

Helicobacter pylori -
Molecular mechanisms
for variable adherence properties

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To my husband and son

/ Jens & Oscar

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Abstract

More than half of all people worldwide are infected with *H. pylori*. The infection always cause a gastric inflammation that may develop into peptic ulcer disease or gastric cancer. Attachment proteins, adhesins, mediate specific adherence of *H. pylori* to receptor structures on the human gastric mucosa. The best-characterized *H. pylori* adhesin-receptor interactions are the BabA adhesin and the binding to the fucosylated blood group antigens ABO/Lewis b (Leb) and the SabA adhesin and its binding to the inflammation associated sialyl-Lewis x antigen. During *H. pylori* infection the availability of receptor structures on the human gastric mucosa changes as a consequence of the host inflammatory and immune responses. Consequently the bacterial population need to adjust its adherence properties to stay colonized. This thesis describes mechanisms that generate *H. pylori* populations with variable adherence properties and mechanisms for adjustment of adhesin expression levels.

In *H. pylori* strains devoid of Leb-binding, we found bacterial cells with Leb-binding. Isolation of such *H. pylori* clones demonstrated that the change in receptor binding phenotype was obtained via the mechanisms of homologous recombination and slipped strand mispairing (SSM).

Disease presentation in relation to BabA expression was studied in *H. pylori* infected Mongolian gerbils. We showed that BabA was not essential for colonization but caused severe injury to the gastric mucosa and was turned off during long-term infection by nucleotide changes within the *babA* gene. Gerbils infected with BabA-weak-expressing strains maintained BabA expressing clones for a longer period than gerbils that were infected with BabA-high-expressing strains. Studies of the gerbil gastric mucosal glycosylation showed that gerbils respond in a similar way as humans and Rhesus monkeys which support gerbils to be a model suitable for studying *H. pylori* infection and disease outcome in relation to adherence.

We studied the SSM mechanism of SabA phase variation and the cognate shift in sLex-binding phenotype and we show sLex-binding activity to be growth phase dependent.

H. pylori vesicles were characterized for the major phospholipid and protein components. Virulence factors *e.g.*, VacA, and CagA were identified and both the BabA and the SabA adhesins was shown to be located on the vesicle surface and to mediate specific binding to their cognate receptors present on the human gastric mucosa.

H. pylori generate bacterial cells with different receptor binding phenotypes via the mechanisms of homologous recombination, SSM and nucleotide changes. These mechanisms will probably contribute to bacterial fitness by the generation of quasi species populations where some of the clones will be better adapted to the environmental chances during persistent infection.

Keywords: *H. pylori*, adherence, BabA, SabA, Leb, sLex, phase variation, recombination, vesicles

List of publications

Papers included in the thesis

- I. Metastability of *Helicobacter bab* adhesin genes and dynamics in Lewis b antigen binding.
Bäckström A, Lundberg C, Kersulyte D, Berg DE, Borén T and Arnqvist A.
Proc Natl Acad Sci U S A (2004) 101:16923-8

- II. Effects of BabA expression during *Helicobacter pylori* infection in Mongolian gerbils.
Ohno T, **Vallström A**, Wu MJ, Rugge M, Ota H, Graham DY, Arnqvist A and Yamaoka Y.
Submitted manuscript

- III. Phase variation and expression mechanisms of the sialic acid binding adhesin SabA in *Helicobacter pylori*.
Öhman C*, **Vallström A***, Olofsson A, Johansson P, Larsson C, Aspholm M and Arnqvist A.
Manuscript

- IV. Characterization of *Helicobacter pylori* vesicles and their cognate properties for intimate host interactions.
Olofsson A, **Vallström A**, Petzold K, Schleucher J, Carlsson S, Haas R, Backert S, Wai SN, Gröbner G and Arnqvist A.
Submitted manuscript

* These authors contributed equally to the work.
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Papers not included in the thesis

1. Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin.
Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, Vikström S, Sjöström R, Lindén S, **Bäckström A**, Lundberg C, Arnqvist A, Madhavi J, Nilsson UJ, Velepato B, Gilman RH, Gerhard M, Alarcon T, López-Brea M, Nakazawa T, Fox JG, Correa P, Dominguez-Bello MG, Perez-Perez GI, Blaser MJ, Normark S, Carlstedt I, Oscarson S, Teneberg S, Berg DE and Borén T.
Science (2004) 305:519-22

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Abbreviations

ALeb	A Lewis b
ArsRS	acid-responsive signaling system
BabA	blood group antigen binding adhesin
BLeb	B Lewis b
CagA	cytotoxin-associated gene A protein
Hop	<i>Helicobacter</i> outer membrane protein
HP-NAP	<i>H. pylori</i> neutrophil activating protein
<i>H. pylori</i>	<i>Helicobacter pylori</i>
IARC	International Agency for Research on Cancer
IM	inner membrane
Leb	Lewis b
Lex	Lewis x
Ley	Lewis y
MALT	mucosa-associated lymphoid tissue lymphoma
MMR	mismatch repair
OM	outer membrane
OMP	outer membrane protein
R/M	restriction/modification
SabA	sialic acid binding adhesin
sLea	sialyl-Lewis a
sLex	sialyl-Lewis x
SSM	slipped strand mispairing
T4S	type IV secretion
Th	T helper
VacA	vacuolating cytotoxin A
WHO	World Health organization

Introduction

During bacterial infections, bacterial, host and environmental factors contribute to infection outcome and disease presentation. Bacterial infections can proceed in various directions: (1) the bacteria are cleared by the host's immune system, (2) the host is killed or (3) a persistent infection is established where the host and the bacteria can live in equilibrium for the hosts lifetime. For establishment of a persistent infection the bacteria must have the capacity of adapting to a variety of environmental changes that occur throughout the infection. *Helicobacter pylori* is an example of a bacterium that causes persistent infections in the human stomach. This thesis focuses on molecular mechanisms that cause variable adherence properties in *H. pylori*.

Introduction to *Helicobacter pylori*

In 2005, the Nobel Prize in Physiology or Medicine was awarded for the discovery in the early 1980's of a spiral shaped Gram-negative bacterium and its association to gastritis and peptic ulcer disease (Warren and Marshall 1983; Marshall and Warren 1984). The discovery was first met with skepticism because the general view at the time was that bacteria were incapable of living in the acidic stomach environment. Reports of spiral shaped gastric bacteria were made 100 years earlier but these reports were also met with skepticism since the organism could not be cultured (reviewed by Dubois 1995). To convince the world, Dr Marshall drank a bacterial suspension and as a consequence developed acute gastritis (Marshall et al. 1985). The bacteria was named *Campylobacter pyloridis*, which later was changed to the new genus *Helicobacter pylori* (Goodwin et al. 1989). In 1994 *H. pylori* was the first bacterium to be classified as a class I human carcinogen by the WHO International Agency for Research on Cancer (IARC) because of its epidemiological relationship to gastric cancer (IARC 1994). Today the link between *H. pylori* and gastritis, peptic ulcer disease and gastric cancer is well established in research society and in medical care.

Transmission

H. pylori is human- and primate-specific and no reservoirs outside these hosts have been identified. More than half of the world's population is infected with *H. pylori* but the route of transmission has not been fully elucidated. Epidemiological studies showed that the majority of *H. pylori* colonization occurs during early childhood through close person-person contact. Gastro-oral and fecal-oral transmission routes have been suggested to be the primary transmission routes (reviewed by Malaty 2007).

Clinical outcome and gastric diseases

The *H. pylori* prevalence is estimated to be 50% worldwide and as high as 90% in some populations, particularly in East Asia, South America, Alaska and Southern Europe. The infection causes a lifelong inflammation of the gastric mucosa, gastritis, in all infected individuals although usually clinically asymptomatic. The superficial gastritis progress towards chronic active gastritis characterized by infiltrating neutrophils, B cells, T cells, lymphocytes, macrophages and plasma cells. About 10-20% of the *H. pylori*-infected individuals will develop peptic ulcer disease and 1-2% gastric cancer (Figure 1).

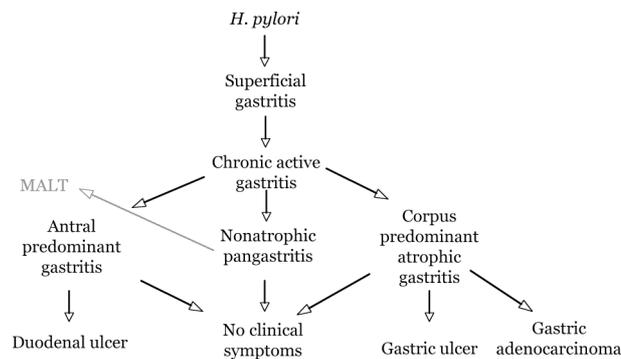


Figure 1. Progression of *H. pylori* infection and disease.

Peptic ulcer disease includes both gastric and duodenal ulcers, which are ulcers that develop in the corpus region and in the transition between the pylorus and duodenum, respectively (Figure 2). Gastric cancer is the fourth most common cancer worldwide with 934,000 cases each year; 490,000 of these cases are reportedly caused by *H. pylori* (IARC 2008). *H. pylori* caused gastric cancer includes gastric adenocarcinomas and the more uncommon gastric mucosa-associated lymphoid tissue (MALT) lymphoma which affect B cells. About 80% of all gastric MALT lymphomas are caused by *H. pylori* and the majority will have a complete regression if the *H. pylori* is eradicated with antibiotics (Parsonnet et al. 1994; Bayerdörffer et al. 1995).

Not all *H. pylori* infected individuals will develop peptic ulcer disease or gastric cancer and why some infections progress into disease and others not is not completely understood. However, some factors are known to contribute to the clinical outcome *i.e.*, the location of the *H. pylori* colonization in the stomach, hormonal changes, acid secretion, polarization of the T helper (Th) type of immune responses, virulence of the bacterial strain and life-style associated factors (reviewed by Atherton 2006).

The location of the *H. pylori* infection in the stomach is dependent on the level of acid produced in the infected person. In a high-acid producing person, the infection will localize predominantly in the less acidic antrum. This antral-predominant infection and associated inflammation leads to even higher acid production levels because of the stimulation of parietal cells in the uninflamed corpus to produce more acid stimulated by the hormone gastrin. Hence, *H. pylori* infection established in the lower part of the stomach predisposes individuals to duodenal ulcer formation. In contrast, individuals with low acid production *e.g.*, individuals with inhibition of parietal cell acid production by IL-1 β will develop severe hypochlorhydria and corpus predominant gastritis (pangastritis) which predisposes them to gastric ulcers and gastric adenocarcinomas (reviewed by Atherton 2006) (Figure 2).

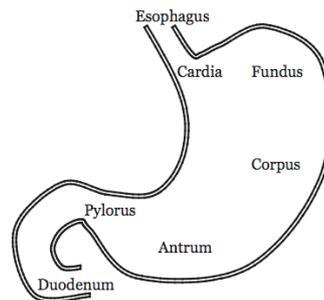


Figure 2. Anatomy of the human stomach

Th1 and Th2 cells are subgroups of CD4⁺ lymphocytes that regulate different immune effector mechanisms via the production of specific cytokines although it is known that other CD4⁺ subtypes including Tregs, Tr1, Th3 and Th17 are also involved in various aspects of immune function and immune-mediated pathology. Unexpectedly, *H. pylori* which is a predominantly extracellular bacteria elicits a Th1-mediated immune response thought to be typical of intracellular pathogens (Bamford et al. 1998; reviewed by D'Elios and Andersen 2007). Host genetic polymorphisms in genes *e.g.*, IL-1 β and TNF- α can affect IL-8 secretion levels resulting in a more pronounced Th1 inflammatory response that in turn causes more gastric mucosal damage, inhibition of acid secretion and increased cancer risk (El-Omar et al. 2000; El-Omar et al. 2003; Machado et al. 2003). Recently, the *H. pylori* neutrophil activating protein (HP-NAP) has been suggested to contribute to the polarization of the Th1 response (Amedei et al. 2006). In contrast, more balanced Th1 and Th2 immune responses have been associated with milder gastritis, higher *H. pylori* density, and lower atrophy and lower cancer risk (reviewed by Atherton 2006).

Colonization and virulence factors

H. pylori chronically infects the harsh environment in the human stomach and have evolved mechanisms to uniquely colonize and persistently survive in this niche. The majority of these bacteria are free living in the gastric mucus layer although about 20% is in close contact with epithelial cells (Hessey et al. 1990). *H. pylori* are 1-4 μm long, Gram-negative, spiral shaped, slow-growing, microaerophilic, flagellated bacteria that grow primarily extracellularly (Figure 3). The ability to regulate gene expression and the ability to adapt to different conditions is probably very important for colonization, survival and the establishment of persistent infections.

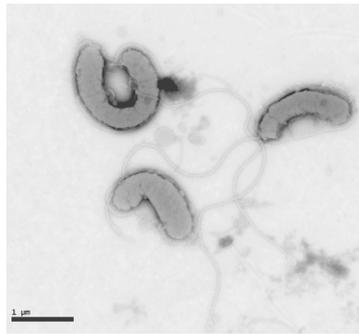


Figure 3. Electronmicrograph of *H. pylori*. Picture: Annelie Olofsson

H. pylori have been found intracellularly in host cells and capillaries of the lamina propria *in vivo* (Semino-Mora et al. 2003; Aspholm et al. 2006; Necchi et al. 2007). Approximately 1% of *H. pylori* cells infecting epithelial cells *in vitro* survived gentamicin treatment (Amieva et al. 2002). The intracellular localization aids to protect against antibiotic treatment and the immune system. Additional factors, which protect *H. pylori* from host responses are the low antigenicity of both the *H. pylori* LPS and flagellar proteins, which do not initiate appropriate innate immune system activation signals via toll-like receptors (TLR) (Muotiala et al. 1992; Gewirtz et al. 2004). In addition, the adaptive immune system is modulated and down-regulated by *H. pylori* effector molecules, *e.g.*, VacA cytotoxin and γ -glutamyltranspeptidase (Molinari et al. 1998; Boncristiano et al. 2003; Gebert et al. 2003; Sundrud et al. 2004; Schmees et al. 2007; Torres et al. 2007). As a consequence of these immunomodulatory mechanisms, the host immune response is not capable of clearing *H. pylori* infections in the human stomach which establishes as a persistent infection for the host lifetime.

Motility and chemotaxis

Motility is important for *H. pylori* and over 50 proteins are involved in the organization of the *H. pylori* flagellar apparatus (Niehus et al. 2004). In the human stomach, pH range from very acidic conditions in the gastric lumen to almost neutral pH close to the gastric epithelial cells. *H. pylori* sense, orient and move away from acidic environments towards the epithelial cells where most of the *H. pylori* localize during persistent infection (Schreiber et al. 2004). Genes mediating chemotaxis and motility are up regulated in *H. pylori* colonizing Mongolian gerbils (Scott et al. 2007) and the directed movement is required for full colonization in animal models (Eaton et al. 1996; Foynes et al. 2000).

Acid acclimation

H. pylori is a neutrophilic bacterium with the ability of surviving and colonizing the human stomach by buffering both its periplasm and cytoplasm to near neutrality in contrast to other neutrophilic bacteria which can only survive transit through the stomach *e.g.*, *Vibrio cholerae* and *Escherichia coli*. An important player in acid acclimation is the urease system which is composed of an inner membrane (IM) channel UreI and the urease enzyme. In acidic environments, urea from the gastric juice passes through porins located in the outer membrane (OM) into the *H. pylori* cytoplasm via the UreI channel. The urease enzyme in the cytoplasm converts urea into carbon dioxide and ammonia which then diffuse into the periplasm (Sachs et al. 2005). An additional enzyme also aids *H. pylori* during acidic conditions, the α -carbonic anhydrase that converts carbon dioxide into bicarbonate thereby buffering the periplasm of *H. pylori* (Chirica et al. 2002; Marcus et al. 2005). Both the urease system and the α -carbonic anhydrase are essential for efficient colonization (Eaton and Krakowka 1994; Weeks et al. 2000; Bury-Moné et al. 2008). Several studies have focused on genes that are regulated in response to pH changes (Merrell et al. 2003; Wen et al. 2003; Bury-Moné et al. 2004; Scott et al. 2007). Several of these genes are controlled by the acid-responsive signaling (ArsRS) regulon (Pflock et al. 2004; Pflock et al. 2006; Wen et al. 2007).

The vacuolating cytotoxin A (VacA)

Supernatants from *H. pylori* cultures induced cytoplasmic vacuoles in *in vitro* grown cells as a result of the secreted vacuolating cytotoxin A (VacA) (Leunk et al. 1988; Cover and Blaser 1992). Besides being secreted, VacA can also localize to the bacterial surface from where it can be transferred to host cells (Ilver et al. 2004). The *vacA* gene is present in almost all human clinical isolates but the VacA protein is only expressed in about 50% of cases (Leunk et al. 1988; Atherton et al. 1995). Cytotoxic activity is linked to allelic variation in the signal peptide (s1 and s2 type), the mid region (m1 and m2

type) and the intermediate region (i1 and i2) of the *vacA* gene. The most virulent allele type of the *vacA* gene is the s1/m1/i1, which are associated with gastric adenocarcinoma (Atherton et al. 1995; Rhead et al. 2007).

The pore-forming VacA cytotoxin has been suggested to have multiple functions. The most studied property is the effect on cellular vesicle trafficking and endosomal maturation leading to cytoplasmic vacuoles in epithelial cells. VacA also induces cytochrome c release from mitochondria resulting in gastric epithelial cell apoptosis (Galmiche et al. 2000; Cover et al. 2003). Another function of VacA is to increase the membrane permeability for small molecules, which has been hypothesized to facilitate nutrient uptake by *H. pylori* (Papini et al. 1998). Immunosuppressive effects, such as inhibition of antigen presentation (Molinari et al. 1998), inhibition of T cell activation and proliferation have also been described (Boncristiano et al. 2003; Gebert et al. 2003; Sundrud et al. 2004; Torres et al. 2007) although additional studies further defining the effects of the VacA cytotoxin *in vivo* are needed and discussed by Schmees and coworkers (Schmees et al. 2006).

The cytotoxin-associated gene A (CagA)

CagA is the first bacterial protein that has been described to be an oncoprotein. It is translocated into host cells via a type IV secretion (T4S) system and CagA interferes with host cell signaling system. Disturbances in these signal transductions are associated with carcinogenesis (reviewed by Hatakeyama 2008). It was recently described that transgenic mice that expresses CagA in the stomach and small intestine develop gastric polyps and adenocarcinomas in the absence of *H. pylori* (Ohnishi et al. 2008). Gastric mucosal damage prevalence is higher in populations where the majority are infected with *cagA*⁺ strains, such as in the East Asia, compared to around 60% *cagA*⁺ *H. pylori* clinical isolates from Western countries (reviewed by Hatakeyama 2009).

The *cagA* gene is located in the *H. pylori* *cag* pathogenicity island (*cagPAI*) (Covacci et al. 1993; Tummuru et al. 1993). The *cagPAI* contains about 30 genes, at least 14 are essential for the T4S system and many of the *H. pylori* unique proteins have not yet a definite function (Fischer et al. 2001; Kutter et al. 2008). Other bacterial species also have T4S systems which they use to translocate proteins or DNA into host cells. The prototype T4S system that has been well defined is that of *Agrobacterium tumefaciens* (Fullner et al. 1996), which have been used to extrapolate more information regarding the *H. pylori* T4S system. Another *cagPAI*-encoded protein CagF, has been suggested to be a chaperone-like molecule associated with CagA. The function of CagF is thought to ensure the integrity of the CagA structure

(Couturier et al. 2006; Pattis et al. 2007). Other *cagPAI* encoding proteins suggested to be involved in the CagA translocation are the CagD and CagN (Bourzac et al. 2006; Cendron et al. 2009).

In vitro the T4S apparatus in *H. pylori* is suggested to dock to the host cell's $\alpha 5\beta 1$ integrin via the tip adhesin CagL (Kwok et al. 2007) and translocate the CagA into gastric epithelial cells (Segal et al. 1999; Asahi et al. 2000; Backert et al. 2000; Odenbreit et al. 2000; Stein et al. 2000). These events affect intracellular signaling networks and induce cytoskeletal changes and secretion of the pro-inflammatory chemokine IL-8 (Censini et al. 1996; Segal et al. 1997). Translocated CagA is initially phosphorylated by Src family kinases and then continuously by the Abl family of kinases (Selbach et al. 2002; Stein et al. 2002; Poppe et al. 2007; Tammer et al. 2007). Phosphorylated CagA affects several host cell signaling pathways, causing cytoskeletal changes known as the hummingbird phenotype (Segal et al. 1999; Higashi et al. 2002b; Selbach et al. 2003). The number of the CagA phosphorylation sites, EPIYA motifs, has been correlated to the development of gastric cancer (Higashi et al. 2002a; Basso et al. 2008). CagA can also act in a phosphorylated-independent manner stimulating different transcriptional factors (Mimuro et al. 2002; Churin et al. 2003) involved in both cellular proliferation and apoptosis. An additional effect of the unphosphorylated CagA is the disruption of tight junctions that effect cell polarity likely supplying the bacteria with nutrients (Amieva et al. 2003; Bagnoli et al. 2005; Saadat et al. 2007). Unphosphorylated CagA also interacts both with E-cadherin and the cadherin-associated protein β -catenin, pathways that may contribute to intestinal metaplasia (Franco et al. 2005; Murata-Kamiya et al. 2007).

Vesicles

Gram-negative bacteria shed 50 to 250 nm diameter vesicles *in vivo* and *in vitro* consisting mainly of OM proteins, phospholipids and LPS. In addition, both DNA and RNA have been found within vesicles. The current view of how vesicles are shed includes budding of the OM and that components of the periplasmic space are trapped within the vesicles (reviewed by Beveridge 1999; Kuehn and Kesty 2005; Mashburn-Warren et al. 2008b). Several recent studies have shown, however, that vesicles also carry proteins of cytoplasmic origin as well (reviewed by Lee et al. 2008). Under normal conditions vesicles are shed constantly from Gram-negative bacteria. During bacterial stress more vesicles are shed. The relation of vesicle shedding and stress is supported by the study of Button and coworkers where the expression of the stress-related σ^E was tightly coupled to the amount of vesicles that were produced in *E. coli* (Button et al. 2007). In *Pseudomonas*

aeruginosa the quorum sensing signal PQS has been shown to stimulate the production of vesicles and to be used for communication between bacteria by packing quorum sensing molecules within the vesicles (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008a; Mashburn-Warren et al. 2009).

Because of the diverse cargo of vesicles multiple roles for the vesicles have been suggested for them although the common theme is that the vesicles enhance survival of the bacteria or damage the host. Vesicles derived from pathogenic bacteria often contain toxins and host-affecting molecules *e.g.*, heat labile enterotoxin (LT), (Horstman and Kuehn 2000; Kesty et al. 2004) ClyA, CNF1 and α -haemolysin (Wai et al. 2003; Balsalobre et al. 2006; Kouokam et al. 2006). *Moraxella catarrhalis* vesicles are covered with the complement factor C3 via interaction with the UspA1/A2 surface proteins, a clever strategy to aid its co-infection partner *Haemophilus influenzae* from complement-mediated killing (Tan et al. 2007). *Pseudomonas aeruginosa* vesicles contain hydrolytic enzymes and have been suggested to have antimicrobial activity because the vesicles have the ability to lyse a variety of both Gram-negative and Gram-positive bacteria (Li et al. 1998). In addition, bacterial vesicles are promising candidates for vaccine development. Vesicle-derived vaccines against *Neisseria meningitidis* obtained the best efficacy against serogroup B meningococcal disease. The strain specificity of the vaccine lasted for decades, which is promising in terms of epidemic control discussed in (reviewed by Holst et al. 2009).

H. pylori vesicles have been found in human gastric biopsy material and VacA cytotoxin has been shown to be associated with these vesicles (Sommi et al. 1998; Fiocca et al. 1999; Heczko et al. 2000; Keenan et al. 2000). When *H. pylori* vesicles and gastric cells are co-cultured, a decrease in gastric cell viability, vacuolization and IL-8 secretion occur (Ismail et al. 2003). Similar experiments with co-culturing of host cells and *H. pylori* vesicles induced apoptosis (Ayala et al. 2006). This suggested that *H. pylori* vesicles induce host responses and may contribute to development of gastric disease. For further characterization of *H. pylori* vesicles see paper IV.

Adherence

To initiate and maintain infection microorganisms often attach to host cells. Specific proteins on the bacterial and viral surfaces, so called adhesins, mediate adherence. In some cases by a general adherence mode and in other cases to specific structures, so called receptors or ligands. Receptors are present in the host tissue such as on host cell surfaces, connective tissue or to specific proteins in circulation *e.g.*, complement factors and

immunoglobulins (reviewed by Kline et al. 2009). Receptors constitute proteins, glycoproteins, glycolipids or polysaccharides. The adhesin-receptor interaction is often specific and provides targeted localization of a microorganism to a dedicated host tissue, or subset of cells, *i.e.*, tissue tropism.

In *H. pylori*, the majority of bacterial cells are free living and motile in the gastric mucus layer whereas about 20% adhere to the gastric epithelial cells (Hessey et al. 1990). Besides to resist the peristaltic movements, adherence may promote direct and facilitated delivery of effector molecules to host cells and facilitates nutrients availability. On the other hand, shedding of gastric cells and chronic inflammation can result in clearance of the infection. Adaptation and programmed loss of adherence properties can be beneficial for the maintenance of persistent infection.

Receptor structures

Glycoproteins or glycolipids with fucosylated, sialylated and sulphated oligosaccharides and also unsubstituted core chain glycans are suggested binding sites for *H. pylori*. Receptor structures have been identified on gastric epithelial cells and on mucins, which are highly glycosylated proteins present in the mucus layer (Slomiany et al. 1989; Borén et al. 1993; Mahdavi et al. 2002; Teneberg et al. 2002; Aspholm-Hurtig et al. 2004; Aspholm et al. 2006; McGuckin et al. 2007).

The most studied receptor structures for *H. pylori* are the fucosylated ABO blood group antigens derived from the type 1 core chain; A-Lewis b (ALe_b), B-Lewis b (BLe_b) and Lewis b (Le_b) (Borén et al. 1993; Aspholm-Hurtig et al. 2004). Other pathogenic microorganisms such as the Noroviruses also recognize the ABO blood group antigens as receptors (Hutson et al. 2002). Sialylated structures such as the sialyl-Lewis x (sLe_x) and sialyl-Lewis a (sLe_a) antigens is another group of functional receptors identified for *H. pylori* (Mahdavi et al. 2002; Aspholm et al. 2006). The sLe_x antigen is upregulated during periods of inflammation and this glycan binds to selectin molecules involved in the recruitment and migration of leucocytes from the bloodstream to inflamed tissue. In addition, sialylated structures, including both sLe_x and sLe_a are upregulated in humans during cancer (Sakamoto et al. 1989), and the sLe_a antigen (denoted 19-9) is defined as a tumor antigen (Sipponen and Lindgren 1986). In contrast to the limited set of microorganisms that use fucosylated antigens for attachment, many bacteria and viruses are described to make use of sialylated receptors for attachment *e.g.*, influenza virus, adenovirus and *E. coli* (Parkkinen et al. 1986; Sauter et al. 1992; Arnberg et al. 2000).

Glycosylation pattern studies of the Rhesus monkey gastric mucosa during *H. pylori* infection showed a time-dependent shift. Fucosylated Lewis antigens were suppressed during the acute phase of infection while levels of sialylated structures such as sLex and sLea increased. After 10 months post-infection, levels of both fucosylated and sialylated structures had returned to similar levels as in healthy gastric mucosa *i.e.*, expression patterns of rich expression of fucosylated ABO antigens but only minute levels of sialylated inflammation associated antigens (Lindén et al. 2004; Lindén et al. 2008a). The relevance of adherence for disease outcome was illustrated when transgenic mice that express Leb in the gastric epithelial lining and intestine were colonized by *H. pylori* to similar infection load levels, which showed that Leb-dependent adherence was associated with increased tissue damage and increased inflammation (Guruge et al. 1998).

Examples of additional suggested *H. pylori* receptors are the Trefoil factor 1 (TFF1), the Decay-accelerating factor (DAF), fibronectin, lactoferrin, laminin and collagen IV (Trust et al. 1991; Clyne et al. 2004; Walz et al. 2005; O'Brien et al. 2008). *H. pylori* also bind to several components in saliva (Prakobphol et al. 2005; Lindén et al. 2008b; Walz et al. 2009). Thus, it is likely that the different types of adhesion modes described for *H. pylori* will influence the pathogenesis differently. Binding may prevent killing by the complement system; interaction with extracellular matrix proteins may facilitate bacterial attachment to tissues further down in the epithelial cell layer; and attachment to clearance factors within the saliva may be a scavenger for transportation down to the human stomach.

Adhesins

Probably *H. pylori* need to have a variable mode of adherence both in the number of adhesins available and their expression levels to meet the repertoire of receptors and their dynamic expression described above. Despite the small size of the *H. pylori* genome, approximately 4% of the genome encodes for outer membrane proteins (OMPs) and 33 closely related unique *H. pylori* OMPs belong to the *H. pylori* outer membrane protein (Hop) family 1 including Hop and Hop-related (Hor) proteins (Alm et al. 2000). Several of these proteins have been suggested to act as adhesins or to be adherence-associated proteins. In addition, the hop 1 gene family contains many genes with variable gene expression characteristics. Repetitive sequences within the 5' end of genes and similarities in the 5' and 3' ends of these genes suggested that phase variation via slipped strand mispairing (SSM) mechanism and homologous recombination could occur (Alm et al. 1999).

So far, only the blood group antigen binding adhesin (BabA) (Ilver et al. 1998) and the sialic acid binding adhesin (SabA) (Mahdavi et al. 2002) have been described as functional adhesins. Other Hop-proteins, such as the HopZ protein (Peck et al. 1999), the AlpA and AlpB lipoproteins (Odenbreit et al. 1999), and the HorB protein (Snelling et al. 2007) have been described to be important for adherence although their actual function has not yet been completely understood, and no cognate receptors have been described.

Blood group antigen binding adhesin BabA

The BabA adhesin is the first identified and the best-characterized *H. pylori* adhesin, which mediates binding to the ABO blood group antigens; the fucosylated H1, Leb, A-Leb, B-Leb (Ilver et al. 1998; Aspholm-Hurtig et al. 2004), the fucosylated MUC5AC mucin and the gp-340 glycoprotein (Lindén et al. 2008a; Walz et al. 2009). Not all *H. pylori* strains bind ABO/Leb, but among the Leb-binding strains most strains are generalist strains i.e. they have the ability to bind to all the ABO antigens (H, B, A, A-Leb, B-Leb and Leb) although the highest binding affinity is to Leb. Specialist strains, which only bind H and Leb are predominantly found in the native population of South American Amerindian where the majority of individuals are of blood group O phenotype (Aspholm-Hurtig et al. 2004).

Two genes that encode the BabB and BabC proteins with unknown functions, *babB* and *babC*, have high sequence similarities to *babA*. Sequence diversity of in particular the *babA* gene is prevalent; in fact nearly all *babA* and *babB* genes are unique. Studies of the genetic diversity in the *babA* and *babB* genes show greatest diversity in the middle region and in this region different allele groups have been identified. Because of the genetic diversity in *H. pylori*, parts of *babA* and *babB* genes present in the same strain can be more related than two *babA* alleles in two different strains (Alm et al. 2000; Pride et al. 2001; Hennig et al. 2004). In addition, the presence, number and location of the *bab* genes on the chromosome may differ between strains and chimeric *bab* genes have been described (Ilver et al. 1998; Alm et al. 1999; Alm et al. 2000; Pride and Blaser 2002; Solnick et al. 2004; Colbeck et al. 2006; Hennig et al. 2006).

So called, triple positive strains, where *vacAs1*, *cagA* and *babA2* are present, are strongly associated with duodenal ulcer and gastric adenocarcinoma (Gerhard et al. 1999). This result has been followed up in different parts of the world by a series of studies examining the prevalence of *babA* in relation to disease based on PCR. More recent studies have analyzed this relationship in the context of BabA expression since presence of the *babA* gene not necessarily is correlated to BabA expression (Yamaoka et al. 2002; Yamaoka et al. 2006). In one such study BabA low-expressing strains was shown to

contribute to more severe mucosal injury and were more frequently associated with duodenal ulcer and gastric cancer than strains with high-level or without BabA expression (Fujimoto et al. 2007).

Sialic acid binding adhesin SabA

The *H. pylori* sialic acid binding adhesin SabA is polymorphic in its receptor binding specificity and recognize sLex, sLea, sialyl-lactosamine (sLn), sialyllactose, laminin, MUC6 and MUC7 (Mahdavi et al. 2002; Roche et al. 2004; Walz et al. 2005; Aspholm et al. 2006; Lindén et al. 2008a; Walz et al. 2009). In biopsy material *H. pylori* have been found in blood vessels and the SabA adhesin have been shown to mediate interaction with human erythrocytes and cause sialic-acid-dependent *in vitro* haemagglutination (Aspholm et al. 2006).

In vitro, SabA also interacts with sialylated structures present on human neutrophils, induces phagocytosis and oxidative burst suggesting that SabA likely contributes to damage of the gastric epithelium (Unemo et al. 2005; Petersson et al. 2006). The SabA adhesin is regulated by different mechanisms. The ArsRS system down-regulates SabA expression under acidic conditions (Merrell et al. 2003; Yamaoka et al. 2006; Goodwin et al. 2008). Phase variation as a consequence of the number of CT-repeats present within the *orf* as a result of SSM is also known to regulate SabA expression and sLex-binding (Mahdavi et al. 2002; Yamaoka et al. 2006).

SabA-expressing strains have been associated with gastric cancer and negatively associated with duodenal ulcers (Mahdavi et al. 2002; Yamaoka et al. 2006). One study has suggested that a non-functional *sabB* gene correlated with duodenal ulcer (de Jonge et al. 2004), a gene which DNA sequence is highly homologous with *sabA*. The function of the SabB protein is yet not known.

Genetic diversity

The capacity of *H. pylori* to adapt to the continuously changes in the human stomach environment is decisive to cause persistent infection. Although, only a few two-component systems, σ -factors and transcriptional factors have been identified in *H. pylori* (Tomb et al. 1997; Alm et al. 1999) compared to bacteria that lives in more than one ecological niche. This is probably because *H. pylori* reside in the same ecological niche throughout the infection and not need the same regulation for quick responses to dramatic environmental changes. In addition to other regulatory systems than the classical ones can be involved in the adaptation process. So called quasispecies, which constitutes a pool of clones with high diversity and in

such populations there are always clones present that are fit and ready to adapt to changing conditions (Kuipers et al. 2000). Studies of *H. pylori* bacterial cells isolated at different time points of the same hosts, showed that clones exhibited a great genetic diversity, which are likely to have been created by multiple mechanisms *e.g.*, phase variation, spontaneous mutations and recombination events (Kersulyte et al. 1999; Israel et al. 2001).

Phase variation

The denomination of the phenomenon to avoid the host inflammation and immune responses and gain of function needed for specific conditions is not consistent in the literature; both antigenic and phase variation is used. Antigenic variation is often used in terms of expression of alternative forms of an antigen. Phase variation is often used for mechanisms that turn expression ON and OFF. However the mechanisms involved in phase variation are overlapping. Here, the denomination of phase variation will be used. Phase variation occurs in both pathogenic and non-pathogenic bacteria and is responsible for switching gene expression on and off. Phase variation frequencies of certain genes can vary between strains but are also affected by environmental conditions. The randomness of phase variation makes it impossible to predict which bacterial cells within a population will be subject to change. Phase variation occurs with high frequency switch rates; is reversible and most often affects cell surface composition. Different phase variation mechanisms include the SSM mechanism within short sequence repeats, homologous recombination particularly the site specific recombination and also epigenetic regulation without any changes in the DNA sequence *e.g.*, alternation in the methylation status. The net result of phase variation are populations that consist of clones with different phenotypes, *i.e.*, quasispecies, which are populations where some clones will be fit enough to cope for changes in the environment (reviewed by van der Woude and Bäumlner 2004). In the context of adhesion, the impact of phase variation allows bacteria to cycle between adherent and non-adherent phenotypes and the generation of alternative receptor binding phenotypes.

Slipped strand mispairing (SSM)

A common mechanism of phase variation is SSM, which often results in high phase variation frequencies. Homopolymeric and polynucleotide DNA repeats generate slippage and mispairing during replication or DNA repair that changes the number of repeats. These changes in the number of repeats can lead to phase variation if the location of these repeats is such as they affect transcription or translation and thus the expression status. A common location of the repetitive sequence is near the 5' end of the *orf*, which brings the *orf* in or out of frame. The location of the repeats is also commonly found

within the promoter region which then affect transcription initiation (reviewed by van der Woude and Bäumlner 2004).

In silico analysis identified 46 *H. pylori* candidate SSM phase variable genes with the majority of genes involved in LPS biosynthesis, DNA restriction/modification (R/M) systems and OM proteins *e.g.*, *sabA*, *babB*, *sabB* and *hopZ* (Salaün et al. 2004). The *H. pylori* LPS is highly diverse due to serotype variation of the Lewis antigens. Synthesis of Lewis antigens involves the fucosyltransferases *futA*, *futB* and *futC* which add fucose units creating Lewis x (Lex) and Lewis y (Ley) antigens. Phase variation within the *orf* of the fucosyltransferase genes determines the activity of the enzymes and thus the phenotype of Lewis antigen expressed on *H. pylori* LPS (Appelmelk et al. 1999; Wang et al. 1999). This was further studied when clinical isolates were studied over time, which clearly showed that SSM of the *fut* genes had occurred as well as after infection of mice (Nilsson et al. 2006, 2008; Skoglund et al. 2009).

Mutation and homologous recombination

Differences in *H. pylori* genome sizes, gene order and content were evident following DNA sequence and microarray analyses comparing multiple *H. pylori* strains (Tomb et al. 1997; Alm et al. 1999; Salama et al. 2000; Gressmann et al. 2005; Oh et al. 2006; Baltrus et al. 2009). Nearly half of the *H. pylori* strain-specific genes are present in plasticity zones which have a different GC content than the rest of the genome and are suggested to be DNA sequences adapted from other species (Alm and Trust 1999).

H. pylori is efficient in natural transformation competence as a consequence of high uptake of DNA from the environment. RecA-mediated homologous recombination is an essential step for incoming DNA to be integrated into the genome. *H. pylori* has many R/M systems which protect the bacteria from intake of too much and harmful DNA, events that may be deleterious for the bacteria (Tomb et al. 1997; Alm and Trust 1999). When R/M systems is turned off by phase variation, uptake of foreign DNA is thought to be facilitated and thus increase the genetic diversity of *H. pylori* (Aras et al. 2002; de Vries et al. 2002).

In *H. pylori* several enzymes involved in DNA mismatch repair (MMR) are missing, for example, both MutL and MutH are absent (Alm and Trust 1999). An *H. pylori* MutS2 variant was identified and isolated and shown not to be involved in MMR (Björkholm et al. 2001; Pinto et al. 2005; Wang et al. 2005). Frequencies of spontaneous mutation in *H. pylori* vary between 10^{-5} and 10^{-7} , which are rates similar to those reported for *E. coli* mutator strains (Björkholm et al. 2001).

Frequent recombination is observed in *H. pylori* strains (Suerbaum et al. 1998) and the recombination frequency has been calculated to be unusually high *e.g.*, 50% of the *H. pylori* genome could be exchanged by recombination over a forty year infection period (Falush et al. 2001). Homologous and homeologous recombination facilitates deletion, duplication or creation of chimeric genes *e.g.*, *bab* genes (Paper I). The specificity of the *H. pylori* LPS is in addition to SSM also modulated by homologous recombination by creating different numbers of heptad-repeats in the FutA and FutB enzymes (Nilsson et al. 2006, 2008; Skoglund et al. 2009). Recombinations have been reported to be essential for *H. pylori* fitness and establishment of infection. Strains with a 30-fold reduction in homologous recombination rate were rapidly cleared upon infection compared to the *H. pylori* wild-type strains (Loughlin et al. 2003; Robinson et al. 2005).

Aim

This thesis aimed to study mechanisms involved in phase variation and variable adherence properties present during persistent *H. pylori* infections. Specifically, the adhesins BabA and SabA were investigated in addition to studying *H. pylori* vesicle composition and their binding properties.

Specific aims:

- I. To study mechanisms associated with variable BabA expression and Leb-binding properties.
- II. To follow BabA expression during long-term *H. pylori* infection in Mongolian gerbils.
- III. To characterize the *sabA* operon and analyze for mechanisms involved in phase variation and regulation of SabA expression.
- IV. To characterize *H. pylori* vesicles and in particular their cognate adherence properties.

Results and discussion

Paper I

Metastability of *Helicobacter pylori* *bab* adhesin genes and dynamics in Lewis b antigen binding.

Previous reports described different localization of both the *babA* and the *babB* and in addition chimeric *babA/B* genes, which indicated that *babA* might be subjected to recombination events (Ilver et al. 1998; Alm et al. 1999; Pride and Blaser 2002; Solnick et al. 2004). Even though most strains carries the *babA* gene, not all of them exhibited Leb-binding properties (Ilver et al. 1998). These observations together suggested to us that homologous recombination was involved in *babA* gene expression.

When we analyzed the Leb-binding phenotype between strains using fluorescence microscopy we observed that some bacteria within a Leb-non-binding population had acquired the Leb-binding phenotype. This suggested that the differences in receptor binding phenotype might mirror natural changes that occur in *H. pylori* strains with respect to gaining the Leb-binding phenotype. Therefore we decided to examine this low OFF-to-ON Leb-binding shift using a defined genetic system. Strain CCUG17875 carries two *babA* genes: *babA1* that is inactive and *babA2* that encodes for the Baba adhesin. Using a derivative strain with a knocked out *babA2* locus still possessing a silent *babA1* gene we isolated Leb-binding clones using biopanning with the Leb-receptor conjugate and magnetic beads. Using homologous recombination a chimeric *babB/A* gene was generated by recombining the silent *babA1* gene into the highly related *babB* gene. This *H. pylori* strain displayed the Leb-binding phenotype with similar Leb-binding affinity as the wild-type CCUG17875 strain and had thereby confirmed our hypothesis about the ability of *H. pylori* to gain binding phenotype with homologous recombination.

The chimeric *babB/A* gene exhibited lower expression levels further demonstrating that homologous recombination could facilitate changes in expression by introducing genes into a different expression locus *e.g.*, different expression levels between the weaker *babB* promoter compared to the *babA2* expression locus. An additional feature of the *babB/A* chimera relative to *babA2* was the presence of CT repeats within the *babB/A* *orf*. This facilitated gene regulation by SSM-mediated phase variation. SSM is a faster mechanism of driving the ON-to-OFF-to-ON variation with frequencies

around 10^{-3} compared to homologous recombination which has a frequency of approximately 10^{-5} . Solnick and coworkers showed that homologous recombination and SSM were used by *H. pylori* to turn off BabA expression *in vivo* during an acute phase infection in Rhesus monkeys (Solnick et al. 2004). The down-regulation of BabA was observed for all output clones studied. However, strains expressing BabA and the Leb-binding phenotype are found frequently (Ilver et al. 1998; Yamaoka et al. 2006; Fujimoto et al. 2007). It is therefore likely that mechanisms that turn on BabA expression or not to down-regulate BabA expression also operates *in vivo*. The impact of homologous recombination for the *babA* gene and receptor binding properties was highly lightened when changes from a Leb specialist to ABO generalist binding mode was shown to be facilitated via homologous recombination (Aspholm-Hurtig et al. 2004).

We have shown that BabA expression and cognate Leb-binding properties can be affected by homologous recombination and SSM. We believe that these mechanisms are used to modulate adherence properties and contribute to *H. pylori* persistence when it needs to adapt to host responses and environmental changes. Further studies will be needed to elucidate if *babA* can recombine with other *hop* genes creating clones with additional properties and phenotypes, thereby increasing the diversity of the *H. pylori* adherence profiles in the population. Another interesting question is whether or not the presence of receptor structures in the gastric mucosa influences the adaptation of Leb-binding versus Leb-non-binding clones. This may be possible to study by monitoring Leb transgenic and wild-type mice infected by an *H. pylori* strain with a silent *babA* gene and thus analyze the output clones for differences in Leb-binding.

Paper II

Effects of BabA expression during *Helicobacter pylori* infection in Mongolian gerbils.

Leb-binding and the *babA* gene are associated with severe gastric diseases (Gerhard et al. 1999). Among human clinical isolates, *H. pylori* BabA-low-expressing strains have been found to be more frequently associated with duodenal ulcers and gastric cancers than BabA-high-expressing strains (Fujimoto et al. 2007). So far, it has been difficult to perform relevant *in vivo* *H. pylori* infection studies. For ethical reasons controlled infection studies in humans are not permitted, however, the similarities between humans and Rhesus monkeys make this animal model highly applicable although very expensive. It is possible to infect mice but the infection outcome is different than what is observed in humans *i.e.*, gastric cancer are not developed in

wild-type mice without using any carcinogen. In recent years, Mongolian gerbils have been used since they develop ulcers and in some cases also gastric cancer without any carcinogen (Hirayama et al. 1996; Watanabe et al. 1998; Franco et al. 2005). Only a few *in vivo* infection studies with a focus on BabA expression and its impact on disease outcome have been carried out. Solnick and co-workers followed BabA expression in Rhesus monkeys during acute experimental infection and found BabA to be down-regulated (Solnick et al. 2004).

In this study, we followed BabA expression during long-term infection of Mongolian gerbils. Mongolian gerbils were inoculated with a BabA-high-expressing *H. pylori* strain and output clones were recovered 1, 3, 6 and 18 months post infection and analyzed for BabA expression. BabA up-regulation was observed during the first month post infection and thereafter the expression levels declined. BabA expression was undetectable six months post infection because of nucleotide changes *e.g.*, deletions, insertions and point mutations within the *babA orf* resulted in a truncated BabA protein. In addition, gerbil gastric inflammation was evaluated and shown to peak six months post infection.

We and other have previously demonstrated that BabA expression could be modulated by homologous recombination and SSM (Solnick et al. 2004). Here we show an additional mechanism for changes in BabA expression. Down-regulation of BabA occurred with nucleotide changes within the *orf* resulting in a truncated BabA protein. Similar out-of-frame *babA* genes have earlier been observed in clinical isolates (Hennig et al. 2006). It is not possible to conclude if the loss of BabA expression was the result of adaptation to the suppression of H1 receptor antigen expression during infection or a result of host immune and inflammatory response that have killed BabA expressing clones that are in close connection to the epithelium or both.

In a re-inoculation experiment using output strains of different BabA phenotype from the first round of infection we made an additional interesting observation. The outcome of output clones from gerbils infected with a BabA-weak-expressing strain versus gerbils infected with a BabA-high-expressing strain differed and the gerbils infected with a weak-expressing strain maintained BabA expressing clones for a longer period. This suggested that the level of BabA expression was of importance during infection. If the influence on BabA expression was direct or indirect, as a consequence of weak BabA expression, resulting in a population of *H. pylori* less likely to interact with host cells thereby eliciting a weaker immune response is not known. Furthermore, gastric inflammation was most

pronounced in the BabA-high-expressing strains compared to the BabA-weak and BabA-non-binding strains three months post infection.

We investigated the expression of the *H. pylori* receptor structures Leb, H1 and sLex on Mongolian gerbil gastric mucosa. In contrast to human gastric mucosa, the Leb antigen was not present at any time during infection, however, the H1 antigen was present in the gastric mucosa of healthy gerbils, 1 and 3 months post infection but not 6 and 18 months post infection. Gastric mucosa infected for 1, 3, 6 and 18 month expressed sialylated structures. This fluctuation in the glycosylation pattern in response to infection was similar to what has been described for humans and Rhesus monkeys. Lindén and coworkers observed down-regulation of Leb expression and up-regulation in sialylated structures during *H. pylori* infection of Rhesus monkeys. Ten months post infection the levels returned to approximate similar levels as in healthy gastric mucosa (Lindén et al. 2004; Lindén et al. 2008a). The host response with respect to *H. pylori* receptors and changes in available receptors during infection highlighted the relevance of the gerbil model for studying the effects of *H. pylori* adherence in relation to gastric disease outcome.

Paper III

Phase variation and expression mechanisms of the sialic acid binding adhesin SabA in *Helicobacter pylori*.

SSM-mediated phase variation of the SabA adhesin has been shown to be the reason for instability in the *H. pylori* sLex-binding phenotype (Mahdavi et al. 2002; de Jonge et al. 2004; Yamaoka et al. 2006; Goodwin et al. 2008). For this project we wanted to study the regulation of SabA expression in more detail. We analyzed changes in sLex-binding and sLex-non-binding phenotypes in a series of strains and observed differences in the number of bacteria with changed sLex-binding phenotype. The distribution of clones with a different binding phenotype ranged from 1/30 to 1/800. This is in agreement with differences in switch frequencies of phase variable surface exposed proteins which have been reported in other systems *e.g.*, the P-pili of *E. coli* (Holden et al. 2007). We also demonstrated that SabA phase variation was reversible by isolating binding revertants. One of the clinical isolates examined were devoid of sLex-binding properties. Despite screening of 24,000 clones we did not find any ON phase clones, although the *sabA* gene carried CT-repeats. To turn on protein expression, the addition of two CT-repeats or the deletion of one CT-repeat is required. Here we speculated that the CT-repeat region was too short and fidelity was high enough for proper DNA replication without slippage.

We further characterized the *sabA* operon and found that in non-sLex-binding clones only minute amounts of *sabA* mRNA were detected. In the monocistronic *sabA* mRNA the transcriptional start site was identified and the translational start site and promoter region predicted. The -35 region was predicted to be located in close proximity to a mononucleotide stretch of T's. When we DNA sequenced the polyT-tract located in the promoter region, just adjacent to the predicted "-35" promoter region in subclones with differences in sLex-binding activity, we found differences in the number of T's between high and low sLex-binding clones. Slight changes in SabA expression were also monitored. Similar features were recently reported but the relationship between the length of the T-tract and SabA expression was not further studied (Goodwin et al. 2008).

H. pylori is a slow-growing bacteria that causes persistent infections within the same niche. This imply that regulatory systems adapted for changes in the environment maybe not the same as those present in rapidly-growing bacteria that survive in or are exposed to a number of different environments *e.g.*, *E. coli* and *Vibrio cholerae*. It is tempting to speculate that the changes in the T-tract length are used for transcriptional regulation via a mechanism that compensates for the lack of transcription factors resulting in a change in expression levels. The length of the T-tract may affect transcriptional initiation by changing the DNA architecture (Koo et al. 1986; Jáuregui et al. 2003) which changes the ability of the RNA polymerase to dock to the promoter. Changes in a few C's in a mononucleotide tract near the promoter region have been shown to result in great variability in the *Neisseria meningitidis* Opc outer membrane protein expression levels (Sarkari et al. 1994). However, clones with defined set of T's in the *sabA* promoter region must be constructed and analyzed accordingly to establish any effects of transcriptional initiation and the polyT tract. This is currently on-going in the laboratory.

Growth phase and environmental regulation of virulence bacterial genes have been frequently reported over the years. When sLex-binding activity and SabA expression levels were analyzed in relation to growth, sLex-binding activity peaked during the late logarithmic/early stationary growth phase, the increase in SabA expression was not as pronounced. Due to the many reports regarding the virulence-associated genes and their increased expression during stationary growth phase, we were interested in testing growth phase-related factors that might be involved in SabA up-regulation. No stationary phase-specific sigma factor is yet identified for *H. pylori* but the AI-2 system for quorum sensing exists (Forsyth and Cover 2000; Joyce et al. 2000) and has been shown to regulate genes involved in *H. pylori*

motility (Rader et al. 2007). However, introduction of a *luxS* mutant did not have any effects on sLex-binding activity nor SabA expression.

SabA expression and sLex-binding activity increased and peaked during late logarithmic phase. This is an agreement with a microarray study of *H. pylori* gene expression that demonstrated that *H. pylori* was more virulent during late logarithmic phase (Thompson et al. 2003). The increase in sLex-binding activity could not be explained by the modest up-regulation in SabA expression. Observations by the Borén laboratory suggested that sLex-binding properties were dependent on the AlpA and AlpB lipoproteins (Drs Borén and Haas, manuscript in preparation). We were unable to detect any differences in AlpA/B expression levels during the different growth phases. There may of course be other yet unidentified proteins that could be involved in sLex-binding. When *H. pylori* enters stationary phase it changes from a spiral to a doughnut shape (Worku et al. 1999). The changed curvature of the membrane is due to changes in the membrane phospholipid composition (Tannaes et al. 2000). We discuss the possibility that changes in membrane integrity and curvature in aging *H. pylori* may affect presentation and conformation or the local environment of SabA protein and thus its cognate sLex-binding properties.

BabA expression has been shown to be down-regulated during infection by us and others (Solnick et al. 2004) and Mongolian gerbils (Paper II). We measured SabA expression and sLex-binding activity in output clones isolated after 2 and 17 weeks post infection, the same clones from the Solnick study. In one monkey, the levels of SabA expression and sLex-binding were maintained but the output clones from 2 of 3 animals exhibited a significant increase in sLex-binding activity. Our preliminary data points to the direction of differences in host response in terms of selective pressure for the BabA and the SabA adhesins.

Paper IV

Characterization of *Helicobacter pylori* vesicles and their cognate properties for intimate host interactions.

Bacterial vesicles probably have a role in virulence since they have the potential of delivering virulence associated factors to host cells thereby affecting disease presentation. *H. pylori* vesicles have been found in gastric biopsy materials and found to carry VacA (Fiocca et al. 1999). No thorough characterization of *H. pylori* vesicles has been carried out to date.

This work describes the major protein and phospholipid components of *H. pylori* vesicles and describes the mechanisms for intimate vesicle-host interactions. To identify major protein components in the vesicles we used MS analysis. When performing an MS analysis it is important to remember that a proteome is dynamic and that MS provides an imprint of the proteins present during the conditions tested. The generated data is dependent on sample preparation, the MS method used and the database searched. Analysis of our MS data was done against the NCBI genebank using the Mascot program with significance levels set to 99% with at least two matching peptides. Orthologous proteins were deleted from the data such that only the protein with the highest score remained. The obtained results may have been distinctive if the CCUG17875 genome had been available and included in the analysis or if *H. pylori* vesicle samples corresponding to other growth conditions or vesicles from an alternative strain had been included. The effects of growth conditions on the protein pattern obtained were clearly discernable when comparing protein patterns following growth in broth versus agar (Figure 5C in Paper IV).

Cytoplasmic, periplasmic, IM and OM proteins were all found to be associated with *H. pylori* vesicles. OM proteins represented 16% of the total number of proteins that we identified in the vesicles. However, MS analysis was not quantitative and the relative amount of OM proteins in the vesicles was probably much higher. We found a majority (76%) of the *H. pylori* Hop proteins in the vesicles. We also showed that a series of cytoplasmic proteins were natural components of *H. pylori* vesicles. Cytoplasmic proteins associated with vesicles were recently described for *E. coli* vesicles (Lee et al. 2007). Additional analyses of vesicle composition in combination with increased sensitivity of MS methods may change the view of the makeup of these vesicles. Therefore models describing how vesicles are shed are likely to be modified to include the presence of cytoplasmic proteins.

In the MS analysis described we found virulence factors which have been shown to down-regulate the host immune response or induce apoptosis *e.g.*, the VacA cytotoxin, γ -glutamyl transpeptidase and HPO175 (Boncristiano et al. 2003; Basak et al. 2005; Schmees et al. 2007). The most intriguing revelation from these studies was the presence of CagA in vesicles and we speculated that *H. pylori* vesicles could act as a novel mechanism for the delivery of CagA into host cells. Some additional proteins from the *cag* PAI were also identified. The T4S tip adhesin CagL and other components of the T4S system were not identified during the MS analysis. That we did not find CagL can either depend on absence of a complete T4S machinery in strain CCUG17875 or that the expression of CagL was not sufficient. Numerous of T4 pili appear on the surface of *H. pylori* after 30 min of host cell contact (Kwok et al. 2007). Other have argued that T4S machineries can not be associated to bacterial vesicles since they were described not to contain cytoplasmic components (reviewed by Kuehn and Kesty 2005). Clearly, additional studies regarding the presence of the T4S components in the vesicles are needed. Future studies will also elucidate if vesicle-mediated transfer of CagA generate similar effects as if translocated by the T4S machinery *e.g.*, hummingbird phenotype formation or interference with cell-signaling pathways.

We also investigated the adherence of *H. pylori* vesicles to tissue sections of human gastric mucosa. Both the SabA and the BabA adhesins were functionally active on the *H. pylori* vesicle surface and mediated attachment to cognate receptors. When vesicles were incubated with cognate receptors to the gastric tissues was blocked. To our knowledge, such detailed adherence mechanisms for vesicle-host interaction has not been described previously. *E. coli* LT toxin on vesicles interacts with receptors within lipid rafts prior internalization (Kesty et al. 2004). The *H. pylori* VacA toxin is associated to the surface of vesicles and VacA interacts with lipid rafts and in particular sphingomyelin prior to endocytosis (Schraw et al. 2002; Ricci et al. 2005; Gupta et al. 2008). Further studies will elucidate the initial host-vesicle contact and any involvement of the BabA and the SabA adhesins in uptake of VacA or other host effector molecules. The ability to deliver host effector molecules via vesicles and keeping the bacteria at a distance from the dangerous immune cells may balance the inflammatory response and may have a great impact on pathogenesis.

Concluding remarks

Extensive research has resulted in numerous important findings regarding the biology of *H. pylori* since its discovery 26 years ago *e.g.*, almost 30,000 *H. pylori* related articles have been published. Still there are many unknowns that need to be solved regarding virulence and pathogenesis. An intriguing question is why only a subset of infected individuals develops disease following infection? One approach will be to use molecular biology to identify the mechanisms that regulate persistent infections in the human stomach even though immune and inflammatory systems are activated and the gastric milieu is a hostile and ever-changing environment. One survival strategy employed by *H. pylori* facilitating its adaptation to environmental changes is creating highly diverse genetic populations. Several studies have demonstrated that *H. pylori* populations can be defined as a so-called quasi species composed of clones of variable subtypes. Certain subtypes will be selected during a specific condition and dominate for a time. As the infection develops, clones of other subtypes will become more fit and remain dominant until the environment changes again to select a new clone.

The aim of this thesis was to investigate the mechanisms associated with the creation of quasi species in terms of adherence properties. We demonstrated that homologous recombination, SSM as well as mutations in non-polynucleotide stretches all contributed to either switching adhesin expression on or off or modulating their expression levels (Papers I, II, and III). The next step will be to study these mechanisms in relation to selective pressures *i.e.*, host responses. The host responses are likely to be dependent on bacterial factors. Since few animals were included in our infection studies it was difficult to draw general conclusions regarding the results obtained after challenging gerbils with *H. pylori* clones of different BabA expression levels (Paper II). These results demonstrated that bacterial subtypes affected the host responses and as a consequence of various selection pressures different phenotypes were selected. The results presented in Paper II also emphasized the impact of BabA adherence properties on severe inflammatory response during infection.

It is of great interest to study the availability of receptor structures in relation to adaptation of subclones with a certain phenotype in the population. Highly relevant to such studies is the Leb transgenic mouse strain that specifically expresses Leb in the stomach and intestine. Future studies and infection of Leb-mice and wild-type mice with clones possessing

variable BabA expression levels or clones with a silent *babA* gene (described in Paper I) will shed light on the impact of BabA expression, adaptation of subclones and genetic events that occurred to create the clones that are most fit in relation to Leb expression or lack thereof.

Delivery of host effector molecules requires close contact between the bacterium and the epithelial cell surface. Several studies have highlighted the relationship between adherence properties and CagA phenotype and development of severe gastric diseases (Gerhard et al. 1999; Semino-Mora et al. 2003; Camorlinga-Ponce et al. 2004). To date, no studies have used genetically defined mutants and relevant host cell systems to study the interplay between adherence and host effector molecules. Mimuro and coworkers have recently discussed this issue in relation to CagL mediated contact of the T4S pili for translocation of CagA (reviewed by Mimuro et al. 2008). CagL binds to the $\alpha 5\beta 1$ integrin on the host cell surface and the study was performed in an *in vitro* system using non-polarized AGS cells (Kwok et al. 2007). The AGS cell system has been used significantly to study *H. pylori* virulence, however, this model is not ideal for studying adherence since this cell line lacks apical-basolateral polarity. In the unpolarized AGS cell system $\alpha 5\beta 1$ integrins are available for the bacteria in the beginning of infection in contrast to polarized epithelial cells where $\alpha 5\beta 1$ expression is limited to the basolateral side which mimic the *in vivo* situation. A possible alternative adherence model discussed by Mimuro and coworkers suggested that the initial *H. pylori*-host interaction was mediated by the binding of BabA to Leb followed by CagA translocation and CagA-non-phosphorylated triggering. This leads to tight junction disruption, changes in cell motility and integrity allowing *H. pylori* access to the $\alpha 5\beta 1$ integrin present on the basolateral sides of the host cells (reviewed by Mimuro et al. 2008). In relation to this model it is tempting to speculate that *H. pylori* vesicles loaded with BabA and CagA function to initiate contact with the host and uptake of CagA vesicles results in upregulation and exposure of $\alpha 5\beta 1$ integrins, which can be used for *H. pylori* bacteria to attach via the T4S tip adhesin CagL and subsequent translocation of CagA. Future studies using defined *H. pylori* mutants and polarized host cell systems will be used to dissect the interplay between the different adherence properties and delivery of virulence factors.

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