Helicobacter spp. interactions with mucins

- Adhesion and mucin regulation of pathogen proliferation and gene expression

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Cover illustration:

Left: DAPI-stained gastric tissue of a *H. heilmannii* infected mouse.
Middle: *H. heilmannii* infected mouse gastric tissue overlaid with FITC-labeled *H. heilmannii*.
Upper right: *H. pylori* stained by Live/dead stain.
Middle right: *H. pylori* in co-culture with anti-microbial mucin stained by Live/dead stain.
Lower right: Aggregates of *H. pylori* in a co-culture with mucins stained by Live/dead stain.

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ABSTRACT

Helicobacter pylori colonizes the gastric mucosa of approximately half of the world's population and is a risk factor for gastritis, peptic ulcers and gastric cancer. *H. pylori* is surrounded by, and adheres to, the heavily glycosylated mucins that build up the mucus layer. The carbohydrate structures on the mucins that act as ligands for *H. pylori* vary between individuals and change during disease. In this thesis, we investigated how *H. pylori* interacts with differently glycosylated mucins by analyzing adhesion, proliferation, gene expression and the resulting effect on virulence to host cells. We found that mucins can interfere with *H. pylori* proliferation, partly dependent on binding to the mucins and the presence of known antimicrobial structures, but also observed an inhibition of *H. pylori* proliferation independent of these two factors. The gene expression of *H. pylori* varied greatly in the response to differently glycosylated mucins. Expression of the virulence factor CagA increased in response to some mucins, presumably by Fur-dependent regulation as a result of binding via the SabA adhesin. The varying interaction of *H. pylori* and mucins resulted in alterations in the response of infected gastric epithelial cells *in vitro*.

There are several *Helicobacter* species that commonly infects other animals, but can also infect and cause disease in humans. Their modes of interaction with mucins are unclear. We examined the adhesion of two non-*H. pylori Helicobacter* species to differently glycosylated gastric mucins and mucosal tissue from a range of animals. Our results demonstrated that they can adhere to mucins and gastric tissue via specific glycan structures that change during infection, although the binding ability to human mucins are lower than that of *H. pylori*. In addition, there are other bacteria in the stomach that may interfere with mucin interactions of *Helicobacter* spp. We showed that *Lactobacillus* species isolated from the same stomachs as *H. pylori* did not compete for the same mucin ligands and did not markedly change how co-isolated *H. pylori* interact with the mucins.

In summary, *H. pylori* adhesion to human mucins differs from that of other *Helicobacter* spp. and is not affected by co-colonizing *Lactobacillus* spp. The interactions of *H. pylori* to mucins affect proliferation and expression of virulence factors that may influence the colonization ability, virulence and host response and ultimately play a role in the development of symptoms displayed in the host.

Keywords: *Helicobacter*, *Lactobacillus*, mucin, glycosylation, adhesion, proliferation, gene expression, CagA, SabA, BabA, Fur, ArsS
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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Strain-dependent proliferation in response to human gastric mucin and adhesion properties of *Helicobacter pylori* are not affected by co-isolated *Lactobacillus* sp.**
Emma C. Skoog, Mathilda Lindberg and Sara K. Lindén.
Helicobacter. 2011;16(1):9-19.

II. **Human gastric mucins differently regulate *Helicobacter pylori* proliferation, gene expression and interactions with host cells.**
Emma C. Skoog, Åsa Sjöling, Nazanin Navabi, Jan Holgersson, Samuel B. Lundin and Sara K. Lindén.
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III. ***Helicobacter pylori* responses to mucins are dependent on adhesion and gene regulation via ArsS and Fur.**
Emma C. Skoog, Jonathan Gauntlett, Hans-Olof Nilsson, Mohammed Benghezal and Sara K. Lindén
Manuscript

IV. ***Helicobacter suis* and *Helicobacter heilmannii* adhesion to gastric mucins during health and infection.**
Emma C. Skoog, Médea Padra, Bram Flahou, Annemieke Smet, Freddy Haesebrouck and Sara K. Lindén
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ABBREVIATIONS

AmiE	acylamide amidohydrolase (an aliphatic amidase)
ArsS/R	Acid response regulator S and R
BabA	Blood group antigen binding adhesin A
CagA	Cytotoxin associated gene A
CFU	Colony forming unit
CheA/Y1	Chemotactic response regulator A and Y1
EGF	Epidermal growth factor
FlaA/B	Flagellin subunit A and B
Fur	Ferric uptake regulator
GGT	Gamma-glutamyltranspeptidase
GlcNAc	*N*-acetylglucosamine
HopZ	*Helicobacter pylori* outer membrane protein Z
IL	Interleukin
Le	Lewis antigen
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MUC	Mucin
NapA	Neutrophil activating protein A
NHPH	Non-*Helicobacter pylori Helicobacter*
OD	Optical density
OipA	Proinflammatory outer membrane protein A
PCR	Polymerase chain reaction
PTS	Proline-, threonine- and serine-rich
SabA	Sialic acid binding adhesin A
SLe	Sialylated Lewis antigen
Th	T helper cell
UreA/B	Urease subunit A and B
VacA	Vacuolating cytotoxin A

1 INTRODUCTION

A living organism is affected by its environment, and likewise the environment is effected by the organism. Mucins are proteins that are major components of the environment in the stomach at the site of colonization by the pathogen *Helicobacter pylori*. *H. pylori* is a highly variable organism that can influence the host in many ways and result in different disease symptoms. Mucins can also vary greatly between individuals, consequently altering the environment of *H. pylori*. An understanding of the effects of the bacteria on the host, and conversely, those of the host response on the bacteria, may provide insights into how they regulate each other and what role this might play in the development of disease.

1.1 The mucosal surface

The mucosal surface includes all wet areas on our body that protects against foreign insult from the external environment. The largest area of mucosal surface lines the gastrointestinal tract. The outermost part of mucosal surfaces is covered by a mucus layer, which acts as a barrier protecting the underlying cells. It entraps bacteria and foreign particles that can be washed away with the shedding mucus layer, protects from harmful pH and dry air, and lubricates the surfaces to enable, for example, the blinking of eyes and movement of food through the bowel. The main constituents of the mucus layer are mucins, which are exceptionally large, highly glycosylated proteins. In addition there are ~1000 proteins that have been found in mucus (Johansson *et al.* 2009; Rodriguez-Pineiro *et al.* 2013). Mucins provide binding sites for bacteria and contribute to the viscosity of the mucus layer (Georgiades *et al.* 2013), which is important to retain other secreted proteins such as enzymes, antibodies and defensins. In addition, mucus can provide a niche for microbiota to reside in at a distance from the underlying cells (Johansson *et al.* 2008). Commensal bacteria in the gastrointestinal tract can provide us with nutrients from foods that we are otherwise unable to digest, but if the mucus is impaired they can trigger inflammation (Johansson *et al.* 2014). Pathogens can penetrate or break down the mucus to reach the epithelial surface. In addition, mucus production can be regulated by bacteria, which is demonstrated by the findings that germ free mice have a thinner intestinal mucus layer than conventionally raised mice and that there is altered mucin gene expression during bacterial colonization (Petersson *et al.* 2011; Bergstrom *et al.* 2012). Mucosal surfaces can have different shapes with or without microvilli and glandular tissue and crypts of variable length. This results in different microenvironments in the mucus layer with variable functions, which are more or less exposed to the external environment.

Under the mucus layer is a line of epithelial cells that are closely attached to each other via tight junctions to prevent external content from leaking into the tissue. The apical surface, facing the mucus layer, is covered by a glycocalyx built up by membrane bound mucins and other glycosylated proteins and lipids. The glycocalyx provides further hindrance to bacteria coming into contact with the cell surface and can initiate host intracellular signaling when bacteria are bound (Linden *et al.* 2009). The epithelial cells have different functions within and between organs such as secretion of acid, defensins, digestive enzymes, mucins and ions, absorption of nutrients and fluid, sensing of the external environment and linking it to the immune system.

The mucosa between the epithelial cells and underlying muscles in the gastrointestinal tract contains immune cells and mucosal lymphoid tissue that are responsible for local immune responses. It also contains blood vessels, neurons and fibroblasts. Some carnivores, e.g. cats, have an additional region called stratum compactum between the mucosal gland tissue and the muscular tissue consisting of high amounts of collagen and fibronectin (Zahariev *et al.* 2010).

1.2 Mucins

Mucins are large glycoproteins present in all mucosal tissues. Domains rich in proline, serine and threonine (PTS domains) are densely *O*-glycosylated and account for up to 90% of the mucin molecular weight (Perez-Vilar and Hill 1999). PTS-domains of different mucins can vary in length, sequence and glycosylation and are often built up by tandem repeat sequences (Gum 1992; Jonckheere *et al.* 2013). Mucins can be secreted or membrane-bound. The secreted mucins are MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC9 and MUC19, which all are gel-forming mucins except the smaller MUC7 and MUC9 (Bobek *et al.* 1993; Linden *et al.* 2008; Jonckheere *et al.* 2013). Secreted gel-forming mucins contain conserved cysteine-rich domains and von Willebrand D domains that are involved in crosslinking between mucin molecules, forming the network that mucus consists of (Backstrom *et al.* 2013).

There are 11 identified membrane-bound mucins, of which the most studied is MUC1 (Jonckheere *et al.* 2013). Between the transmembrane domain and the extracellular PTS domain in most membrane-bound mucins is a SEA (sea urchin sperm protein, enterokinase and agrin) domain (Jonckheere *et al.* 2013). The SEA domain undergoes autocatalytic cleavage followed by the presentation of the mucins on the cell surface as heterodimers (Palmai-Pallag *et al.* 2005). Binding of bacteria to membrane-bound mucins can lead to disintegration of the heterodimer and shedding

of the PTS domain along with the bound bacteria (Linden *et al.* 2009). There is also an epidermal growth factor (EGF)-like domain next to the PTS domain that is believed to mediate interaction with other proteins (Jonckheere *et al.* 2013). The cytoplasmic tail of membrane-bound mucins varies greatly in size and sequence, indicating different functions in intracellular cell signaling.

1.2.1 Mucin glycosylation

Each mucin can have up to 100 different types of carbohydrates attached to it. The glycosylation of mucins contribute significantly to the viscosity of mucin solutions, i.e. mucus (Slomiany *et al.* 1987), and the dense *O*-glycosylation of the PTS domain results in a straight conformation and inhibits proteolytic cleavage. The interaction between host mucins and microorganisms are to a large extent dependent on glycans (Linden *et al.* 2008). The process of glycosylation is mediated by glycosyltransferases and occurs in the Golgi apparatus (Wasano *et al.* 1988). The glycan chains are built up by one of 8 possible core structures closest to the peptide chain followed by type-1 or type-2 backbone extensions (Gerken 2012). The peripheral region of the glycans can be more variable and structurally complex and the chains are terminated by specific structures e.g. fucose, sialic acid, sulphate, galactose or *N*-acetylgalactosamine. The variable expression of glycosyltransferases in different cells, organs and species result in different mucin glycosylation, and the selective pressure to handle pathogens is believed to have played a role in the development of these differences (Gagneux and Varki 1999). Some of these variations entail differences in blood group type glycan epitopes. The A, B, H and Lewis (Le) structures are examples of common terminal blood group glycans present on mucins. Individuals that produce fucose α1,2 structures important for the formation of Le^b and H1 structures are referred to as secretors (Clausen and Hakomori 1989; Henry *et al.* 1995). A small part of the population are non-secretors, and they have been associated with reduced severity or risk of infection with pathogens such as *H. pylori* and norovirus (Larsson *et al.* 2006; Azevedo *et al.* 2008).

1.3 The stomach

The main function of the stomach is mechanical and chemical digestion of food. Parietal cells secrete hydrochloric acid that give the stomach lumen its low pH and activates proteases (Richter *et al.* 1998; Schubert 2010). Both proteases and the low pH take part in food digestion, but contribute also to the defense against bacteria. The stomach can be divided into regions with differing physiology and anatomy (Figure 1). In the human stomach, food enters from the esophagus via the cardia to the

fundus, which is the name of the upper curvature of the stomach, and corpus, which is the central region of the stomach also called the stomach body. The fundus and corpus are similar and in some animals only referred to as the fundus. They have a relatively thick mucosal tissue and contain the parietal acid secreting cells. The lower curvature of the stomach contains the antrum, or pyloric antrum. As indicated by its name, this is where colonization of *H. pylori* is most common. The antrum has relatively large crypts. Finally, the food leaves the stomach into the duodenum via the pyloric canal or sphincter.

Rhesus monkeys and cats have a similar stomach anatomy as humans, whereas pigs and mice stomach differ substantially. The esophageal non-glandular tissue reaches further into the pig stomach and is called pars oesophagea. This tissue is lined by keratinized squamous epithelia, does not secrete mucus and are commonly ulcerated in *H. suis* infected pigs (Queiroz *et al.* 1996). The following cardiac region takes up a much bigger area than in humans. It lacks parietal cells and the pH is around 5.5-7. Mice have a big part of their stomach similar to the pig pars oesophagea called the forestomach. The rest of the stomach consists of the cardia, fundus and antrum as present in humans.

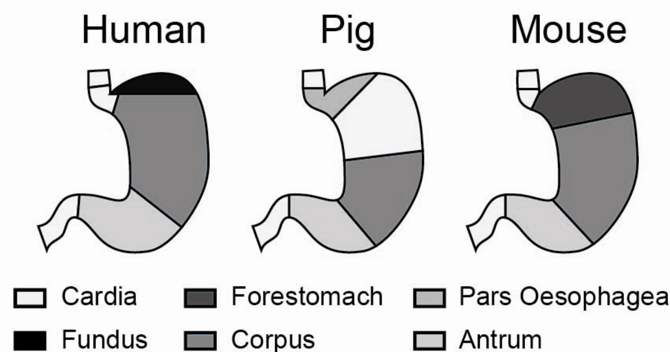

Figure 1. Regions of the stomach of human, pig and mouse.

1.3.1 The gastric mucus layer

The gastric mucus layer is very important in protecting the underlying cells from the low pH in the stomach lumen. There is a gradient of pH within the mucus layer reaching a neutral pH closest to the epithelial cells (Allen and Flemstrom 2005). Ulcers may result when the mucus is insufficient in protecting the gastric wall against acid and pepsin in the lumen, which can be caused by infection, inappropriate diet and stress.

The mucus layer is divided into two layers, one dense stratified layer firmly attached to epithelial cells and one looser layer at the luminal side (Atuma *et al.* 2001). The gastric mucus layer is relatively thin and has little loosely attached mucus, but the inner mucus layer is suggested to be very firmly attached to the epithelial cells. However, both the outer and inner mouse gastric mucus layer is permeable to beads the size of bacteria (Ermund *et al.* 2013). Thus the binding of mucins to bacteria seems to be more important than the organization of mucus to prevent bacteria to come in contact with the epithelia.

Surface epithelial cells secrete MUC5AC, which is the main mucin that builds up the surface gastric mucus layer, arranged in a linear fashion (Ho *et al.* 2004). MUC6 is secreted by neck cells in the gastric glands. MUC6 is the main secreted mucin found in antral glands, but it can also be found as layered sheets interspersed with MUC5AC in the surface mucus (Ho *et al.* 2004). The membrane-bound mucins MUC1, MUC4, MUC12, MUC13 and MUC17 are part of the glycocalyx of the gastric epithelium (Linden *et al.* 2008). In the healthy human stomach, mainly neutral core 2 glycans are attached to the mucins, with 80% of the glycan chains carrying blood group structures (Rossez *et al.* 2012). The glycosylation can, however, be modulated by infection and inflammation.

Rhesus monkeys have similar mucin expression and blood group glycan structures as the human stomach and are good models for human *H. pylori* infection (Dubois *et al.* 1994; Linden *et al.* 2004). Most glycan structures present in rhesus monkey gastric tissue are also present in humans (Cooke *et al.* 2009). Pigs have similar blood group glycan structures as the human stomach, but they have a higher level of sulphation (Thomsson *et al.* 2000). The mucin population in pigs has not been fully characterized. Their mucins in surface and gland mucosa are different from each other and may be equivalent to the human MUC5AC and MUC6 (Nordman *et al.* 1997; Nordman *et al.* 1998). In fact, two major mucins have been found in the pig gastric surface mucosa, PGM-2A and PGM-9B, of which PGM-2A can be recognized by an antibody used to detect human MUC5AC with similar tissue staining patterns (Turner *et al.* 1995; Nordman *et al.* 1998). Mice have similar mucin expression as humans, but a substantially different glycosylation (Thomsson *et al.* 2012). Half of their Muc5ac mucin *O*-glycans are neutral while many glycans are monosulphated and have a low grade of sialylation and fucosylation (Holmen Larsson *et al.* 2013). Mice are not normally colonized by human *H. pylori* strains (Ayraud *et al.* 2002), but there are mouse-adapted *H. pylori* strains used in research models for which modifications of glycan-binding properties have been shown to play a role in the ability to infect the mouse stomach (Yamaoka *et al.* 2002; Chionh *et al.* 2009). In addition, there are transgenic mouse models expressing human Le^b that human *H. pylori* strains can colonize (Falk *et al.* 1995; Guruge *et al.* 1998).

1.3.2 Gastric mucins in disease

In *H. pylori* infection, precancerous lesions and cancer the expression, glycosylation and spatial distribution of mucins change both in humans and in the rhesus monkey infection model (Byrd *et al.* 1997; Linden 2004; Linden *et al.* 2008; Cooke *et al.* 2009). In addition, *H. pylori* can reduce the rate of mucin turnover in a murine model (Navabi *et al.* 2012). *H. pylori* positive individuals display a reduced and spatially different expression of MUC6, expressed also in cells of the surface mucosa, and less expression of MUC5AC and MUC1 (Byrd *et al.* 1997; Kang *et al.* 2008; Shi *et al.* 2013). The degree of reduction depends on the type of disease with less expression in cancer and pre-cancer tissue than in normal gastritis (Kang *et al.* 2008). However, MUC1 can be strongly expressed in some tumors and may act as an oncogene when overexpressed (Boltin and Niv 2013). *De novo* expression of MUC2 can occur in intestinal metaplasia (Reis *et al.* 1999). That *H. pylori* is directly involved in mucin expression changes is demonstrated by the reduced synthesis of MUC5AC and MUC1 within 4 hours of infection in gastric epithelial cell lines (Byrd *et al.* 2000). There is higher expression of *MUC5AC* in cell lines infected with *H. pylori ureB* mutants compared to wild-type, indicating that UreB is responsible for the inhibition of *MUC5AC* expression (Perrais *et al.* 2013).

Experimental *H. pylori* infection in rhesus monkeys results in no change in mucin expression over a 10 month time period, but a transient decrease in Le^b-glycosylation and a stable increase in sialyl-Le^x (Linden *et al.* 2008). A high expression of sialyl-Le^x and sialyl-Le^a in humans is associated with gastric cancer (Fukushima *et al.* 1984; Sakamoto *et al.* 1989). Pathogenic *H. pylori* can induce the expression of the glycosyltransferase beta3 GlcNAc T5 in gastric cell lines, where an overexpression leads to increased levels of sialyl-Le^x (Marcos *et al.* 2008). The mucin *O*-glycosylation has a proposed role in tumorigenesis (Zhang and Ten Hagen 2011).

1.4 Microbes in the stomach

Until recently, the stomach was regarded as devoid of colonizing bacteria. This may be true immediately after birth, but during the early years of life a microbial community is established in the gastrointestinal tract (Adlerberth and Wold 2009). Most bacteria are found in the colon, but the stomach can also be colonized. Recent studies have found up to 262 phylotypes in the human stomach, belonging to 13 phyla of which the most common are Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria (Bik *et al.* 2006; Andersson *et al.* 2008). How many of these bacteria are true colonizers or just swallowed bacteria in passing is unknown. The most common pathogen of the stomach is *H. pylori*, which was likely

first detected in 1893 (Bizzozero 1893), but was established to be a pathogen in 1983 (Warren and Marshall 1983). Other *Helicobacter* species are also pathogenic in the human stomach as well as in cats, dogs, pigs and monkeys among others (Haesebrouck *et al.* 2009). These are commonly termed non-*H. pylori Helicobacter* (NHPH). In total, *Helicobacter* species have been found in 142 different animal species (Schrenzel *et al.* 2010). *H. bizzozeronii* is the only NHPH that has been isolated from human stomachs (Kivisto *et al.* 2010), but *H. suis*, *H. heilmannii*, *H. felis* and *H. salomonis* can also colonize the human stomach (Haesebrouck *et al.* 2009). Besides *Helicobacter*, there are no other bacteria established to be pathogens in the human stomach.

1.4.1 *Helicobacter pylori*

Helicobacter pylori is a gram-negative bacterium of the proteobacteria phylum. They are spiral-shaped rods that grow best under microaerophilic conditions at neutral pH and 37°C. *H. pylori* usually colonize the mucus layer of the stomach antrum, but can also be found in corpus and duodenum (Enomoto *et al.* 1998). They live in the mucus layer near the epithelial cells where pH is close to neutral, but a small fraction of bacteria can invade host cells (Liu *et al.* 2012).

Barry Marshall and Robin Warren were the first to isolate and culture *H. pylori*, and Marshall's self-inoculation experiment confirmed that *H. pylori* induced inflammation of the gastric mucosa (Warren and Marshall 1983). These achievements resulted in a Nobel Prize in Medicine in 2005.

Prevalence and transmission of *H. pylori* infection

H. pylori infects about half of the world's population. In developing countries the prevalence can be as high as 90%, but in developed countries the prevalence is lower and continues to decrease (Fock and Ang 2010). In Sweden, the prevalence of *H. pylori* in the adult population is 30-40% (Storskrubb *et al.* 2005), and only around 2% of children with Scandinavian parents are seropositive (Tindberg *et al.* 2001).

H. pylori is normally acquired in early childhood and can persist throughout the whole life (Mitchell *et al.* 1992). The mechanisms of how *H. pylori* is acquired still remain unclear. Transmission is likely to occur from person to person, mostly parent-to-child, via oral-oral or fecal-oral routes (Urita *et al.* 2013) (Thomas *et al.* 1992). The fact that *H. pylori* can reside in the oral cavity supports an oral-oral transmission (Dowsett and Kowolik 2003). Less common transmission routes can be via contaminated drinking water, contact with pets or non-human primates or intake of contaminated food (Fox 1995; Haesebrouck *et al.* 2009; Vale and Vitor 2010).

Different outcomes of infection

H. pylori is classified as a type I carcinogen, causing gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* is estimated to cause approximately 70% of all gastric cancers, and gastric cancer is the second most common cause of cancer related deaths. However, only 1-2% of infected individuals develop gastric cancers, whereas 10-20% develop gastric and duodenal ulcers (Kusters *et al.* 2006). All infected individuals have gastritis, but the gastritis is clinically asymptomatic in most cases. Acute gastritis can be followed by spontaneous clearance of infection in some patients (Dubois *et al.* 1996; Perez-Perez *et al.* 2003). The outcome of the disease for each individual is hard to predict, but host, bacterial and environmental factors are all involved in determining the risk for disease. A low stomach acid secretion is correlated with colonization and gastritis in corpus as well as in antrum, and leads to enhanced hypochlorhydria and increased risk of gastric ulcer and gastric cancer induced by *H. pylori* (Malfertheiner 2011). Polymorphisms in the interleukin-1 (IL-1) gene cluster that lead to increased expression of IL-1β are associated with hypochlorhydria and increased risk of gastric cancer induced by *H. pylori* (El-Omar *et al.* 2000). Duodenal ulcers are more common in individuals with high acid secretion. Stress, alcohol abuse and use of nonsteroidal anti-inflammatory drugs can cause gastritis and increase the risk of *H. pylori*-induced disease (Kusters *et al.* 2006). The expression of *H. pylori* genes important for colonization and virulence also play a role in the disease outcome. For example, individuals infected with strains positive for *cagA* have a higher risk of developing gastric cancer (Polk and Peek 2010). Risk factors can be additive and enhance each other. For example, several dietary risk factors for gastric cancer directly impact *H. pylori* virulence (Cover and Peek 2013). In addition, we show in this thesis that mucin glycosylation has an impact on *H. pylori* virulence. Differences in mucin expression and glycosylation between individuals also provide variable capacity in inhibiting colonization and adherence to host cells.

H. pylori and mucins

Mucins are major components of the host environment where *H. pylori* is found. *H. pylori* that bind to mucins can be washed away with shedding mucus from the stomach, and mucins provide a physical and mechanical barrier that prevents *H. pylori* from binding to epithelial cells (Linden *et al.* 2009). On the other hand, *H. pylori* that do not bind mucins may not colonize the stomach as successfully, and a positive BabA status has been associated with a more severe disease outcome (Yamaoka 2008). Mucin glycosylation and binding capacity are thus crucial in determining the outcome of infection. Mucins can also interfere with *H. pylori* in ways other than binding and physical interactions. It has been proposed that the carbohydrate structure terminal α1,4-linked *N*-acetylglucosamine (α1,4-GlcNAc) has

antimicrobial activity. This structure inhibits the synthesis of cholesteryl α-glucosides that affect the cell wall of *H. pylori* and thus inhibits their growth (Kawakubo *et al.* 2004). α1,4-GlcNAc can be found on MUC6 expressed in the glands of the stomach, which have a low degree of *H. pylori* colonization. In contrast, commercial porcine gastric mucins increase the proliferation of *H. pylori* (Jiang and Doyle 2000; Cole *et al.* 2004), but the mechanism for this stimulation has so far been unclear.

Some bacteria can cleave the mucin backbone and mucin glycans to retrieve nutrients. This has not been shown for *H. pylori*, and I also performed preliminary experiments that did not indicate that *H. pylori* could cleave mucin glycans (unpublished results). However, a putative polysaccharide lyase, recently identified in *H. bizzozeronii* and suggested as a potential virulence factor (Schott *et al.* 2011), may hypothetically be able to cleave mucin glycans.

The viscosity of mucus is a physical barrier against many bacteria. The combination of a spiral shape and unipolar flagella helps *H. pylori* to be successfully motile in mucus. By increasing the pH around itself, *H. pylori* reduces the viscosity of the mucus gel, which further enables motility (Celli *et al.* 2009). *H. pylori* has a chemotactic response to commercial hog gastric mucins (Foynes *et al.* 2000). Mutants of *cheA* and *cheY1*, which encode for the members of a two-component system for chemotactic response regulation, reduce the chemotactic response to commercial porcine gastric mucins and fail to colonize mice. This two-component system can interact with flagellar motor proteins and regulate the rotation of the flagella (Foynes *et al.* 2000). Chemotaxis to mucins thus seems to be crucial for *H. pylori* to find its niche.

H. pylori adhesins

H. pylori can bind to several structures present in mucus, the extracellular matrix and on the epithelial cell surface (Illustrated in Figure 2). The genome of *H.* pylori contains 32 outer membrane proteins that could be putative adhesins, but of which only a few have been characterized as adhesins (Tomb *et al.* 1997).

Adhesion of *H. pylori* to mucins is highly relevant for colonization, mucin-mediated defense, host cell response and development of disease (Sheu *et al.* 2003; Yamaoka 2008; Linden *et al.* 2009; Linden *et al.* 2010). *H. pylori* can bind to both secreted and surface-bound mucins. The attachment is mainly mediated through the *H. pylori* blood group antigen binding adhesin (BabA) and the sialic acid binding adhesin (SabA) that bind via the glycans to the heavily glycosylated mucins (Lindén *et al.* 2002; Linden *et al.* 2004; Linden *et al.* 2008). BabA binds to Le^b and H-type 1 and is an important adhesin for binding to non-sialylated mucins during colonization (Ilver

et al. 1998). There are regions in the *babA* gene that are highly variable and found in different numbers of repeats. The function and expression of BabA can be modified by genetic remodeling during infection (Styer *et al.* 2010). There are strains that do express *babA*, but have a poorly or non-functional BabA (Odenbreit *et al.* 2009). Strains that can bind via BabA are classified into generalists and specialists. Generalists can bind to fucosylated blood group A, B and O structures, while specialists bind only fucosylated blood group O structures (Aspholm-Hurtig *et al.* 2004).

SabA binds to sialyl-Le^x and sialyl-Le^a, which is present at a higher level in inflamed tissue (Mahdavi *et al.* 2002). Similarly to *babA*, the *sabA* gene is also variable and its expression can be modulated by genetic rearrangements (Yamaoka 2008; Talarico *et al.* 2012). The presence of *sabA* is more common in strains also positive for *babA* and *cagA* (Mahdavi *et al.* 2002). SabA is suggested to bind to and activate neutrophils, and can thus be regarded as a virulence factor (Unemo *et al.* 2005; Aspholm *et al.* 2006).

Both BabA and SabA-mediated binding decrease with lower pH. Instead, *H. pylori* can bind to mucins via a charge/low pH-dependent mechanism at low pH. This binding mechanism is dependent on the presence of charged structures, which can be found on sialylated and sulphated mucins, but is independent of any known mucin-binding adhesin and has its optimal function at pH 3-4 (Linden *et al.* 2004).

The *H. pylori* neutrophil activating protein (NapA or NAP) is a major secreted virulence factor and an adhesin that binds to sulphated carbohydrates on human granulocytes and on salivary mucins at neutral pH. Sulphated carbohydrates can also be present on gastric mucins after infection. Secreted NapA is chemotactic for neutrophils and monocytes and increases the adhesion of neutrophils to endothelial cells (Dundon *et al.* 2002). It also stimulates neutrophils to produce oxygen radicals and promotes a T-helper cell-1 (Th1) type immune response (de Bernard and D'Elios 2010).

H. pylori can present glycans at its surface that are similar to mucin glycans. These provide mimicry and can be used for adhesion to host epitopes. *H. pylori* Le^x have been suggested to bind to galectin-3 (Fowler *et al.* 2006). Galectin-3 is upregulated and released from the cell membrane in cell lines after *H. pylori* infection. In addition, the core oligosaccharide of *H. pylori* lipopolysaccharide (LPS) is involved in the interaction with trefoil factor 1 (TFF1), which promotes mucus colonization (Clyne *et al.* 2004). The expression of TFF1 is increased in cell lines infected with *H. pylori* (Matsuda *et al.* 2008).

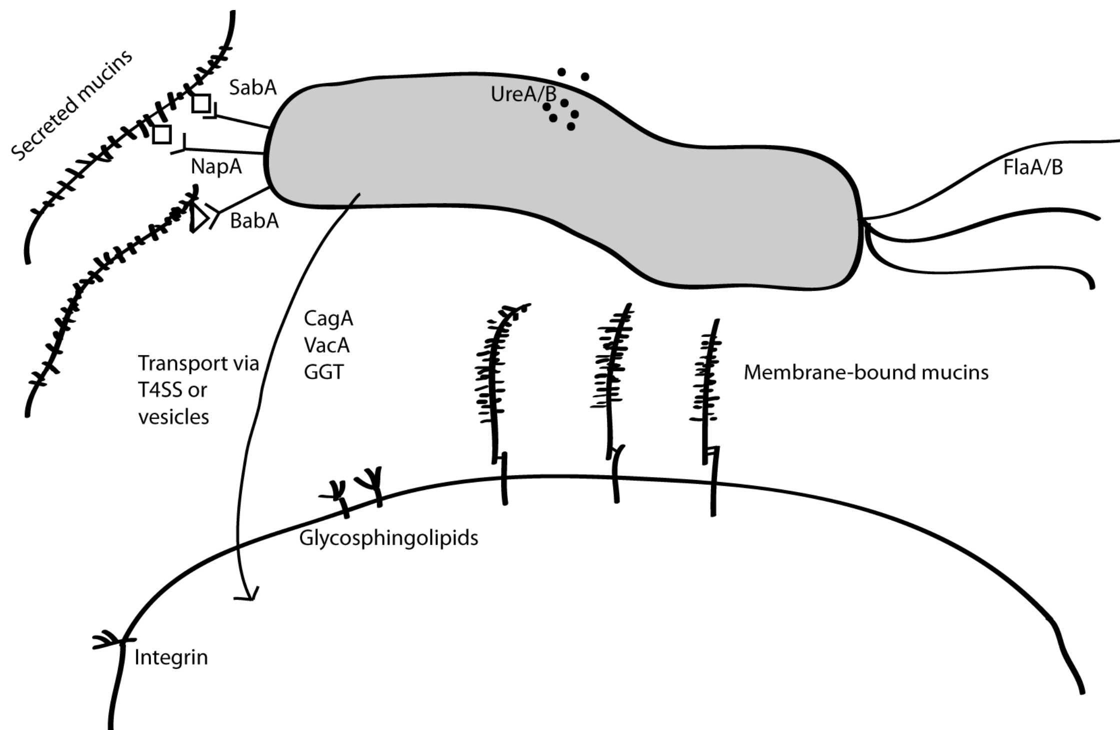

Figure 2. Schematic picture of H. pylori *interactions with the host, with the best characterized adhesin and virulence factors indicated. (T4SS= type IV secretion system)*

Glycosphingolipids are a group of molecules present in the epithelial cell membrane, which can carry similar as well as different glycostructures to mucins. *H. pylori* can use many adhesins for binding to glycosphingolipids, for example BabA, SabA, NapA and vacuolating cytotoxin A (VacA) (Borén *et al.* 1993; Miller-Podraza *et al.* 1997; Teneberg *et al.* 1997; Roche *et al.* 2004; Roche *et al.* 2007; Benktander *et al.* 2012). There are also a range of identified glycosphingolipid ligands for which the adhesin used for binding is unknown (Teneberg 2009). The implication of binding to glycosphingolipids is unknown.

AlpA and AlpB are present in almost all clinical isolates (Odenbreit *et al.* 2009). They were identified as adhesins involved in binding to gastric tissue in 1999, but their ligands were unknown (Odenbreit *et al.* 1999). Recently both AlpA and AlpB has been suggested to bind to laminin (Senkovich *et al.* 2011). Laminin is a glycoprotein present in the extracellular matrix, similar to other proposed *H. pylori* ligands such as vitronectin and fibronectin (Ringner *et al.* 1994), but the relevance of binding to extracellular matrix proteins is unclear. Surprisingly, mutants lacking AlpA and AlpB causes more severe inflammation in gerbils than wild-type (Senkovich *et al.* 2011).

H. pylori can bind to integrin α5β1 via type IV secretion system proteins, e.g. CagL, which triggers CagA delivery and activates kinases for CagA phosphorylation in the host cell (Kwok *et al.* 2007). There are also other *H. pylori* outer membrane proteins for which the host cell receptors are still unknown. Several research projects have studied proinflammatory outer membrane protein A (OipA) and *H. pylori* outer membrane protein Z (HopZ), which are suggested to be adhesins (Peck *et al.* 1999; Dossumbekova *et al.* 2006). The presence of *oipA* is associated with peptic ulcers and a positive status of *cagA* and virulent genotypes of *vacA* (Odenbreit *et al.* 2009; Markovska *et al.* 2011).

Pathogenesis and virulence factors of *H. pylori*

Adhesins are important virulence factors as they are involved in colonization ability and can directly affect the immune response of the host. In addition, adhesion to host structures can result in regulation of other bacterial genes important for growth and virulence (Johnson *et al.* 2012). Virulence can be defined as the competence of any infectious agent to produce pathologic effects. There are many *H. pylori* factors that have been examined for association with disease, but if their function is unknown it cannot be established if the specific factor is a primary virulence factor, or if it is co-regulated or associated with the function of other virulence factors. The following is a summary of commonly studied confirmed or proposed virulence factors.

Cytotoxin-associated gene A (CagA) is probably the most studied virulence factor. It is expressed from the Cag pathogenicity island gene cluster, which encodes for several other proteins involved in CagA translocation. These are responsible for forming the type IV secretion pilus that injects CagA into host cells and binds to the host integrin α5β1 (Kwok *et al.* 2007). Adhesion to host cells via BabA also seems to be important for CagA translocation (Ishijima *et al.* 2011). When injected into the host cell it activates inflammatory responses via nuclear factor κB and enhances characteristics associated with inflammation and cancer, e.g. alters morphology and motility and prevents apoptosis (Polk and Peek 2010). CagA is associated with more severe inflammation and a higher risk of gastric cancer (Plummer *et al.* 2007; Umit *et al.* 2009). The cytoplasmic tail of MUC1 is suggested to decrease CagA-induced IL-8 production by interacting with β-catenin and CagA and thereby preventing translocation of β-catenin into the nucleus (Udhayakumar *et al.* 2007; Guang *et al.* 2012).

Another well studied virulence factor is the vacuolating cytotoxin VacA. VacA is secreted via a type V secretion system and subsequently bound and internalized into host cells, where it causes vacuolation and pore forming in intracellular membranes (Kim and Blanke 2012). In contrast to CagA, VacA promotes apoptosis and has

immunosuppressive properties. These properties have also been demonstrated for gamma-glutamyltranspeptidase (GGT) (Oertli *et al.* 2013). GGT induces apoptosis by the degradation of host cell glutathione, increasing the sensitivity to oxidant damage (Flahou *et al.* 2011). Both VacA and GGT promote persistent infection. Immunosuppression by *H. pylori* is believed to prevent development of asthma and allergy (Arnold *et al.* 2012).

Urease and flagella can be regarded as virulence factors, as they have essential functions for *H. pylori* to reach its niche and be able to colonize the host. Urease and flagella are found in all gastric *Helicobacter* species identified (Haesebrouck *et al.* 2009). Urease is composed of two subunits, UreA and UreB. While urease on the outside of the bacteria can affect the viscosity of the mucus gel (Celli *et al.* 2009), it may be the intracellular urease that maintains a stable neutral pH inside the bacterium when subjected to the low pH of the gastric lumen and outer mucus layer (Stingl *et al.* 2002). The survival at low pH is also promoted by the generation of ammonia by the aliphatic amidases AmiE and AmiF (Bury-Mone *et al.* 2003). The extracellular part of flagella is also composed of two subunits, FlaA and FlaB, of which FlaA is the major one. The flagellar motor and assembly proteins are situated intracellularly. Unlike flagella from other bacteria, *H. pylori* flagella evade recognition by Toll-like receptor 5 and have no role in immune system activation, thus contributing to a persistent colonization (Gewirtz *et al.* 2004).

The duodenal ulcer-promoting gene A (*dupA*) is, as its name suggests, associated with duodenal ulcer disease. But its importance for disease varies in different populations, which may be explained by genome plasticity and the presence or absence of genes clustered with *dupA* (Jung *et al.* 2012). Gastric mucosal IL-8 levels were significantly higher in individuals having strains positive for the *dupA* gene cluster.

Cholesteryl α-glucosyltransferase (αCgT), whose function is inhibited by α1,4-GlcNAc, has recently been identified as a virulence factor (Ito *et al.* 2013). The activity of αCgT from clinical isolates is highly associated with the degree of gastric atrophy, which is suggested to result from the recognition of cholesteryl α-glucosides by invariant natural killer T (iNKT) cells. Mice deficient in iNKT cells showed a higher bacterial load and decreased Th1 and Th2 characteristic cytokine production, compared to wild-type mice, when infected with *H. pylori* with a highly active αCgT (Ito *et al.* 2013).

IceA (induced by contact with epithelia) is also regarded as a virulence factor, although its function is unknown. *H. pylori* strains can carry one or both of the two main alleles, *iceA1* and *iceA2*. As the name suggests, transcription of *iceA* can be

induced by adherence to gastric epithelial cells in vitro (Peek *et al.* 1998; Donahue *et al.* 2000). A meta-analysis performed in 2012 concluded that *iceA1* and *iceA2* were associated with duodenal ulcers by a higher prevalence of *iceA1* and a lower prevalence of *iceA2* than controls, but they were not associated with gastric ulcer or cancer (Shiota *et al.* 2012). Most studies (12/15) show no association of *iceA* and *cagA* status, but some studies have shown that *iceA1* is correlated with mucosal IL-8 levels (Peek *et al.* 1998; Donahue *et al.* 2000).

Gene regulation in *H. pylori*

The regulation of gene expression in *H. pylori* can occur via interaction of transcription factors, changes in DNA arrangement and epigenetic modulation. In addition, regulation of translation can occur via mRNA decay and anti-sense RNA (Akada *et al.* 2000; Sharma *et al.* 2010). Compared to many other gram-negative bacteria, relatively few regulatory functions have been characterized in *H. pylori* (Vries *et al.* 2001). In most cases either the precise mechanism of regulation or the stimuli causing it is unclear.

Several two-component systems have been identified in *H. pylori* that sense environmental stimuli and respond thereafter. The two-component systems can regulate *H. pylori* behavior by direct effects on proteins, as seen for CheA/CheY, or by regulation of gene expression. ArsR/ArsS is an acid-responsive two-component system that can regulate gene expression of many factors involved in surviving in low pH, such as amidase and urease (Pflock *et al.* 2006). In addition, ArsR/ArsS can affect gene expression at neutral pH and can also regulate proteins involved in motility, oxidative stress and other functions (Loh *et al.* 2010). SabA as well as many flagella proteins are downregulated by ArsS both in acid and neutral pH (Forsyth *et al.* 2002; Loh *et al.* 2010). ArsR/ArsS may thus be involved in adhesion and motility within mucus. ArsS can be expressed in different isoforms, which may contribute to the complexity of its regulatory mechanisms (Hallinger *et al.* 2012).

The ferric uptake regulator (Fur) is also involved in acid-resistance, but is mostly associated with the control of iron homeostasis (Pich and Merrell 2013). Iron forms a dimer with Fur, which then binds to promoters and blocks gene transcription. Iron restriction has been shown to induce expression of several outer membrane proteins and VacA (Worst *et al.* 1995; Szczebara *et al.* 1999). Other metal ions can also act as cofactors for Fur, for example the interaction of nickel with Fur is involved in regulation of urease expression. Fur has recently been shown to induce *cagA* and reduce *ggt* expression (Pich *et al.* 2012), and is shown to be involved in the upregulation of both *cagA* and *vacA* in response to host cell contact (Raghwan and Chowdhury 2013).

The expression of *babA*, *sabA*, *oipA*, *hopZ*, LPS synthesis genes and others can be regulated by phase variation (Appelmelk *et al.* 1998; Backstrom *et al.* 2004; Salaun *et al.* 2004; Dossumbekova *et al.* 2006; Goodwin *et al.* 2008; Kennemann *et al.* 2012). Phase variation works as an on/off switch for expression by slipped strand mispairing between multiple nucleotide repeats situated upstream or within the gene sequences. It is regarded as a rapid mechanism of gene regulation in response to different microenvironments, but the stimuli that induce phase variation are largely unknown.

There are studies reporting correlation of gene or protein status among virulence factors. A correlation may indicate co-regulation of genes or that a certain stimuli, e.g. adhesion to mucins, pH, etc., cause a similar regulation pattern of genes. For example, a positive CagA-status has been shown to correlate well with BabA and OipA production (Odenbreit *et al.* 2009).

Detection and treatment of *H. pylori* infection

Several techniques can be used to detect *H. pylori* infection. The techniques vary in sensitivity and specificity, but the use of two or more techniques can validate results if needed. A simple and very common technique is the Urea Breath Test. It is based on the unique characteristics of *Helicobacter* spp. to produce urease. After 10-30 min after ingestion of carbon isotope labeled urea, the presence of the isotope in exhaled carbon dioxide is measured. The detection of isotopes indicates that the urea has been metabolized by *Helicobacter* urease. The breath test cannot distinguish between species of *Helicobacter* as all gastric *Helicobacter* spp. express urease (Haesebrouck *et al.* 2009). Culture from biopsies is specific, but can have lower sensitivity (Choi *et al.* 2012). Cultures can be used to characterize the infecting species or strain and test for antibiotic sensitivity. Histology from gastric and duodenal biopsies is regarded as the "gold standard" for detection of *H. pylori*. In addition, this method can detect inflammation and atrophy. Many patients also undergo endoscopy to evaluate the inflammation, which is then combined with collection of biopsies for histology and culture. *Helicobacter* antigens and antibodies can be measured and detected in blood and stool samples, but these tests cannot be reliable when evaluating the success of treatment, as antigens from dead bacteria and antibodies will be present even after treatment.

Treatment of *H. pylori* is made with a "triple therapy" consisting of two antibiotics and one proton pump inhibitor. The therapy is applied for two weeks and can be very unpleasant. *H. pylori* eradication in gastric ulcer patients reduces the risk of developing gastric cancer (Take *et al.* 2005), but *H. pylori* can be difficult to eradicate and antibiotic resistant strains exist and are becoming more common. *H.*

pylori present in the oral cavity is harder to eradicate than those in the stomach and can be a source for reinfection (Gebara *et al.* 2006; Jia *et al.* 2012). Dental plaque control has been associated with lower risk of reinfection (Jia *et al.* 2012). There are several ongoing research projects for the development of *Helicobacter* spp. vaccines. Promising results have been reported for immunization with whole cell lysate or single protein or combinations of, for example, recombinant BabA, HpaA, GGT and urease subunits (Bai *et al.* 2004; Flach *et al.* 2011) (Ihan *et al.* 2012; Vermoote *et al.* 2013).

1.4.2 *Helicobacter heilmannii* and *Helicobacter suis*

Helicobacter heilmannii has been used as the common name for all long spiral-shaped bacteria other than *H. pylori* found in the stomach. These were later divided into 2 taxa, type 1 and type 2. Type 1 was identified as the bacterium that is now known as *H. suis*, whereas type 2 comprises a group of different *Helicobacter* species found in the stomach of cats and dogs (Haesebrouck *et al.* 2009). Most cats and dogs have single or mixed infections with NHPH species, but with no clear association to disease (Van den Bulck *et al.* 2005). These type 2 *Helicobacter* species are referred to as *H. heilmannii* sensu latu (s.l.), to separate them from the recently characterized species *H. heilmannii* sensu strictu (s.s.) that was isolated from cat (Haesebrouck *et al.* 2011; Smet *et al.* 2012).

Helicobacter suis naturally infects pigs, and it has been reported that around 60% of pigs are infected at slaughter age (Choi *et al.* 2001; Park *et al.* 2004). The presence of spiral bacteria in pig stomachs was described in 1990 (Mendes *et al.* 1990; Queiroz *et al.* 1990), but *H. suis* was first isolated and identified in 2008 (Baele *et al.* 2008). *H. suis* can cause gastritis and ulceration of the non-glandular stomach and antrum in pigs, associated with a decrease in daily weight gain and sudden death (Ayles *et al.* 1996; Queiroz *et al.* 1996; Choi *et al.* 2001; De Bruyne *et al.* 2012). Therefore, in addition to causing pain in the pigs, this infection also results in economical losses for the pig industry.

Continuous contact with infected animals and consumption of undercooked meat are sources for human NHPH infection (Meining *et al.* 1998; Chung *et al.* 2013; De Cooman *et al.* 2013). Up to 6% of *Helicobacter*-infected human individuals with gastric disease are infected with an NHPH, and of these, infection with *H. suis* is most common (Haesebrouck *et al.* 2009). However, the exact proportion varies between studies with different detection methods. Three separate studies, which each examined around 100 biopsies from individuals with a suspected NHPH infection, detected *H. suis* in 14%, 37% and 80% respectively (Trebesius *et al.* 2001; De Groote *et al.* 2005; Van den Bulck *et al.* 2005). The symptoms of NHPH infection in

humans include chronic gastritis, peptic ulcers and MALT lymphoma, and they may also be associated with gastric adenocarcinoma (Morgner *et al.* 1995; Debongnie *et al.* 1998; Okiyama *et al.* 2005; Flahou *et al.* 2013).

The genome of two pig isolates of *H. suis* (strain HS1 and HS5) was sequenced in 2011 (Vermoote *et al.* 2011), and one *H. heilmannii* s.s. (strain ASB1) genome sequence was announced in 2013 (Smet *et al.* 2013). Both *H. heilmannii* s.s. and *H. suis* lack the widely studied virulence factors and adhesins CagA, BabA and SabA, which are commonly found in *H. pylori*, but they have genes with conserved VacA regions and they also have genes for other known *H. pylori* virulence factors, namely NapA, GGT and some of the type IV secretion system proteins. In addition, homologs for the *H. pylori* flavodoxin (FldA) and its electron donor, the pyruvate-oxidoreductase enzyme (POR) complex is present in *H. suis* and *H. heilmannii*. *H. pylori* FldA has been suggested to play a role in the pathogenesis of MALT lymphoma (Chang *et al.* 1999). *H. suis* is also positive for the putative adhesin HpaA and the genomes of both NHPHs code for several outer membrane proteins, of which some could be adhesins. Unlike many *H. pylori* stains, all *H. suis* strains tested (9/9) were able to persist in mice and elicited an immune response which differed from that of mouse-adapted *H. pylori* strains (Flahou *et al.* 2012). Different *H. heilmannii* s.s. strains showed variable success of colonizing Mongolian gerbils and there was a diversity in host immune responses to *H. heilmannii* s.s. strains compared to that of *H. pylori* (Joosten *et al.* 2013).

1.4.3 The Kalixanda Study

The Kalix Haparanda Upper Endoscopy Study (The Kalixanda Study) was a Swedish study performed between 1998-2001 with professor Lars Agréus as project leader (Aro *et al.* 2004). A random selection of 3000 individuals, aged 20-81, in Kalix and Haparanda were given a questionnaire addressing gastrointestinal symptoms, and a random subsample of 1001 individuals was examined by upper endoscopy and more extensive questionnaires. From each of these individuals, 22 biopsies were collected for histology and microbiology and blood samples were taken, including a serology test for *H. pylori*. The three main research lines of the study were epidemiology of symptoms and diseases of the upper gastrointestinal tract, epidemiology and history of lower esophageal findings in endoscopy, and *H. pylori* infection in non-patients. Of the 1001 examined individuals in the Kalixanda study one-third had an ongoing *H. pylori* infection and another 10% had signs of past infection (Storskrubb *et al.* 2005). Corpus-dominant gastritis was found mostly in individuals with ongoing infection, while corpus atrophy was found more often in individuals with past infection.

The Kalixanda study has also given rise to many spin-off projects, some still ongoing today. In a project by Lindberg *et al.* (unpublished, submitted manuscript) the presence of *Lactobacillus* spp. and relation to *H. pylori* infection was examined. 170 individuals from the Kalixanda study were included in this project, of which 59 returned for a second endoscopy four years later. Both *H. pylori* and *Lactobacillus* could be cultivated from 24% of the 170 individuals. The presence of either bacteria was more common in co-existence with the other than alone. In *H. pylori* positive individuals, there was no effect on inflammation due to the presence of *Lactobacillus* spp., but reduced mucus depletion was seen. In 4 of the 59 individuals returning for a second endoscopy, the same *Lactobacillus* strain was isolated. These 4 individuals were also *H. pylori* positive. This indicates that some *Lactobacillus* strains can colonize the stomach over long time periods and that *Lactobacillus* and *H. pylori* can co-exist in the same stomach. The four pairs of co-isolated *H. pylori* and *Lactobacillus* strains were used in Paper I in this thesis, which aimed to characterize the binding and proliferative response of these strains to purified mucins.

1.4.4 *Lactobacillus* spp.

Lactobacillus is a genus of gram-positive rod-shaped bacteria that are anaerobic with varying oxygen tolerance. They belong to the group of lactic acid bacteria, which all produce lactic acid through fermentation. *Lactobacillus* produce lactic acid from lactose and other sugars. In the mouth, *Lactobacillus* have been associated with dental caries (Badet and Thebaud 2008), but in the gastrointestinal tract they are mostly benign and are part of the commensal flora. *Lactobacillus* are found in most human individuals throughout the gastrointestinal tract (Andersson *et al.* 2008; Matsuda *et al.* 2009). Some *Lactobacillus* species are regarded as probiotic bacteria, which by definition confer a health benefit to the host, such as contributing to a healthy flora and inhibiting the growth of pathogens. In addition, the conversion of lactose can be beneficial for people who cannot break down lactose sufficiently. For these reasons, *Lactobacillus* spp. are commonly added to yoghurt and probiotic food. As *Lactobacillus* are ingested with food, it must be determined if they are true colonizers or just passing through. Longitudinal studies have found several species of *Lactobacillus* to colonize the stomach (Reuter 2001; Roos *et al.* 2005). However, when the stomach is infected with *H. pylori* the microbial community is dominated by the *H. pylori*, whereas other bacteria exist only in low abundance (Andersson *et al.* 2008).

Lactobacillus* and *H. pylori

The interaction between *Lactobacillus* and *H. pylori* has been examined in several studies, and there are promising reports on inhibiting effects of *Lactobacillus* spp. on

H. pylori colonization in vivo (Michetti *et al.* 1999; Felley and Michetti 2003; Cui *et al.* 2010; Lionetti *et al.* 2010). These effects have been suggested to result from competition of binding sites in the mucosa and from inhibition of *H. pylori* growth (Michetti *et al.* 1999; Sgouras *et al.* 2004; Uchida *et al.* 2006; Ryan *et al.* 2008). The production of bacteriocins, lactic acid and hydrogen peroxide has been proposed to account for anti-inflammatory and growth inhibitory effects on pathogenic bacteria by *Lactobacillus* supernatants (Bhatia *et al.* 1989; Michetti *et al.* 1999; Felley and Michetti 2003; Otero and Nader-Macias 2006; Corr *et al.* 2007).

***Lactobacillus* and mucins**

Several *Lactobacillus* species have been shown to adhere to blood antigens on human colonic mucins. The cell surface expressed *Lactobacillus* proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP-binding cassette (ABC) transporter and integral membrane protein-2 are examples of proteins thought to be involved in mucin binding (Kinoshita *et al.* 2008; Watanabe *et al.* 2010; Liu *et al.* 2011). Several bacteria expresses either two GAPDH proteins with separate functions or one GAPDH with dual functions; partly expressed in the cytoplasm where it acts as an enzyme and partly at the cell surface involved in adhesion (Fillinger *et al.* 2000; Dumke *et al.* 2011; Kinoshita *et al.* 2013). Additional *Lactobacillus* proteins are involved in binding to mucus, but their ligands within the mucus are unknown (Van Tassell and Miller 2011). It has also been shown that *Lactobacillus* spp. can induce upregulation of mucin gene expression (Mack *et al.* 2003) and that *Lactobacillus* species inhibits the mucus depletion detected in *H. pylori*-positive individuals (Pantoflickova *et al.* 2003). *L. rhamnosus GG* can use mucin-associated carbohydrates as a nutrient source, indicating that *Lactobacillus* spp. can cleave off these structures from mucins (Sanchez *et al.* 2010). In addition, biofilm cells of *L. fermentum* have enhanced proteolytic activity when grown on a MUC5B surface and can degrade this mucin (Wickstrom *et al.* 2013).

2 AIMS OF THE THESIS

General aim:

The overall aim of this thesis was to identify adhesion and responses of *Helicobacter* spp. to differently glycosylated mucins.

Specific aims:

Paper I:

- To investigate if *Lactobacillus* spp. found to colonize stomachs, could bind to gastric mucins.
- To investigate if binding ability and proliferation of co-isolated *H. pylori* were affected by the *Lactobacillus* spp.

Paper II and III:

- To identify and characterize the proliferative response of *H. pylori* to differently glycosylated mucins.
- To examine changes in gene and protein expression by *H. pylori* caused by mucins, and to identify factors that are involved in these changes.
- To study if gastric epithelial host cells were differently affected by *H. pylori* pre-cultured with different mucins.

Paper IV:

- To examine adhesion patterns of *H. suis* and *H. heilmannii* to differently glycosylated mucins from various host species, with or without experimental infection.

3 METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in the attached papers and manuscripts. Below follow explanations and discussions regarding the choice of methods.

Mucin isolation and characterization (Paper I, II, III and IV)

Mucins were isolated from isopycnic density centrifugation as previously described (Nordman *et al.* 1997). Some studies have used crude mucus extractions to analyze interaction with bacteria. These extractions contain a mix of proteins and were thus not appropriate for our aim of studying the specific interactions between bacteria and mucins. The centrifugation separates mucins from other proteins as they have a greater density because they are so heavily glycosylated. DNA is also a large molecule that sometimes co-localizes with mucins in the gradient fractions, but can be separated by a second density centrifugation step.

The glycan structures attached to the mucin backbone remain intact after isolation. We analyzed the presence of total carbohydrates and MUC5AC in all density gradient fractions. The fractions containing mucins were pooled and analyzed for relative abundance of the glycan structures Le^b, sialyl-Le^x, sialyl-Le^a and terminal α1,4-GlcNAc as well as the mucin subtypes MUC5AC, MUC6, MUC5B and MUC2 by enzyme-linked immunosorbent assay (ELISA). Mucin glycosylation was also analyzed by mass spectrometry, but these results are beyond the scope of this thesis and yet unpublished, except for the results of two of the samples (Kenny *et al.* 2012). The sample referred to as P10 Healthy in the papers attached to this thesis is claimed to be positive for Le^b in Paper II. That result is based on current ELISA analysis. However, this mucin sample has previously been reported as Le^b-negative (Lindén *et al.* 2002). Mass spectrometry results in Kenny *et al.* also indicate that this sample is negative for Le^b (Kenny *et al.* 2012). We thus present this mucin sample as Le^b-negative in subsequent manuscripts (Paper III and IV). The inconsistency is likely to be due to cross-reaction by the antibody used in Paper II.

Analysis of adhesion to mucins (Paper I, II, III and IV)

Microtiter-based methods were used for the analysis of bacterial binding to purified mucins. In Paper I and II we biotinylated bacteria and detected bacteria bound to coated mucins by the reaction between horse radish peroxidase-conjugated streptavidin and its substrate. The mild biotinylation did not affect the binding ability of *H. pylori*, and it is unlikely that potential effects on *Lactobacillus* spp. binding ability would totally hinder mucin binding. The reason for biotinylating the bacteria

was because antisera against bacteria often cross react with mucins, including several of the mucins used in this study. Later, we received a range of anti-*H. pylori* sera (kindly provided by Thomas Borén, Umeå University), whereof we found one that showed very low cross-reactivity with the mucins. This removed the need for biotinylation and excluded its possible effect on binding ability. This anti-*H. pylori* serum was found to also detect both *H. suis* and *H. heilmannii*.

Infection-dependent changes affecting adhesion in experimental infection of mice were investigated via binding of fluorescently labeled *H. heilmannii* to tissue sections. Thus, these results do not only reflect binding to mucins, but also binding to other potential ligands. In addition, it revealed the tissue location where most binding is possible. Similar changes in the infection-dependent binding pattern of *H. heilmannii* to mouse tissue as that of *H. suis* to purified pig mucins, indicated that the changes of binding to epithelial cells in tissue sections also reflected changes of mucin binding.

The binding to mucins were analyzed at a range of pH values and adhesion of *H. pylori* to mucins with charged structures (e.g. sialylated or sulphated) was highest at low pH. Binding of NHPH was low at neutral pH, but higher to both neutral and charged mucins at low pH. Proteins can denature at low pH, but the structure of the immobilized and heavily glycosylated domains of the mucin protein are likely unaffected. The *Helicobacter* spp. adhesins might be resistant to low environmental pH due to the secretion of urease by the bacteria. However, the response to low pH might change the conformation or expression of adhesins, or cause changes on the mucin glycan chains, enabling binding to neutral mucins at low pH. *Lactobacillus* spp. do not produce urease and their adhesins may thus be more sensitive to low pH. The lack of adhesion of *Lactobacillus* spp. also indicates that the low pH binding is specific for *Helicobacter* spp. and not just a result of nonspecific binding of denatured proteins.

Analysis of proliferation response to mucins (Paper I, II and III)

To analyze how proliferation of *H. pylori* was affected by mucins we added purified mucin to liquid cultures of *H. pylori*. Some studies have added mucins to the culture plates to study proliferation, but this would be less practical when handling several strains and mucin samples simultaneously. Also, if mucins are added in or on top of the agar, more of the samples would be required to reach the concentration obtained in wells of a 96-well plate, and this would not be practical as the amount of each sample obtained from the isolation is very limited. However, a drop test on agar plates was performed with one mucin sample, but the degree of inhibition exerted by this sample was too small to be detected by this method. Measures of optical density

(OD) of liquid cultures are more sensitive to detect both decreases and increases of bacterial proliferation.

OD is a widely used measure of bacterial growth, and has been used both by us and others previously with the interpretation that an increase in OD after bacterial culture with mucins corresponds to an increase in the number of bacteria (Kawakubo *et al.* 2004; Skoog *et al.* 2012). However, during the work of this thesis we discovered some inconsistencies between different methods of measuring bacterial proliferation for a subset of the samples. There are some issues with optical density measurements that have to be considered. Dead intact bacteria cannot be distinguished from live non-replicating bacteria. It is thus not possible by optical density measurement to determine if the mucins have a direct killing effect or if they merely prevent the bacteria from replicating, which might be temporal and reversed when the bacteria and mucin lose contact. To determine if the bacteria were alive or dead, we stained them after culturing with mucins with a live/dead stain, which has a component that only penetrates the damaged membranes of dead bacteria.

In addition, bacteria can form aggregates with added test substances, which can affect the optical density. Aggregation has previously been analyzed by a decrease in OD over time (Ericson *et al.* 1975). In contrast, we found that bacterial binding to mucins resulted in aggregation which caused a higher OD read. The increase in OD was reversed by mechanically disassociating the aggregates by pipetting. The aggregation was visible by eye as well as by microscopy of fluorescently labeled bacteria by live-dead staining. We complemented the analysis by assessing the metabolic activity of the bacteria as measured by adding alamarBlue®, which can be used as a measure of health status/viability (Ahmed *et al.* 1994). The results after addition of alamarBlue® showed the same trend as pipette-mixed cultures without alamarBlue®, and thus seems to accurately measure *H. pylori* proliferation.

The number of bacteria in a suspension is also commonly quantified by plating the bacteria and counting the colony forming units (CFU). This is also less practical when handling several strains and mucin samples simultaneously. We performed this for a subset of samples and found that the CFU count was in line with the optical density after pipette mixing. However, one major problem with conditions that cause bacteria to aggregate that could cause inaccurate results is if they are not fully dispersed prior to the plating for CFU, as several bacteria in one spot would appear as one colony.

In summary, both optical density and CFU counts as a measure of bacterial proliferation can be misleading when molecules that aggregate bacteria are present. Extrapolating metabolic activity, which can be measured in robust and convenient

96-well plate based assays, into viability and proliferation appears more accurate in this context although one cannot separate proliferation and viability from each other. Stains determining the number of live vs. dead cells can add additional information, but are more time consuming and impractical if large numbers of samples are to be compared.

Analysis of gene expression (Paper II and III)

The gene expression of *H. pylori* was analyzed by quantitative real-time polymerase chain reaction (PCR) and by quantitative mass spectrometry after 24 h culture in the presence of mucins. The responses in gene expression can be acute or long-term, which are both seen in the response to low pH (Marcus *et al.* 2012). We believe that the acute response to mucin binding is less important than the response to low pH as the changes in mucin environment may be slower and less critical than the changes in pH. Changes in *H. pylori* gene expression after adhesion to AGS cells can be seen already after 30 min, with some changes remaining stable and other changes increasing over a 24 h time period (Raghwan and Chowdhury 2013). Thus, 24 h was determined to be an appropriate time point to measure gene expression in the response to mucins, as long as the amount of mucins present were enough to meet an increased number of bacteria due to the 24 h proliferation. Our proteomic studies indicated that mucins were still bound to bacteria after 24 h as peptides from mucins were discovered by mass spectrometry. In addition, the viability of the bacteria can easily be measured after 24 h, whereas at earlier time points it is harder to discriminate if the bacteria are viable in the new environment and what effect the mucins have on proliferation. The 24 h time window also allows the bacteria to recover from the handling.

We normalized the real-time PCR expression data to the expression of 16S rRNA, which is regarded as a housekeeping gene and used as such in many studies of bacterial gene expression. However, the ribosomal content in bacterial cells can vary considerably dependent on growth phase (Vandecasteele *et al.* 2001). Another drawback is that the expression level may be much higher than the mRNA you want to study, making the system more sensitive to changes as small as those caused by technical differences between replicates etc. For human gene expression, normally a set of housekeeping genes is used simultaneously. There have been attempts to characterize housekeeping genes in bacteria (Savli *et al.* 2003; Gil *et al.* 2004), but determination and development of how to calculate gene expression using a standardized set of housekeeping genes are needed.

4 RESULTS AND DISCUSSION

4.1 No role of gastric colonization of *Lactobacillus* spp. for mucin binding and proliferation of co-isolated *H. pylori* (Paper I)

Lactobacillus spp. normally colonizes the intestine but have recently been discovered to also colonize the human stomach (Roos *et al.* 2005). Infection by *H. pylori* was shown to be more common in stomachs also colonized with *Lactobacillus* (Lindberg *et al*. unpublished). We received four pairs of co-isolated *Lactobacillus* spp. and *H. pylori* from the Kalixanda study to investigate their interactions with mucins, with a focus on binding mechanisms. The *Lactobacillus* strains were one *L. gastricus*, two *L. fermentum* and one *L. gasseri*. Binding was analyzed by a microtiter-based assay where biotinylated bacteria bound to immobilized mucins were detected by horse radish peroxidase-coupled streptavidin. Mucins used in the assay were isolated from two human gastric tissue specimens. One specimen was from a full gastric wall of antrum from a healthy part of a gastric cancer affected stomach. Mucins from this specimen represented mucins from healthy gastric tissue with functional binding sites for the *H. pylori* blood group binding adhesin BabA. The other specimen was from gastric tumor (intestinal type) to represent mucins with functional binding sites for the *H. pylori* sialic acid binding adhesin SabA. The *Lactobacillus* strains did not bind to Le^b, sialyl-Le^x or any of the glycan structures Le^a, Le^y, GlcNAc, blood group A or B, H-type-1, or H-type-2, which were all present in either one or both mucin samples, at pH 7.5 (Table 1). *H. pylori* can also use a charge/low pH-dependent mechanism to bind to charged structures at low pH, which can be examined by analyzing binding to DNA at low pH. The binding of *Lactobacillus* strains at pH 2.5 to both mucin ligands and DNA was negligible compared to that of *H. pylori* (Table 1). Thus the *Lactobacillus* strains are not likely to compete with *H. pylori* for binding sites on the mucins either at neutral or acidic pH. Theoretically, another way *Lactobacillus* could inhibit *H. pylori* in the stomach would be to cause *H. pylori* to change its mode of attachment. The *babA* gene is highly variable and a modification of the gene and/or Le^b binding affinity has been shown to occur in experimental infection in animal models (Solnick *et al.* 2004; Styer *et al.* 2010). In 27% of clinical *H. pylori* isolates the *babA* gene is absent, whereas only 46% of the isolates show binding to Le^b (Colbeck *et al.* 2006). We found that the presence of the studied strains of *Lactobacillus* in the stomach did not cause *H. pylori* to change its mode of attachment, as 3 of the 4 co-isolated *H. pylori* strains bound to fucosylated and sialylated mucins at neutral and acidic pH (Table 1), similar to other previously characterized *H. pylori* strains.

It has previously been shown that *L. rhamnosus* GG can use mucin-associated carbohydrates as a nutrient source (Sanchez *et al.* 2010). *Lactobacillus* spp. may thus be able to cleave off carbohydrate structures that might have a role in *H. pylori* binding or proliferation. The growth of bacteria in liquid cultures supplemented with the tumor mucin sample was analyzed. Again the *Lactobacillus* strains showed no interaction with the mucins, i.e. the proliferation was not affected by the mucins. The *Lactobacillus* strains used here probably do not use mucin-associated carbohydrates as a nutrient source, as the proliferation would most likely have been stimulated when more nutrients were added. The *Lactobacillus* strains, live, dead or supernatant thereof, did not have a general effect on *H. pylori* proliferation in brain heart infusion media. Dead bacteria of one of the *L. fermentum* strains slightly inhibited proliferation of the co-isolated *H. pylori*. In the co-culture with *H. pylori* and live bacteria of the other *L. fermentum* strain there was a reduction in the optical density, presumably caused by reduced proliferation of one or both bacteria or a low level aggregation between the bacteria.

Strain	Binding to fucosylated mucin at pH 7.5	Binding to fucosylated mucin at pH 2.5	Binding to sialylated and fucosylated mucin at pH 7.5	Binding to sialylated and fucosylated mucin at pH 2.5	Binding to DNA at pH 2.5	Proliferative response to mucins
H. pylori HP 201	Yes	Yes	Yes	Yes	Yes	Yes
H. pylori HP 364	No	Yes	No	Yes	Yes	No
H. pylori HP 1008	Yes	Yes	Yes	Yes	Yes	-
H. pylori HP 1172	Yes	Yes	Yes	Yes	Yes	Yes
L. gastricus LB 201	No	No	No	No	No	No
L. fermentum LB 364	No	No	No	No	No	No
L. fermentum LB 1008	No	No	No	No	No	No
L. gasseri LB 1172	No	No	No	No	No	No

Table 1. Co-isolated strains of H. pylori and Lactobacillus spp. interactions with mucins: binding to sialylated and/or fucosylated mucins at pH 2.5 and and pH7.5, binding to DNA at pH 2.5 and proliferative response to mucins in liquid cultures. (–not determined)

In summary, we did not detect a general mechanism for how gastric *Lactobacillus* may affect *H. pylori*, although some strains may be able to inhibit *H. pylori* proliferation. That the presence of either bacteria was more common in co-existence with the other than alone, however, indicates that colonization by both of them may be mutually beneficial to each other. Alternatively, some individual hosts could provide a gastric environment that benefits colonization of both *Lactobacillus* and *H. pylori*, dependent on such as the variable glycosylation of mucins. Interestingly, it takes longer for germfree mice than for specific pathogen-free mice to develop symptoms upon *H. pylori* infection (Lofgren *et al.* 2011). The underlying mechanism for the increased pathogenicity is unknown, but can be due to the difference in environment caused by the microbiota, such as the altered expression and glycosylation of mucins.

4.2 Proliferation of *H. pylori* in the presence of mucins is dependent on mucin glycosylation and adhesion (Paper I, II and III)

Mucins are major components of the mucus layer that *H. pylori* colonizes. Differences in mucin glycosylation in health and disease as well as between individuals provide variations in the environment, which affects the presentation of ligands for *H. pylori*. Mucins can also affect the growth of bacteria. For example, terminal α1,4-GlcNAc that can be found on stomach gland mucins inhibits the growth of *H. pylori* (Kawakubo *et al.* 2004). We have studied how differently glycosylated mucins affect *H. pylori* proliferation. In line with previous findings, mucins derived from antrum glands of 3 individuals inhibited the proliferation of *H. pylori*. In contrast, mucins from fundus glands, isolated from one individual, did not inhibit growth, although it contained similar levels of terminal α1,4-GlcNAc as the antrum gland mucins from the same individual. In addition, mucins derived from the full gastric wall of healthy stomachs inhibited the growth of *H. pylori* although they did not contain terminal α1,4-GlcNAc. Thus additional glycan structures other than terminal α1,4-GlcNAc may be able to inhibit *H. pylori* proliferation. The dense glycosylation of mucins may physically hinder bacterial contact with neighboring glycan structures. This might be an explanation for the lack of growth inhibition by the fundus derived mucin sample.

H. pylori responses to the mucin sample P1 TS, which is derived from gastric tumor tissue, was extensively studied in Paper I-III (referred to as tumor mucin in Paper I). We concluded that *H. pylori* viability (measured as metabolic activity) was decreased by the P1 TS mucins, and that the increased OD_{560} values seen in Paper I and II was

misleading due to the heavy aggregation formed in these cultures. Similar results were obtained for cultures of *H. pylori* with Le^b and Sle^x glycoconjugates. The viability and OD_{560} were only affected for *H. pylori* strains with functional BabA and SabA adhesins and there was no effect on proliferation of *Lactobacillus* spp. by the P1 TS mucins sample. Thus, an inhibition of proliferation was not due to general anti-microbial functions, but was dependent on functional binding to the mucin sample. The level of binding to the P1 TS mucin sample was relatively low, when analyzed to coated mucins in microtiter plates, but the degree of aggregation in liquid media was highest with this sample, indicating different binding abilities to immobilized and soluble states of the mucin. The decreased proliferation may be a response of binding via BabA and SabA or a result of physical hindrance due to the binding and aggregation of bacteria. In contrast, Jiang *et al*. (2000) hypothesize that mucin binding and aggregation increase *H. pylori* proliferation due to protection from the surrounding environment (Jiang and Doyle 2000). Both arguments can be true depending on the environment. In a good environment, aggregation might hinder proliferation, while in a hostile environment as found in the gastric lumen, aggregation might protect the bacteria.

Some mucin samples were able to considerably increase the optical density with much less aggregation than to the P1 TS mucins and Le^b-glycoconjugate. These samples may stimulate proliferation. This stimulation of proliferation seems also to be dependent on mucin binding, as there was little effect on J99Δ*babA*Δ*sabA*. Previous studies have reported stimulation of *H. pylori* proliferation by commercial porcine gastric mucins, as measured by optical density in combination with viability assays or CFU counts (Jiang and Doyle 2000; Cole *et al.* 2004). Another study showed increased OD in cultures with mucins from human gastric surface mucosa, but the presence of aggregates is not mentioned (Kawakubo *et al.* 2004).

If and how proliferation of *H. pylori* can be stimulated by human gastric mucins remains unclear. However, there appears to be several mechanisms for how mucins can inhibit *H. pylori* proliferation. We confirmed that mucins with terminal α1,4-GlcNAc can inhibit proliferation, but suggest that surrounding glycans can counteract or sterically hinder the effects of growth modulating structures. Our results also imply that adhesion to mucins slow down proliferation. Mucins that bind *H. pylori* can thus inhibit growth concurrently with the bacteria being washed away with the shedding mucus, increasing the efficiency of this host response. Additionally, we observed a reduction in proliferation in response to some mucins despite low *H. pylori* binding and the absence of terminal α1,4-GlcNAc, indicating that other glycan structures can also be inhibitory to growth. In conclusion, mucins can inhibit *H. pylori* proliferation in several ways, contributing to the host response against this pathogen. Mucins with different glycosylation have variable ability in modulating *H.*

pylori growth, and thus the glycan-mediated defense inhibiting colonization can vary between individuals and be involved in determining the susceptibility and outcome of infection.

4.3 Gene and protein expression in *H. pylori* are regulated by mucins (Paper II and III)

Paper II was the first study showing that gene expression in *H. pylori* can vary in the presence of mucins from different individuals. By real-time PCR we showed that expression of *babA*, *sabA*, *cagA*, *flaA* and *ureA* were increased by some of the mucins. There was a correlation between the expression of *babA*, *sabA*, *cagA* and between *flaA* and *ureA* indicating co-regulation of genes in the response to mucins. We also reported that the *babA* and *ureA* expression correlated negatively to proliferation levels and that *cagA* expression correlated negatively to OD_{560} and binding levels. In this publication, we interpreted the changes in the OD_{560} value as reflecting the proliferation. However, as we later found that the increase in OD_{560} does not always represent an increase in proliferation, our interpretation that the gene expression of these genes are downregulated concurrently with an increasing proliferation may not be accurate. In contrast, the *cagA* expression was dependent on mucin binding, probably via SabA, as the increase in *cagA* expression was seen also when culturing *H. pylori* in the presence of SLe^x-glycoconjugate and was lacking when *sabA* was deleted (Figure 3A and B). There was no upregulation of *cagA* in the presence of Le^b-glycoconjugate and the impact of *babA* deletion is so far preliminary and unclear. However, the lack of response of J99Δ*babA*Δ*sabA* to mucins indicate that the mucin regulation of all genes examined is dependent on binding, either by direct interaction between adhesins and their ligands or by enabling closer contact to other glycan structures.

Preliminary data show that the *cagA* upregulation in response to mucins is absent in *fur* deletion mutants (Figure 3B). This is in line with the result of a previous study showing that binding to cells upregulated *cagA* expression via a Fur-dependent pathway (Raghwan and Chowdhury 2013). Thus, binding via adhesins such as BabA or SabA to both cells and mucins may result in Fur-dependent upregulation of *cagA*. In addition, ArsS may also play a role in gene regulation in response to mucins. ArsS is known to repress the expression of *sabA* and we have found that binding of J99Δ*arsS* is increased to SLe^x but decreased to Le^b. However, the deletion of *arsS* had no effect on *babA* expression. Similarly, J99Δ*babA* and J99Δ*sabA* showed higher binding to SLe^x and Le^b respectively, but no change in expression of the respective adhesin not deleted has been detected. Instead, these results may be due to changes in steric hindrance when competition of space on the bacterial outer

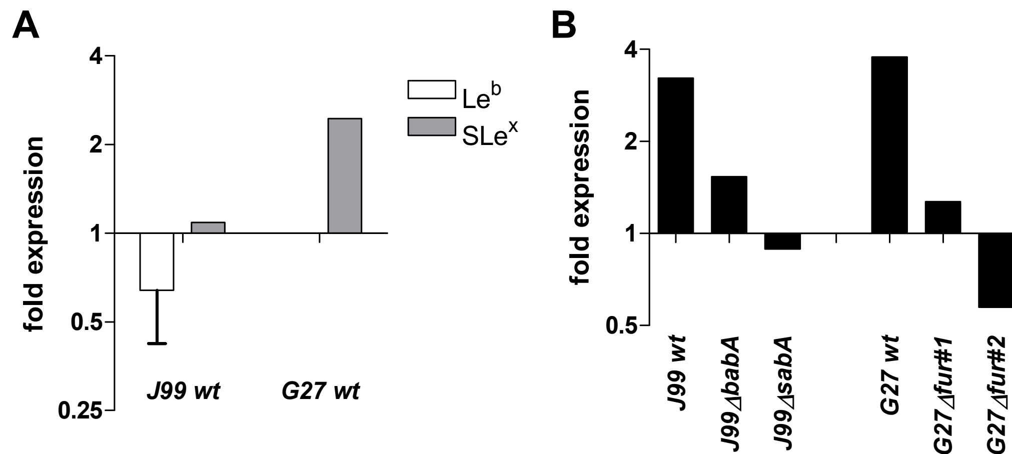

Figure 3. Expression of cagA *in* H. pylori *cultured for 24 h with mucins or glycoconjugates. A) J99 wt and G27 wt in response to* Le^b *and* SLe^x *HSA-glycoconjugates, and B) J99 wt, Δ*babA*, Δ*sabA *and G27 wt, Δ*fur*#1, Δ*fur*#2 in response to P1 TS mucin sample (positive for* Le^b*,* SLe^x *and* SLe^a*).*

membrane is skewed when the expression of one of the adhesins is changed. The regulation of adhesion expression and binding ability by ArsS can thus interfere with mucin interaction and response of *H. pylori*.

The proteome of *H. pylori* cultured with 9 different mucins was studied using quantitative mass spectrometry with chemical labeling (iTRAQ). Of the around 600 proteins detected, 55 were significantly changed in abundance, but peptides of low abundant proteins like BabA and SabA were not detected. No *H. pylori* protein was found to change in abundance similarly in response to all the different mucin samples. This indicates that changes in *H. pylori* protein expression vary with different glycosylation of the mucins that they are cultured with. However, in response to many of the mucin samples, and similar to the real-time PCR results, an upregulation, or a tendency thereof, of FlaA expression was seen, highlighting the importance of motility through the mucus layer. The motility in combination with resistance to low pH is essential for *H. pylori* colonization, and inhibiting *H. pylori* acid resistance in the outer mucus layer near the stomach lumen would be a good host defense to decrease colonization ability. All three samples from cultures with mucins isolated from healthy stomachs and one sample from a diseased stomach had a lower abundance of a urease accessory protein detected. In addition, a tendency for lower abundance of AmiE was detected with all mucins. But the expression of urease subunits (UreA/B) were unaffected or even increased, as detected by proteomic and real-time PCR analysis. The tumor-derived mucin sample P1 TS was the only one where there was an increased abundance of several members of a protein complex involved in protein secretion and trafficking to membranes (the Sec-complex). This

complex is suggested to take part in CagA and VacA secretion (Kim *et al.* 2012). In addition, many other virulence and colonization factors are secreted or membrane-bound, and a regulation affecting their spatial expression would have great impact on pathogenicity.

In summary, we show that differently glycosylated mucins have variable effects on *H. pylori* gene expression, most likely leading to modulations of virulence. It has previously been shown that mucins and associated glycans can regulate virulence of other bacteria. MUC2 affects expression of genes with various functions including colonization and pathogenicity in the intestinal pathogen *Campylobacter jejuni* (Tu *et al.* 2008), and the sensing of fucose by enterohemorrhagic *Escherichia coli* can regulate genes for both virulence and metabolism (Pacheco *et al.* 2012). Thus, mucins can act as a cue for bacteria to modulate their gene expression and adapt to the environment, and the presence of specific glycan structures may have great influence on the virulence of pathogenic bacteria.

4.4 Host cell responses and adhesion to host cells varies for *H. pylori* pre-cultured with mucins (Paper II)

As mucin-associated ligands for *H. pylori* can be differently abundant and presented, mucins are likely more or less competent in preventing *H. pylori* binding to epithelial cells. We hypothesized that mucins would also interfere with the host response to *H. pylori* due to the ability of mucins in modulating *H. pylori* viability and gene expression. Infection of confluent gastric MKN7 cells, mimicking the gastric epithelial surface cell layer, demonstrated that *H. pylori* J99 bound to the host cells, reduced their viability and induced apoptosis as well as the secretion of IL-6 and IL-8. *H. pylori* J99 co-cultured for 24 h with different concentrations of the P1 TS mucin sample showed a concentration-dependent reduction in binding, protection of host cell viability and reduced induction of apoptosis, but an increased production of IL-6 and IL-8. There was variable ability of other mucins to inhibit *H. pylori* binding and to maintain viability, which partly reflected the ability of mucins to bind the bacteria. However, some mucin samples, to which *H. pylori* had low binding ability, also protected the host cells, indicating a role for the inhibition of *H. pylori* viability exerted by these mucins. Co-culture of *H. pylori* J99 with SLe^x prior to the infection had no effect, whereas co-culture with Le^b also inhibited binding, but not the induction of apoptosis or cytokine production. An effect on the host cell response in cytokine production was not detected for most samples, and was not associated with any other parameters tested. The absence of a correlation for all samples between the

degree of binding and induction of apoptosis and cytokine production indicates that the cells also can respond to unbound bacteria, but then without a major decrease in viability. As demonstrated in Paper III, upregulation of *H. pylori cagA* expression was induced by the mucin sample P1 TS, but not by Le^{b}, and only marginally by SLe^{x}. We also show in Paper II that other genes were differentially expressed in the presence of these mucins, and there may likely be changes in genes not identified in our studies. Thus, *H. pylori* treated with an increasing concentration of this mucin sample, but not with Le^{b} and SLe^{x}, may be more virulent, causing an increase in host cytokine production independent of the number of bound bacteria.

4.5 *H. suis* and *H. heilmannii* can adhere to gastric mucins via glycan structures that are modulated by infection (Paper IV)

The *H. pylori* adhesins BabA and SabA are crucial for the ability to bind to mucins (Lindén *et al.* 2002; Linden *et al.* 2008). The closely related *H. suis* and *H. heilmannii* s.s., both capable of infecting humans, are negative for *babA* and *sabA* and it is unknown if and how they bind to mucins and other host structures. In Paper IV, we examined the mucin binding of two *H. suis* strains isolated from pig and two *H. heilmannii* s.s. strains isolated from cat.

First, we verified that there was no binding to Le^{b} or SLe^{x} of any of the tested strains, demonstrating that they do not have BabA, SabA or any other homologous adhesin capable of binding to these structures. We then examined their ability to adhere to a range of mucins isolated from human and animal hosts with differing health status, to present a large range of glycosylated structures. All *H. suis* and *H. heilmannii* s.s. strains bound to human and monkey gastric mucins with a binding pattern similar to each other. The binding was highest at pH 2, whereas there was almost no binding at pH 7 and at pH 5. Even at pH 2 there was no binding of *H. suis* and *H. heilmannii* s.s. to some mucins that *H. pylori* otherwise have higher binding to at low pH, and overall the binding was much lower than that of *H. pylori* (Figure 4). The highest binding of all non-*H. pylori Helicobacter* strains was to a mucin sample with glycosylation diverging from other samples in that it contained higher amounts of terminal N-acetylgalactosamineβ1-4N-acetylglucosamine (LacdiNAc) (Figure 4). This structure is involved in termination of glycan side chains, which has been suggested to prevent more complex glycosylation (Kenny *et al.* 2012). The availability of less complex glycan structures may be beneficial for pathogen adhesion, as there then would be less steric hindrance to interfere with binding of other possible ligands. Alternatively, LacdiNAc might be a ligand itself that

Helicobacter spp. can bind to at low pH. The binding of *H. pylori* to mucins at low pH is less dependent on BabA and SabA, but more dependent on the charge/low pH-dependent binding mechanism (Linden *et al.* 2008). By analyzing binding to DNA at low pH we found that *H. suis* and *H. heilmannii* s.s. can use the charge/low pH-dependent binding mechanism, but to a lesser extent than *H. pylori*.

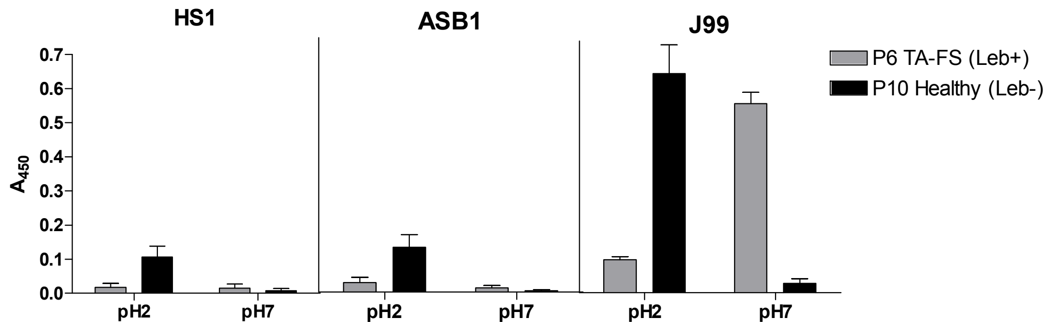

Figure 4. Binding of H. suis *(HS1),* H. heilmannii *(ASB1) and* H. pylori *(J99) to a* Le^b*-positive mucin derived from human fundus surface mucosa (grey) and a* Le^b*-negative mucin with high amounts of LacdiNAc derived from human antrum surface+gland mucosa (black).*

The binding level differed to mucins from different individuals and between species. The highest degree of *H. suis* binding was to some of the pig mucins, which were almost 5 times higher than to human and monkey mucins. *H. heilmannii* s.s. showed more binding to cat mucosal surface epithelium than to that of mice. The overlay of *H. heilmannii* s.s to gastric tissues demonstrated binding to components in the mucus, at the surface of epithelial cells and in the extracellular matrix in the submucosa. All of these locations contain molecules that previous studies have suggested to be ligands for *H. pylori*.

There was higher binding of *H. suis* to mucins from uninfected pigs than to mucins from pigs with 4 weeks experimental *H. suis* infection. Likewise, *H. heilmannii* s.s binding to the mucosal surface epithelium of uninfected mouse gastric tissue was more pronounced than to that of mice with 4 weeks experimental *H. heilmannii* s.s. infection. The binding to mucins and epithelial tissue of surface mucosa was higher than the binding to mucins and epithelial tissue of gland mucosa, whereas the opposite was found in specimens from infected tissue for both *H. heilmannii* s.s. and *H. suis*. Mucins from infected pigs were found to have more charged structures (e.g. sialylated and sulphated) than those from uninfected pigs. This demonstrates that infection with these NHPH induced changes in mucin and tissue glycosylation affecting the adhesion pattern of the pathogen, which has similarly been reported to occur in *H. pylori* infection. In addition, it proves that both *H. heilmannii* s.s. and *H.*

suis can bind to mucins by additional mechanisms other than the charge/low pH-dependent mechanism, as the binding was higher to the less charged mucins and tissue of uninfected individuals.

In summary, we show that *H. suis* and *H. heilmannii* s.s. can bind to mucins and that the binding was highest at low pH. The binding was mediated by an unknown mechanism which is affected by infection-induced changes such as the differential glycosylation of mucins. In addition, the adhesion pattern of *H. heilmannii* s.s. to gastric tissue slides indicated on ability to bind to other structures in the tissue. There might also be adhesin-ligand interactions between mucins and *Helicobacter* spp. that not has been characterized yet and the genome sequence of *H. heilmannii* s.s. and *H. suis* suggests the presence of several outer membrane proteins, that may act as adhesins (Vermoote *et al.* 2011; Smet *et al.* 2013). However, BabA and SabA-positive *H. pylori* appears to have much higher binding affinity, or more targets, to human and monkey mucins at neutral pH compared to *H. heilmannii* s.s. and *H. suis*. *H. pylori* is also superior to *H. suis* and *H. heilmannii* s.s. in binding to human mucins at low pH. This might reflect host species preferences for different *Helicobacter* spp.

5 CONCLUDING REMARKS

The topic that was the focus of this thesis is how the gastric pathogen *H. pylori* responded to differently glycosylated mucins, with an emphasis on proliferation and gene expression. In addition, we have analyzed mucin interactions of two non-*H. pylori Helicobacters* capable of infecting humans, as well as *Lactobacillus* spp. isolated from *H. pylori* infected stomachs. Our main findings were that:

- Proliferation of *H. pylori* can be inhibited by mucins containing the previously characterized antimicrobial glycan structure terminal α1,4-GlcNAc, but also additional glycan structures, and by forming aggregates with the bacteria due to mucin binding.

- Mucins regulate *H. pylori* gene expression, and different mucin glycosylation can cause variations in expression.

- Interaction with mucins results in upregulation of CagA, probably via Fur-mediated regulation following binding of SLe^x to SabA.

- Differently glycosylated mucins can modulate *H. pylori* interactions with epithelial host cells, resulting in variable pathogenicity.

- *H. suis* and *H. heilmannii* can bind to mucins, and this binding is modulated by infection. Their binding ability to human mucins are, however, much lower than that of *H. pylori*.

- Gastric *Lactobacillus* spp. could not compete with, or modulate, *H. pylori* mucin binding, and showed limited effect on *H. pylori* proliferation.

We have performed the first studies investigating changes in the expression of several virulence factors in response to purified human mucins from different individuals. Our conclusion is that different glycosylation of mucins have affects on *H. pylori* that can play a role in its virulence. The mucin glycosylation can also affect the adhesion and proliferation of *H. pylori*. It was already known that *H. pylori* can influence the host with the result of both temporary and more stable alterations in mucin glycosylation. In combination with this, our results demonstrate a bidirectional regulation of adhesion to mucins, as well as a dynamic interaction between the pathogen and its host that can influence the bacterial virulence and the host immune response.

H. pylori seem to have remarkably high binding ability to human gastric mucins in comparison to other bacteria, which may result from excellent adaptation to this host niche and be related to the fact that *H. pylori* is the dominant gastric bacteria present in infected individuals (Andersson *et al.* 2008). It may also be an explanation for the high number of human individuals infected with *H. pylori* rather than with other *Helicobacter* spp. (Haesebrouck *et al.* 2009). In line with previous studies, this suggests that binding to mucins is important for colonization of the gastric mucus layer. However, other *Helicobacter* spp. can also cause severe disease in humans, and a protective affect against *H. pylori* by the gastric microbiota cannot be ruled out.

The development of severe symptoms like ulcers and malignant disease is probably a result of imbalance of responses of host and pathogen to each other as well as of commensal bacteria and environmental risk factors. Our research contributes to the understanding of the host-pathogen interactions of *H. pylori*. The mucin glycan epitopes important for this interaction may eventually be exploited for screening for risk factors or for developing therapies to decrease colonization and risk of disease.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Denna avhandling behandlar olika perspektiv på interaktioner mellan *Helicobacter* spp. och muciner. Huvudsakligen studeras *Helicobacter pylori*, som infekterar magen hos ca 50% av jordens befolkning. Infektionen kan vara symptomfri eller resultera i magsår eller magcancer. *H. pylori* lever mestadels i magens slemlager som skyddar den underliggande vävnaden. Slemlagret är till stor del uppbyggt av muciner, vilka kontinuerligt utsöndras och tvättar vävnaden. Bakterien kan binda till kolhydratstrukturer som finns på dessa muciner. Kolhydratstrukturerna (glykosyleringen) samt uttrycket av muciner varierar mellan individer och ändras under infektionens gång och i utvecklingen av cancer. Genomgående i denna avhandling studerar vi bindningen av olika stammar och arter av *Helicobacter* till muciner från olika individer, med eller utan magcancer. Vi har också studerat vilka effekter olika muciner kan ha på bakteriernas tillväxt och genuttryck. I första delarbetet har vi studerat egenskaper hos *H. pylori* och *Lactobacillus* som för första gången funnits kolonisera samma magar och isolerats från dessa. Ingen av de tre arterna av *Lactobacillus* spp. som isolerats kunde binda muciner och dess tillväxt påverkades inte av muciner, medans de *H. pylori* som isolerats inte skiljde sig från tidigare karakteriserade stammar i bindningskapacitet eller tillväxtrespons till muciner. I fjärde delarbetet har vi jämfört bindningen till muciner av *H. pylori*, *H. suis* och *H. heilmannii*. Alla dessa kan infektera människor, men de vanligaste värdarna för *H. suis* och *H. heilmannii* är grisar respektive hundar och katter. Dessa Non- *H. pylori Helicobacters* (NHPH) har inte de kända adhesinerna som *H. pylori* använder för att binda till muciner. Vi upptäckte att dessa NHPH kan binda till muciner, men att bindningsmönstret mestadels skiljer sig från *H. pylori*, och att de binder bäst till både isolerade muciner och fixerad vävnad vid lågt pH. I andra och tredje delarbetet har vi grundligt undersökt hur både tillväxt och gen/protein-uttryck av *H. pylori* påverkas av olika muciner. Vi har också relaterat detta till hur muciner påverkar dess virulens då de infekterar celler in vitro. Tillväxten av bakterier visades bero på bindningen till mucinerna men också på kända och okända tillväxthämmande och stimulerande faktorer. Vi visar som första studie att gen- och proteinuttryck för *H. pylori* påverkas av muciner, samt påverkas olika av muciner från olika individer. Vi har också identifierat faktorer hos *H. pylori* som påverkar dess respons till muciner. Tillsammans visar dessa studier att muciners moduleringar kan påverka bindning, tillväxt och virulens av *H. pylori.* Variationen av mucinglykosylering mellan individer kan vara en orsak till varierande mottaglighet för infektionen samt påverka vilka symptom som infektionen resulterar i.

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