

**Swedish Institute for Infectious Disease Control  
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**DIVERSITY AND PERSISTENCE OF  
*HELICOBACTER PYLORI***

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**SMITTSKYDDSinSTITUTET**  
*Swedish Institute for Infectious Disease Control*

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**Things are not always as they seem.**

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## ABSTRACT

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*Helicobacter pylori* is an exceptionally diverse bacterial species, and every infected individual harbors a unique strain, apart from the strain concordance found in some families. The infection is usually acquired during childhood and once established, persists for life unless treated with antibiotics. When infecting a new host, the bacterium causes inflammation in the gastric lining, which in most cases is asymptomatic, but may progress to chronic gastritis, gastric or duodenal ulcers or gastric cancer.

We have performed extensive genetic analyses of *H. pylori* to give further insight in this immense diversity with a focus on the cytotoxin associated gene pathogenicity island (*cag* PAI), encoding a type IV secretion system (TFSS). First we studied two subclones, one *cag* PAI positive and one negative, isolated from the same biopsy. These isolates differed genetically, as determined by microarray genotyping, but were more similar to each other than to any other analyzed strain. In addition, both subclones colonized germ-free transgenic Lewis B expressing mice to an equal density, but the *cag* PAI negative strain did not colonize conventionally raised mice, while the *cag* PAI positive did. Investigating reisolates from mice infected for up to 10 months with the *cag* PAI positive strain revealed genetic stability of the *cag* PAI. In the second paper we determined the nucleotide sequence of the *cag* PAI in four clinical isolates, two strains isolated from patients with duodenal ulcer, and two from patients with gastric cancer. These strains all harbored a functional TFSS and the overall genetic structure of this 40 kb region was similar, with some interesting exceptions. One completely new hypothetical gene, named HP0521B, was found in three of these strains. This gene was present in about half of the Swedish clinical isolates in our study. In addition, in one duodenal ulcer strain a large insertion or rearrangement in the intergenic region between HP0546 and HP0547 was found. This genomic change did not seem to affect the function of the TFSS.

The genetic composition of the *H. pylori* population in two individuals was examined in paper three. Subclones isolated from two time points with a nine-year interval were used. When sequencing ten loci (~6,000 bp) in three subclones from each time point, only two substitutions were found in one patient and no differences in the other. Further microarray genotyping revealed distinct differences between the subclones within the patients regardless of time of collection, indicating a potent micro-diversity.

In the fourth paper we characterized a putative Nudix hydrolase, NudA, in *H. pylori*. The preferred substrate of NudA was Ap<sub>4</sub>A, a molecule found at elevated concentrations in cells exposed to oxidative and heat stress. When challenging an isogenic mutant of *nudA* and a wild type with hydrogen peroxide, the mutant was less capable to survive. NudA was found to be an abundant enzyme in *H. pylori*, expressed at equal amounts at all stages of growth and during stress exposure. This work has increased our understanding of the complex genetic diversity of *H. pylori*, and revealed the function of a Nudix hydrolase likely to be important for the persistence of this bacterium in the human stomach.



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## LIST OF PAPERS

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

- I. Björkholm B., Lundin A., Sillén A., Guillemin K., Salama N., Rubio C., Gordon J. I., Falk P. and Engstrand L. 2001. Comparison of genetic divergence and fitness in two subclones of *Helicobacter pylori*. *Infect. Immun.* 69 (12), p. 7832–7838
- II. Blomstergren A., Lundin A., Nilsson C., Engstrand L. and Lundeberg J. 2004. Comparative analysis of the complete *cag* pathogenicity island sequence in four *Helicobacter pylori* isolates. *Gene* 328, p. 85–95
- III. Lundin A., Björkholm B., Kupersmidt I., Unemo M., Nilsson P., Andersson D. I. and Engstrand L. When and where is the *Helicobacter pylori* genome variability generated? Submitted manuscript
- IV. Lundin A., Nilsson C., Gerhard M., Krabbe M., Andersson D. I. and Engstrand L. 2003. The NudA protein in the gastric pathogen *Helicobacter pylori* is an ubiquitous and constitutively expressed di-nucleoside polyphosphate hydrolase. *J. Biol. Chem.* 278 (14) p. 12574–12578

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## ABBREVIATIONS

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AGS	human gastric adenocarcinoma cell-line
AP-PCR	arbitrary primed PCR
bp	base pair
<i>cag</i>	cytotoxin associated gene
CAG	chronic atrophic gastritis
Cy3	indocarbocyanine
Cy5	indodicarbocyanine
DNA	deoxyribonucleic acid
EST	expressed sequence tag
Grb-2	growth factor receptor bound 2
IFN $\gamma$	interferon gamma
IL	interleukin
IS	insertion sequence
kb	kilo base pairs
kDa	kilo Dalton
Le <sup>a, b, x, y</sup>	histo-blood group antigen Lewis a, b, x or y
MALT	mucosa associated lymphoid tissue
Mb	mega base pairs
NF- $\kappa$ B	nuclear factor kappa B
Nod-1	nucleotide-binding oligomerization domain-1
ORF	open reading frame
PAI	pathogenicity island
PCR	polymerase chain reaction
PZ	plasticity zone
RFLP	restriction fragment length polymorphism
SHP-2	Src homology 2 domain
SEM	scanning electron microscopy
Src	tyrosine kinase protein family
TFSS	type IV secretion system
TNF $\alpha$	tumor necrosis factor alpha
Trx	thioredoxine
UBT	urea breath test



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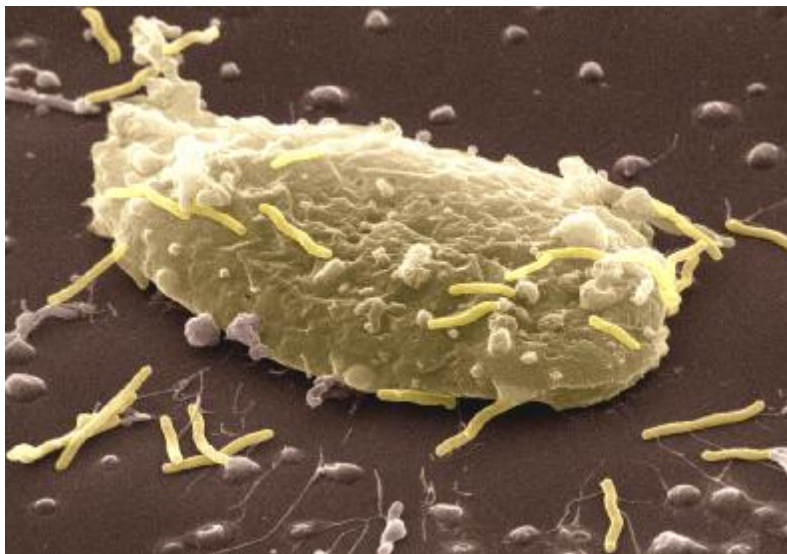
## INTRODUCTION

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### History

In 1879 Robert Koch postulated that microorganisms could be responsible for some severe human diseases. A few years later he discovered an association between microbes and diseases like cholera and tuberculosis, where the latter gave him the Nobel Prize in 1905 (Porter, 2001). Some years later Dr. Bizzozero, an anatomist in Turin, observed Gram negative spirochetes within parietal cells and gastric glands from dog tissue. We now know that he had discovered the *Helicobacter* species (Marshall, 2001). Several observations by pathologists of spirochetes in human stomach tissues have been reported since. However, it was not until the early 1980s that a correlation between the bacterium *Helicobacter pylori* and gastroduodenal diseases was established (Doenges, 1938; Freedburg and Barron, 1940; Marshall *et al.*, 1985). Dr. Warren and Dr. Marshall were able to culture a slow growing microaerophilic bacterium in the laboratory, by accidentally leaving agar plates containing samples from stomach biopsies in the incubator over the Easter Holidays (Marshall and Warren, 1983). They named the newly discovered organism *Campylobacter pyloridis*, a name that was later changed to *Campylobacter pylori* and finally to the current name *Helicobacter pylori* (Goodwin *et al.*, 1989). To really establish a correlation between *H. pylori* and disease, and thereby fulfill the Koch's postulate, Dr. Marshall ingested a liquid culture of the bacterium and immediately suffered from acute gastritis (Marshall *et al.*, 1985).

The co-evolution of *H. pylori* and its human host has probably progressed for more than 10,000 years. DNA of the bacterium has been detected in mummies (Allison *et al.*, 1999). Furthermore, population studies reveal relatedness between Amerindian and East Asian strains. These human populations separated approximately 11,000 years ago (Ghose *et al.*, 2002; Yamaoka *et al.*, 2002; Falush *et al.*, 2003).



*H. pylori* interacting with a cultured epithelial cell (SEM kindly provided by Mats Block).

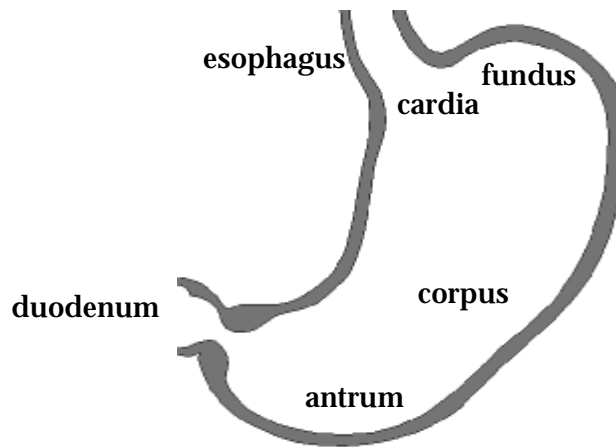
## General microbiology, transmission and prevalence

The *Helicobacter* species belongs to the  $\epsilon$ -proteobacteria. A continuously increasing number of subspecies, to date about 35, are found in humans and animals (Fox, 2002; Gueneau and Loiseaux-De Goer, 2002; NCBI, 2004). *Helicobacters* reside in the gastrointestinal tract and are Gram negative, microaerophilic, and spiral shaped bacteria with polar flagella allowing motility.

*Helicobacter pylori* colonization is restricted to primates with no other natural reservoirs (Dubois *et al.*, 1994; Dubois *et al.*, 1995; Mitchell, 2001; Solnick *et al.*, 2003). This bacterium is highly adapted to the human stomach, where few competing organisms can survive. It is generally considered to be an extracellular bacterium. However, *in vitro* studies on primary cells and cell lines suggest a mechanism of possible intracellular invasion of *H. pylori* (Björkholm *et al.*, 2000; Kwok *et al.*, 2002). The infection is usually acquired before the age of ten and persists for life unless treated. However, there are some reported cases of spontaneous clearance in children (Granström *et al.*, 1997; Klein *et al.*, 1994). Less than 1% of adults in developed countries acquire *H. pylori* per year (Ernst and Gold, 2000). The mode of transmission is still unclear, but the most probable route is familial fecal-oral or oral-oral transmission with the strongest positive correlation of mother to child transmission (Kivi *et al.*, 2003; Björkholm *et al.*, 2004). During starvation *H. pylori* converts to a non-culturable coccoid form. Whether this form is a dormant stage, possibly involved in transmission, or simply non-viable cells, is still under debate (Eaton *et al.*, 1995; Kusters *et al.*, 1997; Hultén *et al.*, 1998; Enroth *et al.*, 1999a). The prevalence of *H. pylori* infection is decreasing in high-income areas, ranging between 20–50% depending on age, explained by a birth-cohort phenomenon with higher incidence in the past (Banatvala *et al.*, 1993). The proportion of infected individuals in low-income countries is however higher, with up to 90% of the population being infected (Mitchell, 2001). Thus, the social-economical status and house crowding are positive transmission factors.

## *H. pylori* associated diseases

The human stomach is a harsh environment with a high concentration of hydrogen chloride (~pH 2), which is required for digestion of lipids and proteins in food. The acid protects against ingested microorganisms, but is on the other hand fundamental in the development of gastroduodenal diseases. In order to protect the gastric epithelial cells against the acid, there is a thick mucus layer, consisting of mucins and glycopeptides. The acid is transported from the gastric pit units, located in corpus, through the mucus layer in thin channels (Johansson *et al.*, 2000). In the mucosa, the acid concentration gradually decreases from the luminal pH 2 to neutral pH close to the epithelial cells (Schade *et al.*, 1994). The gradient is explained by secretion of bicarbonate, neutralizing the acid in the close vicinity of the epithelial cells that serve as an additional protective barrier (Phillipson *et al.*, 2002). *H. pylori* is believed to initially colonize the antrum where no acid producing parietal cells are present, which makes it a less acidic environment than the corpus.



Anatomy of the human stomach.

### ***Early infection***

When *H. pylori* enters the stomach the initial, acute phase of infection is triggered by bacterial outer membrane components such as lipopolysaccharide (LPS) structures. LPS has chemotactic properties that recruit monocytes and neutrophils and activate mast cells (Innocenti *et al.*, 2001). The mast cells release inflammatory mediators that increase vascular permeability and expression of leucocyte adhesion molecules on endothelial cells. Macrophages release the proinflammatory cytokines  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  that in turn increases the levels of IL-8, recruiting additional neutrophils. Degenerative changes of the protective mucin layer also occur (Newton *et al.*, 1998; Henriksnäs, 2003; Slomiany and Slomiany, 2003)

### ***Chronic active gastritis***

Once an infection is established it will remain as an asymptomatic chronic infection in the majority of infected individuals, if not treated with antibiotics. However, in some cases, an accumulation of chronic inflammatory cells will result in acute neutrophil gastritis or chronic active gastritis. A cell-mediated response, activating  $\text{CD8}^+$  T cells and stimulating formation of autoantibodies (Th1) is triggered, as well as a secretory immune response that attempts to reduce the bacterial load (Th2). The response is strongly directed toward Th1 in most patients, but is dependent on both host genetic and bacterial factors (Bamford *et al.*, 1998; Lindholm *et al.*, 1998).

### ***Duodenal ulcer***

Patients with duodenal ulcer have high acid production due to an increased sensitivity to gastrin (Gaskin *et al.*, 1975; Chen *et al.*, 1994). They also have an impaired ability to secrete protective mucosal bicarbonate (Hogan *et al.*, 1996).

Generally, *H. pylori* does not colonize the duodenum, but in hyperchlorhydric individuals acid leaks into the duodenum, resulting in replacement of intestinal cells with gastric cells that allow *H. pylori* to colonize.

### ***Gastric cancer***

Atrophy is characterized by loss of glandular tissue, i.e. the specialized and functional cells of the pit-gland units of the corpus are replaced by fibrous tissue as a consequence of long-term mucosal damage. This will increase the pH and *H. pylori* can colonize corpus to a larger extent. Intestinal metaplasia is a potential reversible stage where the gastric epithelial phenotype is changed to small or large intestinal phenotype. Atrophy and intestinal metaplasia are both pre-cancer stages present in about 20% of European patients with chronic active gastritis (Filipe *et al.*, 1985; James, 1997). Intestinal metaplasia is most frequent in the antrum part of the stomach and in older patients (Eidt and Stolte, 1994).

Gastric cancer is the third most common cancer after lung and breast cancer, and the second most common cause of cancer-related mortality after smoking-related lung cancer. There are two distinct types of gastric cancer, intestinal and diffuse. The intestinal type progresses in a series of well-defined histological steps and occurs late in life, predominantly in men (Parkin *et al.*, 2001). The diffuse type arises earlier in life, at an equal frequency in both men and women, and is characterized by infiltration of neoplastic cells.

In 1994, *H. pylori* was classified as a class I carcinogenic agent by the International Agency for Cancer Research (IARC, 1994). Convincing evidence of a positive correlation between *H. pylori* infection and increased risk of gastric cancer is presented in a number of case-control studies (Nomura *et al.*, 1991; Parsonnet *et al.*, 1991). This has been confirmed in additional human and animal studies (Enroth *et al.*, 2000; Uemura *et al.*, 2001). In the Mongolian gerbil model up to one-third of the animals develop gastric atrophy, intestinal metaplasia and intestinal type adenocarcinoma after one to two years of *H. pylori* infection (Honda *et al.*, 1998; Watanabe *et al.*, 1998).

### ***MALT lymphoma***

Mucosal associated lymphoid tissue (MALT) lymphomas were first described in 1983 as a distinct pathologic entity of the non-Hodgkin's type B-cell lymphomas (Isaacson and Wright, 1983). Of all gastric MALT lymphoma cases, about 90% have a *H. pylori* associated gastritis, linking the bacterium to this type of cancer. This is further evident since approximately two thirds of the cases will have a regression of the lymphoma after *H. pylori* eradication (Nagashima *et al.*, 1996; Morgner *et al.*, 2001).

### ***Esophageal cancer***

Gastroesophageal reflux disease (GERD) is a significant risk factor of esophageal adenocarcinoma (Labenz *et al.*, 1997; Lagergren *et al.*, 1999). There are epidemiological studies supporting that *H. pylori* reduce the risk of developing GERD and esophageal cancer (Chow *et al.*, 1998; Vicari *et al.*, 1998; Ye *et al.*, 2004). However, evidence for a protective role of *H. pylori* infection in this type of cancer is



still missing and contradicting studies have been presented (Werdmuller and Loffeld, 1997). By reducing the prevalence of smoking, gastroesophageal reflux, and overweight, and by increasing the consumption of fruits and vegetables the incidence of esophageal cancer may be reduced (Engel *et al.*, 2003).

#### ***Polymorphisms in host genetic factors related to H. pylori associated cancer***

It is the degree of activation of the inflammatory response in the stomach that determines the *H. pylori* associated pathology. There seems to be a synergistic effect between host factors and *H. pylori* factors determining the level of inflammation. As mentioned, early *H. pylori* infection induces production of the pro-inflammatory cytokine IL-1 $\beta$ . This cytokine is a potent acid secretion inhibitor, i.e. high levels of IL-1 $\beta$  leads to pan-gastritis, reduced acid production and gastric atrophy (El-Omar, 2001; Takashima *et al.*, 2001). Genetic polymorphisms in the gene encoding IL-1 $\beta$  affecting the cytokine expression, has been studied in relation to *H. pylori* infection and related diseases, where a correlation between certain alleles and disease has been reported (El-Omar, 2001; El-Omar *et al.*, 2001; Figueiredo *et al.*, 2002; Rad *et al.*, 2003). In addition, certain alleles of the genes encoding the IL-1 $\beta$  receptor antagonist IL-1ra, IL-10, and TNF $\alpha$  have been correlated to *H. pylori* related cancer (El-Omar *et al.*, 2003; Rad *et al.*, 2003).

## **Detection and treatment**

There are several ubiquitous enzymes in *H. pylori*, urease, oxidase and catalase, used for biochemical detection and verification in the laboratory. Catalase, along with superoxide dismutase (SOD), protects against the toxic effects of oxygen (Hazell *et al.*, 1991; Pesci and Pickett, 1994). Urease is an important enzyme neutralizing the acidic environment surrounding the bacterium by converting urea to ammonia and carbon dioxide (CO<sub>2</sub>) (Eaton *et al.*, 1991).

Detection of *H. pylori* infection in patients can be classified into two groups; non-invasive and invasive methods (Knigge, 2001). Non-invasive methods are serological tests detecting specific *H. pylori* IgG or IgA antibodies and urea breath tests (UBT). The UBT method is based on the presence of the abundant urease enzyme in *H. pylori*, where labeled (<sup>13</sup>C or <sup>14</sup>C) urea is converted by the urease to ammonium and labeled CO<sub>2</sub> is detected in the exhalation air (Parente and Bianchi Porro, 2001).

The invasive method, through collection of stomach biopsies, is readily used to detect gastroduodenal diseases and *H. pylori* infection. Triple staining using Hematoxyline and eosin, Alcian blue, and Genta or El-Zimaity, on histological sections allows visualization of both the gastric morphology and presence of *H. pylori*. However, culturing from biopsies is also routinely used (Graham and Qureshi, 2001).

The standard treatment regimen of *H. pylori* infections is a triple therapy using acid secretion inhibitors (H<sub>2</sub> antagonists or proton pump inhibitors e.g. omeprazole), clarithromycin, and amoxicillin or metronidazole (Gisbert and Pajares, 2001). Treatment is controversial mainly due to the emerging antibiotic resistance problems

therefore treatment in preventive purpose is not recommended. To date, there are no known bacterial markers that can predict whether the infection will stay exclusively asymptomatic or progress to disease.

### General genetics of *H. pylori*

*H. pylori* harbors a relatively small and compact genome, in concordance with other bacteria only present in restricted niches. *H. pylori* was the first bacterium where two complete genomes were sequenced and compared. The first strain, 26695, isolated from a patient in United Kingdom suffering from gastritis, was sequenced in 1997 by The Institute for Genomic Research (TIGR) (Tomb *et al.*, 1997). This was a laboratory strain passaged numerous times prior to sequencing. Sequencing of the second strain, J99, was completed in 1999 by AstraZeneca and Genome Therapeutic Corporation. J99 was isolated from a patient in the United States with duodenal ulcer and only passaged a few times before being sequenced (Alm *et al.*, 1999). The G+C content is 39% in both strains. The genome size of strain 26695 is 1.67 Mb with 1,590 predicted ORFs, whereas strain J99 is 1.64 Mb and contains 1,496 ORFs. A high number of strain specific genes were identified in the first comparison of these two genomes, 89 and 117 for J99 and 26695, respectively, corresponding to 6–7% of the ORFs (Alm *et al.*, 1999). In the same comparison, approximately 60% of the ORFs were found to have a predicted function, 24% were conserved unknown ORFs present in other bacteria and 17% were *H. pylori* specific with unknown function in the current databases. Later, when the complete genome of the close relative *Campylobacter jejuni* was published, 86 of the J99 specific ORFs were found as orthologues in the *C. jejuni* genome (Parkhill *et al.*, 2000). Recent reannotation of strain J99 and 26695 reduced the number of unknown genes from approximately 40% to 33% (<http://genolist.pasteur.fr/PyloriGene>) (Boneca *et al.*, 2003). A high proportion of the strain specific genes found in 26695 and J99 are located in two plasticity zones (PZ) of the genome. When genotyping different *H. pylori* strains with microarray, these PZ are highly variable (Salama *et al.*, 2000; Björkholm *et al.*, 2002).

Insertion sequence (IS) elements are short mobile DNA sequences that promote genetic rearrangements. These are often present in multiple copies at different chromosomal locations. Four different IS elements (IS605–608) have been identified in various *H. pylori* strains (Kersulyte *et al.*, 1998; Kersulyte *et al.*, 2000; Kersulyte *et al.*, 2002). IS elements usually encode two transposases, *orfA* and *orfB*, and in the IS elements identified in *H. pylori*, *orfA* is homologous in three of the IS elements (IS605, IS606 and IS608) and *orfB* in all four. The *orfA* gene in IS607 shows protein homology to the regulatory gene *merR* and a resolvase in IS1535 of *Mycobacterium tuberculosis* (Kersulyte *et al.*, 2000). The gene order in *H. pylori* is relatively conserved when comparing strain J99 and strain 26695. Only ten regions have a different chromosomal position in J99 compared to 26695, ranging in size from 1 to 83 kb (Alm *et al.*, 1999). However, results from PFGE suggest that the gene order is more variable among other strains (Taylor *et al.*, 1992; Jiang *et al.*, 1996).

## Generation of genome diversity

The genomes of prokaryotes are almost endless in their variation. The smallest known bacterium *Mycoplasma genitalium* lacks a cell wall and is therefore obligate intracellular. *M. genitalium* has only 480 genes and a genome size of about 0.58 Mb (Fraser *et al.*, 1995). This bacterium is highly adapted to a parasitic lifestyle and restricted in several biosynthesis pathways. More complex and free-living species like *Escherichia coli* and *Pseudomonas aeruginosa* harbor larger genomes of about 4.6 (strain K-12) and 6.3 Mb, respectively, and can survive in a broad range of niches, also where nutrients are scarce (Blattner *et al.*, 1997; Stover *et al.*, 2000). In relation to other bacteria, *H. pylori* has a relatively small and compact genome of approximately 1.65 Mb (Tomb *et al.*, 1997; Alm *et al.*, 1999). There are various ways in which genomic diversity is generated, but homologous recombination is a prerequisite for microbial evolution and genome plasticity, where the driving force is selection pressure in the form of nutrients and other factors affecting the growth conditions, including competition from other organisms. The recombination rate of *H. pylori* is extremely high, allowing an elevated level of genetic exchange (Suerbaum *et al.*, 1998; Achtman *et al.*, 1999). Moreover, intra-chromosomal recombination through direct repeats has also been suggested as a mechanism of diversification (Aras *et al.*, 2003b).

Genetic adaptation may occur by selection of advantageous randomly acquired mutations anywhere on the chromosome, or as duplications of genes where the additional duplicated gene may evolve by random mutations and subsequently achieve a completely new function. The most efficient way of diversification is to acquire DNA through horizontal transfer, donated from other organisms in the surroundings. The acquired DNA may be incorporated into the chromosome through homologous recombination or freely replicate in the cytoplasm in the form of plasmids.

### *Horizontal gene transfer*

Horizontal transfer of DNA occurs in three major ways, conjugation, transduction and natural transformation. A DNase resistant conjugation-like mechanism, transferring plasmids, has been suggested to occur between *H. pylori* strains (Kuipers *et al.*, 1998; Ando *et al.*, 2000; Aras *et al.*, 2002). About 50% of *H. pylori* strains harbor naturally existing plasmids of various sizes, ranging from 1.5 to 148 kb, but their role remains to be determined (Heuermann and Haas, 1995; Minnis *et al.*, 1995; Penfold *et al.*, 1988; Hofreuter and Haas, 2002). Both rolling-circle and theta replicating plasmids are found (Kleanthous *et al.*, 1991; De Ungria *et al.*, 1999). Transduction, i.e. phage transfer has not been described. Natural transformation, or uptake of naked DNA from the surroundings is common in both Gram positive and Gram negative bacteria. This is also the major way in which *H. pylori* attains foreign DNA and two thirds of the strains are accessible *in vitro* (Nedenskov-Sorensen *et al.*, 1990; Haas *et al.*, 1993; Tsuda *et al.*, 1993; Wang *et al.*, 1993). The transformation frequency varies in the range of  $10^{-3}$ – $10^{-8}$  between different *H. pylori* strains and is most efficient in early log phase (Haas *et al.*, 1993; Tsuda *et al.*, 1993; Wang *et al.*, 1993; Ando *et al.*, 2000; Israel *et al.*, 2000). Bacteria like *Neisseria gonorrhoeae* and *Haemophilus influenzae* use

specific uptake signals, but such signals has not yet been described in *H. pylori* and thus the mechanism of natural transformation in *H. pylori* remains to be clarified (Danner *et al.*, 1980; Correia *et al.*, 1986; Saunders *et al.*, 1999). Gram negative bacteria commonly use type IV pilin for DNA transfer, but no such system has been found in *H. pylori* (Dubnau, 1999). Instead, the *comB* locus (*comB7-10*), related to type IV secretion systems, has been described to form a possible transmembrane pore that may substitute for the pilin (Odenbreit *et al.*, 1996; Hofreuter *et al.*, 1998; Hofreuter *et al.*, 2003).

Additional homologues, *drpA* and *comL*, are found in the *H. pylori* genome and these genes are involved in transformation competence in other bacteria. A mutant of *H. pylori dprA* is 100-fold less efficient in DNA uptake, but the *comL* gene does not seem to be involved (Smeets *et al.*, 2000b). A *comEC* homologue is present in *H. pylori* but the function of this gene remains to be determined. The *comH* gene is *H. pylori* specific and is essential for uptake of both chromosomal DNA and plasmids *in vitro* (Smeets *et al.*, 2000a).

### **DNA repair**

All organisms have more or less complex systems for DNA repair, both during and after replication. When these systems are impaired, an accumulation of random mutations occurs. Strains with deficient repair systems are called mutator strains and there is an unusually high number of naturally occurring mutator strains of *H. pylori* (Björkholm *et al.*, 2001). This would indicate that this bacterium is deficient in repairing DNA damages, which is a possible explanation of the exceptional genetic diversity. One could speculate that the mutator phenotype is needed for rapid adaptation when the physiology of the stomach is changing in an aging human host.

There are several repair mechanisms studied in bacteria, recombinational, mismatch, base excision and nucleotide excision repair. RecA, involved in SOS response and homologous recombination, is an abundant protein in many organisms. *H. pylori* seem to lack a SOS response, since no homologues of *lexA*, *umuCD* and *dinB* have been identified. RecA, on the other hand, is important in both chromosomal and plasmid DNA transfer in *H. pylori* (Schmitt *et al.*, 1995; Thompson and Blaser, 1995). One unusual feature of *H. pylori* RecA is the need of post-translational modifications, i.e. glycosylations, for full activity in DNA repair. The glycosylations are possibly executed by two proteins encoded downstream of *recA* (Fischer and Haas, 2004). Additional genes, involved in homologous recombination in *E. coli*, are found in the *H. pylori* genome. These are *recG*, *recJ*, *recR*, *recN*, *ruvA*, *ruvB* and *ruvC* (Alm *et al.*, 1999). The function of these genes in *H. pylori* needs to be verified, except for the studied *ruvC* gene encoding a Holiday junction resolvase. A *ruvC* mutant has a 17- to 45-fold reduced frequency of homologous recombination and an increased sensitivity to DNA-damaging agents, e.g. oxidative stress and antimicrobial agents (Loughlin *et al.*, 2003). Homologues to *recBCD* are not found in *H. pylori*.

The methyl directed mismatch repair (MutHLS) system acts both during and after replication, but homologues of *mutL* or *mutH* have not been identified in *H. pylori*, suggesting a deficiency in mismatch repair. Furthermore, the *mutS* homologue is of

*mutS2* type, more likely to be involved in chromosomal segregation than in DNA repair (Eisen, 1998).

Nucleotide excision repair homologues, *uvrABCD*, have been identified in *H. pylori*. The Uvr proteins act upon various types of damage. A dimer of UvrA binds to UvrB in an ATP-dependent fashion. This complex translocates along the DNA, hydrolyzing ATP and screening for damaged nucleotides. Upon binding to a damaged site, ATP is hydrolyzed, UvrA is released from the complex and UvrB recruits UvrC. UvrB nicks the DNA 3' and UvrC 5' to the damage site. Subsequently, UvrD (helicase II) and DNA polymerase I displace UvrBC and the damaged nucleotide and fill the gap (Grossman and Yeung, 1990). Both *uvrA* and *uvrB* mutants in *H. pylori* are sensitive to acid, and the latter is also sensitive to UV radiation and sulfonate (Thompson *et al.*, 1998; Bijlsma *et al.*, 2000), indicating that the nucleotide excision repair system is functional in *H. pylori*.

Base excision repair proteins are often glycosylases that remove damaged bases followed by additional processing by AP endonucleases, widening the gap that is subsequently repaired by DNA polymerase and ligase. Homologues of *ung*, a uracil DNA glycosylase and *nth* (HP0585 and HP0602), an endonuclease III acting on pyrimidin hydrates, are present in *H. pylori* (Tomb *et al.*, 1997). The endonuclease III homologue encoded by the HP0602 gene has been characterized biochemically, and is named MagIII as this protein is a 3-methyladenine DNA glycosylase that protects against alkylation (O'Rourke *et al.*, 2000). The second homologue, HP0585, is important for colonization, and thus is believed to protect against the host oxidative response in mice, therefore suggested to be the actual *nth* homologue (O'Rourke *et al.*, 2003). In addition, *mutY* and *mutT* (HP1228) genes are found, but no *mutM* homologue. MutY and MutM act in synergy and are both glycosylases. MutM excises the toxic compound 8-oxo-dGTP (generating A-T/C-G transversions) when paired with cytosine, whereas MutY removes miss-incorporated adenine that has paired opposite 8-oxo-dGTP (Fowler *et al.*, 2003). MutT in *E. coli* hydrolyzes 8-oxo-dGTP, but the MutT homologue in *H. pylori* seems to have a different function (paper IV).

### ***R-M systems***

The *H. pylori* genome contains an unusually high proportion of restriction modification (R-M) systems and every strain harbors its own unique set of these, contributing to the strain diversity (Salama *et al.*, 2000; Xu *et al.*, 2000; Lin *et al.*, 2001; Takata *et al.*, 2002). There are three types of R-M systems (I, II and III) all consisting of a methyltransferase and a corresponding endonuclease. The R-M systems protect the endogenous DNA by adding a methyl group on specific target sequences, which thereby prevent the cognate endonuclease to cut, as well as protects against invading non-methylated foreign DNA. Since many *H. pylori* strains are naturally competent (see above) this would be a way to keep the genetic integrity. In addition, there are orphan methyltransferases in the *H. pylori* genome and these are suggested to play a role in gene regulation (Low *et al.*, 2001; Donahue *et al.*, 2002).

## Nudix hydrolases

The first protein characterized in the Nudix hydrolase protein family was MutT in *E. coli*, hydrolyzing the toxic compound 8-oxo-dGTP, as mentioned above (Maki and Sekiguchi, 1992). The Nudix hydrolases is a large family of proteins that use a variety of substrates e.g. ADP-ribose, di-nucleoside polyphosphates, NADH, nucleotide sugars and ribo- and deoxyribonucleoside triphosphates (Dunn *et al.*, 1999). Some homologues have incorrectly been assigned the MutT function and later been found to use other substrates, as in the case of HP1228 in *H. pylori* (paper IV). Nudix hydrolases are ubiquitous proteins present in a broad range of organisms, both prokaryotes and eukaryotes, and to date there are over 800 homologues in the databases (Xu *et al.*, 2002). They all have the Nudix box in common, forming the catalytic epitope G<sub>5</sub>XEX<sub>7</sub>REUXEEXGU, where X represents any amino acid and U represents Ile, Leu or Val. The biological role of Nudix hydrolases is still unclear, but when degrading nucleoside polyphosphates, e.g. di-adenosine tetraphosphate, Ap<sub>4</sub>A, they are believed to reduce the oxidative stress and heat shock responses. Ap<sub>4</sub>A is a signaling molecule, present at increased intracellular levels during stress (Lee *et al.*, 1983; Garrison *et al.*, 1986; Nishimura *et al.*, 1997). Ap<sub>4</sub>A is also suggested to be a signaling molecule for cell division and apoptosis, whereas Ap<sub>3</sub>A, a co-inducer of cell differentiation, is a physical antagonist (Vartanian *et al.*, 1999). Furthermore, the Nudix hydrolase IalA in *Bartonella bacilliformis* and Ygdp in *E. coli*, are suggested to play a role in invasion of erythrocytes and microvascular endothelial cells, respectively (Mitchell and Minnick, 1995; Badger *et al.*, 2000). Similarly, the Nudix hydrolase InvA in *Rickettsia prowazekii* is upregulated in early infection of host cells (Gaywee *et al.*, 2002a; Gaywee *et al.*, 2002b). An unusually high number of Nudix hydrolases is found in *Deinococcus radiodurans*, a radiation resistant bacterium with high ability to keep the DNA integrity and restore DNA damages (Makarova *et al.*, 2000; Xu *et al.*, 2001).

## Polymorphic virulence genes involved in pathogenesis

### *VacA*

The vacuolating cytotoxin A, VacA, is an *H. pylori* specific auto-transporter assembled as multimeric, flower shaped pores in gastric epithelial cells (Leunk *et al.*, 1988; Cover and Blaser, 1992; Cover *et al.*, 1997). The pore causes substantial vacuolization by alteration of the intracellular vesicular trafficking of the host cells and allows urea and ions to exit through the channel into the lumen (Iwamoto *et al.*, 1999; Szabo *et al.*, 1999; Tombola *et al.*, 2001). The major effect of VacA is suggested to be apoptosis of the epithelial cells, as shown by *in vitro* studies (Kuck *et al.*, 2001; Cover *et al.*, 2003). Other functions of this cytotoxin are loosening of epithelial tight junctions, which release nutrients, and a potential effect on T cell proliferation and immune suppression (Molinari *et al.*, 1998; Gebert *et al.*, 2003). VacA seems to play a role in the initial colonization of mice and may be important for transmission (Salama *et al.*, 2001). However, there is no effect of VacA on bacterial load or degree of inflammation in mice, gerbils or piglets (Eaton *et al.*, 1997; Wirth *et al.*, 1998;

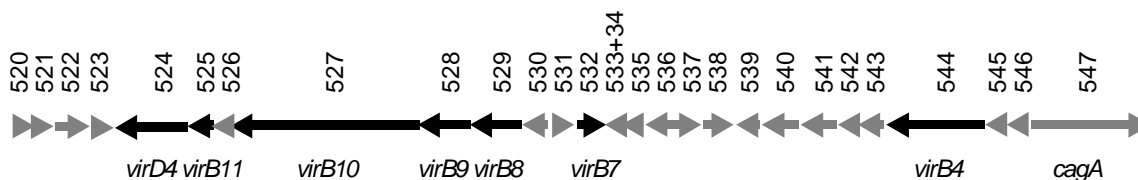
Salama *et al.*, 2001). All these effects connected to VacA positive strains facilitate *H. pylori* persistence in the gastric lumen.

The *vacA* gene is highly polymorphic with two variable parts, the N-terminal signal sequence (s-region) and the mid-region (m-region) encoding the cell-binding domain. Two main alleles have been identified of both regions s1 (a, b, c), s2 and m1 (a, b, c), m2 (Atherton *et al.*, 1995; Xiang *et al.*, 1995; van Doorn *et al.*, 1998; van Doorn *et al.*, 2000). The s1 allele is required for the toxin to be fully active and is correlated to more severe disease (Letley and Atherton, 2000; Letley *et al.*, 2003). VacA containing the s2 allele has an N-terminal extension that blocks the vacuole formation and this allele is rare in patients with peptic ulcers and gastric adenocarcinoma (Atherton *et al.*, 1995; Atherton *et al.*, 1997). The m2 allele induces less damage, with a narrower range of vacuolization than the m1 allele (Letley *et al.*, 2003).

### cag PAI

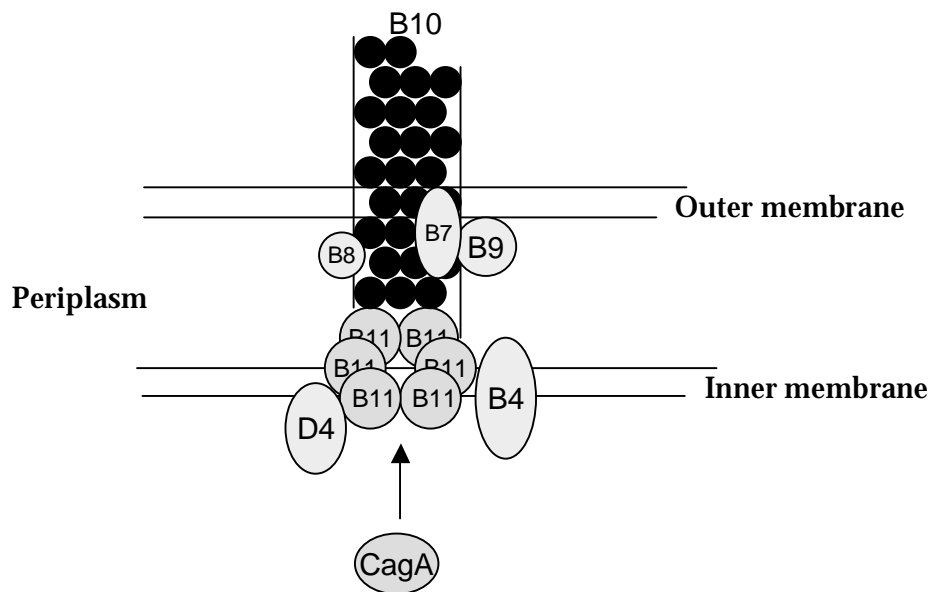
Many Gram negative bacterial pathogens harbor DNA loci called pathogenicity islands (PAI). These are horizontally acquired, instable genetic elements often inserted inside or close to tRNA genes and contain IS elements and virulence genes. These distinct islands of DNA have a different G+C content compared to the rest of the genome and are often flanked by direct repeats (Hacker *et al.*, 1997). Pathogenicity islands may encode secretion systems that have evolved from either the flagella (type III) or the conjugation (type IV) apparatus.

The cytotoxin associated gene A, *cagA* (HP0547), in *H. pylori* was discovered in 1990 as an immunodominant protein linked to VacA production (Cover *et al.*, 1990). Later, when sequencing the flanking regions of *cagA*, it was found that this gene was part of a PAI that contains 27 ORFs (HP0520–547), therefore named the *cag* PAI (Censini *et al.*, 1996; Akopyants *et al.*, 1998). This PAI is *H. pylori* specific, horizontally acquired and inserted into the conserved glutamate racemase (*glmM*) gene. The 31 bp direct repeats flanking the *cag* PAI allows loss or gain of the complete 40 kb locus through homologous recombination (Kersulyte *et al.*, 1999), but partially deleted *cag* PAIs are also found in clinical isolates (Nilsson *et al.*, 2003). The *cag* PAI and VacA are located far apart on the chromosome, but there is a statistical linkage between presence of the *cag* PAI and s1 genotype of VacA (van Doorn *et al.*, 1999). Strains with this genotype are called type I strains and are generally more virulent than type II strains that lack the *cag* PAI and harbor the less virulent *vacA* s2 genotype (Xiang *et al.*, 1995; Censini *et al.*, 1996).



Overview of the *cag* PAI locus (nomenclature according to strain 26695).

Structural homologues of the *vir* genes in the well-known type IV secretion system (TFSS) in *Agrobacterium tumefaciens* causing plant tumors are found in the *cag* PAI, (Christie, 1997). Three genes, HP0524 (*virD4*), HP0525 (*virB11*) and HP0544 (*virB4*) encode structural proteins with ATPase activity located in the inner membrane (Yeo *et al.*, 2000; Savvides *et al.*, 2003). The *virD4* homologue is suggested to be an adapter protein for transfer of the effector molecule CagA (Selbach *et al.*, 2002b), HP0532 (*virB7*) is a lipoprotein, forming complex with HP0528 (*virB9*) in the periplasmic space, where also HP0529 (*virB8*) is located. The HP0527 (*virB10*) gene is large, 5–6 kb, and contains a highly repetitive region resulting in antigenic variability (Aras *et al.*, 2003a). The VirB10 protein is the main component of the pilus structure, and polymerization of the pilus is induced when the bacterium adheres to host epithelial cells.



Schematic view of the *vir* homologues in the *cag* PAI forming the secretion apparatus.

The TFSS enhances an IL-8 response in cultured cells and thereby contributes to the inflammatory response through neutrophil recruitment and activation (Rieder *et al.*, 1997; Fischer *et al.*, 2001). Mutagenesis of individual *cag* PAI genes in strain 26695 has shown that all but seven genes (HP0520, HP0521, HP0534, HP0535, HP0536, HP0543 and *cagA*) are necessary for the IL-8 response in cultured AGS cells (Fischer *et al.*, 2001). However, a previous study show a diminished IL-8 response in an HP0521 mutant of strain 26695, when assessing IL-8 expression using a IL-8 promoter coupled to a luciferase reporter system in transfected Kato-III cells (Li *et al.*, 1999). Nevertheless, this would indicate that it is the structure of the TFSS rather than effector molecules responsible for the IL-8 induction.



CagA is currently the only known effector molecule of the *cag* PAI, but additional proteins or components are possibly transported by the same mechanism (Segal *et al.*, 1999; Asahi *et al.*, 2000; Backert *et al.*, 2000; Odenbreit *et al.*, 2000; Stein *et al.*, 2000). Interestingly, Viala *et al.* has suggested that peptidoglycan may be injected via the TFSS encoded by the *cag* PAI, subsequently leading to activation of Nod1 and induction of NF- $\kappa$ B and IL-8 (Girardin *et al.*, 2003; Viala *et al.*, 2003). When CagA is injected into host cells through this system, it is subsequently phosphorylated by host tyrosine kinases of the Src family (Selbach *et al.*, 2002a; Stein *et al.*, 2002). Phosphorylated CagA activates SHP-2 through complex formation and the signaling cascade that follows is associated with cytoskeletal rearrangements and elongation of cultured epithelial cells. Non-phosphorylated CagA also interferes with the host signaling system by interacting with Grb-2 in the c-Met/Ras pathway (Mimuro *et al.*, 2002). The *cagA* gene is highly variable with several repetitive regions in the 3' end. One to seven tyrosine phosphorylation motifs, EPIYA, are located in this repetitive region that varies among strains. A correlation between the presence of *cag* PAI with certain *cagA* genotypes and more severe disease has been presented (Yamaoka *et al.*, 1998; Yamaoka *et al.*, 1999; Azuma *et al.*, 2002). The CagA repeat region in isolates from East Asia that are genetically distinct compared to Western isolates, have been suggested to bind SHP-2 more avidly and generate a stronger host response than Western isolates (Higashi *et al.*, 2002). However, Backert *et al.* found no correlation between specific patient groups and the cellular responses induced by CagA, when studying *H. pylori* strains from 75 German patients with gastritis, duodenal ulcer or gastric cancer (Backert *et al.*, 2004).

## Outer membrane components

### *Flagella*

There is a pH gradient in the stomach mucosal layer, where the pH is almost neutral close to the epithelial cells and approximately two near the interior of the stomach (Schade *et al.*, 1994). In order to survive and establish persistent infection, *H. pylori* needs the unipolar sheathed flagella to swim down to the more neutral environment. The flagellar apparatus is complex, involving more than 50 proteins in its expression, secretion and assembly (Tomb *et al.*, 1997; Alm *et al.*, 1999). Two proteins form the flagella filament, FlaA and FlaB. Both are needed for motility, but FlaA usually predominates (Kostrzynska *et al.*, 1991; Eaton *et al.*, 1996). Chemotactic agents, e.g. amino acids (Glu, His, Lys and Ala), mucin, urea, sodium carbonate and sodium chloride, direct the swimming towards the epithelial cells (Spohn and Scarlato, 2001).

### *Adhesins*

Bacteria commonly use structures on the surface to facilitate close contact to host cells, in order to establish persistent colonization and cause an infection. Only a small proportion, about 1%, of the *H. pylori* population is proposed to adhere to the epithelial cells, whereas most of the bacteria reside in the mucus layer (Blaser and Kirschner, 1999).

There are two well studied adhesins in *H. pylori*, the blood group antigen binding adhesin A, BabA, and the sialic acid-binding adhesin, SabA (Ilver *et al.*, 1998; Mahdavi *et al.*, 2002). A proportion of *H. pylori* strains express the BabA adhesin that binds to Lewis b epitopes present on the gastric epithelial cells (Borén *et al.*, 1993; Falk *et al.*, 1993). BabA is encoded by the *babA2* gene, while the close homologue *babA1* is silent due to lack of a translational start and signal sequence (Ilver *et al.*, 1998). A correlation between more severe disease and the presence of the *babA2* gene has been presented (Gerhard *et al.*, 1999). *H. pylori* is capable of altering expression of adhesions, e.g. through gene conversion of the two *babA* alleles or through deletions (Pride and Blaser, 2002; Solnick *et al.*, 2004). SabA binds to sialic acids e.g. sialyl Lewis x and Mahdavi *et al.* have proposed an adhesion model for *H. pylori* where initial binding is generated through BabA and Le<sup>b</sup> interactions. During persistent infection and chronic inflammation *H. pylori* SabA binds to sialyl-Le<sup>x</sup>, that is up-regulated in inflamed tissue. Through frame-shift mutations in the genes encoding the two adhesins *H. pylori* may alter the binding capacity and thereby loosen the tight contact to the epithelium and escape from the immune system (Mahdavi *et al.*, 2002). Furthermore, the outer membrane proteins AlpA and AlpB have been suggested to be involved in *H. pylori* binding (Odenbreit *et al.*, 1999), as well as a haemagglutinin encoded by the *hpaA* gene, that binds to erythrocytes (Evans *et al.*, 1993). In addition, *H. pylori* binds to mucins and the extracellular matrix components such as collagen, laminin and fibronectin in *in vitro* models (Israel and Peek, 2001).

### **LPS**

Lipopolysaccharides (LPS) or endotoxins are present on the surface of Gram negative bacteria, with the major function to keep the integrity of the bacterial membrane. LPS consists of the immunodominant lipid A part, anchoring the structure in the bacterial membrane, an oligosaccharide core region specific for each bacterial species, and a variable O-antigen polysaccharide situated on the tip of the structure. *H. pylori* is unique in its ability to express human Lewis blood group antigens on the O-antigens of the LPS. About 85% of the strains express Lewis antigens, where Le<sup>x</sup> and Le<sup>y</sup> are predominating, but H type 1, Le<sup>a</sup> and Le<sup>b</sup> have also been detected (Simoons-Smit *et al.*, 1996; Wirth *et al.*, 1996; Heneghan *et al.*, 2000). *H. pylori* strains may simultaneously express more than one Lewis phenotype (Aspinall and Monteiro, 1996; Monteiro *et al.*, 1998a; Monteiro *et al.*, 1998b; Monteiro *et al.*, 2000). There are three genes encoding fucosyltransferases in *H. pylori*, two  $\alpha$  1,3(4)-fucosyltransferases, FutA and FutB generating Le<sup>x</sup> and/or Le<sup>a</sup> and one  $\alpha$  1,2-fucosyltransferase FutC generating either Le<sup>y</sup> and/or Le<sup>b</sup>. All three fucosyltransferases may undergo a reversible phase variation by DNA slippage on single nucleotide repeats during replication (Appelmelk *et al.*, 1998; Appelmelk *et al.*, 1999; Wang *et al.*, 2000). This is a fast way of generating diversity in a bacterial population and the frequency of Lewis antigen phase variation in *H. pylori* is 0.2–0.5% *in vitro* (Appelmelk *et al.*, 1998). *H. pylori* Lewis antigens are suggested to play a role in adhesion, colonization and immune evasion through molecular mimicry (Sherburne and Taylor, 1995; Moran *et al.*, 1996; Valkonen *et al.*, 1997; Heneghan *et al.*, 2000). Animal experiments in monkeys and mice suggest that the Lewis antigen expression of *Helicobacter* spp. is dependent on the Lewis phenotype of the host (Monteiro *et al.*, 1997; Croinin *et al.*,

1998; Wirth *et al.*, 1999). LPS of the close relative *C. jejuni* generate autoantibodies directed against ganglioside structures in nerve tissue that may lead to the Guillain-Barré-syndrome (Moran *et al.*, 1996). However, there are to date no strong evidence that the LPS of *H. pylori* cause autoimmunity.

## Gene regulation

Exploring the *H. pylori* genome, there are few apparent regulatory genes and yet this bacterium needs to adapt to environmental changes in the stomach. There is one extensively studied regulator, the Fur repressor, involved in iron homeostasis repressing ferritin transcription under low iron concentrations (Bereswill *et al.*, 2000; Delany *et al.*, 2001; van Vliet *et al.*, 2002). Fur is also involved in acid resistance, nickel induced urease expression and amidase- and formamidase ammonia production (van Vliet *et al.*, 2001; Bijlsma *et al.*, 2002; van Vliet *et al.*, 2003).

*H. pylori* has only few genes sensing changes in the surrounding, as compared to *E. coli* that harbors more than 60 different homologues belonging to two-component systems (Mizuno, 1997). Three histidine kinases and five response regulator homologues are found in *H. pylori*. One two-component sensor kinase, HP0166, has been studied and a mutant of this gene shows altered expression compared to the wild type (Forsyth *et al.*, 2002). Four response regulators, HP0165, HP1365, HP0244 and HP1364, seem to be required for colonization in mice (Panthel *et al.*, 2003). Furthermore, *H. pylori* lack homologues for the stress related sigma factors RpoS ( $\sigma^{38}$ , stationary phase) and RpoH ( $\sigma^{32}$ , heat shock)(Alm *et al.*, 1999). Therefore, other ways of transcriptional regulation such as slippage-strand regulation and methylation have been proposed to compensate for the missing regulators. Despite the low number of regulators, expression analysis *in vitro* using microarray has shown that there is a large variation in gene expression at different growth phases, as well as when comparing growth in neutral versus acidic pH (Merrell *et al.*, 2003; Thompson *et al.*, 2003). Thompson *et al.* presented an induction of virulence genes, a so-called switch in expression profile between late log phase and stationary phase (Thompson *et al.*, 2003).

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## AIMS OF PRESENT INVESTIGATION

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The aims of this work were to:

- I. Investigate the genotypic and phenotypic diversity of two *H. pylori* isolates with different *cag* PAI status isolated from the same biopsy in one individual, and study the genetic evolution of these isolates during experimental infection in mice.
- II. Compare the complete *cag* PAI sequence in *H. pylori* strains isolated from four patients suffering from either gastric cancer or duodenal ulcer.
- III. Study the genetic composition of the *H. pylori* population in two individuals and determine the genetic changeability of the bacterium over a prolonged time *in vitro* and *in vivo*.
- IV. Determine the biochemical and biological function of a putative Nudix hydrolase in *H. pylori*.

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## METHODS

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For complete description of material and methods please refer to the corresponding sections in the individual papers.

### *H. pylori strain background (paper I–IV)*

*H. pylori* strains Ca34, Ca52, Ca73, Du23:2, Du52:2, CAG7:8, 67:20 and 67:21 were isolated from patients as single colonies from primary cultures of two pooled antral biopsies. The patients were selected from a Swedish case-control study performed by Enroth *et al.* where strains isolated from cancer patients are referred to as Ca#, duodenal ulcer patients as Du# and chronic atrophic gastritis patients as CAG# (Enroth *et al.*, 2000). Hp1 is a clinical isolate obtained from a Peruvian patient with gastritis (Guruge *et al.*, 1998). This strain has a partially deleted *cag* PAI only containing the first seven *cag* PAI genes (HP0520 to HP0527), but has a high colonization success rate in experimental mice and is therefore commonly used. Strain 2808 is an easily transformable mouse passaged strain that originates from a German patient diagnosed with gastric ulcer. In paper IV the strain originate from two patients in a Swedish follow-up study by Gustavsson *et al.* (Gustavsson *et al.*). Single colonies of strain 67:21 isolated after 10 months of growth in a mouse are referred to as 3:9:1–10.

### *Animal model (paper I)*

The animal experiments were performed using protocols approved by the animal studies ethical committee of Stockholm North. The transgenic mice of FVB/N background express a human  $\alpha$ 1,3/4-fucosyltransferase gene under the control of transcriptional regulatory elements from a *Fabp* gene (Falk *et al.*, 1995). This fucosyltransferase utilizes the mouse H-type-1-like structures, expressed on surface mucous cells and gastric pit cells, to produce histo-blood group Le<sup>b</sup> (Fuc $\alpha$ 1,2Gal $\beta$ 1,3[Fuc $\alpha$ 1,4]GlcNAc $\beta$ ) antigens. The fucosylated epitopes serve as receptors for the *H. pylori* adhesin BabA.

Mice raised under conventional conditions harbor a large and complex gastric microflora, while mice raised in a germ-free setting are sterile. It should be noted that the physiology of a mouse stomach is different from a human stomach. Mice have a large fore-stomach and are colonized with *Lactobacillus* spp. to a great extent, unlike the nearly sterile human stomach, which only harbors a low number of transient infecting bacteria, except for a possible persistent *H. pylori* infection. *Lactobacilli* have been shown to inhibit growth of *H. pylori* in mice (Kabir *et al.*, 1997).

### *Whole-genome microarray (paper I and III)*

The main advantage of using microarray is the possibility to study a large amount of genes, ORFs or ESTs in one experiment. This technique is mainly used as a comparative tool for gene expression, but was in this work used as a genotyping tool. Briefly, the probe DNA is covalently linked (spotted) to a solid surface (glass slide or

nylon membrane) in small circular areas, whereupon a mix of labeled sample and control DNA is hybridized. Fluorescent labeling is most commonly used, but for membrane arrays radioelements are primarily used. The probes are based on either synthesized oligomers (usually 50 or 70 bp long) or PCR amplified fragments of different length. The advantage of using oligomers is that the complete sequence is not needed for all genes and they are comparably inexpensive to purchase. A fixed melting temperature ( $T_m$ ) can be set for all oligos to achieve optimal hybridization. The disadvantage is that several oligos are needed to cover a complete gene. When using PCR-products as probes, full-length genes can be represented in single spots. The PCR-products are usually of different lengths resulting in different  $T_m$ , which may cause problems with different optimal hybridization temperatures.

In paper I, we used a PCR product based microarray system developed at Stanford University (Salama *et al.*, 2000). A set of 1660 PCR products spotted in duplicates representing 98.9% of the ORFs in the 26695 and J99 genomes were used on this array. Strain 26695 was arbitrarily set as the reference strain and ninety-one ORFs were unique to J99. Two microgram DNA of each strain and control (an equimolar ratio of J99 and 26695) was labeled directly with fluorophores Cy3 or Cy5. All hybridizations were performed in duplicate. Data from duplicate datasets were merged and analyzed according to Salama *et al.* Previous control experiments, using Cy3-26695 or Cy3-J99 DNA alone, have established that the hybridization conditions employed for the microarray analysis results in 0% false positives with the 26695 probe, and 7% with the J99 probe. The false negative rates are 1% and 2% for the 26695 and J99 probes, respectively.

In paper III a custom-made oligonucleotide based microarray was used. Optimal 50-mer oligonucleotides with minimal cross-hybridization and similar melting temperature were designed to cover the complete set of known genes in the two fully sequenced *H. pylori* strains 26695 and J99. The oligonucleotides were designed and produced by MWG Biotech (Ebensberg, Germany) using the Oligos4Array software. Forty-two *Arabidopsis thaliana* controls were also included in the oligo set. In total, 1920 oligonucleotides were spotted in triplicates on epoxy-coated slides at the Swedish Royal Institute of Technology, KTH, using a Genetix Qarrayer. Two micrograms of DNA from each strain was labeled indirectly with either of the two fluorophores Cy3 or Cy5 and the control, an equimolar mix of DNA from 26695 and J99, was labeled with the opposite dye. Labeling was performed by incorporation of amino-allyl coupled dUTP followed by incubation with the fluorophore. Two hybridizations were performed for each sample.

#### ***Substrate specificity analysis using HPLC (paper IV)***

The substrate specificity of NudA was determined by ion-exchange high performance liquid chromatography, HPLC, on a Resource Q column. The enzyme was incubated with various substrates at 37°C, snap-frozen and stored at -20°C before use. The reaction products were eluted and separated by gradient elution. The proportion of hydrolyzed substrate was calculated by dividing the peak areas from samples lacking enzyme with the samples containing enzyme. Each value represents the mean of two individual analyses with an experimental difference of 7% or less.

The reaction products were determined using the controls AMP, ADP and ATP, to which the retention time of the sample products were compared.

***Enzyme kinetics (paper IV)***

The Michaelis-Menten kinetics was applied to calculate the catalytic constants of the preferred substrate Ap<sub>4</sub>A of the NudA enzyme. The dissociation constant,  $K_m$ , was determined using Eadie-Hofstee plots from three independent measurements using an assay based on conversion of a phosphatase-insensitive substrate to phosphatase-sensitive product. The level of free phosphate,  $P_i$ , was assayed according to the sensitive method described by Ames and Dubin (Ames and Dubin, 1960). In each measurement, different concentrations of NudA were incubated at 37°C with the substrate in a total volume of 0.3 ml. An ascorbic-molybdate mixture (1 part of 10% ascorbic acid and 6 parts of 0.42% ammonium molybdate  $\times$  4 H<sub>2</sub>O in 1 N H<sub>2</sub>SO<sub>4</sub>) of 0.7 ml was added to each tube and incubated for 20 min at 45°C and a color change from transparent to yellow was read at 820 nm spectrophotometrically. The first-order rate constant  $K_{cat}$ , or turnover number, was calculated from the HPLC measurements where 0.3  $\mu$ mol Ap<sub>4</sub>A was hydrolyzed per min and mg enzyme.

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## RESULTS AND DISCUSSION

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### *Paper I*

A previous study has shown that a 90-year old patient, suffering from gastritis, harbor an *H. pylori* population consisting of a mixture of *cag* PAI positive and *cag* PAI negative subclones that show identical banding pattern on AP-PCR (Enroth *et al.*, 1999b; Enroth *et al.*, 2000). AP-PCR is a rather rough method, but due to the high genetic diversity of *H. pylori*, strains isolated from different individuals renders specific banding pattern. One exception is strains isolated from individuals within some families that may give indistinguishable banding patterns. To further assess whether subclones from the 90-year old patient originated from the same ancestral strain we performed AP-PCR and RFLP on the *flaA* gene on 10 and 24 such subclones. The banding patterns were identical for these subclones and thus they were concluded to originate from the same ancestral strain.

Two subclones, 67:20 (*cag* PAI negative) and 67:21 (*cag* PAI positive) were selected for genetic investigations using the PCR-product-based microarray at Stanford University. The microarray analyses revealed additional differences where 67:20 harbored 29 genes absent in 67:21, and 67:21 contained 10 genes and the *cag* PAI that were absent in 67:20. In addition, a total of 133 genes present in strain 26695 and J99, were not detectable in either of the subclones. It must be stressed that the genes defined as absent need to be verified by PCR or Southern blot to distinguish whether they are truly missing, or if they are present but contain such large sequence differences that poor or no hybridization to the microarray was possible at the conditions used. Furthermore, there may be additional genes present in these two subclones that are not present on the chip, since it is based on the genes present in the two fully sequenced strains 26695 and J99. Nevertheless, when clustering the two subclones with 15 unrelated strains from a different study (Salama *et al.*, 2000), they were more similar to each other than to any other strain.

When knowing that these subclones were of distinct genotypes, even though isolated from the same biopsy in one individual, we continued to study whether these differences also conferred distinct phenotypes, by investigating the colonization ability in transgenic Le<sup>b</sup> expressing FVB/N mice. Remarkably, only the *cag* PAI positive subclone 67:21 was able to colonize conventional mice, while both subclones colonized germ-free mice to an equal density. About 70% of the conventionally raised mice were colonized with strain 67:21 after three or ten month of infection. An *in vitro* binding assay showed that the difference found in the mice experiments was not an effect of binding, since both subclones bound equally well to stomach sections from Le<sup>b</sup> expressing transgenic mice. Collectively, these results indicate that the fitness of the *cag* PAI positive subclone 67:21 was higher than for the *cag* PAI negative subclone 67:20, when a competing microflora was present in the mouse stomach. It is known that patients with intestinal metaplasia are colonized with intestinal microbes in the stomach, and one may speculate that *cag* PAI positive strains may have a fitness advantage over *cag* PAI negative strains in these patients. The positive



influence of the secretion system apparatus on colonization is supported by a study of Marchetti and Rappuoli (Marchetti and Rappuoli, 2002). In this work, conventionally raised mice were infected with the wild type strains SS1 or Iris1, or with isogenic mutants of the *vir* homologues HP0524, HP0525, HP0527, HP0528 and HP0544. A strong reduction in number of recovered bacteria was seen in mice infected with the mutants. Furthermore, Ogura *et al.* found a milder inflammation when Mongolian gerbils were colonized with a HP0544 (*virB4*) mutant or a *cag* PAI deleted mutant, compared to gerbils colonized with the wild type strain TN2 (Ogura *et al.*, 2000; Akanuma *et al.*, 2002). Using germ-free piglets, Eaton *et al.* found no effect on colonization when comparing *cag* PAI deleted mutants of strain 26695 or SS1 and the corresponding wild type strains, supporting our results (Eaton *et al.*, 2001). However, one should be cautious when interpreting these results since these authors did not verify a functional TFSS, encoded by the *cag* PAI, in the wild type strains. We know from paper II that 67:21 harbor a functional PAI translocating CagA into cultured AGS cells. There is an ongoing discussion in the *H. pylori* research community whether mice should be used when studying the function of the *cag* PAI. Mice do not produce IL-8 and the pathology, when challenged with *H. pylori*, differs from that in humans (Crabtree *et al.*, 2002).

We were also interested in the genetic stability and evolution of these subclones, and studied reisolates after prolonged experimental infections. First we assessed potential loss of the *cag* PAI, by PCR detection of the *cagA* gene, in 140 and 152 reisolates of strain 67:21 after 3 or 10 months infection of conventionally raised mice, respectively, as well as in 40 reisolates from ex-germfree mice after 3 month of infection. All subclones harbored *cagA*, indicating presence of the *cag* PAI. Since partial deletion of the PAI may occur, a total of 30 reisolates were analyzed on a smaller custom-made PCR-product based microarray, containing only the *cag* PAI genes (Nilsson *et al.*, 2003). They all harbored the complete *cag* PAI, reflecting stability during these experimental conditions. No nucleotide substitutions were detected when sequencing the *cagA* (only in 67:21), *recA* and 16S rRNA genes in 5 reisolates after three months conventional and ex-germ-free infection and 10 month ex-germ-free. In summary, this study indicates that the *cag* PAI is stable in single strain experimental infections and that distinct subclones are present with specific genotypes and phenotypes after prolonged infection in a human host.

### **Paper II**

We sequenced the complete *cag* PAI in four *H. pylori* strains isolated from two duodenal ulcer patients (Du23:2 and Du52:2) and two gastric cancer patients (Ca52 and Ca73) included in a Swedish case-control study (Enroth *et al.*, 2000). The general structure of the *cag* PAI in these strains was similar, but some unexpected features were discovered. Strain Ca52 had an IS606 element in the intergenic region between HP0523 and HP0524. This strain also lacked HP0521, a gene with unknown function that is not required for the type IV secretion machinery in *in vitro* studies using AGS cells (Fischer *et al.*, 2001). The remaining three strains contained a new allele at the position of HP0521. This 750 bp gene, named HP0521B, did not match any sequence in the database. To render a more detailed description of this locus, twenty-one

additional strains of Swedish origin were sequenced, and a variation of translational start and stop sites was discovered in both HP0521 and HP0521B, when comparing these strains. A total of 63 strains were analyzed by PCR for presence of the HP0521 locus, and about half of these strains contained HP0521B instead of HP0521. The HP0521 locus has previously been described as variable in microarray analyses (Björkholm *et al.*, 2002; Kim *et al.*, 2002).

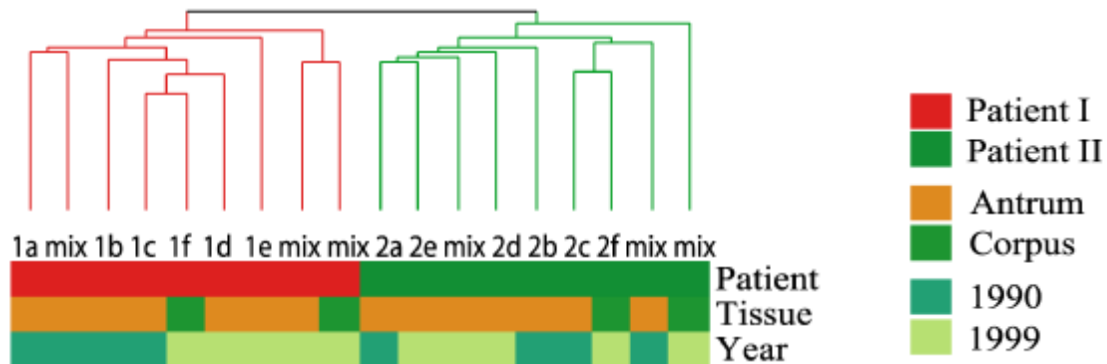
Strain Du23:2, Du52:2, Ca52 and Ca73 all harbored a functional type IV secretion system, encoded by the *cag* PAI, as determined by detection of intracellular tyrosine phosphorylated CagA and IL-8 induction in cultured AGS cells. Strain Du52:2 had a large insertion or rearrangement between HP0546 and HP0547 (*cagA*) that did not affect the functionality of the type IV secretion apparatus. We were not able to obtain the sequence of this region, since it was larger than four kb and primer walking was not possible because of sequence similarities to other loci on the chromosome. The 5' end of this locus was similar to two genes upstream of the *cag* PAI; HP0509 and HP0510, and the 3' end contained a mini IS605 element, the HP0511 gene and part of the variable intergenic region between *cagA* and HP0549.

The CagA protein is known to be highly variable and East Asian isolates have a distinct genotype compared to Western isolates in the repetitive C-terminal region. A high number (>3) of tyrosine phosphorylation motifs (EPIYA) have been correlated to more severe disease and increased morphological changes in cultured cells (Yamaoka *et al.*, 1999; Yamaoka and Graham, 2001; Stein *et al.*, 2002). In our study, Du52:2 possessed five Western type EPIYA motifs and the remaining three strains contained one motif, which is the most frequent type. When calculating  $K_S$  and  $K_A$  values, describing the rate of synonymous and non-synonymous differences in the coding sequence, HP0520, HP0521B, HP0538 and *cagA* were the most variable genes of the *cag* PAI in these four strains. As expected, the *vir* homologues were highly conserved. A correlation between certain sequences and disease outcome could not be elucidated in our study due to the low number of strains analyzed. In a recent study, Backert *et al.* found no association between specific patient group and the *H. pylori cag* PAI induced host responses, when using 75 isolates from three different patient groups, gastritis, peptic ulcer and gastric cancer (Backert *et al.*, 2004). Therefore, one can conclude that there are likely several factors, including the *cag* PAI, important for the progression of *H. pylori* associated diseases.

### ***Paper III***

The *H. pylori* population was studied genetically in two patients in an attempt to calculate the rate of changes over a time period of nine years. One antral biopsy was collected in 1990 and two biopsies, one from antrum and one from corpus, were collected in 1999 from each patient. The methods used were *i*) AP-PCR, *ii*) PCR of four repetitive regions, *iii*) sequencing of ten different loci and *iv*) whole genome microarray. In *i*) and *ii*) we used ten subclones (single cell isolates) from each biopsy. In *iii*) and *iv*) six subclones were randomly selected from each patient where the clones *a*, *b* and *c* were isolated from the antral biopsy collected in 1990, *d* and *e* from the antral biopsy from 1999 and *f* from the corpus biopsy from 1999.

The banding patterns, using two different AP-PCR primers, were highly similar within the two patients, when studying ten subclones from each biopsy and patient. It was concluded that both patients carried only one *H. pylori* strain. When sequencing ten different loci (~6,000 bp), only two base pair differences were found in patient I, and no differences in patient II. DNA microarray analysis, using 50-oligomer probes covering all ORFs in strains 26695 and J99, and PCR of the repetitive region in the *amiA* gene, revealed substantial genetic variation among subclones. Notably, this variation was as high within as between the two time points in both patients.

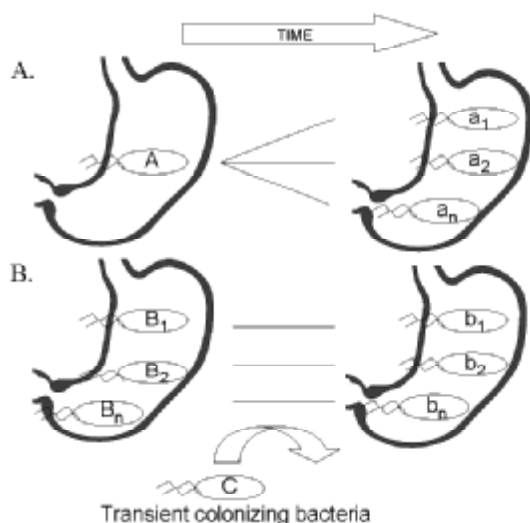


Hierarchical clustering and whole-genome comparison of six subclones (clones *a*, *b*, *c* from biopsy 90A, *d*, *e* from biopsy 99A and *f* from biopsy 99C) from each patient and the total population (Mix) from each biopsy.

We also studied genetic changes of *in vitro* growth of strain 26695 for 2,500 generations and colonization of mice with strain 67:21 for 10 months. Microarray analyses showed a low but significant degree of variability. However no differences were found when sequencing five loci of the *in vitro* passaged strain 26695. It should be stressed that additional genomic changes in the form of intra-chromosomal recombination events, known to occur frequently in *H. pylori*, are not detected using these methods. Only nucleotide substitutions and deletions are found. The variation already existing at the first time point in both patients, and the absence of nucleotide differences, excluded the possibility to calculate the rate of changes between the two time points. However, such calculations have been presented previously (Falush *et al.*, 2001). These authors did not take into account the possible clone variability present at each time point of collection since they only used one subclone from each time point. Therefore, their calculations of rate of change may be overestimated.

Considering the comparably small number of differences accumulated over nine years in our two patients, it is difficult to reconcile the fact that every person harbors his or her own unique *H. pylori* population. For this reason, we propose two possible

scenarios for the generation of *H. pylori* variability. In the first scenario, transmission is monoclonal and the single clone initially diverges rapidly within the micro-niches of the stomach to adapt to the new environment, but as soon as the niches are occupied, the rate of change is reduced. Alternatively, transmission is polyclonal and the transmitted clonal variants have evolved as divergent populations in several generations of hosts. These subclones may use DNA from the total *H. pylori* gene pool, as well as DNA from transiently infecting organisms, to gain or lose genes for further diversification.



Model of two possible ways of generating *H. pylori* diversification. In case A, a transfer bottleneck results in one genetic variant that spreads to the different compartment of the stomach and rapidly adapts to the local environments. Once equilibrium is reached the rate of genetic change is reduced and adaptation is slow. In B, a mixed population is acquired, shaped during several generations of hosts that slowly continue to diverge in the new host.

#### **Paper IV**

In this paper we sought to determine the biochemical and biological function of a putative Nudix hydrolase, NudA, in *H. pylori*. The *nudA* gene (HP1228) is abundant in this bacterium, as all of the 70 strains analyzed by PCR harbored the gene. When sequencing the *nudA* gene in four strains and comparing these to the fully sequenced strains 26695 and J99, 48 of 465 nucleotides were variable of which 23% corresponded to non-synonymous substitutions. The Nudix box encoding the catalytic domain of this enzyme was comparably conserved, with only one amino acid varying. The *nudA* gene is the first gene in an operon of five. The downstream genes encode a putative aspartate kinase (HP1229), a hypothetical protein (HP1230), a DNA polymerase III delta prime subunit (HP1231) and a dihydropteroate synthase, *folP* (HP1232). HP1231 is the only DNA polymerase III delta prime subunit homologue in *H. pylori* and is therefore likely to be essential.

By recombinant expression of the 465 bp *nudA* gene in *E. coli*, tagged with thioredoxine (Trx) and histidine (His), we purified the protein and determined the enzymatic function using a colorimetric method and HPLC. The preferred substrate was di-adenosine tetraphosphate, Ap<sub>4</sub>A, asymmetrically hydrolyzed to ATP and AMP. Other di-nucleoside polyphosphates were hydrolyzed as well, but to less extent. NudA has a broad pH range, between 7 and 9, and requires divalent cations, e.g. Mg<sup>2+</sup>, as described for other Nudix hydrolases. The catalytic constants K<sub>m</sub> and k<sub>cat</sub> were determined to 80 mM and 0.1/s for Ap<sub>4</sub>A.

We constructed a mutant by inserting a kanamycin resistance cassette upstream of the Nudix box in the *nudA* gene in strain 2808, to study the biological role of the enzyme in *H. pylori*. The mutant and wild type grew equally well at normal growth in broth, but differed in ability to survive at an oxidative stress condition. There was a 2- to 7-fold reduction in survival of the mutant after two hours of hydrogen peroxide exposure, despite probable protection by the two enzymes catalase and superoxide dismutase (SOD), two potent enzymes in *H. pylori* that protect against oxidative stress. No differences in survival rates were found after short time exposure to high temperature (55°C).

The Nudix hydrolase homologue MutT in *E. coli* hydrolyzes the toxic compound 8-oxo dGTP causing A-T/C-G transversions. Therefore, an increased frequency of spontaneous mutations of the NudA mutant would indicate that NudA and MutT are functional homologues. We used rifampin as a marker of spontaneous mutations and determined the frequencies in the wild type and the corresponding NudA mutant. There was no increase of spontaneous mutations in the mutant compared to the wild type, suggesting that NudA does not hydrolyze this compound. However, since the intracellular levels of 8-oxo dGTP were not measured, we cannot exclude or prove this function of the enzyme.

Nudix hydrolases in *B. bacilliformis*, *E. coli* and *R. prowazekii* seem to be involved in invasion of host cells and intracellular survival (see introduction). When comparing the invasion frequency of the *nudA* mutant and the wild type, using the gentamicin protection assay, we could not detect any difference. However, the invasion frequency of *H. pylori* is much lower than for other bacteria and the assay used might not be sensitive enough.

Using Western blot analysis with polyclonal antibodies produced against recombinant NudA, we concluded that the protein is constitutively expressed in similar amounts regardless of strain or growth phase. We found no difference in expression levels of NudA when the bacteria were challenged with oxidative stress or heat shock. Notably, the NudA protein from different strains migrated differently on a SDS-PAGE, even though the genes were of the same size. Therefore, we speculate that NudA might undergo post-translational modifications in *H. pylori*. Since other Nudix hydrolases are found to be more efficient than the recombinant NudA enzyme, such modifications may be needed for NudA to be fully active.

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## CONCLUDING REMARKS AND FUTURE DIRECTIONS

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*Helicobacter pylori* is specifically adapted to the human stomach, a niche with few competing organisms. It resides in the stomach for decades, in most cases without causing severe damage to the host. This adaptation has progressed for more than 10,000 years, which has resulted in a compact genome with unique features like the vacuolating cytotoxin, the *cag* PAI encoding a type IV secretion system, and fucosyltransferases responsible for expression of Lewis antigens on the LPS. Our knowledge regarding the role of these specific loci in the development of *H. pylori* related diseases is increasing, but further studies are required to understand the complete picture of the pathogenesis of this bacterium.

In this thesis, the genetic complexity of *H. pylori* was investigated. Single isolates from one individual was shown to differ both in genotype and phenotype, even though being isolated from the same biopsy. When comparing subclones collected from biopsies nine years apart from two patients, these all exhibited distinct genetic profiles regardless of time point, supporting a micro-diversity in this bacterium. However, the substitution rate in ten sequenced loci was low. We propose two possible ways of how the genetic diversification of *H. pylori* takes place. Either one strain variant is transmitted to a new host that rapidly adapts to the new environment, or a mixture of genetically distinct subclones that have evolved in several generations of hosts are all successfully transmitted. The transmission of *H. pylori* is not fully understood, but in order to test the two scenarios we are currently investigating one family where the mother and three children carry similar *H. pylori* strains. We also aim to study four adult volunteers that have been experimentally infected with a clinical strain. In these individuals biopsies were collected after five and eight months. This is a short time span, but if the adaptation is rapid and dependent on host factors in the initial phase of colonization, we expect to find large genetic differences in the strains reisolated from these subjects.

We also compared the complete *cag* PAI in *H. pylori* strains isolated from four patients. Most of the 27 genes in this PAI have unknown function, apart from the seven *vir* homologues that form a type IV secretion apparatus and the *cagA* gene, encoding an effector protein CagA. A new gene, HP0521B, was found in three of the sequenced strains. We aim to further investigate the proportion of HP0521 and HP0521B in other human populations as well as study their role in *H. pylori* pathogenesis.

The relative contributions of the factors important for the initiation and progression of gastroduodenal pathology are still largely unknown. This work has given additional insight into the genetic diversity of *H. pylori* and revealed that there are a variety of genetically distinct subclones in the population in one stomach. In order to find specific bacterial genetic markers associated to disease, one has to account for the large variation within the bacterial population residing in each individual. Whether such variations determine the disease outcome remains to be studied.

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