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# Mechanisms of genetic adaptation in *Helicobacter pylori*

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# Abstract

*Helicobacter pylori* is one of the most common bacteria worldwide, estimated to infect half of the human population. The infection is usually established in childhood and persists for a lifetime, unless treated. Most individuals never experience symptoms from *H. pylori* infection and are not aware of that their stomachs are invaded by spiral-shaped bacteria. However, about 20% of the infected individuals develop severe diseases such as peptic ulcers and gastric cancers. Why some persons are asymptomatic while others develop disease is not fully understood, but it seems to be a combination of bacterial, host and environmental factors. Since *H. pylori* was discovered in 1982 research has been devoted to solving the mystery of *H. pylori* associated disease development, and much has been learned, but still there are many unanswered questions. *H. pylori* is described as a panmictic population where the clonal structure is lost due to high recombination frequencies, which makes genetic studies challenging. This thesis aimed to investigate potential virulence associated genes: Restriction-modification (R-M) systems and Lipopolysaccharide (LPS), and to describe how these genes are expressed, what function they may have and the underlying mechanisms of *H. pylori* adaptation. In paper I, the *H. pylori* type II MTase, M.*Hpy*AIV, was characterized and functionally described. The frequency and distribution of GANTC sites in the fully sequenced *H. pylori* strains was mapped. In a M. *Hpy*AIV knockout mutant strain, the *H. pylori* catalase was significantly down-regulated, which implies that M.*Hpy*AIV affect gene expression. In paper II, the *H. pylori* type I R-M systems were in focus. The presence of the different subunits was investigated and we found that these R-M systems were rather conserved in our clinical isolates. However, genetic analyses revealed high allelic diversity of the specificity subunits involved in DNA recognition. In paper III intra-individual strains from biopsies obtained at different regions of the stomach were investigated. A molecular ruler model for how fucosylation of O-antigens may occur was suggested and correlated to genetic structures of the fucosyltransferase genes. In paper IV, isolates in different environmental settings were described and we showed that the phenotype of O-antigen chains altered when *H. pylori* strains were passaged *in vivo* in mice. Furthermore, we revealed that recombination events occurred between the two orthologous α1,3-FucTs in intra-individual isolates, which may alter their activity. The studies of this thesis contribute to the understanding of inter- and intra- individual diversity of *H. pylori* isolates and how these bacteria may evolve in order to adapt to new hosts and new environments. The way *H. pylori* alters its genotype has an impact on the expression and activity of the enzymes, which in turn may be of importance in *H. pylori* pathogenesis.

# List of Publications

I. **Anna Skoglund**, Britta Björkholm, Christina Nilsson, Anders F. Andersson, Cecilia Jernberg, Katja Schirwitz, Cristofer Enroth, Margareta Krabbe and Lars Engstrand.
Functional Analysis of the M.*Hpy*AIV DNA Methyltransferase of *Helicobacter pylori*.
J Bacteriol, 2007, 189(24):8914-8921.

II. **Anna Skoglund**, Sönke Andres, Christina Nilsson, Margareta Krabbe, Britta Björkholm and Lars Engstrand.
Type I Restriction-Modification loci reveal High Allelic Diversity in Clinical *Helicobacter pylori* isolates.
Submitted manuscript.

III. Christina Nilsson**, Anna Skoglund**, Anthony P. Moran, Heidi Annuk, Lars Engstrand and Staffan Normark.
An enzymatic ruler modulates Lewis antigen glycosylation of *Helicobacter pylori* LPS during persistent infection.
Proc Natl Acad Sci USA, 2006, 103(8):2863-2868.

IV. Christina Nilsson, **Anna Skoglund**, Anthony P. Moran, Heidi Annuk, Lars Engstrand and Staffan Normark.
Lipopolysaccharide Diversity Evolving in *Helicobacter pylori* Communities through Genetic Modifications in Fucosyltransferases.
Submitted manuscript.

# Contents

# List of abbreviations

| | |
|---|---|
| AP-PCR | arbitrary primed PCR |
| BabA | blood group antigen binding adhesin |
| bp | base pairs |
| *cag* | cytotoxin associated gene |
| CAG | chronic atrophic gastritis |
| CC | chief cells |
| CcrM | cell-cycle regulated methyltransferase |
| Dam | DNA-adenine methyltransferase |
| DNA | deoxyribonucleic acid |
| ECL | enterochromaffin like cells |
| ELISA | enzyme-linked immunosorbent assay |
| FucT | fucosyltransferase |
| GERD | gastroesophageal reflux disease |
| IceA | induced by contact with epithelial cell |
| IFN | interferon |
| IL | interleukin |
| IS | insertion sequence |
| kb | kilo base pairs |
| Le | Lewis |
| LPS | lipopolysaccharide |
| MALT | mucosa-associated lymphoid tissue |
| Mbp | mega base pairs |
| MNC | mucus neck cells |
| MSC | mucus surface cells |
| MTase | methyltransferase |
| NAP | neutrophil-activating protein |
| Nf-κB | nuclear factor- κB |
| NOS | nitrogen species |
| OMP | outer membrane protein |
| PAI | pathogenicity island |
| PC | parietal cells |
| PCR | polymerase chain reaction |
| R-M | restriction-modification |
| RNase | restriction enzyme |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| SabA | sialic acid binding adhesin |
| SNP | single nucleotide polymorphism |
| SP-D | surfactant D-protein |
| Th | T helper |
| TNF | tumor necrosis factor |
| UBT | urea breath test |
| VacA | vacuolating cytotoxin |

# 1. *Helicobacter pylori*

## 1.1 HISTORY

In 1893, novel spiral-shaped bacterium was discovered in the gastric mucosa of dogs and its appearance in the mucosa was documented in hand-drawn pictures by Bizzozero (20). This was the first description of a *Helicobacter* species. After that, several reports indicating Helicobacter findings were described, but presumably due to a common opinion that the stomach is sterile and no living organism could survive in the harsh acidic environment, these observations were not further investigated. A whole century passed before *H. pylori* was cultured for the first time (91). In Perth, Australia, an agar plate containing culture from a stomach biopsy was forgotten in an incubator over the Easter holiday 1982. To their great surprise, when the investigators came back, tiny transparent colonies were growing on these plates. The newly discovered bacterium was named *Campylobacter pyloridis* and was eventually accepted as a bacterium that actually colonizes the stomach. The two explorers Robin Warren and Barry Marshall detected the bacterium in patients suffering from stomach diseases and suggested a correlation between presence of the novel bacterium and gastric illness, but there was not enough evidence to prove this (91, 152). In 1985, Marshall decided to confirm this relationship. He ingested a suspension of the bacterium and was shortly after this suffering from acute gastritis (90). Thanks to this unconventional method to fulfil Koch´s postulate, an enormous amount of people could be cured from ulcers, which was earlier believed to be caused mainly by stress. Later, the bacterium was renamed *Campylobacter pylori* and in 1998 it obtained the current name, *Helicobacter pylori* (61). In year 1994, due to its association with gastric cancer, the World Health Organization classified *H. pylori* as a type I carcinogen (IARC 1994). In 2005, there was a big moment for *H. pylori* enthusiasts all over the world, when Warren and Marshall were awarded the Nobel Prize in Physiology or Medicine for their important discovery (116).


**Figure 1.** Electron microscopy picture showing *Helicobacter pylori* strain 26695.

## 1.2 MICROBIOLOGY

*H. pylori* is a gram-negative spiral shaped bacterium which is 2.5-4.0 µm long (62). It is a slow growing bacterium, requiring a $37^{O}$C microaerophilic (low oxygen levels) atmosphere and a rich medium for laboratory growth. A coccoid inactive form of *H. pylori* has also been described but it is not known whether this is a nonviable or dormant form (62). The spiral-shaped viable form of *H. pylori* has four to six flagella, attached at one end that renders the bacterium motile and enables it to penetrate the mucus layers in the stomach, where a less acidic environment is found. To withstand the acidic environment in the stomach *H. pylori* produces an enzyme called urease, which converts urea to ammonia and carbon dioxide (42). This leads to an increase in pH of the micro-environment that surrounds the bacterium and enables bacterial colonization of the harsh gastric environment. Besides urease, other proteins produced by *H. pylori* to help colonization include thioredoxin, catalase and superoxide dismutase (94, 130).

## 1.3 TRANSMISSION AND PREVALENCE

The only known source of *H. pylori* is the human being. The transmission route is believed to be fecal-oral, oral-oral or gastro-oral, prominently between family members (77, 113). The infection is acquired during childhood and usually persists for lifetime, unless treated. High risk factors for infection are low socioeconomic status, large family size and familiar connections to high prevalence countries (60, 77). It is estimated that about 50% of the world´s population is infected. Although the incidence today is less than 10% in children living in high income countries, the incidence in low income countries the is about 80% (146). In Western countries the prevalence of *H. pylori* infection is higher in older individuals, ranging from 20-80%, which reflects a birth-cohort phenomenon (111). The usually worse sanitary conditions and lower socioeconomic status when these individuals were children has resulted in higher infection prevalence among the elderly.

## 1.4 DETECTION AND TREATMENT

There are both invasive and non-invasive methods to detect *H. pylori*. The invasive methods; culture, histology or rapid urease test requires gastric endoscopy and sampling of biopsy specimens (104). To reach a high sensitivity, two biopsies are necessary for culture, due to the patchy colonization of *H. pylori*. Gram-negative staining and positive urease, catalase and oxidase tests identify the cultured bacteria as *H. pylori*. Giemsa, or other staining methods directly on tissue sections can be performed as alternative detection methods (48).

Non-invasive methods include serological tests, and the urea breath test. *H. pylori* infection elicits IgG and IgA anti-*H. pylori* antibodies that can be used for diagnosis (120). The urea breath test (UBT) takes advantage of the enzymatic activity of the *H. pylori* urease. The patient ingests $^{13}$C-urea and if *H. pylori* urease is present, converts this to $^{13}$C-labelled carbon dioxide which is exhaled and used for detection (57). Non-invasive methods are generally less expensive and more rapid.

Eradication treatment of *H. pylori* infection is performed by using two antibiotics, generally combinations of clarithromycin and either amoxicillin or metronidazole (88,

138). In addition, a proton pump inhibitor (e.g. omeprazole) is given to heal the ulcer and because antibiotics can have an impaired effect in low pH. The treatment lasts for one to two weeks and is usually successful.

Resistance to antibiotics sometimes occurs, which can result in treatment failure. Antibiotic resistance towards clarithromycin and metronidazole has been found in clinical samples (54). The resistance mechanisms are due to genetic mutations, which may appear rapidly due to the high mutation frequencies in *H. pylori* (22).

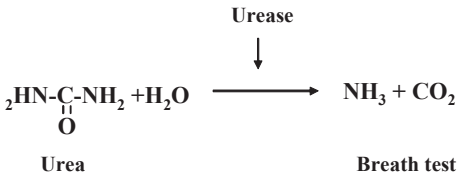


**Figure 2.** Urease converts urea to carbon dioxide. This enzymatic reaction is taken advantage of in urea breath test diagnostics.

## 1.5 GASTRIC DISEASE DEVELOPMENT IN *H. PYLORI* INFECTION

All individuals infected by *H. pylori* establish gastritis. First, an acute inflammation occurs, which later turns into a less symptomatic variant of chronic superficial gastritis. Host immune mediators keep the infection under control, but are still unable to clear the invader from the host. This results in a persistent infection for life, unless treated with antibiotics. Usually the infection is asymptomatic and the persons infected are not harmed by *H. pylori* carriage. However for the 10-20% that develop disease the consequences are serious and sometimes fatal (139).

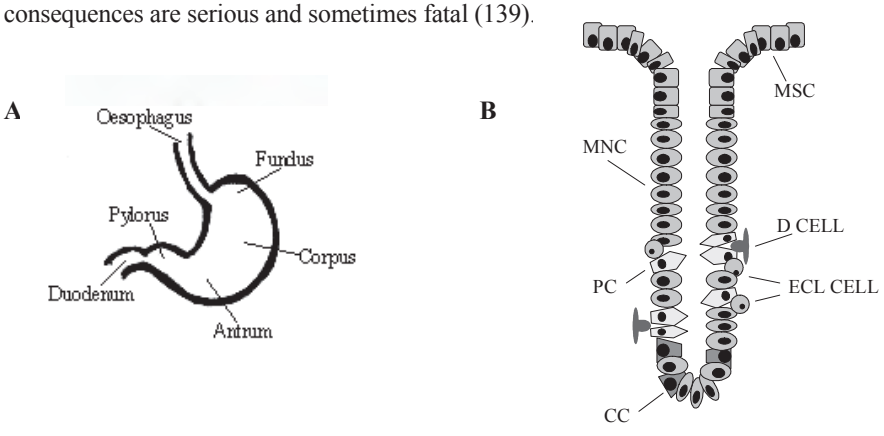


**Figure 3.** A) The anatomy of the stomach. B) Schematic picture of a gastric gland showing, mucus surface cells (MSC), mucus neck cells (MNC), parietal cells (PC), enterochromaffin-like (ECL) cells, chief cells (CC) and somatostatin-containing D cells.

### 1.5.1 Gastritis

The acute inflammation in the gastric mucosa that occurs upon infection with *H. pylori* after a while continues to a more superficial inflammation. In volunteer studies the symptoms of acute infection were nausea, vomiting and/or fever and biopsies from these individuals revealed gastric inflammation (2). Other symptoms described from acute inflammation are cramping, gastric pain, headaches, anorexia and hypochlorhydria - an increase of pH in the stomach (2). After a period with acute gastritis presumably the immune system controls the infection, and a majority of the individuals become asymptomatic. However, in *H. pylori* positive gastric biopsies there are always signs of inflammation in the tissue, whether the individual suffers from symptoms or not. When the normal mucosa is disturbed due to an active chronic gastritis this can result in chronic atrophic gastritis (CAG). CAG is characterized by loss of acid-producing parietal cells and pepsinogen-expressing chief cells, and is believed to be a precursor of stomach cancer (110).

### 1.5.2 Ulcers

Ulcer disease is caused by an imbalance of aggressive factors such as acid or pepsin and defensive factors such as mucus, bicarbonate and blood flow. This balance may be disturbed due to *H. pylori* infection, since the bacterium is found in approximately 94% of duodenal ulcer and 84% of gastric ulcer cases (80). Gastritis predominately localized to the antrum is associated with duodenal ulcers and high acid output, whereas gastritis in the corpus is associated with gastric ulcers and low acid output. One proposal is that the local acid output of the individual may determine *H. pylori* colonization density and virulence in the site, which in turn correlates to the degree of associated inflammation (40).

**Duodenal Ulcers**

Increased acid levels are found in patients with antral gastritis. This is explained by elevated gastrin release from the antrum, which in turn activates the acid producing parietal cells situated in the corpus (58). The elevated acid output result in acid leakage and affects the duodenal cells to undergo gastric metaplasia – the transformation of the duodenal cells to gastric epithelial cell morphology. The transformed cells in the duodenum then serve as a new niche where *H. pylori* may colonize. The occurrence of gastric metaplasia in combination with inflammatory response due to *H. pylori* colonization of this site may be the contributing factors resulting in duodenal ulcer disease. Patients suffering from duodenal ulcer disease seem to be protected against development of gastric cancer (148).

**Gastric Ulcers**

In a gastric ulcer patient there is a low output of acid, due to impaired functions of the parietal cells. How this is accomplished and why *H. pylori* cause gastric ulcers is not fully understood. The tissue damage enzymes caused by *H. pylori* and the harmful effects of the immune system may result in increased epithelial-cell proliferation, which induces an imbalance of the defensive factors. The damaged tissue may then become more sensitive to acid secretion and gastric ulcers may arise. Gastric ulcer patients are at risk of developing cancer.

### 1.5.3 Gastric adenocarcinoma

There are two types of gastric adenocarcionomas defined, intestinal and diffuse. The intestinal type is usually found in older men and has relatively well defined histological morphology. The diffuse type appears in both sexes and in a wider age-range. The hallmark of the diffuse type of gastric cancer is infiltrating neoplastic cells that do not form glandular structures, and it is not associated with intestinal metaplasia (129). Strong evidence for the correlation between *H. pylori* and gastric cancer were presented by Uemuera *et* al. in a large prospective study including 1526 Japanese patients suffering from different gastric diseases (148). Gastric cancers developed in 2.9% of the *H. pylori* positive patients, meanwhile no cancer was detected in patients that were *H. pylori* negative. In addition, *H. pylori* infection of Mongolian gerbils showed an increased risk of developing cancers. The different stages in development of the intestinal type cancer through *H. pylori* infection where corpus-predominant gastritis progress to atrophic gastritis, intestinal metaplasia, dysplasia and finally gastric cancer have been described by Correa *et* al. (31). Oxidative stress is one factor suggested for cancer development (30). The inflammation process due to *H. pylori* inflammation continues for decades, delivering reactive oxygen species (ROS) and nitrogen species (NOS) targeting epithelial cells. This combined with other destructive bacterial factors, the host´s capability of handling the stress and amount of antioxidant intake may be the players important whether or not cancer develops.

### 1.5.4 MALT-mucosa associated lymphoid tissue lymphoma

Unlike most other lymphomas that occur in lymph nodes, gastric MALT arises from lymph tissue present in the gastric lining. *H. pylori* infection significantly increases the risk of gastric MALT lymphoma, and 72 to 98% of patients with MALT lymphoma were *H. pylori* positive (112, 158). There is evidence for regression of gastric MALT lymphoma due to eradication of *H. pylori*, which strengthens the correlation (16).

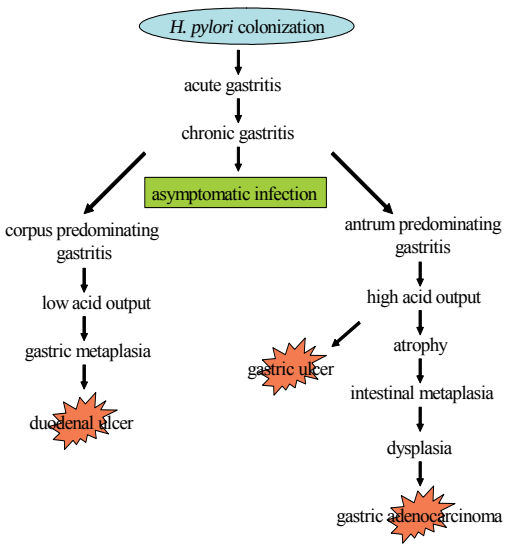


**Figure 4.** Model for how disease development of *H. pylori* infection, adapted from Correa *et* al. (31).

### 1.5.5 Effect of acid homeostasis in disease development

It is interesting how the bacterium *H. pylori* can contribute to such different types of diseases. Even though the peptic ulcer diseases of corpus and antrum have similar characteristics, duodenal ulcer patients are at low risk for development of gastric ulcer and gastric cancer, while gastric ulcer patients are at risk for cancer development but are at low risk of developing duodenal ulcers (26). This may be explained by the interplay between the inflammatory process and cells involved in acid homeostasis (58). In duodenal ulcer patients the acid secretion is high. This may be caused by the up-regulation of gastrin production in the antral G cells, which in turn is affected by impaired synthesis and release of somatostatin by the D Cells. As an answer to the high gastrin level, the parietal cells situated in the corpus start the production of gastric acid. Enterochromaffin-like (ECL) cells also situated in corpus are affected by gastrin and release histamine, which also positively regulates acid production. The mucosa of the duodenum reacts to the overload of acid production in the stomach and gastric metaplasia develops. When inflammation is initiated in the corpus, the same cascade of reactions occurs, but in addition the inflammation has a direct effect on the parietal cells and ECL cells, down-regulating histamine and acid production. The net effect of corpus predominated gastritis is then a low acid output. The ulcers established in the corpus are suggested to be formed due to a weakening of the tissue caused by the inflammation and high cell-turnover. In the light of acid homeostasis, the use of proton pump inhibitors in ulcer disease may be discussed. If the ulcers are caused by an *H. pylori* infection, the lowering of acidity due to the drug may enhance colonization of the corpus, which in turn may be a risk factor for cancer development. Also, intestinal metaplasia of the gastric cells that causes overgrowth with intestinal bacteria may be and additional risk.

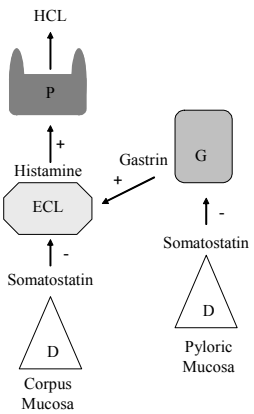


**Figure 5.** The acid homeostasis in the stomach is of significance in ulcer development. The cells involved in acid homeostasis include acid producing parietal cells (P), somatostatin producing D cells (D), gastrin producing G cells (D) and histamine producing ECL cells (ECL). Picture made by Mathilda Lindberg.

## 1.6 HOST RESPONSE

A strong immune response is triggered by *H. pylori* infection and both cellular and humoral host responses are activated. Despite this, *H. pylori* remain at high densities in the stomach and persist for decades. Several *H. pylori* proteins and other structures, such as the Lewis antigens, probably affect the immune response to become more beneficial for the bacterium.

In a volunteer study where twenty individuals were infected with *H. pylori*, antral biopsies obtained after two weeks of infection were infiltrated by monocytes and macrophages and showed a significantly increased expression of the pro-inflammatory cytokines interleukin (IL)-8, IL-6, and IL-1β (132). This is in concordance with another study where the abovementioned cytokines and in addition tumor necrosis factor (TNF)-α were found in increased levels in gastric *H. pylori* infected tissue specimens (34, 114). IL-1β and TNF-α inhibit acid secretion and high expression level of these cytokines is suggested to contribute to atrophic gastritis and increased risk of adenocarcinoma development. IL-6 promotes a humoral host response, and antibodies such as IgA, IgG and IgM that are reactive against *H. pylori* antigens have been detected in infected individuals (153). High amounts of IL-12 production, which is required for differentiation of naive T-cells into activated Th1 cells, has been observed and associated with *H. pylori* infection (15). Lewis antigens are suggested to interact with the receptor DC-SIGN on dendritic cells and depending on which Lewis antigens are expressed, different cytokines promoting Th1 or Th2 response are expressed. This study suggests that a more balanced Th1/Th2 response is acquired through the interaction of Lewis antigens (18). Other *H. pylori* structures have also been reported to affect the immune response. Neutrophil-activating protein (NAP) promotes Th1 polarization by stimulating IL-12 and IL-23 from neutrophils and monocytes (6). Interestingly, a high Th1 host response has been reported due to *H. pylori* infection. Th1 is usually activated in the presence of intra-cellular organisms. Although some studies reports intra-cellular *H. pylori* (108), the main location is outside the cells in the mucus layers. The *cag* PAI enhances the induction of IL-8 in host cells and may contribute to disease development (33). Nitric oxide, immunological effector molecules of macrophages, has been shown to be up-regulated by *H. pylori* both *in vitro* and *in vivo* (55, 59). Another interesting study showed that *H. pylori* can escape from the macrophages by glycosylation of cholesterol (160).

Bacterial factors may affect the immune response to dysregulate its function, in order to successfully establish a persistent infection of the stomach. The host immune response is normally unable to clear *H. pylori* colonization, although it probably succeeds occasionally, and the inflammation continues for decades. The constant triggering of the inflammatory processes probably contributes to pathogenesis and may be the major cause of *H. pylori* disease development.

## 1.7 BACTERIAL FACTORS

One definition of bacterial virulence factors is that they are essential for disease development in the host, when present in the colonizing bacteria disease progression occurs, but when absent the bacterium is harmless to the host and unable to cause

disease. True virulence factors according to this definition have so far not been discovered in *H. pylori*. A long evolutionary relationship between *H. pylori* and the host has been suggested and argues for an adaptation to a more commensal relationship (53, 137). Some studies even suggest it is beneficial to carry *H. pylori*. These are for example epidemiological data showing that *H. pylori* infection is protective against development of gastric esophagus reflux disease (GERD) (25). Immunocompromised individuals are not at higher risk for development of *H. pylori* related diseases than individuals with normal immune systems, indicating that the bacterium is not primarily a pathogen. Also, since the only reservoir found is in primates it seems likely that a commensal relationship would be an advantage for the survival of *H. pylori*. However, the immune system is activated upon colonization and there are evidence of different *H. pylori* proteins and structures that are harmful for the host, such as the *cag* pathogenicity island (PAI) and vacuolating cytotoxin (VacA). Even if there is no obvious advantage for *H. pylori* to establish disease in the human host, sometimes the expressed bacterial genes and host and environmental factors unfortunately result in pathogenesis. Some of the bacterial determinants involved in *H. pylori* infection and disease development are described below.

### 1.7.1 Colonization

When entering a new host, *H. pylori* requires enzymes to adjust to the environmental surroundings and has to withstand the different challenges of a new host in the fluctuating environment in the stomach. The multiple unipolar flagella attached to *H. pylori* allow the bacteria to swim towards a more neutral pH in the mucus layer via chemotactic signals (163). The flagella are essential for *H. pylori* colonization. To achieve protection from the acidic environment, an enzyme called urease neutralizes the acidic environment by converting urea to carbon dioxide and ammonia. The urase activity is essential for survival in acidic pH and for colonization in mice (44, 147). As protection against reactive molecules, produced by host immune cells, *H. pylori* synthesizes enzymes like superoxide dismutase and catalase to handle oxidative radicals. Moreover, enzymes like phospholipase and thioredoxin assist in colonization by degenerative actions on the mucosa (89, 154).

### 1.7.2 Attachment

When *H. pylori* reaches the less acidic mucus layers close to the epithelial surface, adherence of the bacterium is necessary in order to multiply and for persistent colonization. About thirty putative homologous of outer membrane proteins (OMPs) belonging to the Hor and Hop families are present in the sequenced *H. pylori* strains (3, 109, 145). The function of most of these proteins is largely unknown, although some of them are known as adhesins and porins. BabA and SabA are the most studied of these and are adhesins belonging to the *hop* gene family. BabA bind to ABO/$Le^b$ and SabA binds to sialyl-$Le^x$/sialyl-$Le^a$ antigens, respectively (73, 87). Both the *babA* and the *sabA* genes are frequently turned on/off due to phase variation of repeated CT nucleotides. Interestingly, the receptors recognized by SabA are up-regulated in inflamed tissue (12). This suggests that when inflammation occurs, strains with SabA expression are better fit to the new gastric environment. AlpA and AlpB are two other investigated adhesins that when mutated showed colonization defects in guinea pigs (36). Many of the *H. pylori* OMP genes encompass repeated regions and are suggested

to phase vary by a slipped-strand mechanism (127). Also, recombination events seem to occur between these genes which contribute to the diversity of *H. pylori.* Probably several of the OMP proteins with yet unknown functions play important roles in attachment and persistence in *H. pylori* infection.

### 1.7.3 Vacuolating cytotoxin

Vacuolating cytotoxin (VacA) is a secreted 95 kDa exotoxin that causes the formation of vacuoles in human cells. There are *vacA* genotypic variants, due to alterations in the signal sequence (s1, s2) and /or the middle region (m1, m2), between different *H. pylori* strains (14). Most strains with s1/m1 genotype and some of the s1/m2 express the cytotoxin while strains with s2/m2 genotype do not express a toxic VacA. Studies have shown that strains that express the toxin correlates with severity of disease in the host, suggesting a role for the VacA protein in pathogenisis (161). Although, simultaneous presence of VacA and CagA (see below) is correlated to disease, there seems to be no genetic linkage between them (162) and purified VacA alone is able to induce ulceration in the stomach of mice (144). The capability to induce ulcers in mice models strongly suggests a role for VacA in development of epithelial pathology in the stomach.

### 1.7.4 The Cag-pathogenicity island

Presence of an immunodominant protein in *H. pylori* was found to be associated with peptic ulcer disease in infected patients, and the corresponding gene was named *cagA* (cytotoxin-associated gene), due to its observed correlation to cytotoxic activity by VacA. This virulence marker has together with VacA been used to classify the *H. pylori* strains to (i) type I strains containing the *cagA* gene and expressing a toxic VacA protein and (ii) Type II strains that lacking *cagA* and express a non-toxic form of VacA. The *cagA* gene was later found to be part of a pathogenicity island (PAI), containing 31 different genes. Some of these genes compose a secretion system that is homologous to the *Agrobacterium tumefaciens* type IV secretion system (28). This type of machinery allows for introduction of molecules into the host cells. The effector molecule of the *cag* PAI type IV secretion system is the CagA protein, which is injected into epithelial cells and subsequently becomes tyrosine phosphorylated and interacts with the host SHP-2 tyrosine phosphatase (72). This event triggers a cascade of intracellular reactions in the host cells. In cultured epithelial cells this phenotype is called hummingbird due to the morphological appearance of the cells. It is the EPIYA motifs in CagA that become phosphorylated. These motifs are found repeated in various numbers in the CagA protein and there are also genetic differences of these which have been correlated to geographical regions (71). Depending on the number of repeats and which genetic variants of EPIYA motifs are present, the phosphorylation of CagA is more or less pronounced, which in turn leads to more or less inflammation (10, 71). Induction of the cytokine IL-8 is also initiated by the *cag* PAI. The signaling between bacterium and host resulting in IL-8 induction involves the extracellular signal-regulated kinase (ERK) pathway, which upregulates the transcription factor NF-κB (106).

The entire *cag* PAI can be inserted of deleted in the *H. pylori* genome, due to 31 bp repeats surrounding the *cag* PAI, and both *cag* PAI negative and *cag* PAI strains can be

found in the same individual (21, 66, 75). Analysis of the entire *cag* PAI in correlation to disease has shown that partial deletions of the *cag* PAI should be taken into consideration. When the isolates carried only parts of the *cag* PAI they were less likely to be associated with disease, independent of if they were *cagA* positive or *cagA* negative (105). Presence of the CagA molecule in *H. pylori* isolates has been correlated to peptic ulcer and gastric cancer disease development in the host in several studies (24, 35). However, there are patients that are infected by *H. pylori* isolates lacking the *cag* PAI that still develop disease (105). Moreover, about 70-95% of all strains are *cagA* positive but since only about 10% of *H. pylori* carriers develop disease, there are significantly more *cagA* positive strain carriers never develop disease (51). Thus, the *cagA* gene cannot serve as a marker to predict disease outcome, and neither can other genetic markers or combinations of different genetic markers in *H. pylori* tested so far.

## 1.8 HOST AND ENVIRONMENTAL FACTORS

In bacterial pathogenesis it is often not the pathogen *per se* that is the causative agent of disease development, but the host response that is triggered as a consequence to the infection. Inflammatory cells and mediators are assigned to eliminate the invading pathogen but sometimes the toxic actions from these can result in serious health problems and may even be life-threatening to the host. In a human host, single nucleotide polymorphisms (SNPs) occur throughout the genome. When such SNPs are present within inflammatory-related genes, expression of certain allelic variants can substantially affect the level of inflammation that is invoked in response to a bacterial pathogen. Some have been described as important for *H. pylori* associated disease development. Environmental factors such as diet, can also contribute to either protect against or enhance disease development.

IL-1-β is an important pro-inflammatory cytokine and a potent acid inhibitor. Different polymorphisms in the IL-1 gene cluster have been identified, where genetic variants with enhanced IL-1β production were associated with an increased risk of both hypochlorhydria and gastric cancer (46). Other cytokines have also been investigated and examples are TNF-α, which also affects gastric acid secretion, and IL-10, which is an anti-inflammatory cytokine. Both of these were reported as risk factors for noncardia gastric cancer (47).

Dietary factors have been linked to disease and may also play a role in *H. pylori* disease development (32). High salt intake is positively associated with the neoplastic process. Adequate fruit and vegetable intake is associated with reduced risk of gastric cancers. The antioxidants in fruits and vegetables may contribute to the prevention of carcinogenesis in the stomach by neutralizing reactive oxygen species. Smoking and alcohol intake are factors that modestly increase the risk of gastric cancer, but this relation is controversial (32).

## 1.9 LIPOPOLYSACCHARIDE

The lipopolysaccaride (LPS) of gram-negative bacteria is composed of an O-antigen polysaccharide chain, a core oligosaccharide and a lipid moiety, lipid A, that anchors the LPS molecule to the outer membrane (118, 122). A typical O-antigen of *H. pylori* consists of polymeric *N*-acetyl–β-lactosamin (LacNAc)units that are partly fucosylated mimicking Lewis (Le) antigens also found on human epithelial and glandular cells (13). The last step of *H. pylori* O-antigen synthesis is performed by three fucosyltransferase (FucTs), encoded by three genes *futA* (HP0379), *futB* (HP0651) and *futC* (HP0093-94). The FucTs of *H. pylori* and the human equivalent are only homologous in a short region of a catalytic site, but appear to have functional similarities (86). FutA and FutB are paralogous enzymes with either α1,3- and/or α1,4-FucT activity and are required for $Le^x$ or $Le^a$ antigen expression, respectively. In a second step, the transfer of a second fucose may be accomplished by FutC, an α1,2-FucT, resulting in expression of $Le^y$ or $Le^b$. Type 2 chains express precursor i-, $Le^x$ – and $Le^y$ - antigens and are the most commonly detected Lewis antigens in the O-antigens of *H. pylori*. Type 1 chains (precursor H1, $Le^a$, and $Le^b$ antigens) are present at lower frequencies and are sometimes found simultaneously expressed together with type 2-chains in *H. pylori* (95). However, geographical variations of Lewis antigen expression have been suggested (96). All three FucT genes contain poly-C tracts that enable the genes to be switched on and off by a slipped-strand mispairing mechanism (8). Also, in the C-terminal region, a sequence of seven amino acids is repeated in FutA and FutB. The number of repeated heptads varies in different strains and they also match to create an α-helical coiled-coil structure that may be involved in dimer formation (93). In this thesis we suggest a functional mechanism for the variable heptad domains (paper III).

Bacterial LPS is also called endotoxin, due to the usually toxic activities of Lipid A as strong stimulator of the immune response in gram-negative bacteria (118). The *H. pylori* LPS has weak endotoxic and immunological activities as compared to other gram-negative bacteria. Structural analyses of the *H. pylori* Lipid A suggest that this may be explained by different patterns of acetylation, phosphorylation and substitution by long chain fatty acids (97, 101). Several implications of *H. pylori* LPS involvement in pathogenesis have been suggested, and especially the role of altered *H. pylori* Lewis antigens expression has been investigated.

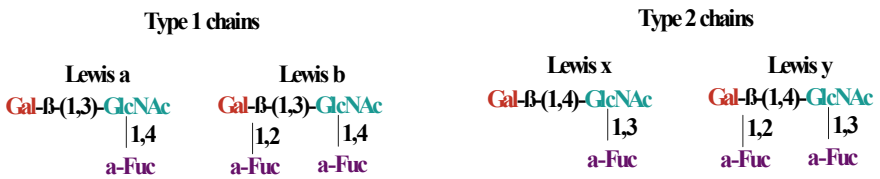


**Figure 6.** Structures of the type I chains $Le^a$ and $Le^b$, and the type II chains $Le^x$ and $Le^y$.

Expression of *H. pylori* $Le^x$ has been correlated to symptomatic *H. pylori* infection and strains that do not express Lewis antigens have been associated with asymptomatic carriage (70, 119). Also, in a mouse model an *H. pylori* strain that lacked Lewis antigen expression failed to induce gastritis compared to the wildtype strain (45).

Lewis antigens are also expressed on various human cells. The pattern of Lewis glycosylation in gastric tissues is dependent on the secretor status of the host, and secretors and non-secretors mainly express di-fucosylated ($Le^b$ and $Le^x$) and mono-fucosylated ($Le^a$ and $Le^x$), respectively. Since Lewis antigens on the LPS of *H. pylori* are similar to those expressed on human cells and can also undergo phenotypic changes, the theory of molecular mimicry has been investigated in several studies. By mimicking the human phenotype, *H. pylori* may prevent production of antibodies towards epitopes that are shared by the host and bacterium. In one study, *H. pylori* Lewis antigen phenotypes adapted to imitate the same secretor phenotype as found in the infected rhesus monkeys (156), suggesting that the host phenotype invokes a selective pressure on the bacterial Lewis phenotype. However, other studies trying to elucidate molecular mimicry of bacteria in human beings are conflicting (69, 143, 155), which may be explained by differences in the studied populations. In our paper III, we describe *H. pylori* populations with different Lewis antigen expression in sub-isolates from the same biopsy, in different regions of the stomach and differently expressed over time. These results provide evidence against a molecular mimicking theory, at least in a persistent infection, and imply that other factors than the host phenotype affect Lewis antigen expression. One such factor may be change in acidity, since the pH is dissimilar in different compartments of the stomach and Lewis antigen expression has been shown to alter in different pH (99).

Lewis antigen involvement in induction of autoantibodies has also been investigated. When similar epitopes are expressed in the bacterium and the host, this results in autoimmune reactions that have a potential role in pathogenesis (9, 69). The β-subunit in the human H+, K+ ATPase (the gastric proton pump) expresses $Le^y$ and human gastric mucin express both $Le^x$ and $Le^y$. Autoreactive antibodies directed against the proton pump have been found and suggests a potential target for *H. pylori* Lewis antigen induced autoantibodies (5). However, subsequent studies demonstrated that human sera lacking Le antigen also reacted with H+ K+ ATPase (29). Eventually, it was revealed that the autoantibodies produced were directed towards protein epitopes and does not involve Lewis antigens (151).

Moreover, LPS and Lewis antigens are suggested to be important for *H. pylori* adhesion and colonization. Mouse-models have been used and shown that inactivation of different genes involved in LPS biosynthesis have an effect on colonization (83, 92, 96, 100). In some studies the inactivation of genes was constructed in a manner that only affected Lewis antigen expression, to investigate if the Lewis antigen by itself was the cause of reduced mouse colonization. Two studies verified reduced colonization due to lack of expression of Lewis antigen (92, 100), but in one study no colonization difference could be found between knockout mutant and wild-type strains (142).

Co-localization of *H. pylori* and Surfactant D-protein (SP-D), involved in antibody independent pathogen recognition and clearance, was demonstrated (102) and this

finding was followed up in mice models. *H. pylori* colonization was more frequent in SP-D deficient mice compared to normal mice (98). To evade interactions with SP-D, *H. pylori* variants with modified O-antigens can arise, which resulted in decreased SP-D interactions (76). Also, Lewis expression regulates the interaction of *H. pylori* with DC-SIGN (an innate immune receptor found on dendritic cells) (18). Monomeric $Le^x$ and $Le^y$ have been shown to bind to DC-SIGN which increased the production of IL-10. This is a Th2 cytokine that hinders Th1 response and promotes Th2 response. In this way alternate Lewis antigen expression can affect the polarization of Th1/Th2 response, which may help the bacterium to persistently colonize the stomach.

The large number of investigations involves several interesting proposals explaining the importance of Lewis antigen expression involvement in disease development, but there are still questions that need to be answered in this field.

## 1.10 RESTRICTION-MODIFICATION SYSTEMS

Restriction-modification (R-M) systems have two enzymatic activities, modification of DNA through transfer of a methyl-group and restriction of DNA by generating a double-stranded break in the DNA strand. The modification event performed by a methyltransferase (MTase), protects the self-DNA from digestion by corresponding restriction enzyme (RNase). R-M systems have been widely described in the literature, especially restriction enzymes due to the use of them as valuable tools in molecular methods. There are three classical R-M systems: type I, II and III. These systems are classified by their enzyme subunit composition, cofactors requirements in the enzymatic reactions, position of DNA-cleavage and structure of their DNA target sequence. There are also recently discovered systems that do not fit into this classification (19, 103).

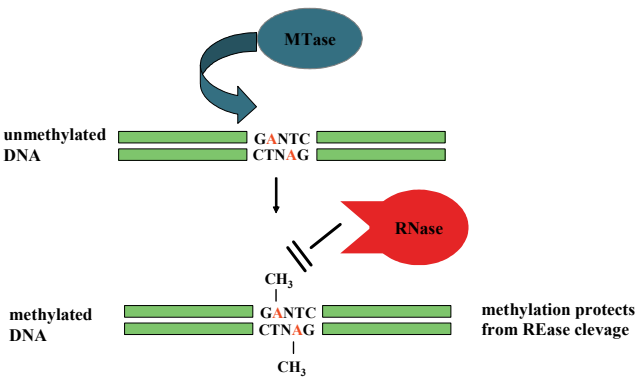


**Figure 7.** Methylation protects the recognition site from cleavage by corresponding restriction enzyme.

### 1.10.1 Type I systems

The type I R-M systems differ to type II and III systems in that they are composed of three subunits encoded by three linked genes: *hsdS*, *hsdR* and *hsdM*. The HsdS specificity subunit determines the target sequence specificity for both restriction and

methylation events and the HsdM protein is the modification subunit that is responsible for DNA methylation (19, 103). The recognition sequence of type I R-M systems consists of two non-symmetrical target half sites, each 3-5 bp long, separated by a 6-8 bp non-specific linker. The HsdS and HsdM proteins are required for methylation, together with the cofactor adenosylmethionine (AdoMet) which serves as the methyldonor. All three subunits compose a multifunctional enzyme with both restriction and modification activities. For restriction events ATP and Mg-ions are necessary as cofactors. Unmodified DNA is cut by the restriction enzyme in a random manner at sites remote from the recognition sequence, at a distance of 400 to 7000 bp. Studier *et al*., described a model for how this may occur, describing the interplay between two R-M enzymes. When the recognition sequences are non-methylated, enzymes bound to these translocate the DNA strand until they are in contact with an adjacent bound enzyme, followed by the introduction of a break of the DNA strand in between the two binding enzymes (136).

The specificity subunits in type I R-M systems can further be divided into different families IA, IB, IC and ID and has mainly been classified by sequence similarities and complementation tests, where the subunits could be interchanged between bacteria. In the specificity subunits of Type IC R-M systems, a repetitive four amino acid motif has been found in *hsdS* genes from several bacterial species (1). In a study performed in *Escherichia coli*, a four amino acid motif TAEL in an *E. coli* strain was repeated in different numbers, which affected the recognition site by altering the number of nucleotides in the non-specific spacer (117). Mutant R-M systems with changes in the number and the length of the central amino acid repeats exhibited decreased restriction activity and relaxed substrate specificity (64). Moreover, recombination events in constant regions of the HsdS subunits can result in fully active hybrid HsdS subunits with new specificites (56, 65). In paper II, we describe the presence of similar four amino acid motifs repeated in varying numbers in the *hsdS* (HP0790) locus of *H. pylori*. We also found high allelic variation of this locus in different strains.

### 1.10.2 Type II systems

Type II R-M systems are the simplest of the different R-M systems and are composed of two independent enzymes, one MTase and one cognate restriction enzyme, which cuts un-methylated DNA within a symmetric recognition sequence. Interestingly, in cognate methyltransferase and restriction enzymes no amino acid sequence homologies in the target recognition are found, although they have the same recognition sequence (81). Also, between different restriction enzymes no homologies are revealed, even though they are isochizomers with the same specificity. This suggests that both MTases and RNases have evolved independently and that the RNases have not evolved from the same precursor.

### 1.10.3 Type III systems

Type III systems represent the smallest group of the R-M systems and consist of two subunits, Mod and Res. Both subunits are required for restriction activity, where the Mod subunit provides DNA recognition. The Mod subunit alone is sufficient for DNA methylation. ATP and AdoMet are required cofactors to the type III systems that cut DNA close to the recognition sequence.

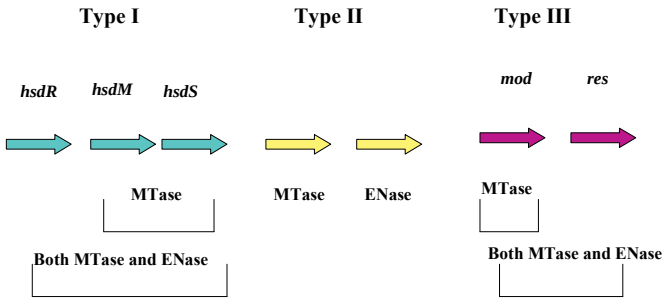


**Figure 8.** Genetic organization of the different genes in the three R-M systems

### 1.10.4 Biological significance of R-M systems in prokaryotes

The most commonly suggested biological role of R-M systems is that they act like primitive immune systems to protect the bacteria from invading phages. Phage plaque assays have demonstrated this phenomenon, where phages containing the specific recognition sequence are eliminated when bacteria can produce the corresponding R-M system (43). Restriction events may also facilitate incorporation or recombination of incoming DNA by introducing double strand breaks (131). A selfish gene theory of R-M systems has been proposed, where these systems are suggested to be small selfish mobile elements (78). However, this theory has been questioned in type I R-M systems (107). Other R-M systems also have implications in virulence and gene expression in *H. pylori* and other bacterial species. Recently, a phase-variable type III *mod* gene in *Hemophilus influenzae* was shown to coordinate the random switching of expression in numerous genes (133). The orphan type II MTases CcrM and Dam are important players in normal cellular physiology, for the regulation of gene expression as well as for and virulence in several bacterial species (84).

The Dam (DNA-adenine MTase) recognizes GATC sequences and has been described in *E. coli* as for gene regulation of several genes, including the *pap* operon that encodes for pili important for virulence in urinary tract infections (67). Gene regulation by different methylation patterns was shown to be formed through interactions between regulatory proteins and differentiations in methylation patterns. These interactions can either up- or down-regulate the expression of genes, depending on the nature of the protein affected by the methylation. Dam also plays an important role in cellular functions such as replication, mismatch repair and transposition (27, 123, 128). An interesting study of Dam in *Salmonella typhimurium* showed that Dam is required for virulence in mouse-models, where a Dam knockout mutant was strongly attenuated (68).

Another important orphan MTase with GANTC specificity that was discovered in α-proteobacteria is named CcrM (cell-cycle regulated methyltransferase), due to its involvement in the regulation of the cell-cycle in *Caulobacter crescentus*. Overexpression of CcrM resulted in changes of the bacterial cell morphology (164).

CcrM expression is restricted to the end of DNA synthesis (s-phase) and is under control of the global cell cycle regulator CtrA (121). Knockout experiments showed that CcrM is essential for viability in *C. cresentus*, *Rhizobium melioti*, *Agrobacterium tumefaciens*, and *Brucella abortus* (74, 124, 134, 159).

### 1.10.5 R-M systems in *H. pylori*

In the sequenced *H. pylori* strains 26695, J99 and HPAG1 an abundance of R-M systems have been found (3, 109, 145). Furthermore, the specificities and activities of the R-M systems in several studies of type II systems have been investigated. From these studies it appears that each *H. pylori* strain has its own unique subset of different R-M genes and these could therefore be suggested as candidates for strain-specific genes involved in pathogenesis. Although no entirely conserved orphan MTase such as Dam or CcrM has been found in the *H. pylori* strains, its involvement in gene regulation by methylation is a possibility. One argument supporting this is that few regulatory homologues are found in the sequenced *H. pylori* strains, as compared to other bacteria, and therefore alternative ways of gene regulation exist. R-M systems in *H. pylori* may also act as protectors from phages, although few *H. pylori* phages have been reported. The common feature of natural transformation may require R-M systems to decrease the amount of foreign DNA insertions into the *H. pylori* genome.

There is evidence of transcriptional regulation by R-M systems in *H. pylori* from a study where a knockout mutant of M.*Hpy*I showed an altered expression pattern of the stress operon *dnaK* in a stationary phase culture and in adherence to epithelial cells (41). In this study, no methylation site of M.*Hpy*I could be found in regulatory regions, which could explain the altered expression due to the methylation pattern, but the authors suggest conformational changes due to methylation, which also has been suggested in other studies. In paper I of this thesis, the *H. pylori* catalase was found to be significantly down-regulated in the M. *hpy*AIV knock-out mutant. A GANTC site is situated 95 bp upstream of the translational start codon of *katA*, which may be a potential promoter-binding site, and could explain the difference in transcription.

In two different studies aiming to identify genes affected by host interactions, two different *H. pylori* RNases were found: *hpyIR* and *hpyC1IR*. A knockout mutant of *hpyC1IR* displayed decreased cell adherence and elongated cell structure (82) and *hpyIR* was up-regulated upon contact with epithelial cells (115). The *hpyIR* has been named *iceA* (induced by contact with epithelial cell) and further investigations showed that *iceA* was correlated to peptic ulcer disease and suggested as a virulence marker. However, other studies have argued against this correlation. Another RNase gene, *hypIIIR*, is in some strains replaced by the *hrgA* gene which has been associated with *cagA* positive strains from Asian cancer patients. Moreover, *hsdS* genes have been correlated to a high host-response in a mouse-model (23).

Several of the R-M genes in *H. pylori* have been suggested to phase-vary by a slipped-strand mechanism (125, 127). This is usually a phenomenon that occurs in order to adapt to changes in the environment and to hide from the immune system. It is especially, genes encoding for structures exposed on the bacterial surface that are usually found to be phase-variable. In a type III R-M system there is evidence for

coordinated expression of the *mod* and *res* genes, a novel mechanism explaining how gene expression may be regulated in *H. pylori* (37).

Why there are so many R-M systems in *H. pylori*, as compared to other bacteria has been subject for speculation about and several studies have implied important correlations and the significance of these. The unique set of R-M systems of each *H. pylori* strain clearly contributes to the diverse genetic content of the strains and these genes may affect transcription and be associated to more or less virulent strains. Their potential role as epigenetic regulators add to the variability of the *H. pylori* strains and may be important in adaptation of strains to the different milieus the bacteria encounter.

## 1.11 THE FLEXIBLE BACTERIUM

Three *H. pylori* genomes have been fully sequenced; in 1997 strain 26695 from a patient with gastritis was sequenced, in 1999 strain J99 from a duodenal ulcer patient and in 2006 strain HPAG1 from a patient suffering from chronic atrophic gastritis (4, 109, 145). The sizes of the genomes were 1.67 Mb containing 1590 predicted ORFs, 1.64Mb containing 1496 ORFs and 1.60 Mb containing 1536 ORFs (in 29955, J99 and HPAG1), respectively. In these three small genomes, 1379 ORFs were common for all three strains and about 10% of the genes were strain specific. Using microarrays, Gressman *et* al. investigated the gene content from 56 global *H. pylori* strains and found that 1111 ORFs were universally present within all strains (63). The group of genes containing most variability, according to the Gressman study, were found in genes of unknown function (44%), genes associated with DNA metabolism (54%, where many of them were R-M system), outer membrane proteins (22%), cellular processes/*cag* PAI (40%) and other categories (100%, including transposons).

The high variability of *H. pylori* strains is not only assigned to strains from different individuals, but variability in intra-strain isolates has also been observed (79). These variable isolates are suggested to originate from the same clonal strain but have during the years changed their genome, presumably to adapt to the gastric environment of a new host. The genetic variability of *H. pylori* may be explained by the bacterium's ability for natural transformation that enables horizontal gene transfer, and the high mutation- and recombination frequencies (52, 140). Imported DNA fragments are on average 417 bp, which is small compared to other bacteria. In a model used to calculate the recombination frequencies, it would only take 48 years to exchange 50% of the gene content in an *H. pylori* genome, assuming there was a mixed strain population. If the assumption considers clonally related strains, the same situation would occur in 2200 years, still a relatively short period of time (52).

Homologues to MutL and MutH involved in the mismatch repair system are missing in *H. pylori*. In addition MutS shows no effect on the mutation frequency in *H. pylori*, which implies lack of mismatch repair function in this species (22). Antibiotic resistance can occur through point mutations. For example, clarithromycin resistance in *H. pylori* can be caused by either of two point mutations in the 23S rRNA gene. Moreover, mutations in *frxA* or *rdxA* genes cause metranidazole resistence. The high mutation frequencies are problematic in *H. pylori* eradication treatment, where

antibiotic resistant clones emerge at rather high frequencies (22). In addition, high allelic diversity of *H. pylori* genes may promote intra-strain recombination. Also, insertion sequence (IS) elements and repeated nucleotides which can turn genes on and off through mismatch slippage are present in the genome (126, 127). This also contributes to the variability in the expression of genes.

In *H. pylori* there are few regulatory genes when compared to other bacterial species. Several of the main regulatory proteins present in other gram-negative bacteria are missing in *H. pylori* and only four genes with perfect helix-turn-helix motifs, which are characteristic for DNA binding proteins, are present (145). Homologues to four histidine kinases and six response regulators in two-component systems responding to environmental stimuli have been found (17). However, sigma factors involved in stress-regulation are missing (3). Proteins involved in iron and nickel homeostasis, Fur and NikR, respectively, have been extensively studied (38, 39, 50, 150). Except for regulating the amount of these important metals, there seems to be a cross-talk between these and regulatory proteins which further has an effect on the expression of urease (149). Due to the variable and unique set up of R-M systems in every strain, and the different methylation patterns in *H. pylori,* strains may affect gene regulation and may contribute to differential expression of genes. In paper I, we knocked out the MTase M.*Hpy*AIV, which in turn affected the expression of catalase, possibly due to a differentiation of GANTC metylation patterns. Blaser and Atherton have presented a model for host-microbial cross-talk during long coevolution (26). They describe a bi-directional signalling and response between the host and bacterium, where a linkage between the two occurs. The host signals with for example changes in pH, oxidative stress etc, and the bacterium answers by an adaptation to the conditions. In addition to two-component systems responding to environmental stimuli, alternative adaptive mechanisms like phase-variation by slipped-strand mechanisms and differential methylation patterns may occur in sub-isolates of clonally related strains. The regulation of gene expression, in addition to the plastic gene pool, may be a survival and persistence strategy for *H. pylori*, where the sub-isolates with the best ability of adaptation have the right genetic set-up to fit in the new conditions encountered.

# 2 AIM

The general aim of this thesis was to increase our understanding of how *H. pylori* diversity is maintained as well as to and to understand how adaptive mechanisms might affect virulence and disease outcome.

**Specific aims:**

**Paper I.**
To investigate the activity and distribution of the type II DNA MTase M.*Hpy*AIV in clinical *H. pylori* isolates. Furthermore, we aimed to investigate if the methylation of these sites influences growth, stress and gene transcription of the bacterium.

**Paper II.**
To examine the distribution of type I R-M genes in clinical *H. pylori* isolates and genetically characterize these genes of isolates in persistent infection.

**Paper III.**
To investigate the expression of Lewis antigens in clinical isolates obtained from different compartments of the stomach and over time. The genes encoding fucosyltransferases involved in Lewis antigen synthesis were further investigated to examine how Lewis antigen phenotype is determined by genetic alterations of these genes.

**Paper IV.**
To investigate Lewis antigen expression in different environmental settings by comparing strains within the same patient, through *in vivo* passages in mice and through *in vitro* passages on plates. Genetic mutations and rearrangements were further examined to understand how genetic variability might occur.

# 3 MATERIALS AND METHODS

## 3.1 *HELICOBACTER PYLORI* STRAINS

*H. pylori* strains were cultured on solid *GC* plates or in liquid cultures and incubated in microaerophilic conditions at 37°C. Liquid cultures of *H. pylori* were grown in Brucella broth, supplemented with Fetal Bovine Serum and Isovitalex Enrichment.

Studies involving human strain material and/or animal experiments were approved by appropriate Ethical committees (UmDnr98-99, Dnr 96/95, Dnr N14/00, Dnr Ni72/96 and Dnr 500:16 342/99).

### 3.1.1 Cancer-control study

The sixty-one clinical *H. pylori* isolates used in paper I and paper II were obtained from a Swedish gastric cancer case-control study performed by Enroth *et* al. (49). The isolates included patients diagnosed with gastric cancer, duodenal ulcer and non-ulcer dyspepsia as well as asymptomatic control subjects.

### 3.1.2 Kalixanda (paper I and II)

The Kalixanda study was set in two communities in northern Sweden: Kalix and Haparanda. When collected, the study aim was to investigate the prevalence, symptoms and risk factors for *H. pylori* disease in a general adult population (11, 135). One-thousand persons volunteered to undergo endoscopy and one-third of these individuals had an ongoing *H. pylori* infection. Serology, histological examination and culture of the *H. pylori* were performed and the *H. pylori* positive patients were followed up for a second gastroscopy four years later. Sub-isolates from this study are used in paper I and paper II to investigate if genetic alterations of R-M genes occurred over time. Samples used in these studies were from two individuals diagnosed with normal gastric mucosa at both consecutive occasions, two individuals who developed atrophy during the four years of study and one individual that progressed from gastric atrophy to gastric cancer development during the study-period.

### 3.1.3 Örebro study (paper III)

Isolates from the two patients in paper III, where collected from a study performed by Gustavsson *et* al., which in total included forty-seven patients from Örebro county, in Sweden (66). Both patients suffered from dyspeptic symptoms. According to the Swedish standard treatment protocol, the patients were not treated for their *H. pylori* infection and underwent a new endoscopy nine years later. Biopsies were obtained from corpus at year 1 and from both corpus and antrum at year 9. Genetics of subisolates from these patients has also been characterized by Lundin *et* al. (85).

### 3.1.4 Small- and large bottleneck *in vitro* passage (Paper II and IV)

Two sub- isolates (67:20 and 67:21) were obtained from a gastric ulcer patient included in the cancer-control study and these isolates were further used for *in vitro* selection by serial passages. The 67:21 isolate contains the entire *cag* PAI, while in the other isolate 67:20, the *cag* PAI was eliminated. These two isolates have earlier been characterized by microarray (21). The two isolates were sub-cultured 50 times on agar plates by *in vitro* passages using two different approaches: small bottleneck and large bottleneck. In

the small bottleneck approach, only one single colony was transferred at each passage. In the large bottleneck methodology, a sweep of bacteria was transferred at each passage. After the 50 passages, single colonies from each set-up were analyzed for LPS profile (Paper IV) and *hsdS* sequence variants (Paper II).

### 3.1.5 Mouse-passage strain (Paper II and Paper IV)

Strain 67:21 containing the *cag* PAI was further used for *in vivo* passage in transgenic mouse models expressing the $Le^b$ receptor. Single colony isolates were isolated from three selected mice and used for analyses in paper II and paper IV. One mouse was raised under germ-free conditions and colonized with the 67:21-strain for three months. Two mice were conventionally raised and inoculated with 67:21 for three and ten months, respectively. The 67:20 isolate did not colonize transgenic mice.

## 3.2 CONSTRUCTION OF KNOCKOUT MUTANTS (PAPER I AND III)

Knockout experiments were performed in paper I and paper III. The genes of interest were amplified by PCR, using primers targeting conserved regions. The amplified gene was ligated into the vector and transformed into competent *E. coli* (DH5α) by heat-shock. The constructed plasmids were linearized at one single site within in the gene using suitable RNase that generated one single site within the gene and had no recognition site in the vector. If no such site was found, mutagenesis PCR was performed. The restriction site was used as insertion point for a kanamycin- or chloramphenicol resistance cassette, with approximately 100-500 bases of the gene at each side. The transformants were selected on LB plates containing the appropriate antibiotic. To obtain *H. pylori* insertion mutants, plamid-constructs were electroporated or naturally transformed into the different strains. PCR and sequencing was used to confirm that the antibiotic resistance marker was inserted into the correct location in the genome.

### 3.2.1 Quantitative real-time reverse transcriptase PCR (paper I)

In paper I, we addressed if the M.*Hpy*AIV had an effect on gene expression in a knock-out mutant compared to the wild-type strain 26695. RNA from the wild-type strain and the isogenic knock-out mutant were isolated for cDNA synthesis. Quantitative real-time reverse transcriptase PCR was performed on seven selected genes that fulfilled the criteria of having GANTC sites present upstream of ORFs in both 26695 and J99. The genes analyzed were: *cag*13 (HP0534), *cag*16 (HP0537), *cag*21 (HP0542), *katA*, HP0835, HP0922 and HP1564. The 16S rRNA gene was used as endogenous control after verification that the expression levels were the same in wild-type and mutant strain. Calculation of the relative gene expression was performed using the Q-gene software (Biotechniques software library, http://www.biotechniques.com).

### 3.2.2 Methylation assays (paper I)

In order to detect activity of GANTC methylation in clinical strains, isolated DNA was digested by the RNase *Hin*fI, digesting GANTC sites. If the DNA was undigested, this indicated active GANTC methylation and if the DNA was digested, this implied absence of GANTC methylation. DNA from wildtype and constructed M.*Hpy*AIV mutant was also analyzed this way. Another *in vitro* methylation assay was performed to detect methylation activity of the purified protein. A PCR fragment containing one GANTC site was incubated together with different concentrations of purified M. *Hpy*AIV protein. The *Hin*fI RNase was used to investigate if the protein was able to

methylate and thereby protect the GANTC sites from being digested. This assay was performed in paper I using a Bioanalyzer (Agilent, Palo Alto,CA).

### 3.2.3 *In silico* genomic analysis (paper I)

In-house developed PERL scripts were used to analyze the occurrence of GANTC sites in the two sequenced genomes 26695 and J99. The genomes and gene annotations were downloaded from NCBI. Randomized shuffling of the nucleotide order was performed by 10,000 pair-wise exchanges of nucleotides within the same intergenic region, whereas intragenic regions were randomized by pair-wise exchanges of codons encoding the same amino acid within the same gene. Analyses of intergenic distribution of GANTC sites were performed by dividing each intergenic region containing one GANTC site into 10- nucleotide non-overlapping windows $\{w_1, w_2\ldots, w_n\}$. This distribution was then compared to those obtained after randomly positioning the GANTC within the same intergenic regions.

### 3.2.4 Lewis antigen detection and analysis (paper III and IV).

Equal amounts of bacteria were harvested as measured by OD 600nm after growth to exponential phase and LPS was isolated using hot/phenol extraction. Isolated LPS was separated on SDS polyacrylamide gel and either used for silver staining or immunoblots. Antibodies towards Lewis antigens used were monoclonal mouse anti CD-15 (i.e $Le^x$) of anti-$Le^y$, and secondary antibody was rabbit anti-mouse, conjugated to horseradish peroxide. Enzyme-linked immunosorbent assay (ELISA) was also performed to detect Lewis antigens. The antibodies used were IgM mouse monoclonal antibodies against $Le^a$, $Le^b$, $Le^x$, $Le^y$ and sialyl-$Le^x$.

# 4 RESULTS AND DISCUSSION

**Paper I: Functional analysis of the M.*Hpy*AIV DNA methyltransferase of *Helicobacter pylori*.**

We investigated a type II DNA MTase in *H. pylori*, previously determined to recognize of GANTC sites (157). The type II R-M system was characterized in clinical isolates and functional analyses were performed using purified M.*Hpy*AIV protein and a knockout mutant. Our results point to the possibility that the methylation by this enzyme affects gene transcription.

The presence of the MTase gene and corresponding REase was investigated in a PCR assay on clinical isolates. The MTase gene was present in 60% (36/60) and the REase in 53% (32/60) of the tested strains, according to this assay. To test if the presence of the gene was correlated to the methylation of GANTC sites, isolated DNA from the same strains was digested with *Hin*fI, a REase that digests GANTC sites. This test was also performed on DNA from an M.*Hpy*AIV knockout mutant. DNA isolated from all of the strains where the MTase gene was absent as well as from the knockout mutant was susceptible to restriction, which indicated that M.*Hpy*AIV is the only MTase methylating GANTC sites. Resistance to digestion was found in 69% of the isolates containing the MTase gene.

Sequencing was performed to identify possible genetic alterations that could explain why not all strains containing the M.*Hpy*AIV gene were able to protect GANTC sites. Strains with inactive MTase activity contained alterations in poly-nucleotide repeats, compared to strains with active methylation. The difference in the repetitive region resulted in translational frame-shifts which may result in truncated proteins. This indicates that the M.*Hpy*AIV gene varies in its expression by slipped-strand mispairing mechanisms. Single cell isolates obtained from the same individual were investigated in attempts to find variations in activity due to slipped-strand mispairing mechanisms but no such variants were found. This may indicate that slippage of the repetitive nucleotides does not occur at high frequencies in this gene. However further studies should be performed to answer these questions. In the intra-strain isolates obtained from the stomach of one individual, most of the isolates contained an active MTase, but in two colonies, where no activity was found, both genes encoding the R-M system were absent. The R-M system has repeated regions surrounding the genes and Takata *et* al. have suggested that it is recombination events that enable the elimination of the R-M system (141).

*In vitro* analyses of purified M.*Hpy*AIV protein showed that the enzyme was DNA-binding and able to protect GANTC sites in a concentration dependent manner. A knockout mutant was investigated and compared to the wild-type strain in *in vitro* growth, heat-shock tolerance and ability to induce IL-8 in epithelial cells. However, no differences were found in these assays.

Methylation has been shown to regulate gene expression in other species. Therefore, we mapped the distribution of GANTC sites in the two sequenced strains of 26695 and J99. Randomized shuffling of the GANTC sites revealed that there are less GANTC sites in the two sequenced strains than expected by chance, especially in intergenic regions. Moreover, when the GANTC sites were present, there was an enrichment of

these sites proximal to the translational start codon. From these data, one could speculate that GANTC sites are avoided in most cases because methylation of these sites might affect gene expression. However, this capability may sometimes be required and therefore when GANTC sites are present they are located in potential promoter regions. To test if GANTC methylation had an effect on gene expression transcripts of seven selected genes were compared between the M.*Hpy*AIV knockout mutant and the wild-type strain by quantitative-real-time reverse transcriptase PCR. Two genes were down-regulated in the knockout mutant: the *H. pylori* catalase and HP0835 (predicted histone-like DNA-binding protein HU) indicating that GANTC methylation has an effect on gene transcription in *H. pylori*.

The M.*Hpy*AIV of *H. pylori* is not universal, but when present, it is in most cases active and protective against REase activity targeting GANTC. There are several genes harbouring GANTC sites in potential promoter regions and there seem to be a selection to where they are situated. There also seems to be an overall avoidance of having GANTC sites in the genome. Interestingly, there are two indications how the M.*Hpy*AIV can lose activity, either by alternating the expression through slipped-strand mispairing or by losing the entire R-M system by a recombination event. Methylation by M.*Hpy*AIV also seems to have an effect on gene expression, but since the MTase is not present or active in all strains this is not essential to the bacterium. Instead this epigenetic regulation may contribute to the strain variability of *H. pylori* clones.

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

In this study our interest was to investigate the incidence and diversity of the genes belonging to the type I R-M systems in *H. pylori*. In-depth analyses of two specific *hsdS* genes were performed by sequence analyses in single colony isolates of different individuals over time, as well as through *in vitro* passages of the isolates on plates and *in vivo* passages in mice. Three subunits in type I R-M systems are necessary to obtain two enzymatic activities, restriction and modification. The specificity subunit HsdS determines the specificity of the DNA sequence being recognized. Together with the HsdM subunit, methylation is accomplished. For restriction events the third subunit HsdR is also required. Previous studies on *H. pylori* imply that some R-M genes may be associated with virulence (7, 23, 115), in the regulation of gene transcription and may be up-regulated upon contact with the host (41). Three Type I R-M systems are found in the fully sequenced strains of *H. pylori*, although the activities of these have not been investigated. In addition there are some orphan *hsdS* genes annotated in the sequenced strains that seem to lack corresponding *hsdM* and *hsdR* genes.

Annotated genes of the type I R-M systems were screened by PCR in 61 clinical isolates. Primers were designed from the fully sequenced strains of 26695 and J99. The *hsdM* and *hsdR* genes were conserved and detected to 75-100% in our strains using solely one primer pair per gene, and the amplified gene products were of expected sizes. However, the *hsdS* genes were more difficult to detect and several primers were designed to target the same gene and PCR amplicons were often of variable sizes. The *hsdS* genes were detected in 0-100% of the clinical isolates. Primers targeting

neighboring genes of the HP1383 locus (an orphan *hsdS* gene present in the fully sequenced strain 26695) were not able to detect the locus in our strain material. The HP0790 locus, another orphan *hsdS* gene was detected in 100% of our strains, also using primers targeting neighboring genes. However, PCR amplicons were of highly variable sizes ranging from 1500 to 4000 bp. Also primers targeting the *hsdS* loci HP0462 and HP0848 resulted in size-variable PCR fragments.

PCR fragments from eight strains with a variable HP0790 locus were subjected to sequence analyses. Results from these showed that this locus was highly variable in the different isolates. Conserved regions were found in the beginning and central parts of the gene, but otherwise all strains seemed to have their own unique variant of this orphan *hsdS* gene. To examine if allelic variants were present in clonally identical strains in different environmental settings, the HP0790 locus was analyzed in strains from the same gastric ulcer patient, *in vitro* plate-passage strains and *in vivo* mouse-passage strains. Sequence differences of the loci were only found in single colony isolates from the gastric ulcer patient. In the central parts of the gene a 12-bp nucleotide sequence was iterated in variable numbers and when translating this sequence it formed the amino acid motif TELN. Similar four-amino acid motifs have been found in *hsdS* genes in other bacterial species (1) and shown to alter the specificity of the R-M system (117). Also different numbers of similar repeats have been shown to alter restriction activity (64).

To investigate if genetic evolution of *hsdS* genes occurs over time in the same individuals, the HP0790 and HP0462 loci were analyzed in isolates from five patients with different disease background (asymptomatic gastritis, atrophy and cancer) at two occasions with a time-interval of four years. Ten single colony isolates from corpus at the two occasions and, when available, five colonies from antral biopsies where analyzed. The TELN motif of the HP0790 locus was found repeated in varying numbers, in all biopsy samples. In two of the individuals diagnosed with atrophy, more extensive differences of the same locus were found. In one of these, only one variant of the gene was found at year 1 (10/10 isolates), but four years later only one isolate (1/10) carried this allelic variant, while in the rest of the isolates a new genotype dominated (9/10). The HP0462 locus was also found in allelic variance in intra-individual strains. As for the case of the HP0790 locus, the HP0462 locus was also found in allelic variants in corpus isolates of the same two persons with atrophy. Also sequence changes in the HP0462 gene was found in antral strains from biopsies with normal mucosa at both time-points.

Usually, the R-M systems are described not to be conserved in *H. pylori*. In our study however both the HsdM and HsdR genes of type I R-M systems seem conserved. Also, the HsdS subunits seem conserved although they are present in several allelic variants and changeable over time in the same individual. We suggest that new allelic *hsdS* variants may rise due to environmental changes in the stomach.

**Paper III: An enzymatic ruler modulates Lewis antigen glycosylation of *Helicobacter pylori* LPS during persistent infection**

In this study we investigated single-cell isolates from two patients to determine how Lewis antigen display on the O-antigens of *H. pylori* LPS is expressed and varies within the same region of the stomach, between different regions of the stomach and between individual human hosts, and over time in a persistent infection. *H. pylori* varies its O-antigen expression on the surface of the bacterium, which has been implicated to be important for pathogenesis in several studies (18, 98, 119). The three FucTs described in *H. pylori* FutA, FutB and FutC are involved in the synthesis of Lewis antigens and have interesting genetic structural variations. Poly-nucleotide repeats are found within all three FucTs, which can switch the genes on and off through slipped-strand mispairing mechanism. The FutA and FutB enzymes also have a heptad repeat region, which is iterated in varying numbers in different strains. These genetic markers were investigated in this study and correlated with the expression and activity of these enzymes in the *H. pylori* isolates from two patients.

From two individuals, *H. pylori* was isolated from the antrum at year 1 and from both the antrum and the corpus at year 9. Ten single cell colonies were obtained from all biopsies (except from the antral biopsy of patient I where only seven isolates could be recovered). Lewis antigen expression was analyzed and compared by immunoblotting and ELISA, using antibodies towards $Le^x$, $Le^y$, $Le^a$, $Le^b$ and H-1 antigens. The isolates expressed either $Le^x$ or $Le^y$ alone or both, but no expression of the other antigens was observed. Isolates from patient I expressed $Le^x$ except for two colonies obtained from corpus that instead expressed $Le^y$. In patient II, more variations of Lewis antigen expression were observed. Antral isolates from year 1 mainly expressed $Le^y$, but nine years later, $Le^x$ was the dominating Lewis antigen expressed on O-antigens in the antrum isolates. Interestingly, in the corpus at year 9 most isolates lacked expression of Lewis antigens, where only one isolate was $Le^y$ positive. These experiments demonstrate that Lewis antigen expression can vary in isolates within the same compartment of the stomach and the dominating phenotype may change over time. Furthermore, different phenotypes may be expressed in different regions of the same stomach at the same time. This may be a way for the bacterium to adapt to the different environments of the stomach, as represented by different individuals and by corpus and antrum.

The poly-nucleotide tracts situated within the three *H. pylori* FucTs, results in the ability to turn the expression on and off in the genes by slipped-strand mispairing mechanisms. An insertion or deletion of a nucleic acid results in a frame-shift mutation, which may result in a premature translational stop and thereby alter the expression of the gene. In our isolates sequencing over the poly-C repetitive region of *futA* and *futB* enabled us to assess the on/off status of these genes. The on/off status of the *futC* gene in patient I was also evaluated by sequencing. In patient II the repeated C-tract contained 13-16 C residues, which was too many to obtain trustworthy sequence data, using conventional methods. Phenotypes obtained from the immunoblots were well correlated with the genetic on/off status data from the sequence analyses of all three FucTs.

In the N-terminal part of FutA and FutB a heptad-region consisting of a repetitive DD/NLRV/INY motif is situated. This motif is suggested to form dimers assembling to a coiled-coil structure (93). We analyzed the numbers of repeated heptads in our isolates. We then compared the amounts of heptad repeats and the on/off status of the FucTs to the banding pattern results from the immunoblots. We found a correlation between the number of heptads in active FutA and FutB to the sizes of fucosylated O-antigen in the isolates. We proposed a model for how the α1,3-FucTs may form homo- or hetero-dimers in relation to the numbers of heptad repeats, where one heptad repeat corresponds to one O-antigen unit. Knockout mutants of *futA* and *futB* resulted in different glycosylation banding patterns consistent with the number of heptads thereby supporting our suggested model. From one of the isolates, which expressed $Le^x$ and contained and active FutA with one heptad as well as an active FutB with seven heptads, *futA* and *futB* knockout mutants were constructed. The *futA* knockout mutant containing an active seven heptad FutB showed a different glycosylation banding pattern compared to the wildtype strain. Only high-molecular-weight O-antigens were detected in immunoblots of the mutant, as compared to the wild-type where a broader Lewis glycosylation pattern including lower molecular low-weight O-antigens were detected. Interestingly, when inactivating *futB*, rendering a strain with solely one heptad in the expressed FutA, $Le^x$ glycosylation were abolished. This supports the idea of hetero dimerization, where a one-repeat heptad FutA was not able to fucosylate the O-antigen by itself, but together with a FutB containing seven heptads a broad spectrum of differently sized O-antigens expressed $Le^x$.

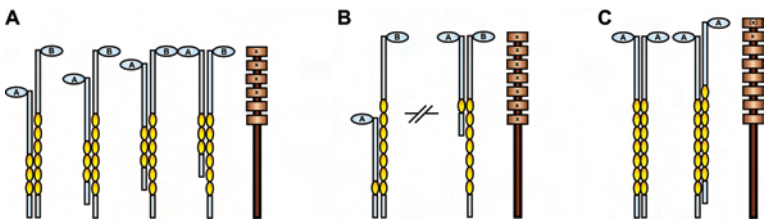


**Figure 6.** Model for how FutA and FutB may form homo- or heterodimers depending on the numbers of heptad repeats present in the two α1,3-FucTs. The $Le^x$ fycosylation pattern of isolates containing: A) FutA with three repeats and FutB with seven repeats. B) FutA with one repeat and Fut B with seven repeats. C) Only active Fut A with seven repeats.

FutC uses $Le^x$ as a substrate and is responsible for the synthesis of $Le^y$. In our experiments we sometimes found that the $Le^y$ glycosylation patterns were not entirely correlated with the number of heptads as in the case for $Le^x$. Instead, one O-antigen size seemed to be preferred as substrate for these isolates. In a wild-type strain, which expressed *futA* with three heptad repeats and *futB* with six heptad repeats, the $Le^y$ glycosylation pattern was indistinguishable from the homologous *futB* knockout, suggesting that $Le^x$ glycosylation corresponding to three heptad sizes was the preferred substrate for $Le^y$ glycosylation. A *futA* knockout mutant in the same strain resulted in

the loss of $Le^y$ fucosylation. Further investigation revealed that in this mutant, slippage of the poly-C tract of *futC* had occurred resulting in an out-of frame genotype. In two other strains expressing $Le^y$ and where only one α1,3-FucTs was active, attempts to knock out the active α1,3-FucT resulted in a frame-shift mutation in the other former inactive gene, resulting in a remaining α1,3-FucT activity and $Le^y$ glycosylation. This may indicate that the expression of $Le^y$ affects the expression of the FucTs. If the right substrate for $Le^y$ glycosylation is not available, either the expression of the α1,3-FucT genes are affected or FutC is turned off.

The three *H. pylori* FucTs can reversibly turn their activity on or off through slipped-strand mispairing mechanisms which result in a variability of Lewis antigen expression. Different Lewis antigen expression can occur within the same micro-environment of the stomach, between different compartments of the stomach and may change over time. We suggested that heptad repeats in the orthologous α1,3-FucTs, determine the sizes of the O-antigen being fucosylated. The heptad may also contribute to the diversity of $Le^y$ expression by altering substrate availability for *futC*. The variable nature of Lewis antigen expression in *H. pylori* strains may be of importance in bacterium-host interactions.

**Paper IV: Lipopolysaccaride Diversity Evolving in *Helicobacter pylori* Communities through Genetic Modifications in Fucosyltransferases**

This paper aims to characterize the LPS O-antigen chains and Lewis antigen glycosylation in *H. pylori* populations in various environmental settings. Single colony isolates were analyzed from a gastric ulcer patient, from *in vitro* sub-cultured bacteria and from *in vitro* mouse-passaged strains.
Intra-strain diversity of LPS profiles was investigated by analyzing twenty single colony isolates from a patient suffering from gastric ulcer by silver staining and immunoblots. The silver staining results revealed that LPS profiles were variable in the isolates from the same stomach, as represented by altered length of the O-antigen chains. Two of the isolates were further passaged 50 times on solid agar medium, using two different approaches with either a small bottleneck, where a single colony isolate is transferred in each passage, or a large bottleneck, where a streak with bacterial mixture was transferred in each passage. Twelve single colony isolates were analyzed from each of the two strains by the methods described above. In *H. pylori* isolates that were subcultured with a large bottleneck passage, no major phenotypic differences were observed in the LPS and most sub-isolates expressed the same characteristic O-antigen pattern as the wild-type strains. In the small bottleneck approach, however, more pronounced differences were found, where high molecular weight O-antigens differed in size and were even lacking in some of the sub-isolates. The small bottleneck approach may select for variants that disappear in a larger population, which may explain the different results obtained from the two subculturing methods. Moreover, in six of the isolates $Le^x$ was simultaneously expressed in combination with $Le^y$, which is in contrast to the wild-type isolates that only expressed $Le^y$. These findings confirm that the Lewis antigen expression can alter *in H. pylori* cells when cultured *in vitro*. In the mouse-passaged strain, even more diversification was found. Only 2 out of 30 isolates expressed the high-molecular-weight O-antigen as seen in the wild-type. This implies

that the high-molecular-weight O-antigens may be important for *H. pylori* in persistent human infection but when transmitted to a new murine host, the bacterium adapt to better fit the new environment.

Sequence analysis of *futA* and *futB* genes in clonal isolates revealed genetic variations at these loci as a result of recombination between the two genes. This is the first time that evidence for intra-strain recombination between these genes has been demonstrated. Frequencies of genetic changes in *futA* and *futB* genes was calculated and compared to four other *H. pylori* genes (*flaA*, *recA*, *ureI* and *16sRNA*). In *futA* and *futB* the observed frequencies were 8.0% and 4.4%, respectively. In the other genes, only three point mutations at the *ureI* gene were identified and corresponded to a frequency of 0.51%. These comparisons reveal high genetic changes in *futA* and *futB* which further support the theory of recombination events between these two orthologous genes.

Low-molecular-weight O-antigens were detected in several colonies subsequent to *in vitro* small bottleneck subculturing and *in vivo* passage in mouse, as compared to isolates from the human gastric biopsy, where all cells expressed high-molecular-weight O-antigens. This finding indicates that there is a preference for different phenotypes depending on where the colonization occurs. In the two $\alpha$1,3-FucTs, we found allelic variants that arise through recombination events within and between the two genes. These alterations may affect the glycosylation activity and contribute to create diversity of the LPS.

# 5 CONCLUDING REMARKS

The genetic variability of the *H. pylori* species makes it a challenge to identify genetic markers that are associated with virulence and disease development. Although extensive studies trying to reveal why only some individuals develop *H. pylori* associated disease have been undertaken, there are still unanswered questions in this matter. So far, no true virulence markers, that is always present in strains that cause disease has been identified in *H. pylori*. However some proteins have been shown to interact with and affect the host, such as the *cag* PAI and VacA. Not only the genetic variation, but also the gene expression and the enzymatic activities have to be taken into consideration when investigating virulence association. In this thesis we describe some mechanisms for how genetic diversity occurs and how protein expression and activity are affected. A few regulatory genes are present in the sequenced *H. pylori* genomes, but the bacterium still needs to adjust to the different environments it encounters when transmitted to a new host, to different regions within the stomach and to physiological changes in the stomach, e.g. after meals. Alternative mechanisms of gene regulation are present in *H. pylori*. Several *H. pylori* genes are subjected to phase-variation by slipped-strand mechanisms, some of them described in this thesis. Phase-variable slipped-strand mechanism can be an effective way of altering gene expression by translational frame-shifting to turn genes on- or off. In the M.*Hpy*AIV knockout mutant, the *H. pylori* catalase was down-regulated. This enzyme is important for handling oxidative radicals introduced by cells of the immune system. An increased or decreased expression of such enzymes may contribute to a more or less fit clonal variant in the stomach. Although the R-M systems are not essential, different methylation patterns may contribute to strain-specific epigenetic gene regulation, and therefore contribute to the strain variability due to differential gene expression. The high variability of the type I *hsdS* and the α1,3-FucTs genes also implies that recombination events between allelic variants frequently occur in *H. pylori*. Recombination events may be a mechanism to gain new activities and specificities of the enzymes. Also, there are repeated amino acid sequences in both the α1,3-FucTs and *hsdS* genes which may determine the activity and specificity for their enzymatic reactions. Our suggested molecular ruler mechanism is an example on how genetic alterations affect phenotype, where the Lewis antigen expressed on the *H. pylori* LPS is affected by alterations of heptad domains. Interestingly, low-molecular weight LPS phenotypes dominated when *H. pylori* was transmitted to mice or when passaged on plates, which suggests adaptation of the LPS when entering a new host or environment. The O-antigens of LPS may be involved in disease development since they are expressed on the surface of *H. pylori* and are proposed to interact with components of the immune system. *H. pylori* disease development is presumably not solely due to presence of absence of virulence genes in the bacterial genome, but rather influenced by expression of certain genes in interplay with the host and environment, where time of infection is an important factor. The constant triggering of the immune system by an *H. pylori* infection may induce changes in the stomach tissues resulting in disease development. Suggested virulence factors such as Lewis antigen, adhesins and the *cag* PAI may affect the *H. pylori*-host interaction, but are probably of different importance

in different individuals, depending on other factors such as genetic polymorphism as well as the age of the host, the time of infection and personal diet.

The scientific contribution of this thesis is to clarify some aspects of how *H. pylori* diversification occurs and how these changes can affect the activity and expression of genes important for the adaptation to the gastric niche of the bacterium, which in turn may be important in *H. pylori* disease development.

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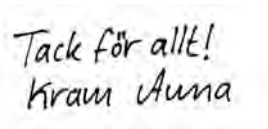

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