaptation.

H. pylori is a genetically diverse species. The work presented in this thesis provides an insight into the effects of this high diversity on immune response to the bacterium, on variation in *H. pylori* virulence as well as on a possible bacterial adaptation process.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Andres S**, Schmidt HM, Mitchell H, Rhen M, Maeurer M, Engstrand L.
Helicobacter pylori defines local immune response through interaction with dendritic cells.
Submitted.

II. Schmidt HM, **Andres S**, Kaakoush NO, Engstrand L, Eriksson L, Goh KL, Fock KW, Hilmi I, Dhamodaran S, Forman D and Mitchell H.
The prevalence of the duodenal ulcer promoting gene (*dupA*) in *Helicobacter pylori* isolates varies by ethnic group and is not universally associated with disease development: a case-control study.
Gut Pathogen, 2009 Mar 11; 1(1):5.

III. Schmidt HM, **Andres S**, Kovach Z, Nilsson C, Kaakoush NO, Engstrand L, Goh KL, Fock KW, Forman D and Mitchell H.
The *cag* PAI is intact and functional but HP0521 varies significantly in *Helicobater pylori* isolates from Malaysia and Singapore.
Eur J Clin Microbiol Infect Dis, 2010 Apr; 29(4):439-51.

IV. **Andres S***, Skoglund A*, Nilsson C, Krabbe M, Björkholm B and Engstrand L.
Type I restriction-modification loci reveal high allelic diversity in clinical *Helicobacter pylori* isolates.
Helicobacter, 2010 Apr; 15(2):114-25

* The authors contributed equally to the work.

CONTENTS

LIST OF ABBREVIATIONS

AA	amino acid
AdoMet	S-adenosylmethionine
AGS cells	human gastric adenocarcinoma cells
ATP	adenosine triphosphate
bab	blood group antigen binding adhesin
cag	cytotoxin associated gene
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DU	duodenal ulcer
dup	duodenal ulcer promoting gene
FD	functional dyspepsia
GC	gastric cancer
GERD	gastroesophageal reflux disease
Hp	*Helicobacter pylori*
hsd	host specificity for DNA
Ig	immunoglobulin
IL	interleukin
IFN	interferon
kDa	kilo Dalton
LPS	lipopolysaccharides
Le^b	Lewis b
MALT	mucosa-associated lymphoid tissue
MAP	mitogen-activated protein
MHC	major histocompatibility complex
MOI	multiplicity of infection
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nod	nucleotide-binding oligomerization domain-containing
oip	outer inflammatory protein
OMP	outer membrane protein
ORF	open reading frames
PAI	pathogenicity island
PCR	polymerase chain reaction
PU	peptic ulcer
R-M	restriction-modification
sab	sialic acid binding
TGF	transforming growth factor
Tcyt	T cytotoxic
Th	T helper
Treg	T regulatory
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRD	target recognition domain
vac	vacuolating cytotoxin

1 INTRODUCTION

When a pathogen enters its host it encounters a well-engineered defense mechanism, the immune system. The activation of the innate and adaptive immune response can result in disease symptoms. Usually, if invader and host survive the first encounter the pathogen is eliminated by the adaptive immune response. However, some pathogens can sustain the **host response and** cause a **persistent infection** such as *Mycobacterium tuberculosis*, *Chlamydia* spp. or ***Helicobacter pylori*** (see Figure 1.1). *H. pylori*, formerly known as *Campylobacter pyloridis*, is a bacterium that since more than 60 millennia has colonized the human gastric mucosa and today infects approximately half of the world's population [2, 3]. Within that span both the bacterium and its host have adapted to each other and developed a complex relationship that has challenged scientists since Warren and Marshall first cultured and isolated the species in 1982 [4]. Spiral shaped bacteria had already been identified in gastric contents and mucosa in 1889 but a causative role of the organisms for disease pathogenesis could not be proven at that time [5]. To prove pathogenicity, Barry Marshall fulfilled Koch's postulates on himself, drank a bacterial suspension and subsequently developed acute gastritis [6]. Thirty years of intense *H. pylori* research have revealed many facts about the versatile bacterium and the host response to it. However, there is still lot of debate about the nature of *H. pylori* infection that stays asymptomatic in the majority of infected individuals but can result in mucosa-associated lymphoid tissue lymphoma, peptic ulcer disease or gastric adenocarcinoma (an estimated 5.5% of all human cancer cases per year are caused by *H. pylori* [7]). After all, the development of the precancerous lesion atrophic gastritis seems to be disadvantageous for both host and bacterium since it leads to the loss of structure and function of the bacterial niche and thus the disappearance of *H. pylori*.

Several bacterial, host and environmental factors determine the nature of *H. pylori* infection. The work presented in his thesis focuses on bacterial factors and mechanisms that modulate persistent *H. pylori* infection and host response.

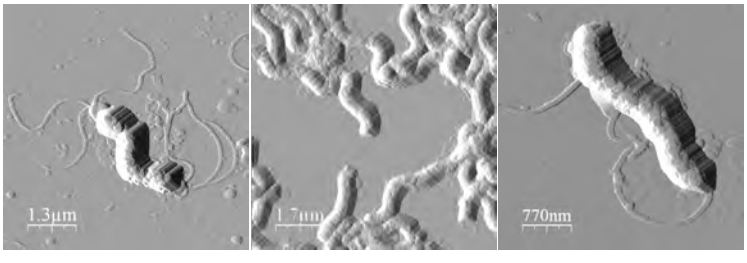

Figure 1.1. Atomic force microscopy images of *H. pylori* (kindly provided by K. Jonas)

1.1 The human stomach

H. pylori has its ecological niche in the mucosa of the human stomach. One of the main functions of the stomach beside the initiation of the digestive process is to eliminate invading microorganisms. *H. pylori* is able to colonize the stomach because it can, in contrast to most other bacteria, overcome the extreme acidity of pH 2 in the lumen (see 1.7).

The stomach is anatomically divided into four regions, the cardia, the fundus, the corpus and the antrum where *H. pylori* is primarily found (see Figure 1.2).

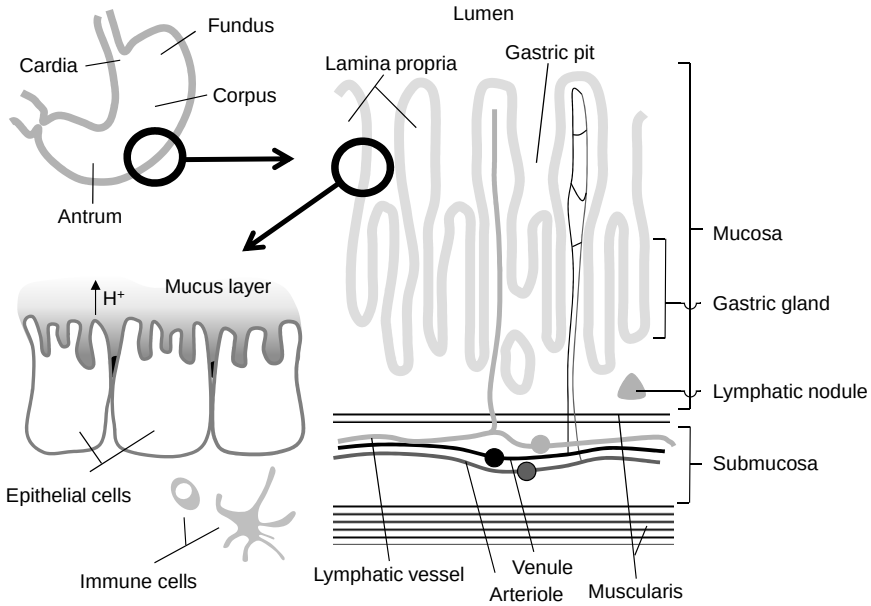

Figure 1.2. The histology of the human stomach
⟶ : The arrow points to a magnification of the circled area.

The outer tissue layer facing the stomach lumen is the gastric mucosa. Its surface is lined by epithelial cells that form the gastric pit. Three types of epithelial cells differentiate from multipotential stem cells of the central part of a gastric unit: the mucus-producing pit cells, pepsinogen-producing zymogenic cells and acid-producing parietal cells. Epithelial cells present a mechanical barrier separating the lamina propria from both commensals and pathogens. The protective function is enabled by a brush border on the luminal surface and tight junctions holding together adjacent cells [8]. A

thick mucus layer, trapping and washing away invading bacteria, completes the protection. *H. pylori* subverts clearance at this stage by adherence to the epithelial surface (see 1.7.4).

Beside the physical barrier, epithelial cells also provide an innate and an adaptive immune barrier and are, together with dendritic cells (DCs), the main cells to interact with luminal bacteria [9, 10]. Immune cells enter, and leave, the site of infection via lymphatic vessels of the lamina propria. The immunology of *H. pylori* infection is described in section 1.5.

1.2 Microbiology of *H. pylori*

H. pylori is a Gram negative, microaerophilic bacterium that belongs to the class of ε-proteobacteria. It measures 2 to 4 µm in length and 0.5 to 1 µm in width and is spiral shaped. The bacterium is motile due to two to six unipolar, sheathed flagella of approximately 3 µm length (see Figure 1.1). *H. pylori* can change morphology and convert to a non-culturable, coccoid form during starvation. It has been suggested that this form represents a dormant stage, but no reports about *in vitro* cultured coccoids exist [11, 12]. The bacterium can survive brief exposure to low pH but grows only at neutral pH, which can be found in the mucus layer of the gastric mucosa (see 1.1).

1.3 Transmission and epidemiology of *H. pylori*

H. pylori infection is commonly acquired during the first years of life. Transmission is thought to occur through direct human-to-human contact. It occurs in the majority of cases within families through the fecal-oral, oral-oral or gastro-oral route [13-18]. Colonization is presumably restricted to primates, since no other natural reservoir has been described [19, 20]. *H. pylori* infection is persistent but can be eradicated by treatment for two weeks with two or three antibiotics, e.g. clarithromycin and amoxicillin, and a proton pump inhibitor [21]. The human stomach is usually colonized by one *H. pylori* strain but co-colonization by multiple strains has been reported [22]. Due to high mutation and recombination frequencies and its natural competence *H. pylori* is genetically diverse [23-25]. This enormous genetic variability leads to the fact that almost every infected individual carries a unique set of strains (see 1.6).

Half of the world's population is chronically infected with *H. pylori* [26]. For children living in high income countries the incidence is low, less than 10%, in contrast to children living in low income countries where the incidence is about 80% [27]. The prevalence of infection is age-dependent particularly in countries with high

socioeconomic status. This is because of a decline of infections in childhood over time and reflects the birth-cohort effect. Improved sanitation, smaller family sizes and the use of antibiotics may be reasons for this development [28, 29].

1.4 Consequences of *H. pylori* infection

Persistent infection is asymptomatic in 80-90% of the infected individuals. However, the clinical course of the infection is influenced by many factors and might result in either duodenal ulcer or a gastric cancer phenotype (see Figure 1.3) [30, 31].

H. pylori has been associated with peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and increased risk of gastric cancer [32-35]. It has been suggested that the bacterium is involved in other conditions such as iron and vitamin B12 deficiency, immune thrombocytopenic purpura (ITP) and a variety of disorders outside of the stomach [36-38]. There have also been beneficial effects of *H. pylori* infection described (see 1.4.4) [39].

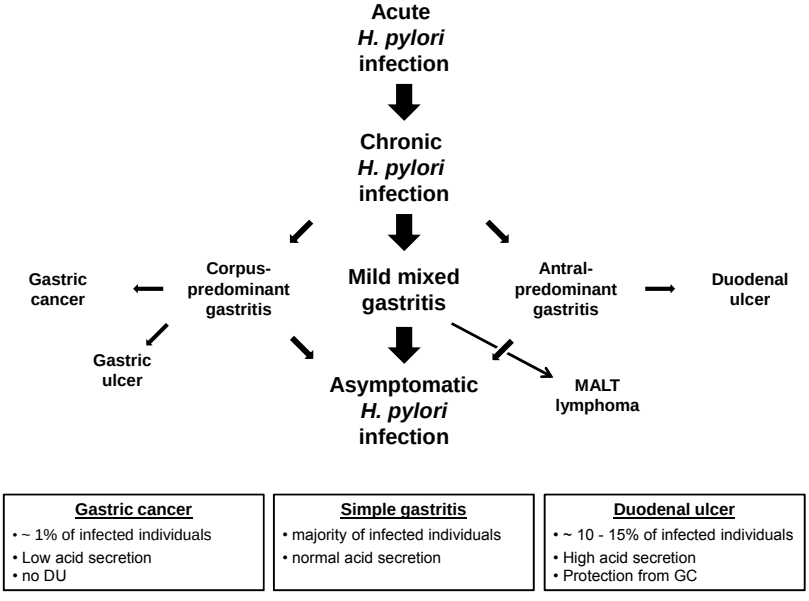

Figure 1.3. Clinical outcomes of *H. pylori* infections (modified from Suerbaum *et al.* [31])
The course of *H. pylori* infection is variable depending on various bacterial, host and environmental factors. High gastric acid output is associated with antral-predominant gastritis and duodenal ulcer development, whereas patients with low gastric acid output are predisposed to gastric cancer progression.

1.4.1 Gastritis

Colonization of *H. pylori* is always associated with an infiltration of inflammatory cells to the gastric mucosa, causing the gastritis [34, 40]. The first encounter with the bacterium is followed by the acute phase of infection, which can be associated with nonspecific dyspeptic symptoms such as fullness, nausea and vomiting. It affects the entire stomach and is often accompanied by low acid secretion called hypochlorhydria [41]. The initial colonization might be cleared at this stage, but a constant infiltration of lymphocytes and granulocytes can also progress into acute chronic gastritis [42-44]. A more pronounced inflammation is characteristic for chronic gastritis. Infiltrating cells together with epithelial cells produce pro-inflammatory cytokines and reactive nitrogen and oxygen types, which contribute to tissue damage (see 1.5) [40, 45].

1.4.2 Peptic ulcer

95% of patients with duodenal ulcer and 80% of patients with gastric ulcer are infected with *H. pylori* [46, 47]. The further development of *H. pylori* associated diseases is dependent on the gastric distribution of the gastritis during infection. Whereas duodenal ulceration is characterized by high acid secretion and antrum-predominant gastritis, gastric ulceration and gastric cancer are associated with low acid secretion and corpus-predominant gastritis (see Figure 1.3) [31, 48]. High acid secretion by parietal cells in the corpus in antrum-predominant gastritis leads to a higher acid output to the duodenum and metaplasia of the epithelial surface. The subsequent colonization of the duodenum by *H. pylori* leads to inflammation and some individuals develop ulceration [41, 49]. In contrast, the less frequently occurring corpus predominant gastritis is associated with a low acid output, partially due to the inhibitory effect of increased levels of IL-1β [50]. The subsequent pronounced colonization by *H. pylori* is followed by an elevated immune response and tissue damage that may lead to gastric ulceration [41]. Especially the predominance of effector Th1 cells and Th1 specific cytokines, such as interferon-γ and tumor necrosis factor (TNF), in the mucosa is associated with peptic ulcer development in *H. pylori* infected patients (see 1.5.2) [51].

1.4.3 Gastric cancer

In 1994, the World Health Organization's Agency for Research on Cancer classified *H. pylori* as a definite carcinogen [52]. The bacterium is estimated to be responsible for approximately 592,000 gastric cancer cases per year with the highest incidence rates in Eastern Europe, Central and South America and Eastern Asia. The five-year survival rate is extremely low, less than 20% after appearance of clinical manifestations [7].

The most common type of gastric cancer is adenocarcinoma, which can be sub-divided into the intestinal and the diffuse type dependent on the cell differentiation [53]. The

precursor of adenocarcinoma is chronic atrophic gastritis, a condition associated with the loss of acid producing parietal cells and pepsinogen-producing zymogenic cells and a parallel increase of gastric stem cells [31]. In addition to *H. pylori* and the accompanied inflammatory response many risk factors for the development of gastric cancer are suggested including: host gene polymorhpisms, dietary factors and smoking [54-57]. The predisposition of genetic polymorphisms in cytokine genes for neoplastic transformations, described e.g. for IL-1β, TNF-α and IL-10, is controversial [58-60].

A mucosa-associated lymphoid tissue (MALT) lymphoma consists of monoclonal proliferation of neoplastic B cells which infiltrate gastric glands [61]. This gastric cancer variant is a rare outcome of *H. pylori* infection. Up to 98% of patients with this disease carry the bacterium and *H. pylori* positive individuals have an increased risk for the development of MALT lymphoma [62, 63]. After the eradication of *H. pylori* patients regressed in 70 to 80% of the cases [64].

1.4.4 Potential benefits

H. pylori is gradually disappearing from a number of populations including Western Europe and the US [29]. At the same time other diseases are becoming more prevalent, e.g. an increased risk of gastroesophageal reflux disease (GERD), type 2 diabetes and asthma. The increase of these and other, in many cases autoimmune and allergic diseases, have been linked directly to the decreased prevalence of *H. pylori* [39, 65-68]. This has lead to speculations about human adaptation to *H. pylori* over thousands of years and a possible imbalance of immune response and physiology in case of its absence [69].

1.5 Immune response to *H. pylori* infection

H. pylori infection always results in an immune response, but the bacterium persists and the inflammation continues if not treated (see 1.2 and 1.4.1). For *H. pylori* a regulated balance of the immune response might be the key to persistence. An excessive inflammation results in loss of the ecological niche while a certain level of tissue disruption seems to be favorable because of the leakage of nutrients into the mucosa [70, 71]. It is the permanent, unbalanced activity of the immune system rather than direct bacterial action that induces *H. pylori* associated pathology [36]. There are many existing theories about the failure of the host response. These involve macrophages, dendritic cells and T cells [72].

The following section focuses on epithelial and dendritic cells in *H. pylori* infection. An overview of other elements of the immune response towards the bacterium is given in Table 1.1.

Table 1.1. Selected cells and cytokines involved in the immune response towards *H. pylori*

Component	Role in *H. pylori* infection and disease development	Ref.
Epithelial cells	**see 1.5.1**	
Neutrophils	- cause cell damage - generate reactive oxygen and nitrogen species - produce IL-12	73-76
Macrophages	- generate reactive oxygen and nitrogen species - produce IL-1, TNF-α, IL-6, IL-12 - *vacA*$^+$ *Hp* avoid effective phagocytosis - reduced antigen presentation - low activation via *Hp* LPS	73-82
DCs	**see 1.5.2**	
Lymphocytes	- *Hp* inhibits lymphocyte proliferation	74, 83
B cells	- increase of Ig producing cells in *Hp* infection - produce autoreactive antibodies	84-86
T cells	- down-regulated by VacA	83, 87
***Th*$_1$**	- more predominant in PU than in chronic gastritis - impaired function in chronic infection - promote epithelial cell death - cells from *Hp*$^+$ subjects respond poorly to *Hp* antigens - produce IFN- γ and IL-2	73, 74, 88-93
Tcyt	- highly present in infected mucosa - produce IFN-γ	92, 93
Treg	- increased in infection - depletion of Tregs in mice increases gastritis and pro-inflammatory cytokine levels - *Hp* specific suppression of memory T-cells	94, 95
IL-1β	- increased level in gastric mucosa - causes epithelial cell damage - host gene polymorphism associated with increased GC risk - inhibits acid secretion by parietal cells *in vitro*	50, 56, 96-99
IL-2	- VacA interferes with T-cell proliferation by blocking IL-2 signalling	83, 87
IL-6	- increased level in gastric mucosa - linked to activation of TLR-4, MAP kinase and NFκB signalling	97, 100, 101
IL-7	- increased level in gastric mucosa	100
IL-8	- increased level in gastric mucosa - increased production towards *cag* PAI$^+$ *Hp* - attracts neutrophils to the site of infection	96, 97, 100, 102, 103
IL-10	- increased level in gastric mucosa - infection of KO-mice resulted in severe gastritis and bacterial clearance	100, 104
IL-12	- increased level in gastric mucosa - linked to PU development in infection with *cagA*$^+$ *Hp* - stimulates Th1 cells and mediates protection	73, 74, 105-107
IFN-γ	- increased level in gastric mucosa - increase MHC II (receptor for *Hp* binding) expression - mediates mucosal damage	91, 97, 108
TNF-α	- increases level in gastric mucosa - causes epithelial cell damage - inhibits acid secretion by parietal cells *in vitro*	96-100, 102

1.5.1 Role of epithelial cells in immune response to *H. pylori* infection

The immune response towards *H. pylori* is initiated via the production of the pro-inflammatory chemokine interleukin-8 (IL-8) by gastric epithelial cells. Compared to other Gram-negative species the induction of pro-inflammatory cytokines via TLR-4 and TLR-5 is weak [109, 110]. Interleukin-8 release is mainly associated with the *H. pylori* cytotoxin associated gene pathogenicity island (*cag* PAI) components, such as the *virB4* homologous ATPase CagE [111, 112]. It is believed that the activation of Nod1→NFκB-dependent pro-inflammatory responses by peptidoglycan leaking through the TFSS is responsible for IL-8 secretion [112]. Direct contact between *H. pylori* and epithelial cells is required for IL-8 induction and is partially mediated by the outer membrane proteins OipA and BabA [113, 114]. It has been described that *dupA* and *vacA* are involved in IL-8 induction as well (see also 1.7.1 - 1.7.4). The release of IL-8 leads to increased infiltration of lymphocytes and granulocytes to the site of infection, resulting in inflammation [115, 116].

The further actions of *H. pylori* virulence factors CagA and VacA can cause tissue disruption and alterations in the morphology of epithelial cells, which enhance the direct involvement of submucosal immune cells in inflammation [71, 117, 118].

1.5.2 Role of dendritic cells in *H. pylori* infection

Dendritic cells can penetrate epithelial barriers to sample bacteria from the gut or gastric lumen [119, 120]. The interplay of *H. pylori* and DCs at this stage is of special importance, since DCs together with macrophages determine the T-cell response after their encounter with the bacterium.

Development of peptic ulcer disease in *H. pylori* infection is associated with Th1 response, while the presence of both cell types Th1 and Th2 is associated with asymptomatic gastritis (see Figure 1.4) [88]. A balanced inflammation is important for *H. pylori* to establish persistent infection (see 1.5). However, *in vitro* stimulation of human monocyte-derived DCs by *H. pylori* generated a Th1-biased response [121-123]. Bergmann *et al.* suggested a role of *H. pylori* LPS phase variation in suppression of Th1 development after active gastritis fades to chronic gastritis. They speculate that *H. pylori* regulates the binding to the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) through phase variable expression of fucosylated O-antigens in the LPS. The resulting attachment to DC-SIGN then affects the release of anti-inflammatory IL-10 and suppresses a Th1 predominant response [124]. DCs can, by generating inhibitory cytokines (such as IL-10), activate regulatory T-cells (Tregs) which in turn down-regulate T-cells and macrophages [125]. It has recently been described that *H. pylori*, due to the expression of fucose on its LPS, actively inhibits

DC-SIGN signaling. As a consequence IL-10 was up- and IL-6 and IL-12 were down-regulated [126].

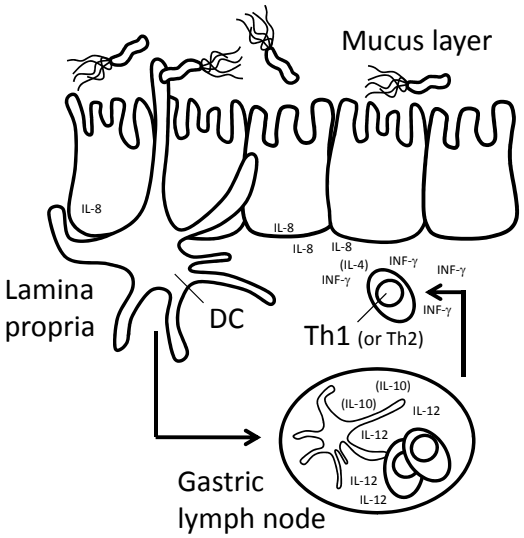

Figure 1.4. DCs bias the specificity of T-cell responses in *H. pylori* infection
After contact with *Hp* in the mucosa DCs migrate to the lymph node and activate T-cells [127]. The T-cell responses differ in peptic ulcer disease and asymptomatic gastritis (see text). → ( ) Th2 cells, IL-10 and IL-4 are involved in mild mixed asymptomatic gastritis in addition to Th1 cells, IL-12 and INF-γ. Macrophages, neutrophils and B-cells have been excluded for simplicity.

1.6 *H. pylori* evolution and genetic diversity

Elevated mutation rates and frequent intra-specific recombination in *H. pylori* are the basis for a high genetic variability, which enables a rapid adaption to its individual host. This is of importance after the transmission to a new host, but also during persistent infection in order to adapt to the continuously altering gastric conditions of the host, as well as the immune response towards *H. pylori* infection over time [25, 128-130]. In addition, the lack of genes involved in DNA repair, as e.g. mismatch repair enzymes, and an abundance of genes, which contain homopolymeric G or C tracts that favor translational phase variation, increase genetic variation in *H. pylori* [131-133]. Due to this variability almost every *H. pylori* infected individual harbors her/his own, genetically distinct set of closely related strains [23, 130, 134, 135]. An additional mechanism to increase genetic diversity is the inter-strain recombination with clonal variants of the strain or with other strains. Inter-strain recombination occurs at a very high frequency within

H. pylori compared to other organisms. The ability of natural transformation facilitates horizontal gene transfer. Falush *et al*. estimated that up to 50% of an *H. pylori* genome could be exchanged by recombination in only 41 years [24, 136, 137]. As a comparison, it takes 10-100 million years to replace 60% of the *E. coli* genome [138]. Interestingly, genetic diversity in *H. pylori* decreases with geographic distance from east Africa, an observation that led to the assumption that humans had already been infected with *H. pylori* before their migration out of the African continent about 58,000 years ago [3]. The high genetic diversity of *H. pylori* affects the whole genome as well as single genes. Several *H. pylori* genomes have been sequenced and an enormous genomic plasticity and diversity of *H. pylori* becomes apparent when analyzing these genomes (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). The comparison of 56 globally representative strains by genome hybridization revealed a conserved core genome that consists of 1111 genes [139]. The sequenced genomes of *H. pylori* contain between 1382 and 1576 predicted coding open reading frames (ORFs). So, approximately 400 genes are only present in a subset of strains. In accordance with this Salama *et al.* described a fraction of 22% genes that were variably present in microarray studies [140]. Highly present among the variable genes were, beside genes of unknown function, those genes that encode for restriction-modification (R-M) systems and outer membrane proteins (OMPs) but also *cag* PAI genes. Differences in the main virulence factors *vacA* and *cagA* related to geographical origins have been described [141, 142]. Genetic diversity of *H. pylori* genes, e.g. *cagA*, *vacA* and *hsdS*, the variability subunit of type I R-M systems, are the focus of papers I-IV.

1.7 *H. pylori* virulence factors

The colonization of the gastric mucus layer by *H. pylori* is the basis for virulence or pathogenicity. Essential for *H. pylori* colonization of the human stomach is its ability to survive the harsh environment. Several factors enable the bacterium to overcome gastric acidity and to find the way through the gastric mucus to the epithelial surface [48, 143, 144]. The enzyme urease is the most prominent one. It converts urea to ammonium and carbon dioxide and thereby neutralizes the local pH environment of the bacterium. The whole protein content of *H. pylori* contains a remarkable 15 % of preformed urease [145, 146]. To reach the protective area close to the gastric epithelial surface *H. pylori* uses unipolar flagella that allow chemotaxis driven motility towards higher pH [147, 148]. In the more neutral environment close to the epithelial cells, densities of 100 million bacteria/ml may be reached [149, 150]. Colonization is the first step in *H. pylori* virulence, but it is not necessarily accompanied by disease development (see section 1.4). Though, there are several virulence factors that are associated with disease development. It is worth mentioning that these disease associations with virulence factors are often

contradictable when comparing isolates from different populations even within the geographic regions. Allelic variants have been described for specific *H. pylori* virulence factors in different geographic regions [151, 152]. However, a virulence factor should show epidemiological consistence across populations and regions [153]. Papers II-III describe prevalence and variability of virulence factors in different geographic regions and ethnicities.

The following section focuses on the most common *H. pylori* virulence factors, with specific emphasis on those analyzed in papers I-III.

1.7.1 *cagA* and *cag* PAI

Pathogenicity islands (PAIs) are horizontally acquired genetic elements that contain virulence genes. They have a different G+C content compared to the rest of the genome and are often flanked by direct repeats [154]. The cytotoxin associated gene (*cag*) PAI of *H. pylori* encodes a type IV secretory system (TFSS) that allows the translocation of effector molecules, such as CagA, into the host cell [155]. *H. pylori* strains that carry the 40 kb DNA fragment induce IL-8 production in epithelial cells (see 1.5.1). Up to 32 genes are encoded on the *cag* PAI, of which 18 genes are required for CagA translocation and *cag* PAI-associated IL-8 induction (see Figure 1.5). The island encodes homologues of almost all of the genes that can be found in the well-defined TFSS of the plant pathogen *Agrobacterium tumefaciens* (*virB/D4* genes) [156]. The gene *cagE* for example is a homologue of *virB4* that encodes an ATPase. CagE is essential for IL-8 induction [111, 157].

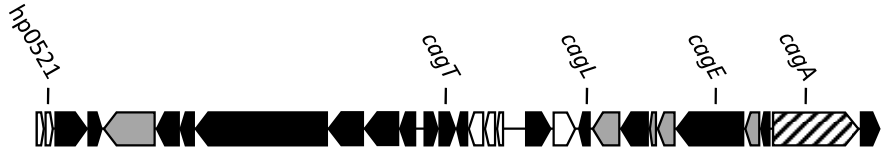

Figure 1.5. The *cag* PAI
The figure illustrates the arrangement of *cag* PAI genes in *H. pylori* 26695. The genes *hp0521, cagT, cagL, cagE* and *cagA* are of interest in paper III. Genes depicted by black arrows are essential for IL-8 induction and CagA translocation. Genes depicted by grey arrows are essential for CagA translocation only. White arrows represent genes that are not involved in these processes [156].

Strains that are positive for *cagA* are associated with a higher risk to develop peptic ulcer and gastric cancer [157, 158]. However, isolates that carry only partial deletions of the

cag PAI are less likely to be associated with disease development, independent of the *cagA* status [159]. Kersolyte *et al.* described a recombination event between strains that resulted in *cag* PAI excision and subsequent positive selection of the *cag* PAI negative strain, which leads to the question how the *cag* PAI might benefit *H. pylori* during persistent infection [160].

The CagA protein of the *cag* PAI was already discovered in the early 1990s, by several groups independently [158, 161, 162]. Despite its name, cytotoxin associated gene A, *cagA* is not chromosomally linked to the vacuolating cytotoxin A (*vacA*) or needed for the production of the toxin. After entry into the epithelial cells CagA is tyrosine-phosphorylated by Src family kinases. Up to five repeated phosphorylation sites with the motif Glu-Pro-Ile-Tyr-Ala (EPIYA) are located at the C-terminus of the gene [163]. The EPIYA-D motif as well as repeats of the EPIYA-C motif have been associated with gastric cancer development [164, 165]. The subsequent interaction of phoshorylated CagA with the tyrosine phosphatase SHP-2 and other intracellular proteins causes elevated cell motility and cellular elongation *in vitro*, a phenomenon described as hummingbird phenotype [166]. Large variations have been revealed in the conformation of the EPIYA segments that affect the extent and duration of CagA-SHP-2 signaling [167, 168]. CagA also communicates with tight junction proteins disrupting barrier function and leading to the loss of cell-cell adhesion [71, 169].

In addition to *cagA* and *virB/D4* homologues the *H. pylori cag* PAI contains several genes with unknown function. HP0521 is one of those genes [156]. In a Swedish study it was found to be allelic with a second variant, HP0521B, in approximately half of the isolates and just one out of 63 isolates lacked this gene locus [170]. Beside main virulence factors and markers for an intact *cag* PAI the HP0521 locus has been investigated in paper III.

1.7.2 *vacA*

The vacuolation cytotoxin VacA is a multifunctional toxin that contributes to colonization and virulence of *H. pylori* [171]. It is present in all *H. pylori* strains, although different strains harbor different allelic variants. These variants vary in cytotoxic activity, which has an impact on the association between *vacA* and peptic ulceration [141]. The *vacA* gene contains three genetically variable regions: the signal region (s), the middle region (m) and the intermediate region (i). Two allelic variants are distinguished for each region. Strains that contain the *vacA* signal sequence s2 fail to induce cell vacuolation *in vitro* [172]. The mid region determines cell-specificity [173]. The s1/m1 variants are most virulent and are associated with high vacuolating activity, while

s2/m2 variants lack activity [141]. The intermediate region variant i1, which was described much later, has been associated with gastric cancer [174].

VacA was initially recognized for the induction of vacuoles in epithelial cells but has many additional functions [175]. One effect is the induction of host-cell death through pore formation in mitochondrial membranes [176]. It furthermore causes leakage of nutrients, such as sugars and amino acids, by disrupting tight junctions [177]. Last but not least VacA plays a direct role in the modulation of immune response. It alters phagosome properties in macrophages which might impair phagocytosis, interferes with antigen presentation by B cells and inhibits production of IL-2 by T cells [79, 81, 83]. Also, pro-inflammatory effects have been described, such as the induction of IL-8 [178].

1.7.3 *dupA*

The duodenal ulcer promoting gene A (*dupA*) was first described in a study examining the relationship between *A. tumefaciens vir* gene homologues in *H. pylori* and clinical outcomes in *H. pylori* infections [179]. It was demonstrated that the *virB4* homologues genes *jhp0917* and *jhp0918* of the plasticity zone of *H. pylori* J99 form one continuous gene that encodes for a functional ATPase. The *H. pylori* genomes possess multiple *virB4* homologues, one of them is *cagE* (see 1.7.1).

The presence of *dupA* was found to be a marker for duodenal ulcer but not for gastric cancer development in patients from Korea, Colombia and Japan. Later studies investigating the possible association between *dupA* and clinical outcomes have been controversial (see Table 1.2).

Table 1.2. Controversial association between *dupA* prevalence and clinical outcome

Region	*dupA*$^+$/studied strains (%)						Ref.
	FD		DU		GC		
Colombia	15/40	(39)	24/45	(55)	6/50	(12)	[179]
Japan	7/50	(14)	11/30	(37)	3/30	(10)	
Korea	2/30	(7)	24/65	(37)	3/50	(6)	
Brazil	133/444	(92)	110/126	(87)	71/81	(88)	[180]
Belgium	26/76	(38)	20/40	(50)	10/19	(53)	[181]
China	3/12	(25)	2/11	(18)	1/1	(100)	
South Africa	11/15	(73)	12/13	(92)	16/18	(89)	
USA	9/20	(45)	9/21	(43)			
India	16/70	(23)	36/96	(38)			[182]
Iran	34/68	(50)	15/30	(50)	6/13	(46)	[183]
Japan	23/78	(30)	17/62	(27)	10/34	(29)	[184]

FD = functional dyspepsia, DU = duodenal ulcer, GC = gastric cancer

1.7.4 Outer membrane proteins

H. pylori expresses numerous adhesins which establish tight contact to the host cells. The attachment to epithelial cells facilitates colonization by escaping peristalsis and enables the bacterium to inject effector proteins into the host cells (see 1.7.1). Although adhesins can mediate cell invasion, it is commonly believed that *H. pylori* typically remains extracellular [185].

The best characterized adhesion protein of *H. pylori* is blood group antigen binding adhesin A (BabA). The 78-kDa protein mediates the binding to the fucosylated blood group antigen Lewis b (Le^b) [114, 186]. The full sized *babA2* allele was described to be associated with peptic ulcer disease and gastric adenocarcinoma [187]. Another outer membrane protein is the 34 kDa outer inflammatory protein A (OipA). OipA is controversially linked to IL-8 induction [188, 189]. Its expression is, similar to that of other outer membrane proteins, such as the sialic acid binding adhesin SabB, or enzymes of LPS biosynthesis, modulated by phase variation, which makes it phenotypically very diverse [113, 132, 190]. BabA and OipA are discussed in papers I (*babA* only) and III. *H. pylori* encodes over 30 outer membrane proteins. SabA, the adherence-associated lipoproteins AlpA and AlpB and the *Helicobacter* outer membrane protein HopZ have specifically been described regarding their roles in attachment or bacterial fitness [191-193].

1.7.5 Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram negative bacteria. It is typically composed of a lipid A anchor, a core oligosaccharide and a fucosylated O-antigen [194]. The O-antigen in *H. pylori* displays Lewis antigens, structures similar to those found on human epithelial cells or erythrocytes [195]. One single LPS molecule can present several O-antigens. Most clinical isolates express Le^x and Le^y in LPS, but other Lewis antigens such as Le^a and Le^b may also be expressed [196, 197]. The distinctive LPS phenotypes are due to translational frame-shifting in homopolymeric tracts within the genes of the three fucosyltransferases that catalyze the transfer of fucose to the carbohydrate precursor of the O-antigen [132, 198]. In addition, two fucosyltransferases, FutA and FutB, contain a variable heptad repeat region that determines the extent of O-antigen fucosylations [199]. *H. pylori* expressing Le^x and Le^y bind DC-SIGN on gastric DCs. The binding results in increased IL-10 release by DCs and a partial inhibition of Th1 activation (see also 1.5.2). It has been suggested that *H. pylori* populations adapt to changes in the gastric environment during disease progression by switches in the Lewis phenotype [200].

1.8 Restriction-modification

Bacterial restriction systems are, to a certain degree, comparable to the immune system of the vertebrates, distinguishing self from non-self elements and attacking foreign molecules. The capability of bacteria to make this differentiation and to protect its own DNA from cleavage is enabled by sequence specific modification. Restriction and modification are by necessity combined into systems called restriction-modification (R-M) systems. More precisely, while unmethylated target sites are substrates for the restriction enzyme, the endonuclease, hemimethylated DNA is fully methylated by the modification enzyme, the methyltransferase, and thus protected from restriction. The classification of bacterial R-M systems is based on their composition, co-factor requirements and the nature of the target sequence [201, 202]. Most of them fit into one of the three well-described classical groups (I, II and III, in order of their discovery). Type I R-M systems are the focus of paper IV and are therefore described in more detail.

1.8.1 Type I R-M systems

The bi-functional type I enzyme is a complex of three subunits encoded by *hsdR*, *hsdM* and *hsdS* (*h*ost *s*pecificity of *D*NA) (Figure 1.6). HsdM and HsdS, whose genes are transcribed from the same promotor, are both required for the S-adenosylmethionine (AdoMet) dependent methylation, or modification. For restriction activity, the third subunit HsdR is necessary as well. Restriction requires ATP and Mg^{2+} as co-factors in addition to AdoMet and occurs randomly at considerable distances, up to several thousand basepairs (bp), distant from the recognition sequence.

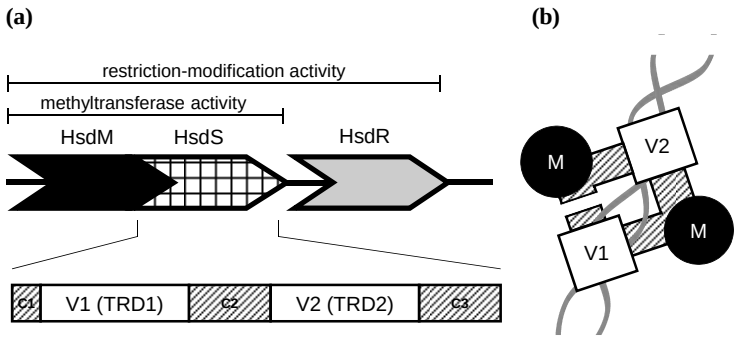

Figure 1.6. Structure and organization of type IC restriction-modification systems
(a) The three subunits of type I R-M enzymes are products of the genes *hsdM*, *hsdS* and *hsdR*. *hsdS*, encoding the type IC specificity subunit, comprises three conserved (C1-C3) and two variable target recognition domains (V1, V2). **(b)** It is assumed that the variable regions of HsdS identify the two DNA recognition sequences while the conserved regions bind HsdM [203]. The pictures are not drawn to scale.

The sequence recognized by type I R-M systems is asymmetric and covers two parts of 3-4 and 4-5 bp respectively, that are separated by a spacer of 6-8 bp [204]. For example: the recognition sequence for *Eco*KI, a R-M enzyme of *Escherichia coli* K12, is AAC(N_6)GTGC [205]. The subunit responsible for target recognition is HsdS (S for specificity). HsdS consists of three regions of conserved amino acids in the center, the C-terminus and, in some cases, the N-terminus, flanking two variable regions, that determine target specificity (target recognition domains, TRDs). The conserved regions of the HsdS subunit are important for the symmetrical configuration of the enzyme and the interaction with the subunits that carry out restriction (HsdR) and methylation (HsdM) [203]. Depending on the genetic organization of the *hsd* loci and the arrangement of conserved and variable regions on the *hsdS* gene, type I R-M systems are subdivided into four families (Type IA-D) [206-208]. Relatedness of subunits from different enzymes in the same family can be demonstrated by complementation tests [209-211].

1.8.2 Non-type I R-M systems

Type II systems are very simple in their structures and consist, in contrast to type I or type III R-M systems, of two independent enzymes, an endonuclease and a methyltransferase. DNA cleavage requires no ATP and occurs within the symmetric recognition sequence [204]. For this reason type II endonucleases became key enzymes in molecular biology and biotechnology [212]. The third group, type III R-M systems, consist of two enzyme subunits called Mod and Res. While Mod is sufficient for DNA methylation, both subunits are required for restriction activity. Type III systems cut DNA close to the recognition sequence [201, 204]. However, some of the identified R-M systems do not fit into any of the three groups mentioned [213, 214].

1.8.3 Relevance of R-M systems

Restriction-modification systems are common among bacteria from all ecological niches and taxonomic groups [202]. Protection from foreign DNA, especially through phage infection, but also by introduction of new plasmids into the bacterial cell, is the first described and most cited role for these systems [202, 215]. Other functions of DNA methyltransferases mainly involve DNA mismatch repair and the regulation of replication and gene expression [216-219]. An interesting consequence of endonuclease activity is that DNA-ends generated by restriction may stimulate recombination events [220]. This is of special interest in the case of type I R-M systems, which affect any kind of gene since they do not cut within the target sequence. In contrast, type II and type III systems generate a small number of “recombinogenic” breaks, since they always induce DNA strand breaks at the same places [221].

The described effects of R-M systems may affect bacterial-host interaction and increase virulence. Such an involvement of DNA methylation in virulence has been described before [216, 222].

1.8.4 R-M systems in *H. pylori*

Restriction-modification systems are highly present in multiple loci in the *H. pylori* chromosome (see Table 1.3 for type I R-M systems) [223-226]. For example the *H. pylori* HPAG1 genome contains 31 putative R-M systems, based on *in silico* analysis of the full genome sequence. As a comparison there are only three in *E. coli*, with an almost threefold genome size, and six in the related *Campylobacter jejuni* with a genome size comparable to *H. pylori* [224, 227]. The average number of R-M systems per genome for all sequenced bacteria is 4.3 [228]. In addition, the genes of *H. pylori* R-M systems comprise

Table 1.3. Annotations of *H. pylori* type I R-M genes (modified from [1]).

J99	26695	HPAG1	Annotation
		HPAG1_0439	*hsdR1*
JHP0416	HP0464	HPAG1_0440	*hsdR1*
JHP0415	HP0463	HPAG1_0438	*hsdM1*
JHP0414			*hsdS1a*
	HP0462		*hsdS1b*
		HPAG1_0437[a]	*hsdS1c*
JHP0784	HP0846	HPAG1_0831	*hsdR2*
JHP0786	HP0850	HPAG1_0833	*hsdM2*
JHP0785	HP0848		*hsdS2*
		HPAG1_0832[a]	*hsdS2b*
JHP1424	HP1402	HPAG1_1466	*hsdR3*
JHP1423	HP1403	HPAG1_1464	*hsdM3*
		HPAG1_1465	*hsdM3*
JHP1422			*hsdS3a*
	HP1404		*hsdS3b*
		HPAG1_1462[a]	*hsdS3c*
JHP0726			*hsdS4*
		HPAG1_0775[a]	*hsdS4/5*
	HP0790		*hsdS5*
	HP1383		*hsdS6*

The table shows the three complete type I R-M systems and additional orphan genes found in the sequenced *H. pylori* strains 26695, J99 and HPAG1. The levels of AA similarities are high between individual HsdR or HsdM proteins, but low between the three different HsdR or HsdM proteins (R1-3, M1-3; data not shown), suggesting the presence of different type I R-M system families.

[a] The numeric designation of the *hsdS* genes is not based on allelic identity but on genomic location. Especially the *hsdS2* and *hsdS4/5* genes show overlapping amino acid similarities.

a remarkable part, more than 50%, of the strain-specific genes and show a high diversity [229-233]. Different sets of R-M systems have been described to be functionally active for different *H. pylori* strains [227, 232].

It is not known why there are so many R-M systems in *H. pylori* and why these are so diverse, both genetically as well as functionally. Few reports about *H. pylori* phages exist, which supports the idea of other roles for R-M systems, e.g. in gene expression [234, 235]. For instance, The *H. pylori* M.HptAIV methyltransferase was shown to down-regulate the transcription of the catalase *katA* [236]. As for R-M systems in general, more attention has been paid to type II systems, and not much is known about the role of type I restriction and modification. The correlation of the presence of various *hsdS* genes with the induction of a robust host response in germ-free mice prompted Björkholm *et al.* to suggest that HsdS proteins affect gene transcription by methylation or direct binding of DNA [237].

Genes of type I R-M systems and putative orphan genes of the three sequenced *H. pylori* strains, 26695, J99 and HPAG1, are listed and arranged in Table 1.3 according to their potential affiliation to different families. In paper IV we characterize type I R-M system diversity and diversification in *H. pylori*.

2 AIMS

The work presented in this thesis has focused on the role of *H. pylori* in persistent infection. It aimed to investigate bacterial factors that, in multiple ways, influence the interaction of *H. pylori* with its host.

These were the specific aims for the papers that are included in this thesis:

Paper I:

To examine the impact of *H. pylori* on dendritic cell response and to evaluate the importance of this interaction on the inflammatory response in persistent infection

Papers II and III:

To investigate established and putative *H. pylori* virulence factors and this combinations as well as their association with disease in different ethnic groups

Paper IV:

To investigate type I restriction-modification systems in *H. pylori* and the role of R-M systems in disease development

3 MATERIAL AND METHODS

In this chapter, the bacterial strains used and the cell culture experiments applied in the present work are described. A more detailed description of the methods can be found in the papers attached. The studies involved the use of *H. pylori* strains isolated from humans. The collection of these strains was approved by ethical committees.

3.1 Bacterial strains

Swedish hospital based case-control study

The 52 and 61 clinical *H. pylori* isolates, that were used in the papers II and IV, to investigate *dupA* prevalence and sequence diversity of type I R-M systems were obtained from a Swedish gastric cancer case-control study [238]. The subjects were enrolled at eight regional hospitals between September 1995 and August 1997 and include patients diagnosed with gastric cancer, duodenal ulcer, non-ulcer dyspepsia and asymptomatic control subjects.

Paper IV included two isolates (67:20 and 67:21) from a gastric ulcer patient of this study. These strains were passaged *in vitro* 50 times using both small- and large bottleneck approaches [239]. Furthermore, single colony isolates of *H. pylori* 67:21 had been passaged *in vivo* in three transgenic, Lewis^b-expressing mice. Two of the mice were conventionally raised and infected with 67:21 for three and ten month respectively. The third mouse was raised under germ-free conditions and colonized with same strain for three months [240]. *H. pylori* 67:20 (*cag* PAI negative) and 67:21 (*cag* PAI positive) were also included as control strains in papers I and II.

Swedish random population based study

In the Kalixanda study, a random population-based study, volunteers from two Swedish communities chosen using a computerized national population register were evaluated. Information was available about the grade of inflammation, gastric diseases and score of *H. pylori* organisms present [241, 242]. After four years *H. pylori* positive individuals were followed up with a second gastroscopy.

Twenty isolates with different host background regarding the grade of inflammation at the site of the biopsy were used in paper I to investigate the cellular immune response against *H. pylori*. In paper IV, isolates from six individuals were used to examine variation in the type I R-M specificity subunit gene *hsdS*.

Australian, Malaysian and Singaporean hospital based studies

In previous studies patients with upper gastrointestinal symptoms were recruited from Sydney (Australia), Kuala Lumpur (Malaysia) and Singapore for routine endoscopy and subsequently diagnosed with gastric cancer (GC), duodenal ulcer (DU) or functional dyspepsia (FD). In paper II isolates from 45 Australian, 52 ethnic Chinese, 42 ethnic Indian and 14 Malay FD patients, 22 ethnic Chinese GC patients and 16 DU patients were used to investigate *dupA* prevalence and sequence diversity. Additional isolates from seven ethnic Indian and four Malay FD patients, but no isolates from Australian patients, were included in paper III to investigate the prevalence of novel combinations of *H. pylori* virulence factors in different ethnic groups.

3.2 Cell culture experiments

Infection of human gastric adenocarcinoma (AGS) cells

AGS cells (ATCC CRL-1739) secrete the inflammatory mediator IL-8 when infected by *H. pylori* [243]. In papers I, II and III the levels of cytokine secretion in response to different *H. pylori* strains were investigated. The ability of *H. pylori* strains to translocate CagA into the AGS cells was assessed in addition. Briefly, cells were cultured in 12- or 24-well plates to 80-90% confluence. *H. pylori* cultures were adjusted to a multiplicity of infection (MOI) of approximately 100 (for IL-8 induction) or 25 (for CagA translocation). AGS cells were infected with *H. pylori* for six hours at 37˚C. Cytokine concentrations were measured by ELISA and CagA translocation was examined by Immunoblot analysis.

Generation and infection of monocyte derived DCs

The impact of *H. pylori* strains on DC activation and maturation was investigated in paper I. Monocytes were purified from standard buffy coats from anonymous, healthy donors at the Karolinska hospital by Ficoll-Hypaque density separation using RosetteSepTM human enrichment cocktail (Stem cell Technologies). Immature DCs were derived in medium supplemented with GM-CSF (75 ng/ml) and IL-4 (75 ng/ml). After 6 days the phenotype was assessed by examination of CD1a and CD11c.

Cells at a concentration of 1 x 10^6 ml^{-1} were infected with *H. pylori* at a MOI of 10. Twenty-four hours later, maturation of DCs was detected by expression of the surface maturation markers CD80 and CD86. Both markers are involved in T cell activation [244]. DC activation was determined by measuring cytokines in the supernatant. Using the Human Inflammatory Kit for Cytometric Bead Arrays (CBA) the amount of IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70 could be detected simultaneously.

4 RESULTS AND DISCUSSION

4.1 Paper I

Helicobacter pylori defines local immune response through interaction with dendritic cells.

Dendritic cells are key players in both innate and adaptive immune response and of great importance in the immunology of *H. pylori* infection (see 1.5.2). This paper describes the cellular immune response, especially that by dendritic cells, towards *H. pylori*. Our *in vitro* results led to the suggestion that *H. pylori* defines the immune response at the site of infection through direct interaction with dendritic cells.

4.1.1 Strain selection and characterization

The strains used for this study were chosen from a random population based study: the Kalixanda study (see 3.1). For each strain several histological parameters were provided, e.g. the level of lymphocyte and granulocyte infiltration and the amount of *H. pylori* at the site where the biopsy was taken. Based on these three criteria, 20 out of 336 strains were selected for further characterization in *in vitro* cell culture experiments (see Table 4.1). Seven of the strains representing bacteria of a low inflammatory potential were isolated from biopsies with slight infiltration of lymphocytes and granulocytes but high amounts of *H. pylori*. A second group included the 6 strains with the highest infiltration of inflammatory cells and only slight amounts of *H. pylori* in the corresponding biopsy, which were considered to be of high inflammatory potential. Seven additional strains from biopsies with medium grade of infiltration of inflammatory cells completed the set of bacteria used. The selection of the strains and the assignment into a low, medium and high inflammatory group might be debatable. The infiltration of the inflammatory cells and the density of *H. pylori* in the biopsy often, but not necessarily, reflect the situation of the entire stomach because of the patchy nature of *H. pylori* infection [245, 246].

Table 4.1. Strain selection

Term	Lc + Gc	*H. pylori*
low	2	3
medium	3-4	1
high	5-6	1

Strains were selected based on the information about lymphocyte and granulocyte infiltration (Lc + Gc) and the density of *H. pylori* at the site of infection and separated into three groups of low, medium and high inflammatory potential. 1 = slight, 2 = moderate, 3 = high grade of infiltration/bacterial density

To characterize the virulence of strains, the presence of the main virulence factors *cag* PAI, *cagA*, *vacA* (s and m region) and *babA* was examined by PCR. In addition, strains were exposed to AGS cells to assess their effect on IL-8 release and their ability to translocate CagA into the epithelial cell (see 3.2). By doing this, the functionality of the *cag* PAI was determined.

All of the 15 *H. pylori* strains that induced a high amount of IL-8 release in AGS cells were able to translocate CagA. The *cag* PAI was therefore considered to be functional in these strains. In contrast, IL-8 induction could not be associated with the presence of any of the other virulence factors or the high, medium and low inflammatory potential of the selected strains.

4.1.2 DC response to *H. pylori* strains of diverse inflammatory potential

To characterize the impact of the different *H. pylori* strains on DCs their potential to initiate maturation and activation was assessed as described in 3.2.

The bacteria differed slightly in their impact on DC maturation. It has been described before that *H. pylori* has the ability to stimulate DC maturation [122, 123]. However, no correlation to the inflammatory potential of the strains was detected here by looking at the maturation profiles. This is in contrast to the results from the activation experiments (see Figure 4.1). Average amounts of IL-12, TNF-α, IL-6 and IL-1β were significantly higher in the supernatant of infections with strains from the medium or the high inflammatory group compared to the amount detected in the supernatant of the infection with strains of the low inflammatory group. By taking only the information about density of *H. pylori* at the site of infection into account, we observed a higher induction of cytokines for those strains that were present in low densities in the biopsies. Beside differences between the inflammatory groups, we described a trend for higher average IL-12 release in response to translocation negative strains compared to the response to translocation positive strains. This has not been described before, but seems reasonable since the *cag* PAI prevents phagocytosis, which is necessary for maximal IL-12 production [247, 248].

A critical point is the discrepancy of the results for the single donors. None of the described effects could be observed in all of the four independent runs suggesting an impact of the host on the results (Figure 4.1). The host's role in disease development has been in the focus of *H. pylori* research for a long time. For example, many polymorphisms in genes coding for proteins of the inflammatory response have been described to increase the risk for gastric cancer. However, some studies could not confirm these results [36, 56, 58].

Paper IV suggests that DCs play an important role in the definition and local response in persistent *H. pylori* infection. It also describes an association between the inflammatory background of *H. pylori* strains and their potential to activate DCs. The infiltration rates in the biopsies of the selected strains could be explained by their potential to define the DC response.

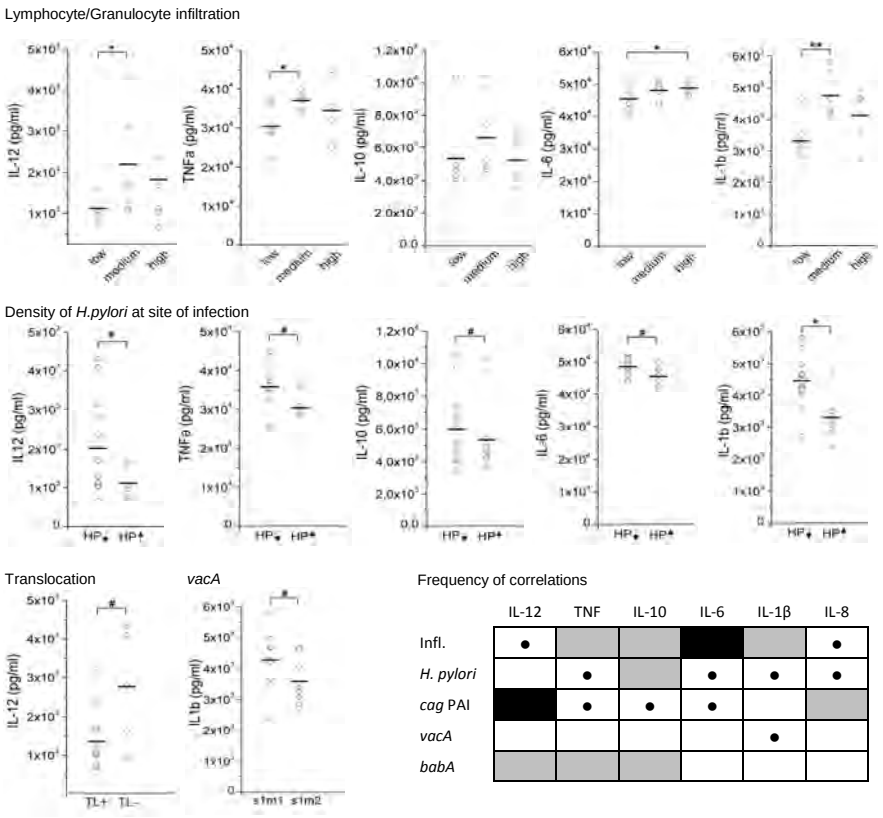

Figure 4.1. Cytokine profile in relation to strain characteristics

The figure shows the average cytokine secretion in relation to the inflammatory potential of the strains, the density of *H. pylori* in the matching biopsy (↓ = slight, ↑ = high), CagA translocation and *vacA* genotype. Data reflect the average from four independent experiments containing all of the 20 strains. The figure contains only those results that revealed differences between the selected groups. # $P < 0.1$, * $P < 0.05$, ** $P < 0.01$.

Differences in cytokine release that were detected for the four donors are depicted in the lower right corner. The table depicts the frequency of tendencies or significant differences. Infl. = inflammatory background; •, ▭, ■ = one, two, three correlations in four independent runs.

4.2 Papers II and III

The prevalence of the duodenal ulcer promoting gene (*dupA*) in *Helicobacter pylori* isolates varies by ethnic group and is not universally associated with disease development: a case-control study.

and

The *cag* PAI is intact and functional but HP0521 varies significantly in *Helicobacter pylori* isolates from Malaysia and Singapore.

In these two papers we described the prevalence of *H. pylori* virulence factors and their combinations in different ethnicities in Malaysia and Singapore. We also investigated the possible association of virulence factors with disease development in this population. In paper II clinical isolates from Australia and Sweden were added for the deeper characterization of the putative virulence factor *dupA*. No universal association with disease development could be observed for any of the tested virulence factors. Paper II describes a high variance of *dupA* prevalence between ethnic groups and geographic regions. A novel association between the allelic HP0521 and CagA EPIYA motifs was found in paper III.

4.2.1 *H. pylori* virulence factors and ethnic diversity

H. pylori is a genetically diverse organism (see 1.6). Strains differ, not only geographically between different countries, but also within countries between ethnic groups [249]. The investigation of *H. pylori* genotype variability in the different ethnic populations in this study was based on *dupA*, *cagA* and EPIYA motifs, *cagE*, *cagT*, *cagL*, HP0521, *babA*, *vacA* and *oipA*. Virulence genotypes of 52 Chinese, 49 Indian and 20 Malay patients with functional dyspepsia (FD), 22 Chinese patients with gastric cancer (GC) and 16 Chinese patients with duodenal ulcer (DU), all of them resident in Malaysia and Singapore, were determined by PCR. In addition, the prevalence of *dupA* was investigated in isolates from 20 Swedish patients with FD, 11 with DU and 21 with GC and 45 isolates from Australian patients with FD. A summary of the prevalence of individual virulence factors is given in Table 4.2.

The results in Table 4.2 show that the main virulence factors *vacA* s1i1m1 and s1i1m2, *oipA*, *babA* and an intact *cag* PAI were highly present through all the groups, independent from ethnicity or disease status of the host. This suggests that these factors are not contributing to disease development in these populations and are therefore no reliable predictors of disease. Differences in the prevalence within ethnic groups could

be detected for the EPIYA motifs and the *hp0521* variants as well as in the prevalence of *dupA* in both ethnic groups and diseases.

Correlation between EPIYA motifs and HP0521

We found an association between HP0521 alleles and EPIYA motifs. The EPIYA-C motif and the *hp0521* A allele were highly present in the Indian and Malay group, whereas EPIYA-D and a restricted deletion over *HP0521* were present in the Chinese group. HP0521 has not been assigned a function yet, but HP0521 domains show similarity to DNA topoisomerase I. The result indicates a functional connection of the locus with *cagA*. HP0521 may play a role in an undescribed function of the type IV secretion system.

Table 4.2. Genotype prevalence in 159 Malay and Singaporean *H. pylori* isolates

Ethnicity	**Chinese**			**Indian**	**Malay**
Diagnosis	**FD**	**GC**	**DU**	**FD**	**FD**
Total Number	**52**	**22**	**16**	**49**	**20**
cagA	100.0%	100.0%	100.0%	100.0%	100.0%
EPIYA-C	9.6%	13.6%	31.3%	89.8%	70.0%
EPIYA-D	90.4%	86.4%	68.8%	10.2%	30.0%
cagE	92.3%	95.5%	93.8%	93.9%	100.0%
cagL	86.5%	100.0%	100.0%	98.0%	100.0%
cagT	96.2%	86.4%	100.0%	98.0%	100.0%
***hp0521* ES**	88.5%	86.4%	100.0%	20.4%	25.0%
***hp0521* A**	7.7%	13.6%	0.0%	73.5%	55.0%
***hp0521* A+**	0.0%	0.0%	0.0%	4.1%	10.0%
***hp0521* B**	3.8%	0.0%	0.0%	2.0%	10.0%
***cag* PAI intact**	7.7%	13.6%	0.0%	73.5%	75.0%
***cag* PAI intact 521 ES**	73.1%	72.7%	81.3%	16.3%	25.0%
***cag* PAI partial**	19.2%	13.6%	18.8%	10.2%	0.0%
***vacA* s1i1m1**	51.9%	50.0%	75.0%	61.2%	60.0%
***vacA* s1i1m2**	48.1%	50.0%	25.0%	26.5%	40.0%
***vacA* s2m2i2**	0.0%	0.0%	0.0%	8.2%	0.0%
babA	100.0%	95.5%	100.0%	100.0%	100.0%
***oipA* ON**	86.5%	86.4%	87.5%	91.8%	95.0%
dupA	32.7%	59.1%	62.5%	14.3%	30.0%

The *cag* PAI including *hp0521*, *cagE*, *cagA* and the EPIYA motifs, *vacA*, *babA*, *oipA* and *dupA* have been described in 1.7. *cagL* and *cagT* are both part of the *cag* PAI and are involved in IL-8 induction and CagA translocation. *hp0521 A* and *hp0521 B* are the different allelic variants that were found in Swedish isolates, *hp0521* ES included *hp0520* and *hp0522* but not *hp0521* detected by empty site (ES) PCR, *hp0521* A+ is a longer variant of *hp0521* A. *cag* PAI intact, intact 521 ES and partial isolates were either positive for all genes of the *cag* PAI, positive for all *cag* genes and the *hp0521* ES PCR or lacked one of the *cag* genes.

Ethnic and geographic differences in *dupA* prevalence

The only *H. pylori* virulence factor tested that was associated with disease development was *dupA* (see Table 4.2). In paper II, FD, DU and GC isolates from Sweden and FD isolates from Australia were added to confirm the disease association and to compare the geographical prevalence of *dupA*. Sixty-five percent of the Swedish FD, 64% of the Swedish DU, 62% of the Swedish GC and 37% of the Australian FD isolates were *dupA* positive (see Figure 4.2). We concluded from the data, that *dupA* prevalence differs between ethnic and geographical groups and that the disease association of *dupA* is inconsistent between the Swedish and the ethnic Chinese population. The data are consistent with previous reports showing that the prevalence of *dupA* and also the association of *dupA* with clinical outcome vary between different countries (see Table 1.2) [179, 181, 182]. Our data suggest that not only geographic but also ethnic aspects should be considered before associating new virulence factors with disease development.

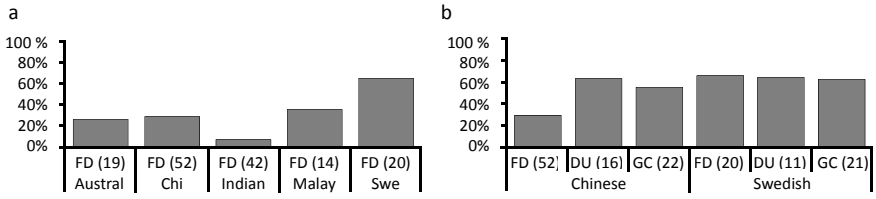

Figure 4.2. Variations of *dupA* prevalence
a: Prevalence of *dupA* differs between ethnic groups (Chinese, Indian, Malay resident in Malaysia and Singapore) and geographically. **b**: *dupA* is associated with DU and GC in ethnic Chinese Malaysians and Singaporeans but not in the Swedish population.

4.2.2 CagA translocation and IL-8 induction

We performed CagA translocation and IL-8 induction experiments on AGS cells to functionally validate the PCR-based classification of intact and partial *cag* PAIs, and IL-8 induction experiments to investigate the previously described association of *dupA* with the chemokine release by AGS cells (see 3.2).

While 90% of the *cag* PAI intact strains (with or without the *hp0521* gene) were fully functional, the two strains classified as *cag* PAI partial did not translocate CagA or induce IL-8 in AGS cells. Therefore we propose that our classification was reliable and the combination of *cagA*, *cagE*, *cagL* and *cagT*, but not *hp0521*, most likely indicates that the *cag* PAI is functional in East Asian populations.

The *dupA* gene is located in a plasticity zone and encodes homologues of the VirB4 ATPase (see 1.7.3). We sequenced the loci in clinical isolates and found *dupA* to be highly conserved, independently of the ethnic or geographic background suggesting an advantage associated with *dupA* for *H. pylori*. However, no association could be reported between the presence of *dupA* in the clinical isolates and the ability of these isolates to induce IL-8. It is not clear which role *dupA* plays for bacterial virulence in ethnic groups or geographic regions.

4.3 Paper IV

Type I restriction-modification loci reveal high allelic diversity in clinical *Helicobacter pylori* isolates.

H. pylori possesses many R-M systems including three complete type I R-M systems and additional orphan genes encoding the specificity subunit HsdS (see Table 1.3). However, the biological role for R-M systems, especially of type I, in *H. pylori* is not understood (see 1.8.4). In this paper, we investigated the genetic diversity and distribution of type I R-M systems in *H. pylori* isolated from patients with different clinical background (see 3.1). We suggest that genetic variability in *hsdS* genes may lead to adaptation to a changing gastric environment.

4.3.1 HsdS diversity in *H. pylori*

We could show in paper IV that the *hsdM* and *hsdR* genes were highly present in the 61 clinical isolates tested. The amplified PCR fragments were of the same, expected sizes. In contrast, *hsdS* genes were highly diverse, detected at lower ranges and showed extensive variation in the size of PCR amplicons. This prompted us to investigate this sequence variation. We selected the only *hsdS* locus which was present at the same genomic position in all of the analyzed strains, the orphan locus *hsdS4/5*, for sequence analysis and picked nine strains that showed large variability in the size of the *hsdS* PCR amplicons. The analysis revealed extreme diversity of the HsdS subunit regarding the presence of conserved and variable regions and the amino acid (AA) sequence of variable regions (see Figure 4.3).

The high levels of sequence similarities of the R and M subunits within the same families were expected and have been previously described for *H. pylori* (see Table 1.3). Diversification of the S subunit seems reasonable because the specificities of both endonuclease and methyltransferase are changed simultaneously. It furthermore provides the bacterium with the ability to increase and change specificities of R-M systems. Genetic variation of the variable regions results most likely due to

recombination events within two conserved regions [250, 251]. It has been described that recombination events are highly frequent during chronic *H. pylori* infection [137] (see 1.6).

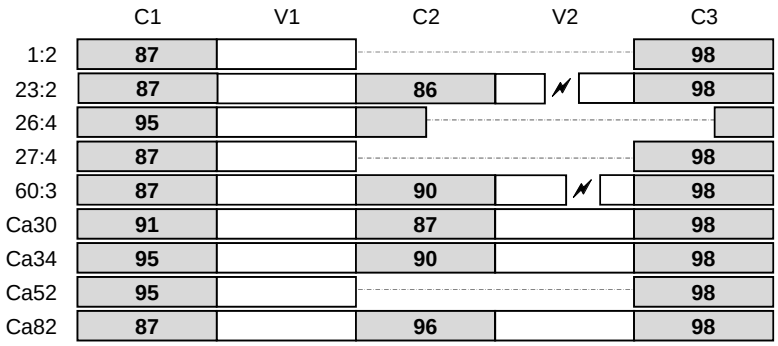

Figure 4.3. Extensive amino acid diversity of HsdS subunits.
The figure shows a schematic illustration of the HsdS4/5 subunits of nine *H. pylori* strains (1:2-Ca82). The specificity subunit is divided into three conserved (C1-3) and two variable regions (V1-2) (see also Figure 1.6). The numbers in the boxes specify amino acid identities between conserved regions and the alignment consensus sequence of the homologues in 26695, J99 and HPAG1. Only three of the nine proteins (in CA30, Ca34 and Ca 82) were complete while the other proteins were either restricted due to a frame shift mutation (23:2 and 60:3) or incomplete with one conserved and one variable region missing (1:2, 26:4, 27:4 and Ca52). The pictures are not drawn to scale.

4.3.2 Intra-strain HsdS diversification in the human gastric environment but not in *in vitro* or *in vivo* experiments

We aimed to test the hypothesis that the above described diversity could be beneficial for the bacterium under certain conditions and speculated if these conditions induce a more excessive diversification of the genetic loci in one strain. To study this, we sequenced the *hsdS* genes of different sets of *H. pylori* with the same origin.

We first analyzed single-cell colonies from the biopsy of a gastric ulcer patient. The sequence analysis of four PCR amplicons of different sizes revealed variation in the central conserved region. The differences were due to a 12 bp repeat encoding the AA motif TELN. The TELN motif was repeated up to five times. Tetra AA repeats are typical for type IC R-M systems [210]. A high level of nucleotide similarity and the presence of the AA repeat indicate that *H. pylori* HsdS2 and HsdS4/5 belong to this family. The repeated motif can give rise to a new configuration of the enzyme, as

described for the *E. coli* specificity subunit EcoR124 where the insertion of the AA repeat TELN increased the length of the two DNA binding domains, thereby changing the specificity of the whole protein [252]. Apart from the repeated motif no further sequence variations were identified.

Next, we investigated isolates that were either plate-passaged 50 times or recovered from experimental infections of mice. No changes in primer binding sequence or gene size were detected when we examined isolates before and after experimental subculture *in vivo* and *in vitro*.

To study if genetic modification of the *hsdS* genes occurs in persistent infection we selected isolates that were obtained from six individuals at two different time-points. Two of the individuals had a normal gastric mucosa at both time-points, two individuals had an ongoing atrophy development, one individual developed atrophy from normal mucosa and one individual developed cancer from moderate atrophy after the four years. Strains from different individuals (inter-strain) showed high sequence diversity. Extensive variations in the amino acid sequence of intra-individual strains were found for individuals with ongoing atrophy development. In one of these individuals two separate *hsdS* genotypes dominated at the two different time-points. Furthermore, intra-strain diversity could be detected for one of the two examined *hsdS* loci in one individual with normal mucosa.

Considering these results we suggest that the changing gastric environment during atrophy development leads to adaptation via increased *hsdS* variation. A selective force could be the lower acidic level in the mucosa, which often leads to the invasion of competing bacteria from the intestine.

5 CONCLUSION AND FUTURE PERSPECTIVE

H. pylori is a burden for humans since it causes serious, often lethal diseases, such as peptic ulceration and gastric adenocarcinoma in a substantial subset of infected individuals. However, most infected individuals will not suffer from any disease. The outcome of *H. pylori* infection is determined by a complex interaction between bacterial, host and environmental factors. This thesis examined bacterial factors and mechanisms that modulate persistent *H. pylori* infection. These are factors that enable the bacterium to adapt to its host and factors, which actively balance, or unbalance, host-bacterium interaction.

In paper I we describe that the IL-8 response of the human epithelial adenocarcinoma cell line AGS to *H. pylori* does not reflect the inflammatory state of the individual bacterial host, which was defined by the infiltration of lymphocytes and granulocytes to the site of infection. This was in contrast to the *in vitro* DC response to *H. pylori* infection represented by their cytokine release. We therefore suggest that DCs play an important role in local immune response to *H. pylori* infection. Variable bacterial factors may promote, or inhibit cytokine release by the immune cells. It might be a mechanism of immune suppression of certain strains that enables persistent infection. One can assume that it is not a single feature that determines the outcome of this interaction, and that it requires more than these bacterial factors to provoke a high inflammatory response over a long period. It remains to be studied whether the mechanisms behind the bacterial induction of cytokine release *in vitro*, which may be the same that define the infiltration by immune cells in *H. pylori* infection, lead to disease development in the susceptible host.

The main virulence factors *vacA* s1i1, *oipA*, *babA* and an intact *cag* PAI studied in paper II were in general highly present in ethnic groups resident in Malaysia and Singapore. No association of these virulence factors with disease development in the ethnic Chinese group could be detected, suggesting that these factors do not contribute to the progress of gastric cancer or duodenal ulcer in this population. However, variations between ethnic groups could be described in the prevalence of the *cag* PAI gene *hp0521* and *dupA*. A correlation of *hp0521* and *cagA* EPIYA-C prevalence in these isolates suggests a functional connection between these loci. The prevalence of *dupA* differs, as shown in paper III, between isolates from geographic regions and among different ethnicities and is associated with disease development in the ethnic Chinese but not in the Swedish population. Its association with IL-8 induction could not be replicated here. The roles of both of *dupA* and *hp0521* need to be studied in more

detail. There is probably no *H. pylori* virulence factor or combination of factors that can predict a certain development of infection. One has to be careful before associating bacterial factors to disease development in a population and also distinguish between geographic and ethnic differences.

The target recognition subunit gene *hsdS* of type I restriction-modification systems has been described to be genetically highly diverse between clinical *H. pylori* isolates. We found a high level of diversity between intra-strain isolates obtained from two patients with ongoing atrophy development over a four year interval. The results propose that variations in the HsdS subunit, which defines the specificity of the enzyme, may be induced by the changing gastric environment. It could be a way for the bacteria to adapt during persistent infection. To study this, a larger number of isolates from patients with disease development is needed. It is difficult to develop a functional assay that can prove this assumption because of the complicated nature of type I R-M systems.

New sequencing technologies will produce massive amounts of data on genetic diversity of *H. pylori*. By using such methods it will be easier to study bacterial virulence factors and to describe allelic variations in the future. However, one has to be careful when putting these data into the context. The work presented in this thesis provides new insights into the bacterial role in persistent *H. pylori* infection. It challenges general ideas about mechanisms of bacterial virulence and adaptation and will hopefully open questions for future research.

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