Helicobacter pylori
outer membrane vesicles
and the host-pathogen interaction

Annelie Olofsson
Till min mormor och farfar
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ABSTRACT

The gastric pathogen Helicobacter pylori chronically infects the stomachs of more than half of the world’s population. Even though the majority of infected individuals remain asymptomatic, 10–20% develop peptic ulcer disease and 1–2% develop gastric cancer. The ability to colonize the harsh environment of the stomach and to persist for decades in the face of both innate and adaptive immune responses makes H. pylori a remarkably successful pathogen. In my thesis I have characterized the membrane vesicles that are released from H. pylori and studied the consequences of vesicle and host cell interactions.

Vesicles are released by Gram-negative bacteria as part of their normal growth cycle and these vesicles consist of outer membrane components and substances from the periplasmic space. I have characterized the major protein and phospholipid components of H. pylori vesicles through mass spectrometry (MS) and nuclear magnetic resonance (NMR). A new technique, semiconstant-time 2D $^{31}$P,$^{1}$H COSY NMR, was developed for analyzing phospholipids in complex mixtures using H. pylori as a model system. Several of the known virulence factors of H. pylori were found to be associated with the vesicles, for example, the blood group antigen binding adhesin, BabA, and the sialic acid binding adhesin, SabA. The vesicle-associated BabA and SabA adhesins were found to mediate specific binding to their cognate receptors, ABO and Lewis b (Le$b$) blood group antigens and sialyl Lewis x/a antigens (sLe$x/a$), respectively, that are present in the human gastric mucosa.

To study the cellular uptake pathways of H. pylori vesicles, we developed a system to specifically detect internalized vesicles. We showed that H. pylori vesicles can enter host cells through clathrin-mediated endocytosis, and that cholesterol is important for vesicle internalization. The oncoprotein CagA has previously been shown to be translocated to host cells through T4S systems and to subsequently interfere with host cell signaling pathways and cellular functions. We found that CagA was associated with H. pylori vesicles and that vesicles localize to junction areas and induce host cell signaling. In summary, my thesis describes the role of H. pylori vesicles in adherence and the delivery of host effector molecules and the effects of the vesicles on host cell responses.
LIST OF PAPERS

Paper I

Biochemical and functional characterization of *Helicobacter pylori* vesicles


Molecular Microbiology, 2010, 77(6), 1539–1555

Paper II

Semiconstant-time P,H-COSY NMR: analysis of complex mixtures of phospholipids originating from *Helicobacter pylori*

Petzold, K., **Olofsson, A.,** Arnqvist, A., Gröbner, G., and Schleucher, J.

Journal of the American Chemical Society, 2009, 131(40), 14150–14151

Paper III

Endocytosis of *Helicobacter pylori* vesicles

**Olofsson, A., Nygård Skalman, L., Petzold, K., Schleucher, J., Gröbner, G., Lundmark, R., and Arnqvist, A.**

Manuscript

Paper IV

*Helicobacter pylori* vesicles and host cell responses


Manuscript
SAMMANFATTNING


Många bakterier och däribland *H. pylori* knoppar av små bläsor från cellytan. Bläsorna är små, mellan 20-300 nanometer i diameter och kallas vesiklar. Vesiklarnas yta liknar bakterieytan och därför har vesiklar och bakterier många gemensamma egenskaper. Hur vesiklarna bildas och vad det är som gör att de lossnar från bakterierna kan inte förklaras på molekylär nivå. Tittar man i vävnader där bakterier finns ser man också vesiklar men det är ännu relativt outforskat vilken roll bakteriers vesiklar spelar i den miljö som de finns i. Vesiklar som knoppas av från sjukdomsframkallande bakterier delar vissa virulenta egenskaper med bakterierna och påverkar celler i dess närhet. Den här avhandlingen handlar om vesiklar som lossnar från bakterien *H. pylori*.


En annan målsättning med den här avhandlingen var att studera om magsäckens epitcelceller tar upp *H. pylori* vesiklar och i så fall hur det går till. Vi utvecklade mikroskopibaserade metoder för att analysera upptag och fann att *H. pylori* vesiklarna kidnappar några av de vägar som cellen använder för att ta upp andra faktorer.

I det sista delarbetet i den här avhandlingen beskrivs några av de sätt som magsäckens epitcelceller reagerar i kontakt med *H. pylori* vesiklar. Vissa av signalvägarna som cellen använder för kommunikation påverkades och vi
såg tecken på att vesiklarna kan inducera reaktioner som är typiska då inflammation uppkommer. Vi såg också att vesiklarna påverkar hur magepitelcellerna sitter ihop med varandra och spekulerar att vesiklarna kanske kan passera mellan cellerna för att nå längre ned i vävnaden eller alternativt öppna upp en väg så att *H. pylori* bakterierna kan simma emellan cellerna.

Sammantaget bidrar resultaten i den här avhandlingen till mer kunskap om hur vesiklarnas komposition ser ut, vilka sjukdomsframkallande egenskaper som är associerade till vesiklarna men också att magsäckens celler påverkas av vesiklarna. Vi tittade främst på proteiner som är kopplade till sjukdomsutveckling men fann även proteiner som man sedan tidigare vet dämpar magepitelcellernas respons. Denna avhandling har även bidragit med kunskap om hur vesiklarna tas upp av magsäckens celler.
# ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>OMV</td>
<td>Outer membrane vesicles</td>
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<td>OMP</td>
<td>Outer membrane proteins</td>
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<tr>
<td>OM</td>
<td>Outer membrane</td>
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<tr>
<td>IM</td>
<td>Inner membrane</td>
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<tr>
<td>PL</td>
<td>Glycerophospholipids</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
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<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
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<tr>
<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
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<tr>
<td>CL</td>
<td>Cardiolipin</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PG</td>
<td>Phosphatidylglycerol</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>CG</td>
<td>Cholesteryl glycosides</td>
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<tr>
<td>LPE</td>
<td>Lysophosphatidylethanolamine</td>
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<tr>
<td>VacA</td>
<td>Vacuolating cytotoxin</td>
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<td>CagA</td>
<td>Cytotoxin associated gene A</td>
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<td>cag&lt;sup&gt;PAI&lt;/sup&gt;</td>
<td>cag pathogenicity island</td>
</tr>
<tr>
<td>T&lt;sub&gt;4SS&lt;/sub&gt;</td>
<td>Type IV secretion system</td>
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<td>BabA</td>
<td>Blood group binding adhesin</td>
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<td>SabA</td>
<td>Sialic acid binding adhesin</td>
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<tr>
<td>Le&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Lewis b</td>
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<tr>
<td>sLex&lt;sup&gt;x/a&lt;/sup&gt;</td>
<td>sialyl-Lewis x/a</td>
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**INTRODUCTION**

Microbes are an essential part of our environment. Humans represent a diverse microbial ecosystem, with each person consisting of ten times more bacterial cells than human cells. These commensal bacteria cause no apparent damage to us and, instead, are appreciated for the numerous benefits they provide. In contrast, pathogenic bacteria are capable of causing significant harm. In the beginning of the 1980s, the bacterium *Helicobacter pylori* was found to survive and persist in the acidic environment of the human stomach (Figure 1). The two Australian doctors Barry Marshall and Robert Warren found a spiral shaped bacteria in gastric biopsy materials from patients with gastritis and peptic ulcer disease, and they succeeded in culturing the bacteria and showed that it caused gastritis after inoculation in humans (Warren and Marshall, 1983). For the discovery that *H. pylori* infection causes gastritis and peptic ulcer disease, they received the Nobel Prize in Medicine or Physiology in 2005. Later *H. pylori* was associated with development of gastric cancer and since 1994 *H. pylori* has been classified as a class 1 carcinogen by the World Health Organization (IARC 1994) (Parsonnet et al., 1991).

![Figure 1. Electron micrograph of *H. pylori*](image-url)
**BACKGROUND**

**The human stomach**

The human stomach is part of the digestive system and is the primary reservoir for *H. pylori*. The stomach can be divided into four major areas including the cardia, fundus, corpus, and antrum (Figure 2), and the pylorus connects the stomach to the duodenum. Within the stomach, digestion of food occurs through peristaltic movements and the activity of the gastric juice. The gastric juice is secreted by cells in the gastric glands and contains mucus, digestive enzymes, and hydrochloric acid. The acid is secreted by parietal cells and contributes to the low pH (<2) in the stomach lumen. A mucus layer covers the gastric lining and protects the epithelial cells from the acidic stomach environment by maintaining a pH gradient with a pH near neutral at the epithelial cell surface. The epithelium continuously undergoes self-renewal to eliminate damaged cells and to maintain tissue homeostasis. *H. pylori* primarily resides within the mucus layer of the less acidic antrum region and a smaller population is found attached directly to the epithelial cells (Hessey et al., 1990; Schreiber, 2004).

![Figure 2. The anatomy of the human stomach. Gastric tissue section stained with hematoxylin.](image)
**Helicobacter pylori**

*H. pylori* is a spiral-shaped, microaerophilic, 2.5–4.0 µm long Gram-negative bacteria that has 2–6 characteristic polar, sheathed flagella. The genome of *H. pylori* is relatively small (Tomb et al., 1997), but its high mutation rates and frequent recombination events make *H. pylori* one of the most genetically variable pathogenic bacteria (Suerbaum et al., 1998; Suerbaum and Josenhans, 2007). Infection with *H. pylori* is primarily human specific and the bacteria infect half of the world’s population. *H. pylori* has developed numerous strategies to survive and persist for decades in the gastric stomach environment and the majority of infected individuals remain asymptomatic. Interestingly, *H. pylori* has coexisted with humans for more than 55,000 years (Linz et al., 2007) suggesting that through evolution colonization by *H. pylori* might have been beneficial for the human host (Blaser and Atherton, 2004). Today, however, *H. pylori* is recognized as a major health issue.

**Epidemiology**

The exact mechanism of *H. pylori* acquisition is still unknown. The primary route of transmission is thought to be from person-to-person, and the model of oral transmission, probably gastro-oral, is often favored (Brown, 2000). Colonization of *H. pylori* typically occurs in childhood via close contact with family members and usually persists for the person’s lifetime unless treated with antibiotics. There is a geographical variation in the prevalence of *H. pylori* infection that also differs with age, ethnicity, and socioeconomic characteristics (Malaty, 2007). Generally the prevalence is higher in developing countries and in older age groups.

**Gastric diseases**

The majority of infected individuals remain asymptomatic, but infection with *H. pylori* give rise to a local inflammation known as gastritis and 10–20% of infected individuals develop peptic ulcer disease (Atherton, 2006). Bleeding peptic ulcers are a serious life threatening condition. The Swedish Council on Health Technology Assessment reported in 2011 that 7–10 new persons in Sweden develop bleeding ulcers every day and that close to 25% of those suffering from bleeding ulcers die within a year of beginning treatment. This high mortality rate comes despite an awareness of the tight coupling between *H. pylori* infection and peptic ulcer disease. Thus, preventive measurements are needed to avoid disease development because in addition to peptic ulcers *H. pylori* infection is associated with gastric adenocarcinoma. Gastric cancer is recognized as one of the most common forms of cancer and develops in 1–2% of infected individuals. Less than 1% of *H. pylori* infected individuals acquire mucosa-associated lymphoid tissue (MALT). During the last
decades, the incidence of gastric cancer has declined in many countries (Bertuccio et al., 2009), but gastric cancer is still the second leading cause of cancer-related deaths worldwide (Ferlay et al., 2010).

There is an on-going debate as to whether *H. pylori* could be viewed as commensal and beneficial to its host (Atherton and Blaser, 2009). This in connection to that *H. pylori* has been associated with protection against asthma and allergies as well as gastroesophageal reflux disease (GERD) and its consequences such as esophageal adenocarcinoma (Blaser, 2006; Chen and Blaser, 2007; Thrift et al., 2013).

**Clinical outcome**

Infiltration of polymorphonuclear neutrophils (PMN) and mononuclear leukocytes into the lamina propria of the stomach is characteristic for the gastritis associated with *H. pylori* infection (Correa and Piazuelo, 2012). Disease progression is slow and, depending on the localization of the inflammation, the development of antral gastritis or pangastritis can occur decades after the initial infection (Atherton, 2006). Peptic ulcer disease comprises both gastric and duodenal ulcers that originate from a sore in the epithelial lining and disruption of the normal wound-healing processes. Duodenal ulcers are located in the duodenum and are associated with an antral-predominant inflammation and an increase in acid secretion (hyperchlorhydria, low pH). Hyperchlorhydria may lead to gastric metaplasia in the duodenum and subsequent *H. pylori* colonization. Gastric ulcers, on the contrary, are associated with a low acid concentration (hypochlorhydria, high pH) and pangastritis and are mainly present along the lesser curvature of the stomach. The low acid secretion is a result of damaged and lost cells in the gastric glands and is also the first step in the precancerous cascade of intestinal-type adenocarcinoma (Correa and Piazuelo, 2012). The intestinal-type gastric adenocarcinomas associated with an *H. pylori* infection develop from pangastritis or corpus-predominant gastritis. The histologically distinct forms in the precancerous cascade include multifocal atrophic gastritis (*gland loss*), intestinal metaplasia (*cell-type conversion*), dysplasia (*abnormal proliferation, change of phenotype*), and cancer (*unregulated cell growth*) (Figure 3). *H. pylori* is also associated with diffuse-type gastric adenocarcinoma and, in rare cases, with MALT lymphoma.
**Treatment and disease determinants**

A high level of inflammation caused by *H. pylori* increases the risk for developing severe disease, and the type and distribution of the inflammation determines which disease is likely to develop. Individuals with duodenal ulcers do not have an elevated risk for developing gastric cancer, and eradication of the *H. pylori* infection may cure patients with gastritis, duodenal ulcers, and low-grade MALT lymphoma. Eradication of *H. pylori* significantly reduces the risk of developing gastric adenocarcinoma in patients without premalignant lesions (Wong et al., 2004). The established therapy to eradicate *H. pylori* is through the so-called ‘triple therapy’ that combines a proton pump inhibitor (PPI) and two of the amoxicillin, clarithromycin, or metronidazole antibiotics. There has been a debate about long-term PPI treatment and the risk of promoting atrophic corpus gastritis among *H. pylori*-infected persons. A recent experimental study described that long-term PPI treatment worsens atrophic gastritis in Mongolian gerbils infected with *H. pylori* (Hagiwara et al., 2011). Treatment efficacy of the triple therapy protocol is currently declining and is now less than 70% in some countries (Georgopoulos et al., 2013). This is due to several factors, especially the development of bacterial resistance to antibiotics. Thus, alternative treatments such as bismuth-containing quadruple therapy and second-line rescue therapies are suggested in some cases (Georgopoulos et al., 2013).
Why some people infected with *H. pylori* remain asymptomatic while others develop ulcers or cancer is not fully understood. However, the following three main factors influence the disease outcome in *H. pylori*-infected individuals:

1. **The virulence of the infecting *H. pylori* strain**
   (described later in the section, *H. pylori* colonization and virulence factors).

2. **Host genetic determinants**
   Gastric inflammation and a reduction in acid secretion are critical host responses that influence the progression of *H. pylori*-induced cancer. Host genetic polymorphism in genes encoding for host inflammatory cytokines, including the interleukin-1 (IL) gene cluster, tumor necrosis factor (TNF), IL-10, and IL-8 have been shown to influence the host response (Amieva and El-Omar, 2008; El-Omar et al., 2003; Wroblewski et al., 2010). IL-1β has acid suppressive properties and overexpression is associated with risk for atrophic gastritis and gastric cancer. Transgenic mice overexpressing human IL-1β in parietal cells develop gastritis and dysplasia that progresses into carcinoma within a year after infection with *H. felis* (Tu et al., 2008). The relative importance of host genetics may, however, vary greatly between populations.

3. **Environmental factors**
   Smoking, a high-salt diet, and a low intake of fresh fruits and vegetables influence the disease outcome and a recent study in rhesus machaques described that the synergetic effect of life style and *H. pylori* factors promote gastric cancer (Liu et al., 2009).

**The gastric environment and host cell responses**

The gut mucosa serves as a foothold for many bacterial species and provides multiple host defense mechanisms against pathogens that are important for disease outcome (Ashida et al., 2011). These include the commensal microbiota, the mucus layer, the epithelial integrity, and cell turnover as well as innate and acquired immune responses. In the gastric environment, the acidic conditions and peristaltic movements of the stomach further limit colonization. The thick mucus layer that covers the gut epithelium is rich in large O-glycosylated proteins (the mucins), glycoproteins, digestive enzymes, antimicrobial peptides, and immunoglobulins. It functions as a protective barrier and is important for bacterial clearance via rapid mucin secretion and mucus shedding. The epithelial integrity also forms an efficient barrier against infection by preventing bacteria from reaching the underlying tissues. Epithelial cell-cell and cell-basal membrane adherence is maintained by various junction complexes. Damaged cells are eliminated through a continuous self-renewal of the gut epithelium to maintain tissue homeostasis, and this helps to avoid bacterial colonization. The innate immune response represents the first line of defense against pathogens along
with subsequent activation of the adaptive immune response. An essential role of the innate immune system is the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRR) such as the Toll-like receptors (TLR) and the intracellular NOD-like receptors (Fukata and Abreu, 2008; Harris et al., 2006). Recognition of PAMPs by these receptors results in intracellular signaling and an activation of the immune system that involves the release of inflammatory mediators and recruitment of immune cells to the infected tissue to efficiently eliminate the pathogen.

*H. pylori* has developed numerous strategies to evade immune responses. For example, *H. pylori* expresses common bacterial virulence factors such as flagellin and lipopolysaccharide (LPS) that have low immunostimulatory activity (Lee et al., 2003; Muotiala et al., 1992). TLR4 recognizes the LPS of Gram-negative bacteria, and TLR2 also recognizes LPS along with other microbial components such as lipoproteins and peptidoglycan. There is an on-going debate in the field concerning the recognition of *H. pylori* LPS and the roles of TLR4 and TLR2 (Cullen et al., 2011; Smith et al., 2011). A mechanism (described in more detail in the section on LPS) was recently suggested for how the *H. pylori*-modified form of lipid A aids the bacteria in escaping TLR4 recognition and, therefore, the innate immune response (Cullen et al., 2011).

Factors thought to be necessary for clearance of many bacterial infections, such as antibodies, complement system components, and phagocytic cells have been detected in the gastric mucosa of persons infected with *H. pylori* (Peek et al., 2010). *H. pylori* infection stimulates PMN, macrophage, and dendritic cell (DC) activation and a Th1/Th17 lymphocyte response along with the production of various pro-inflammatory cytokines such as IL-1β, TNFα, IL-8, and IL-6 (Figure 4) (Lindholm et al., 1998; Peek et al., 2010). Additionally, infection with *H. pylori* is associated with an induction of regulatory T-cells (Treg) that are able to suppress the effector functions of several immune cells (Raghavan et al., 2003; Raghavan and Quinding-Järbrink, 2012).

*H. pylori* also has the ability to manipulate immune responses. *H. pylori* has been shown to reprogram DCs leading to an efficient activation of Treg suggesting that *H. pylori* can skew the immune response towards tolerance instead of immunity (Oertli et al., 2012; Raghavan and Quinding-Järbrink, 2012). In addition, *H. pylori* virulence factors such as the vacuolating cytotoxin (VacA) and γ-glutamyl transpeptidase, have been demonstrated to limit T-cell proliferation and, therefore, to suppress the immune response (Gebert et al., 2003; Schmees et al., 2007).
**Figure 4.** *H. pylori* induced inflammatory response. Simplified schematic with *H. pylori* at the gastric epithelial layer close to the apical junction area, with the induction of various immune cells. Adopted from Peek *et al.* 2010.

**H. pylori colonization and virulence factors**
To establish a successful colonization, *H. pylori* has developed a number of strategies to overcome the obstacles that it faces in the human stomach. For survival and growth in the gastric environment, *H. pylori* converts urea to ammonia and bicarbonate. This is largely mediated by the inner membrane UreI channel, urease, and the $\alpha$- and $\beta$- carbonic anhydrases (Sachs *et al.*, 2011). The UreI channel is closed at neutral pH but opens up at a pH below 6 to increase the access to intracellular urea. At low acidic conditions the urease enzyme, which consists of the UreA and the UreB subunits, becomes activated via insertion of nickel through the activity of accessory proteins. In the cytoplasm, urease hydrolyses urea to ammonia and carbonic acid ($H_2CO_3$), which is further converted by $\beta$-carbonic anhydrase into $CO_2$ and $H_2O$. The $CO_2$ diffuses into the periplasm where it is converted into $HCO_3^-$ by $\alpha$-carbonic anhydrase. This acts as a buffer and maintains a pH of 6.1 in both the bacterial cytoplasm and periplasm. Both the UreI protein and the $\alpha$-carbonic anhydrase have been suggested as possible targets for *H. pylori* eradication, and the structure of the UreI channel has recently been solved (Strugatsky *et al.*, 2013).
Motility and adherence is important for *H. pylori* to overcome the effects of peristaltic movements, liquid flow, shedding of the mucus layer, and low pH. *H. pylori* uses chemotactic-mediated motility to sense the pH and bicarbonate gradient in the mucus layer and swim away from the low acid condition in the stomach lumen towards the neutral environment close to the epithelial cells (Schreiber, 2004). The bacteria’s flagella are essential for motility and its ability to persistently infect the host (Lertsethtakarn et al., 2011; Suerbaum, 1995). Adhesins expressed on the bacterial surface mediate adherence of approximately 20% of the *H. pylori* population to epithelial cells (Hessey et al., 1990), preferably in close proximity to the apical junction complex (Amieva et al., 2003). A small portion also has the ability to invade epithelial and immune cells (Dubois and Borén, 2007; Necchi et al., 2007).

The severity of disease is highly correlated with the virulence of the infecting strain. *H. pylori* harboring the *cag* pathogenicity island (*cag*PAI), the VacA cytotoxin, and the outer membrane blood group binding adhesin (BabA) are strongly associated with an increased risk of developing gastric cancer and peptic ulcer disease (Gerhard et al., 1999; Xiang et al., 1995). As with disease prevalence, the distribution of these genomic loci varies geographically. The *vacA* gene is present in all *H. pylori* strains but there are variations in the gene sequence, and strains possessing the s1/m1/i1 allele of *vacA* are associated with an increased risk of cancer (Rhead et al., 2007). VacA induces large vacuoles in cultured epithelial cells and can also stimulate apoptosis through localization to the mitochondria where it triggers the apoptotic cascade (Rassow and Meinecke, 2012). Moreover, VacA induces immune suppression by inhibiting T-cell proliferation by binding to the β2-integrin receptor subunit (CD18) (Gebert et al., 2003; Sewald et al., 2008) and appears to act both synergistically and antagonistically with the cytotoxin associated gene A (CagA) (Kim and Blanke, 2012). Examples of other bacterial factors that may contribute to disease outcome are the *H. pylori* neutrophil-activating protein (HP-NAP), the outer inflammatory protein (OipA), and the high temperature requirement A protein (HtrA) (Evans et al., 1995; Löwer et al., 2008; Yamaoka et al., 2000).

**cagPAI**

The oncoprotein CagA is part of the *cag*PAI, which is a 40 kb DNA insertion element acquired via horizontal gene transfer to *H. pylori* from a yet unknown ancestor (Akopyants et al., 1998; Censini et al., 1996). The *cag*PAI carries up to 32 genes and encodes a type IV bacterial secretion system (T4SS) that is evolutionarily related to DNA conjugation systems (Backert and Meyer, 2006; Fronzes et al., 2009). A T4SS is commonly found in many pathogenic and non-pathogenic Gram-negative bacteria and, depending on the bacterial species, the different T4SS differ both in transported substrate
(protein or DNA-protein complexes) and in the recipient (bacteria or an organism from a different kingdom). The T4SS typically consist of 11 VirB proteins (encoded by virB1 to virB11) and the VirD protein (an NTPase). The T4SS of Agrobacterium tumefaciens is the best characterized and is the prototype for other T4SS (Christie et al., 2005). Two hypothetical models for how the H. pylori needle assembles upon host cell contact have been suggested and these mainly differ in whether the proteins CagL or CagY are coating the needle structure (Barrozo et al., 2013; Jiménez-Soto et al., 2009; Kwok et al., 2007; Rohde et al., 2003; Shaffer et al., 2011). The components of the H. pylori T4SS needle interact with the host-cell receptor αβ1 integrin (Jiménez-Soto et al., 2009; Kwok et al., 2007). The first described bacterial oncoprotein, CagA, is encoded by the cagPAI and is translocated via the T4SS to host cells (Covacci and Rappuoli, 2000; Covacci et al., 1993; Cover et al., 1990; Crabtree et al., 1991). Because the βi integrins are mainly localized basolaterally, it is not fully understood how H. pylori comes into contact with these integrins to deliver CagA. One recent observation that can explain this is HtrA-mediated cleavage of E-cadherin that disrupts the epithelial barrier (Hoy et al., 2010). Host-cell cholesterol and phosphatidylserine are additional factors involved in the delivery of CagA (Hutton et al., 2010; Lai et al., 2008; Murata-Kamiya et al., 2010).

CagA has been implicated as a bacterial oncoprotein because transgenic mice that overexpress CagA develop gastric epithelial hyperproliferation and gastric adenocarcinoma (Ohnishi et al., 2008). CagA is a 120–145 kDa protein that appears to be unique to H. pylori because it has not yet been found in any other bacterial species. The N-terminal ~100 kDa subdomain has recently been crystallized (Hayashi et al., 2012; Kaplan-Türköz et al., 2012), and the C-terminal is a disordered domain containing a variable number of tyrosine phosphorylation motifs (Higashi et al., 2002). Upon host cell delivery, CagA can become phosphorylated by the Src and Abl kinases in a hierarchical order (Tegtmeyer and Backert, 2011). Phosphorylated CagA (CagAPY) is known to interact with a number of Src homology 2 domain (SH2) signaling proteins. CagAPY mimics a tyrosine-phosphorylated host cell protein and induces actin-cytoskeletal rearrangements, cell scattering and elongation, and proliferative and pro-inflammatory signaling (Segal et al., 1999; Tegtmeyer et al., 2011). The first discovered interaction of CagAPY was with the tyrosine phosphatase SHP-2 (Higashi et al., 2002), which has also been co-immunoprecipitated from gastric biopsy tissues (Yamazaki et al., 2003).
CagA and apical junctions
Several cellular interaction partners are also associated with the non-phosphorylated form of delivered CagA. Non-phosphorylated CagA is associated with disruption of cell-cell junctions, affecting cell polarity and inducing pro-inflammatory and mitogenic responses (Backert and Naumann, 2010; Wessler and Backert, 2008).

An intact gastric epithelial barrier is maintained via cell adherence through junction complexes (Yu and Yang, 2009). There are two types of apical junctions, the tight and adherence junctions, and these consist of assemblies of transmembrane and cytoplasmic proteins that connect the actin cytoskeletons of adjacent cells and regulate paracellular permeability (Figure 4). The primary cytoplasmic proteins of the tight junctions are the zonula occludens (ZO-1, ZO-2, and ZO-3) and the primary transmembrane proteins are junctional adhesion molecules (JAMs), claudin, and occludin (Balda and Matter, 2008). A healthy epithelium is characterized by an apical-basal polarity and the junctions aid in maintaining cell polarity by segregating apically expressed membrane proteins from those expressed on the basolateral membrane (Wang and Margolis, 2007). Adherence junctions are located below the tight junctions and can indirectly regulate the function of the tight junctions. The adherence junctions are responsible for the mechanical adhesion between neighboring cells via calcium-dependent interactions with the main component of the junction, E-cadherin (Baum and Georgiou, 2011). Intracellularly, a complex of several proteins including α-, β- and β120-catenin link E-cadherin to the actin cytoskeleton. Components of the adherence junctions are involved in signal transduction to the nucleus to regulate transcription. Triggering of distinct cell signaling pathways by exogenous stimuli, such as bacterial effectors, can disrupt the integrity of junction complexes and lead to epithelial barrier leakage, which is a sign of inflammation (Wroblewski and Peek, 2011).

CagA delivered to the host cell is able to disrupt intercellular adhesion and affects both the tight and adherence junctions. CagA associates with ZO-1 and JAM-A leading to an incomplete assembly of tight junctions (Amieva et al., 2003). CagA also disrupts adherence junctions by interacting with E-cadherin and causing the cytoplasmic and nuclear accumulation of β-catenin (Franco et al., 2005; Murata-Kamiya et al., 2007). Additionally, CagA binds PAR1b/MARK2 and inhibits its kinase activity leading to loss of cell polarity (Saadat et al., 2007). The epithelial integrity is further affected in various ways by other H. pylori effectors, including VacA, LPS, urease, OipA, LPS, and, as mentioned earlier, the HtrA protease (Franco et al., 2008; Hanson et al., 2011; Hoy et al., 2010; Papini et al., 1998; Wroblewski and Peek, 2011; Wroblewski et al., 2009).
Several T4SS-dependent but CagA-independent cellular effects have been reported, including inhibition of phagocytosis, cell scattering, and motility, as well as pro-inflammatory signaling and the activation of NFkB and AP-1 (Backert and Naumann, 2010). This suggests that other factors can be injected into the host cell via the T4SS. One possible candidate is peptidoglycan that is recognized by the intracellular NOD-1 receptor (Viala et al., 2004). Peptidoglycan can induce the release of IL-8 via both the NFkB and AP-1 signaling pathways (Allison et al., 2009; Viala et al., 2004).

**The *H. pylori* outer membrane**

The bacterial membrane is important as a barrier against the outside environment and helps the bacteria survive by adapting to new environmental conditions. Characteristic of Gram-negative bacteria is the presence of an outer membrane (OM) in the cell envelope that is separated from the inner membrane (IM) by the periplasm. Present in the periplasm is the peptidoglycan layer, which consists of sugar polymers covalently linked via oligosaccharides. The OM and IM have a different structure and composition (Bos et al., 2007). The IM is a phospholipid bilayer, but the OM is an asymmetrical bilayer consisting of LPS on the outside and phospholipids on the inside. Proteins spanning the IM generally form hydrophobic α-helices, but those spanning the OM form β-barrels with a hydrophilic interior and a hydrophobic exterior facing the membrane lipids. The OM also functions in protecting the bacteria from harmful substances like antibiotics.

**Phospholipids and cholesterol**

The amphiphilic nature of lipids is essential for the formation of biological membranes, and several types of lipids can be produced based on different compositions of fatty acid chains combined with different head groups. Glycerophospholipids (PL) contain a polar phosphorous moiety, a glycerol backbone, and hydrophobic fatty acid chains. Bacterial membranes contain a heterogeneous PL composition (Matsumoto et al., 2006), and each bacterial species tends to have a distinct and characteristic lipid composition. The fluidity and stability of a bacterial membrane can be changed by altering the PL composition, and this is an important feature in the response to environmental changes such as temperature (Mansilla et al., 2004). PL can also change the local structure of membranes and membrane curvature is influenced by the size of the head group and the fatty acid chains (Tomsiè et al., 2005). Bacteria have been found to contain lipid rafts with a similar function as in eukaryotes in that they organize proteins involved in signal transduction, small molecule translocation, and protein secretion (LaRocca et al., 2010; López and Kolter, 2010).
The most common PLs of *H. pylori* are phosphatidylethanolamine (PE), cardiolipin (CL), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylglycerol (PG) (Hirai et al., 1995). Upon exposure to acidic conditions, the PL composition of *H. pylori* changes with an increasing amount of lysoPE (LPE) (Bukholm et al., 1997). The increase in LPE appears to correlate with higher membrane permeability leading to an increase in the release of virulence factors, destruction of host membranes, and incidence of peptic ulcer disease (Tannaes et al., 2001; 2005). The unique presence of three cholesteryl glycosides (CG) in *H. pylori* is suggested to stabilize the membrane when the LPL content varies (Hirai et al., 1995; Tannaes and Bukholm, 2005). The composition of PL and CG in the *H. pylori* membrane changes during the morphological shift from spiral to coccoid that occurs in response to environmental stress (Shimomura et al., 2004). *H. pylori* must internalize cholesterol to synthesize CG, and the bacteria are able to follow a cholesterol gradient and extract cholesterol from the plasma membrane of epithelial cells (Wunder et al., 2006). CG has been shown to promote escape from the host immune response and to alter the plasma membrane architecture to facilitate T4SS-mediated functions (Wang et al., 2011; Wunder et al., 2006). *Borrelia burgdorferi* may, in a similar manner to *H. pylori*, acquire cholesterol from the plasma membrane of epithelial cells. Recently it was shown that *B. burgdorferi* also is able to transfer both cholesterol and cholesterol-glycolipids back to epithelial cells, which was suggested to contribute to pathogenesis (Crowley et al., 2013).

**Lipopolysaccharides**

LPS is an essential complex glycolipid that consist of lipid A, a short core oligosaccharide, and the O-antigen polysaccharide (Ruiz et al., 2009). Lipid A is relatively conserved among bacterial species, but the O-antigen is variable and not present in all species. LPS, also referred to as endotoxin, is able to cause a broad range of host immunological responses (Trent et al., 2006). As described earlier, *H. pylori* LPS has a significantly lower endotoxic activity compared to LPS from other Gram-negative bacterial species (Moran, 2007; Muotiala et al., 1992). This is suggested to be due to a dephosphorylation of lipid A that makes the bacterial membrane more resistant to cationic antimicrobial peptides and attenuates TLR4 activation (Cullen et al., 2011).

*H. pylori* also shows great diversity in the expression of its O-chain (Moran, 2008; Nilsson et al., 2008; Skoglund et al., 2009). The bacteria can engage in molecular mimicry by assembling surface polysaccharides that resemble the human blood group antigens Lewis x (Le^x^) and Lewis y (Le^y^). This is suggested to contribute to its ability to evade immune detection (Appelmelk et al., 1998; Wang et al., 2000). A role for *H. pylori* LPS in
adherence and colonization is still a matter of debate (Cullen et al., 2011; Edwards et al., 2000; Hiratsuka et al., 2005; Mahdavi et al., 2003).

**Outer membrane proteins**

Approximately 4% of the *H. pylori* genome encodes outer membrane proteins (OMP) (Tomb et al., 1997), and OMPs have important roles in the transport of molecules across the bacterial membrane and in adherence. Adhesion of *H. pylori* to the epithelium is beneficial because it allows the bacteria to avoid being swept away by peristaltic movements in the stomach and brings the bacteria closer to a source of nutrients. Adhesion can also be a disadvantage because the bacteria comes close to immune cells and is threatened with being eradicated.

*Adhesins and their cognate receptor structures*

The two best characterized *H. pylori* adhesins are the BabA adhesin and the sialic acid binding adhesin SabA (Ilver et al., 1998; Mahdavi et al., 2002). The BabA adhesin mediates binding to ABO/Leb blood group antigens H1 and Lewis b (Leb) (Aspholm-Hurtig et al., 2004; Borén et al., 1993). These antigens are found on red blood cells and in both healthy and inflamed gastric mucosa. SabA recognizes the sialylated structures sialyl-Lewis x (sLex) and sialyl-Lewis a (sLea) (Mahdavi et al., 2002). Colonization of *H. pylori* strains expressing BabA and/or SabA is associated with the development of severe gastric diseases (Gerhard et al., 1999; Ilver et al., 1998; Mahdavi et al., 2002; Yamaoka et al., 2006; 2002). In addition, other OMPs have been suggested to be involved in *H. pylori* adhesion, including OipA, HopZ, HopQ, HorB, and AlpAB (Loh et al., 2008; Odenbreit et al., 1999; Peck et al., 1999; Snelling et al., 2007; Yamaoka et al., 2000), but functional receptors for these proteins have not yet been described.

Bacteria exploit host cell structures to colonize the mucosal surface, and the most common receptors are the carbohydrates found on glycoproteins and glycolipids. The glycocalyx that surrounds the surface of epithelial cells is a result of post-translational glycosylation. Glycosylation is tissue and cell specific and may change in response to internal or external factors and during disease progression (Lindén et al., 2008b; Mahdavi et al., 2002; Moran et al., 2011). For example, only low levels of sLea are expressed in healthy gastric mucosa but these antigens are up-regulated during inflammation (Mahdavi et al., 2002). Healthy gastric mucosa also contains the cell surface bound MUC1 and two secreted gel-forming mucins MUC5AC and MUC6 (McGuckin et al., 2011). *H. pylori* is able to interact via the BabA adhesin to the Leb expressed on MUC5AC and both BabA and SabA can mediate binding to MUC1 (Lindén et al., 2009; 2008a; 2002; Magalhães and Reis, 2010; Van de Bovenkamp et al., 2003).
**Bacterial outer membrane vesicles**

Gram-negative bacteria shed outer membrane vesicles from their surface, a feature that was first observed almost 50 years ago (Bishop and Work, 1965; Chatterjee and Das, 1967). Vesicles are created through bulges appearing in the bacterial OM that subsequently pinch off to form spherical, bilayered membrane structures (Figure 5). Thus, the components of vesicles resemble those of the OM and include such things as LPS, PL, and OMPs. Vesicles also contain periplasmic substances that are entrapped as the vesicles are formed (Beveridge, 1999). Recently bacterial vesicles have been given more attention, and today they are recognized to be involved in a number of biological processes such as bacterial virulence and communication (Ellis and Kuehn, 2010; Mashburn-Warren et al., 2008a). Bacteria secrete vesicles during infection with a notable case being a patient with fatal meningococcal septicemia who was found to have a high endotoxin level in his plasma and *Neisseria meningitides* vesicles (Namork and Brandtzaeg, 2002). Importantly, *H. pylori* vesicles carrying the VacA cytotoxin have been detected in gastric biopsies (Fiocca et al., 1999).

**Figure 5.** Vesicle biogenesis. Schematic representation of vesicle release and a transmission electron micrograph of *H. pylori* shedding vesicles.

**Vesicle biogenesis and composition**

The molecular details concerning vesicle shedding are not completely understood. Vesicle biogenesis is thought to involve the following four key features: 1) breaking contacts between the OM and peptidoglycan layer, 2) inducing localized membrane curvature, 3) enriching/excluding certain components, and 4) the release of vesicles (Kulp and Kuehn, 2010).
Membrane curvature is suggested to be initiated by several processes such as breakage of the associations with the peptidoglycan layer, the accumulation of substances in the periplasm, and/or accumulation of curvature-inducing molecules (Deatherage et al., 2009; Mashburn-Warren and Whiteley, 2006; Mcbroom and Kuehn, 2007; Wessel et al., 2013). In *Pseudomonas aeruginosa*, an opportunistic human pathogen associated with lung infections in patients with cystic fibrosis, the negative-charged nature of the OM and interactions between LPS and the quorum-sensing molecule, PQS, has been shown to be important for membrane curvature and vesicle production (Mashburn and Whiteley, 2005; Mashburn-Warren et al., 2008b).

Vesicle biogenesis results in vesicles of different sizes ranging from 20 to 300 nm in diameter and with different compositions (Balsalobre et al., 2006; Horstman and Kuehn, 2000; Kesty and Kuehn, 2004). They are released during all stages of bacterial growth and are not thought to be products originating from bacterial lysis (Kuehn and Kesty, 2005). Vesicles are produced from bacteria grown *in vitro*, both in liquid and solid medium, within biofilms, and during intracellular infections (Bauman and Kuehn, 2006; Galka et al., 2008; Palsdottir et al., 2009). Furthermore, vesicles have been detected in nasal swabs of a child suffering from *Moraxella catarrhalis* sinusitis (Tan et al., 2007). The shedding and composition of vesicles are influenced by environmental conditions and the bacterial growth phase with more vesicles being produced during times of bacterial stress (Mcbroom and Kuehn, 2007; Olofsson et al., 2010; Sabra et al., 2003; Tashiro et al., 2011a). For example, antibiotic treatment with gentamicin affects vesicle composition and increases shedding of *P. aeruginosa* vesicles by about three fold (Kadurugamuwa and Beveridge, 1995; Nguyen et al., 2003).

Vesicles from various species have been characterized in terms of their protein and lipid contents (Lee et al., 2007; Nevot et al., 2006; Post et al., 2005; Tashiro et al., 2011a). The main PL present in vesicles resembles that of the parent bacteria and differs between vesicles from different bacterial species. For example, the main PL in vesicles from *N. meningitidis* is PE and PG (Post et al., 2005), from enterotoxigenic *E. coli* (ETEC) vesicles it is CL, PG and PE (Kesty et al., 2004), and from *P. aeruginosa* vesicles it is PE, PG and PC (Tashiro et al., 2011a). Interestingly, PG is enriched in vesicles from *P. aeruginosa* compared to the OM and the rigidity that comes along with PG is suggested to influence vesicle biogenesis.

Some proteins and lipids seem to be enriched in vesicles while others are excluded suggesting a selective sorting of vesicle material (Haurat et al., 2011; Horstman and Kuehn, 2000; Kadurugamuwa and Beveridge, 1995; Kato et al., 2002; Tashiro et al., 2011a). Vesicles have been shown to contain
adhesins, toxins, and other virulence factors as well as DNA (Lee et al., 2008; Renelli et al., 2004). Proteomic profiles of bacterial vesicles derived from different bacterial species demonstrate that the majority of the OMPs are associated with vesicles (Choi et al., 2011; Elmi et al., 2012; Olofsson et al., 2010; Sharpe et al., 2011). At the moment there is an ongoing debate in the literature concerning the presence of IM and cytoplasmic material within vesicles (Kulp and Kuehn, 2010).

In *H. pylori* vesicles, the lipid composition resembles that of the bacterial OM with PE and CL being the two most dominant types (Olofsson et al., 2010). Two proteomic studies have identified a large number of proteins that are associated with the vesicles (Mullaney et al., 2009; Olofsson et al., 2010). The majority of the OM proteins are found in the vesicles along with several proteins considered to be important for *H. pylori* pathogenesis, including VacA, BabA, SabA, AlpB, OipA, HP-NAP, CagA, and urease. The BabA and SabA adhesins were found to be functionally expressed on *H. pylori* vesicles and contributed to their ability to adhere to gastric tissue sections (Olofsson et al., 2010). The vesicles are enriched in LPS compared to the bacterial OM, and vesicle LPS has the same altered lipid A moiety that is a characteristic phenotype of *H. pylori* LPS (Hynes et al., 2005; Keenan et al., 2008). Similar, the bacterial heterogeneity in the LPS O-chains is reflected in the vesicle LPS phenotype and can be altered with changing environmental conditions. For example, bacterial growth under iron-limiting conditions results in vesicles with less LPS, shorter O-chains, and a structural alteration as well as reduced expression in the Lewis antigens (Hynes et al., 2005; Keenan et al., 2008).

To date no bacterial mutant has been identified that links a specific protein to vesicle production. Bacterial strains have been associated with an increase or a decrease of vesicle production but none that is devoid of vesicles (Mashburn and Whiteley, 2005; Mcbroom and Kuehn, 2007; Mcbroom et al., 2006; Song et al., 2008). For example, vesicle shedding increased 100-fold when the $o^e$ envelope pathway was disrupted in *E. coli*, and this correlated with an increased rate of bacterial survival upon challenge with stressing agents (Mcbroom and Kuehn, 2007; Mcbroom et al., 2006). In contrast, mutants unable to synthesize PQS in *P. aeruginosa* shed a significantly lower amount of vesicles compared to the wild-type strain (Mashburn and Whiteley, 2005).

The production of membranous vesicles is conserved in microbes from prokaryotes, eukaryotes, and archaea (Deatherage and Cookson, 2012; Macdonald and Kuehn, 2012), and vesicle production has recently been identified in Gram-positive bacteria (Lee et al., 2009). Due to the absence of
the characteristic OM present in Gram-negative bacteria, these vesicles derive from the cytoplasmic membrane, contain cytosolic proteins, and are enriched in extracellular or surface-associated virulence proteins (Lee et al., 2009). For example, vesicles containing α-toxin have been isolated from *Staphylococcus aureus* (Thay et al., 2013). Strikingly, there appears to be an overlap not only in how vesicles are formed but also in their function between prokaryotes, eukaryotes, and archaea (Deatherage and Cookson, 2012; Macdonald and Kuehn, 2012; van der Pol et al., 2012).

**Biological consequences of vesicle shedding**

Bacterial vesicles have been suggested to be important in activities such as pathogenesis, nutrient acquisition, quorum sensing, and horizontal gene transfer (Figure 6) (Ellis and Kuehn, 2010; Kulp and Kuehn, 2010). They can deliver their cargo over a long distance and due to their ability to concentrate and protect bacterial effectors from extracellular enzymes they are considered an alternative form of secretion and contribute to bacterial colonization. In fact, vesicles have been shown to interact and communicate with both bacterial and eukaryotic cells (Berleman and Auer, 2012).

![Figure 6. Suggested consequences of vesicles](image)

**Figure 6. Suggested consequences of vesicles**

Bacteria in natural environments usually form biofilms that facilitate bacterial survival (Kostakioti et al., 2013). Vesicles have been found within biofilms where they act as a bridging factor and contribute to biofilm formation by mediating adherence via electrostatic surface-bound DNA and the delivery of extracellular material (Schooling and Beveridge, 2006; Schooling et al., 2009). *H. pylori* vesicles have been detected within biofilms and have been suggested to contribute to the formation of the biofilms
(Yonezawa et al., 2011). Many bacteria are capable of regulating biofilm formation via quorum sensing that allows the bacteria to regulate certain genes based on the bacterial cell density (Li and Tian, 2012). Vesicles from *P. aeruginosa* have been found to contain PQS molecules and this has been suggested to contribute to bacterial communication (Mashburn and Whiteley, 2005).

Vesicles can contribute both to intra- and interspecies communication. DNA has been found in vesicles from several species, including *P. aeruginosa*, *E. coli*, and *M. catarrhalis* (Renelli et al., 2004; Schaar et al., 2011; Yaron et al., 2000). The DNA in *P. aeruginosa* derived from the periplasm of the cell or from the extracellular space (Renelli et al., 2004). Interestingly, vesicles from *E. coli* could facilitate the transfer of genes both to another *E. coli* strain and to other bacterial species such as *Salmonella enterica* and these genes were functionally expressed by the recipient (Yaron et al., 2000). The cytotoxicity of the transformed bacteria increased and the vesicles also delivered genes for antibiotic resistance.

Vesicles from pathogenic bacteria are known to interact with eukaryotic cells and deliver biologically active toxins and virulence factors (Ellis and Kuehn, 2010). For example, *P. aeruginosa* vesicles contain alkaline phosphatase, β-lactamase, hemolytic phospholipase C, hemolysin, pro-elastase, and conductance regulator inhibitory factor (Cif) (Tashiro et al., 2011b). Other examples of toxins present in vesicles are cytotoxic necrotizing factor type 1 (CNF1) in uropathogenic *E. coli*, cytolysin A (ClyA) in enterobacterial *E. coli*, and α-haemolysin in extraintestinal pathogenic *E. coli* (Balsalobre et al., 2006; Kouokam et al., 2006; Wai et al., 2003). Thus, vesicles are suggested as a vehicle for toxin delivery and consistent with this *H. pylori* vesicles alter epithelial cell morphology, induce apoptosis and the vacuolization that is characteristic of the delivery of VacA (Ayala et al., 2006; Ismail et al., 2003; Keenan et al., 2000; Ricci et al., 2005). Interestingly, vesicles may also transfer lipids to host cells, for example, *B. burgdorferi* vesicles may, as their parent bacteria, transfer both cholesterol and cholesterol glycolipids to host cells (Crowley et al., 2013).

**Endocytosis and uptake of bacterial vesicles**

Vesicles from numerous species have been detected intracellularly in human cells, for example vesicles from *H. pylori*, *Porphyromonas gingivalis*, *Haemophilus influenzae*, and ETEC (Furuta et al., 2009; Kesty et al., 2004; Parker et al., 2010; Sharpe et al., 2011). For cellular internalization, vesicles may either fuse with the plasma membrane or enter via endocytosis (Amano et al., 2010). Upon membrane fusion, vesicles deliver their content directly into the cytoplasm. In endocytosis the entire vesicle is internalized and is subject to further intracellular trafficking.
Endocytosis is an essential process for cell survival and is required for nutrient uptake, regulation of surface receptors, and receptor signaling as well as for cell adhesion, migration, and polarity (Doherty and McMahon, 2009). It involves internalization of extracellular molecules in tubular or vesicular membrane carriers that are formed by distinct protein machineries that re-sculpt the plasma membrane. The carriers are “pinched off” from the plasma membrane to form vesicles that are further trafficked to sub-cellular compartments. Endocytosis can be divided into several endocytic processes distinguished by the size of the vesicle formed, their cargo, and the machinery involved (Figure 7) (Doherty and McMahon, 2009). The most studied and well-characterized pathway is mediated by the formation of vesicles coated with clathrin protein (Rosenbluth and Wissig, 1964; RothPorter, 1964). Other pathways independent of clathrin coating have been described more recently but their molecular details and cargo specificity are not as well defined (Hansen and Nichols, 2009).

Figure 7. Endocytosis can be divided into phagocytosis and pinocytosis. Pinocytosis can further be subdivided into different pathways as depicted in the figure. Internalized material is transported via early endosomes to subcellular compartments from all pathways (here shown from CME). PM: plasma membrane

Clathrin-mediated endocytosis
Clathrin-mediated endocytosis (CME) is a conserved process that relies on a complex network of adaptor (e.g. AP-2) and accessory proteins to be recruited to the plasma membrane (McMahon and Boucrot, 2011). The GTPase dynamin assembles at the neck of a budding vesicle and through conformational changes promotes scission so that the vesicle is released
from the plasma membrane (Ferguson and Pietro De Camilli, 2012). After a vesicle is formed, the proteins involved in the process are recycled back to the cytoplasm for reuse in another cycle of endocytosis. The internalized cargo is sorted to endosomes and either transported further to subsequent cellular compartments or recycled back to the surface. A classic example of a receptor that is selectively internalized via CME is the transferrin receptor that within minutes is recycled back to the plasma membrane (Mayle et al., 2012).

The size of the clathrin-coated vesicle depends on its cargo and has an upper limit of a diameter of 200 nm. Thus, CME is an ideal uptake route for vesicles due to their small size, and CME has been shown to be partially responsible for the internalization of *Brucella abortus* vesicles in human monocytes (Pollak et al., 2012). Also, vesicles from *H. pylori* are internalized into gastric epithelial cells via CME (Parker et al., 2010), and Parker and colleagues have found that the presence of VacA enhanced the association of vesicles and epithelial cells.

**Clathrin-independent endocytosis**

Clathrin-independent pathways are less characterized, but small GTPases such as Cdc42, Arf6 and RhoA, and proteins involved in carrier formation such as flotillins, GRAF-1, and caveolin-1 are suggested to be involved and form carriers that are either tubular or vesicular (Doherty and Lundmark, 2009; Doherty et al., 2011; Howes et al., 2010; Lundmark et al., 2008; Sandvig et al., 2011). The *H. pylori* VacA cytotoxin, for example, is internalized into epithelial cells through a Cdc42-dependent pathway, independent of clathrin, and trafficked to late endosomal/lysosomal compartments (Gauthier et al., 2005). Dynamin has been implicated to be involved in some of the clathrin-independent processes, but this is still not fully understood (Doherty and McMahon, 2009; Ferguson and Pietro De Camilli, 2012; Parton and del Pozo, 2013; Sandvig et al., 2011). Clathrin-independent pathways are thought to relay on a specific lipid composition. Lipid rafts are formed in the plasma membrane by fluctuating nanoscale assemblies of cholesterol, sphingolipids and proteins (Simons and Gerl, 2010), when these are stabilized into larger platforms, they can function in membrane signaling and trafficking.

*P. gingivalis* vesicles are internalized through an actin-dependent, lipid raft-mediated pathway that is independent of clathrin and dynamin (Furuta et al., 2009). *H. pylori* vesicles are also suggested to enter gastric cells via lipid rafts (Kaparakis et al., 2010).
Caveolae

Caveolae-mediated endocytosis is a specialized type of lipid rafts and the most studied clathrin-independent pathway. Caveolae are characteristic small invaginations of the plasma membrane forming bulb-shaped structures with a diameter of 50-80 nm and a wide opening (Bastiani and Parton, 2010). They are cell type-specific and heterogeneously distributed within individual cells, for example, they are concentrated at the basolateral surface of epithelial cells (Scheiffele et al., 1998). They are enriched in cholesterol, sphingolipids, and proteins, such as Caveolin 1-3 and Cavin 1-4, that are essential for their formation and stabilization at the plasma membrane (Bastiani and Parton, 2010). They are described as relatively immobile structures, that recently was shown to be stabilized by the assembly of EHD2 proteins (Daumke et al., 2007; Morén et al., 2012; Stoeber et al., 2012).

Vesicles have functional adhesins on their surface and some toxins have been demonstrated to act as adhesins in allowing vesicles to enter host cells via a receptor-mediated endocytic pathway (Kesty et al., 2004; Shoberg and Thomas, 1993). For example, ETEC vesicles carrying the heat-labile enterotoxin LT invade epithelial cells in a time-, temperature-, and receptor-dependent manner in contrast to vesicles lacking LT (Kesty et al., 2004). These vesicles were internalized through cholesterol-rich lipid rafts and sometimes found to co-localize with caveolin. Another example of receptor-mediated uptake is that of *M. catarrhalis* vesicles that bind TLR2 in lipid rafts of host cells with subsequent uptake (Schaar et al., 2011). Lipid rafts and caveolae are also entry points for *H. influenzae* vesicles (Sharpe et al., 2011).

Once internalized, the intracellular routes that vesicles take have been reported to differ. For example, *P. gingivalis* vesicles are sorted to lysosomes after 90 min, and this is in contrast to vesicles from *E. coli* and *P. aeruginosa* that are sorted to non-acidified compartments (Bauman and Kuehn, 2009; Furuta et al., 2009; Kesty et al., 2004).

Vesicles are also suggested to fuse with lipid rafts in the cell membrane and hence deliver there content directly into the cytoplasm. For example, vesicles from *P. aeruginosa* carrying multiple virulence factors deliver these factors to different sub-cellular locations within the host cytoplasm (Bomberger et al., 2009), as do vesicles from *Aggregatibacter actinomycetemcomitans* that deliver the cytolethal-distending toxin (CDT) to the nucleus, whereas the protein OmpA is distributed perinuclear (Rompikuntal et al., 2011).
Modulation of host cell defenses and responses

Vesicles are likely to be a key factor in the host inflammatory response against pathogens because besides bacterial toxins vesicles also carry several of the known PAMPs, including LPS, peptidoglycan, flagellin, and CpG DNA. Accordingly, vesicles have been shown to trigger both the innate and adaptive immune responses from a variety of cell types (Unal et al., 2011).

Vesicles from *H. pylori*, *P. aeruginosa*, and *N. meningitides* were shown to activate the innate immune response through the delivery of peptidoglycan to epithelial cells. The peptidoglycan was recognized by the intracellular NOD1 receptor and this was followed by NFκB activation and subsequent IL-8 release (Kaparakis et al., 2010). In addition, *H. pylori* vesicles delivered intra-gastrically to mice evoke both innate and adaptive immune responses via a NOD-1 but TLR-independent mechanism.

*M. catarrhalis* vesicles may function as decoys and redirect the inflammatory response because they stimulate B-cells but the resulting antibodies are not specific for the parent bacterium. Thus, these vesicles delay the immune response to the infection (Vidakovics et al., 2010). In addition, *M. catarrhalis* vesicles are able to protect the respiratory pathogen *H. influenzae* from complement-mediated killing (Tan et al., 2007).

*Salmonella typhimurium* produces vesicles that stimulate macrophages and DCs, which results in a strong proinflammatory response with increased expression of MHC class II molecules and production of TNFα and IL-12 (Alaniz et al., 2007). Also, these vesicles prime both B-cells and T-helper cells *in vivo* to stimulate protective immunity.

Recently an interesting study revealed that the human commensal bacteria *Bacteroides fragilis* secretes vesicles that mediate immune tolerance in the gastrointestinal tract. The vesicles contain a capsular polysaccharide (PSA) and are internalized into DCs and program them in a PSA-dependent manner to induce Treg cells that suppress Th17 proliferation and the production of inflammatory mediators, hence preventing experimental colitis (Shen et al., 2012).

Vaccines are considered an important strategy in the treatment of bacterial diseases due to the rapid spread of antibiotic-resistant bacteria. Vesicles as a non-replicating vaccine have become a major interest in the field of immunotherapeutics (Unal et al., 2011). *N. meningitides* is responsible for major epidemic outbreaks of meningitis and vesicles have successfully been used as a vaccine against serogroup B meningococci for more than 20 years (Holst et al., 2009).
AIM OF THESIS

The aim of this thesis was to study host-pathogen interactions that occur via bacterial outer membrane vesicles shed from the gastric pathogen *H. pylori*.

Specific aims:

I. Characterize *H. pylori* vesicles concerning their protein content, with a particular interest in the adherence abilities of the BabA and SabA adhesins

II. Characterize *H. pylori* vesicles concerning their lipid content, via the development of a novel NMR method, semiconstant-time 2D 31P, 1H NMR COSEY

III. Analyze possible routes of cellular entry of *H. pylori* vesicles

IV. Analyze host cell responses mediated by *H. pylori* vesicles
RESULTS AND DISCUSSION

Paper I

Biochemical and functional characterization of *Helicobacter pylori* vesicles

Outer membrane vesicles are naturally shed from *H. pylori* during infection (Fiocca et al., 1999). The aim of this study was to isolate vesicles from *H. pylori* and characterize them in terms of their protein and lipid content. We had a particular interest in the BabA and SabA adhesins and their role in bacterial adherence to the gastric mucosa.

With electron micrographs, we confirmed that *H. pylori* shed vesicles during all stages of bacterial growth and that vesicle production increased during the stationary phase. We could show that environmental conditions affected the vesicle composition. We established protocols to isolate *H. pylori* vesicles and used density gradient centrifugation to purify vesicles from contaminating soluble proteins, broken flagella, and bacterial cell debris. The procedure of purifying vesicles was of critical importance because we wanted to study vesicles without worrying about contaminants that could interfere with our results.

Highly purified vesicles were characterized in terms of their protein and lipid content by mass spectrometry and NMR, respectively. PL present in *H. pylori* vesicles were identified with a newly developed method called 2D $^{31}$P,$^1$H COSY NMR (Paper II). The lipid profile in the vesicles resembled that of the outer membrane, with PE and CL being the dominating lipids, and we found that cholesterol represented 10% of the total lipid content in the vesicles.

The proteomic profile of the vesicles was determined with the highly sensitive nanoflow LC FT-ICR MS/MS technique. Proteins from purified vesicles were separated on an SDS-PAGE gel and >30 bands were analyzed with MS. Analysis of the MS data was performed against all species in the NCBI Genebank using the Mascot program and a significance level set to 99% with at least two matching peptides. We identified more than 300 proteins associated with the vesicles, including cytoplasmic and periplasmic proteins as well as OMPs and IM proteins. A majority of the OMPs were represented in the vesicles (77%), and OMPs accounted for 16% of the total protein content of the vesicles.

According to our MS analysis it is possible that cytoplasmic and IM proteins constitute a natural, albeit minor, part of the vesicles. We had loaded a large sample of vesicles when we separated the vesicle proteins by SDS-PAGE prior to the MS analysis. That, in combination with the high
sensitivity of the MS method, may explain the vast number of proteins we found associated with the vesicles. In addition, MS is not a quantitative method, thus it is possible that some cytoplasmic and IM proteins, although present in the vesicles, may correspond to impurities and actually account for a very small fraction of the total protein content. From previous studies it is well established that OMPs make up the majority of the proteins, but there is an on-going debate in the literature concerning the presence of cytoplasmic and IM proteins because the molecular mechanism of how vesicle shedding occurs are still not well understood (Kulp and Kuehn, 2010).

We identified a heterogeneous vesicle population with vesicles of varying sizes, densities (i.e. protein/lipid ratios), and protein contents. In our second MS analysis, we identified 126 proteins associated with vesicles having a narrower density than those isolated in the first analysis, and 93% of these proteins were overlapping with those identified in the first analysis. Mullaney et al. have also analyzed the protein content of vesicles from two *H. pylori* strains and identified 91 and 162 proteins in the two respective strains, both of which were enriched in OMPs (Mullaney et al., 2009). Hence, heterogeneity in the vesicle population, environmental conditions, bacterial strain, and growth phase influence the vesicle proteome.

Several known virulence factors of *H. pylori*, including VacA, HtrA, AlpB, and the BabA and SabA adhesins, were also present in the vesicles. The adhesins were detected on the vesicle surface with electron micrographs using immunogold labeling and cognate receptor conjugates. Interestingly, the vesicle size correlated with the presence of Leb-binding BabA adhesins, the larger the vesicle the more likely the Leb receptor conjugate was bound to the vesicle. The vesicles mediated binding to gastric tissue sections that could be blocked by the respective receptor conjugate. In addition, we showed that vesicle-associated BabA binds to the Leb-receptor conjugate with a similar affinity as BabA present in intact bacterial cells. The fact that the vesicle-associated BabA and SabA adhesins exhibited identical adhesion properties as those on the bacterial surface argues that the respective protein folding is the same. To our knowledge this is one of the most thorough studies of the mechanisms regarding vesicle adhesion and provides new details in regards to vesicle-host cell interactions. Recently it was described that the VacA cytotoxin present in *H. pylori* vesicles enhances vesicle association with host cells (Parker et al., 2010). It is likely that the BabA and SabA adhesins mediate vesicle adherence to host cells, which is followed by subsequent cellular uptake and host cell responses (Papers III & IV).

The *H. pylori* oncoprotein CagA is translocated into host cells via the T4SS. Thus, it was unexpected but very interesting to find the CagA protein associated with the vesicle surface. We found CagA in the MS analysis and
identified the protein with antibodies both in immunoblots and in electron micrographs with immunogold labeling. Only a few components of the T4SS were present in the vesicles so we suggest that the vesicles could constitute an alternative way of delivering CagA to host cells (Paper IV).

In conclusion, in Paper I we established protocols to isolate highly purified vesicles from \textit{H. pylori} and we characterized them in terms of their protein and lipid contents. We found a number of virulence factors associated with the vesicles, among them the oncoprotein CagA. In addition we found that the BabA and SabA adhesins were present in functional forms on the vesicle surface and could mediate binding to gastric tissue sections.

\textbf{Paper II}

\textit{Semiconstant-time P,H-COSY NMR: analysis of complex mixtures of phospholipids originating from Helicobacter pylori}

Biological membranes play vital roles in cellular compartmentalization, communication, and metabolic processes and are, in general, complex mixtures of proteins and PLs. The aim of this work was to establish a novel NMR method – semiconstant-time 2D $^{31}$P,$^1$H COSY – to be able to analyze and identify PLs in complex biological samples. The new NMR method was developed using \textit{H. pylori} as a model system. The advantages to using NMR as a method to analyze PLs is that it can simultaneously analyze each component in a complex mixture, provide quantitative measurements, and give structural information of yet unknown lipids.

To determine if the PLs present in the vesicles originate from the OM of \textit{H. pylori}, we analyzed vesicles along with whole bacterial cells and samples enriched with OM or IM samples (Paper I). The OM and IM could not be isolated with detergents due to disturbances this would cause in the NMR spectra. Instead, bacterial cells were lysed by mechanical force and the OM and IM were separated from each other through density gradient centrifugation. By definition, the IM contain less protein relative to lipids than the OM and this allows for their separation in a density gradient. Fractions enriched with OM were identified by immunoblotting with antibodies recognizing OMPs (\textit{\alpha}BabA, \textit{\alpha}SabA, and \textit{\alpha}OipA) and fractions enriched with IM were identified with antibodies raised against an IM protein (\textit{\alpha}ComB10) (Paper I, Fig. 4). Unfortunately, we could not completely separate the two membranes from each other with this method. We analyzed samples that were enriched with either OM or IM, and we did, indeed, see a difference in PLs between the two samples of membranes. The IM contained
PE and CL which was in contrast to the bacterial cells, OM, and vesicles that all contained PE, CL, PG, PC, and LPE. Thus, we conclude that the PL composition of the vesicles mostly resembles that of the OM where the most abundant PLs are PE and CL.

An increased membrane curvature is associated with both lyso-PL and CL (Huang et al., 2006). Interestingly, the PL composition of \textit{H. pylori} vesicles differed between vesicles of different sizes (Paper III). In addition, previous studies of \textit{P. aeruginosa} vesicles have found higher ratios of PG in the vesicles compared to the OM and suggest that membrane rigidity is important for vesicle biogenesis (Tashiro et al., 2011a). Thus, the bacterial PL composition might influence vesicle shedding and the resulting vesicle size. Because the PL composition in \textit{H. pylori} changes in response to environmental stress (Shimomura et al., 2004), it will be interesting in the future to analyze if the PL composition in vesicles changes along with that of the bacteria and, if so, how this might influence vesicle shedding.

In conclusion, in Paper II we identified major and minor PLs present in \textit{H. pylori} with our newly developed 2D $^{31}$P,$^1$H COSY NMR method that provides high sensitivity and quantification. We believe that this method will be applicable for the analysis of any organic phosphorous compounds.

**Paper III**

**Endocytosis of \textit{Helicobacter pylori} vesicles**

Internalization of vesicles and subsequent delivery of virulence factors can be achieved either through vesicle fusion with the host cell membrane or via endocytic pathways. In this study we aimed to analyze the routes by which \textit{H. pylori} vesicles are internalized by their host cells.

In the initial phase of this project, we used highly purified vesicles to study vesicle uptake in gastric epithelial cells with fluorescence microscopy. It was, however, difficult to distinguish vesicles that were associated with the host cell surface from vesicles that were internalized due to the high background of vesicles bound nonspecifically to the glass slide and to the cell surface. These nonspecifically bound vesicles were difficult, if not impossible, to remove using standard washing procedures. Thus, we explored an alternative approach based on a cleavable linker to specifically analyze and quantify internalized vesicles. Other studies concerning vesicle uptake are mainly based on microscopy, which is time consuming for quantification. We compared vesicle uptake both with fluorescent confocal microscopy and our new quantitative assay and found that the new method allows us to analyze a
significantly larger number of cells simultaneously. With this method, multiple replicates can be analyzed simultaneously and vesicle uptake by cells treated differently, such as with chemical inhibitors or with vesicles derived from different bacterial strains, can easily be compared. This is important because cellular behavior can differ, e.g. the rate of endocytosis is affected by cell density. Also, in comparison to other methods used to quantify vesicle uptake, we do not need to detach adherent cells for analysis and this minimizes the risk of disrupting the system and affecting the results of the experiment.

In this paper we showed that *H. pylori* vesicle internalization is time and temperature dependent. Vesicle internalization increased with time, but at very long time points the vesicles tended to aggregate at the surface and edges of the cells. This has not been reported previously, and we can only speculate if it is of importance or if the phenomenon occurs in nature. Vesicles did not bind to the cells at temperatures below 18° C, implying that membrane fluidity and cholesterol are important for vesicle adherence and internalization.

*H. pylori* vesicle internalization was affected by chemical treatments that deplete cholesterol but not sequester cholesterol. Depletion of cholesterol has been performed in previous studies of *H. pylori* vesicle uptake in where it affected uptake in one study but not in the other study (Kaparakis et al., 2010; Parker et al., 2010). The time of incubation, origin of the vesicles, cell medium, and method of analysis all influenced the results, and this showed that the experimental setup must be taken into consideration when drawing conclusions from the results. In our studies we found that our results differed depending on whether or not the drug was present during uptake and on the drug concentrations used. It was important in the experiments that concentrations of the drugs used to deplete or sequester the cholesterol and the time of treatment were monitored to ensure that they were not toxic to the cells. In addition, we always included transferrin as a control in these experiments. In regards to the other two publications studying *H. pylori* vesicle uptake after 4 h and 16 h, it seems rather unlikely that the cells would survive such a long drug treatment (ours did not). Also, if the drug is not present during uptake, the cells will probably recover and the effect of the drug treatment will be abolished.

We used both chemical inhibition and RNA interference to study vesicle internalization via specific endocytic pathways. We found that vesicle internalization decreased after acute depletion of dynamin, thus we have confirmed that *H. pylori* vesicles can enter epithelial cells via endocytosis. With fluorescent microscopy, we confirmed that *H. pylori* vesicles could enter cells via endocytosis because we saw co-localization of vesicles with a
subset of markers for endocytosis including dynamin, clathrin, caveolin-1, early endosomes, and lysosomes. To our knowledge we are the first to demonstrate that dynamin is important for vesicle internalization. Through chemical inhibition we showed that vesicles could enter host cells via CME. However, we did not see a decrease in vesicle internalization after knockdown of specific proteins such as AP-2 and dynamin II that are involved in CME. To explain this contradictory result, we suggest that in the heterogeneous vesicle population different vesicles use different endocytic pathways and that this occurs simultaneously. As a consequence, we do not detect an effect in vesicle internalization after knockdown because blockage of one pathway may upregulate others during long-term siRNA treatment. In addition, neither of our chemical treatments diminished vesicle uptake completely. This is difficult to achieve in any case, but we would expect the decrease to be more pronounced than it was if the vesicles only used one route for cellular entry.

We showed that there is a great variability in vesicle size, and we speculate that this might influence the endocytic route. In the future it will be interesting to study a subset of *H. pylori* vesicles to determine if vesicle size or the content of the vesicles influence the route of entry. It will also be interesting to analyze if and how specific proteins influence vesicle entry and to which cellular compartment certain proteins are transported. For example, in ETEC vesicles the presence of the LT toxin influences binding, internalization, and toxicity (Kesty et al., 2004) and *P. aeruginosa* vesicles are known to deliver their contents to different subcellular compartments (Bomberger et al., 2009). It has been suggested that the presence of the VacA cytotoxin in *H. pylori* vesicles contributes to the vesicles’ ability to exploit more than one pathway for internalization (Parker et al., 2010). Furthermore, it will be interesting to determine if the high affinity interaction of the BabA and SabA adhesins with their cognate receptors influences vesicle internalization and if these vesicles are associated with a specific route. For example, a large vesicle is more likely to carry the BabA adhesin (Paper I), suggesting that these vesicles can adhere to host cells, which is followed by subsequent internalization. In addition, analyzing vesicle internalization in a system with polarized epithelial cells would mimic the *in vivo* situation and might lead to a different result because endocytosis is differently regulated at the apical and basolateral poles (Sandvig et al., 2011).

In conclusion, we have found in Paper III that vesicles from *H. pylori* are composed of a heterogeneous population in which the proteins on the vesicle surface provide a number of targets for adherence and can potentially influence internalization. A previous report has shown that *H. pylori* vesicles are internalized via lipid rafts (Kaparakis et al., 2010), but another study
concluded that *H. pylori* vesicles enter via CME but not via lipid rafts (Parker et al., 2010). Here we have shown that both routes of entry are possible for *H. pylori* vesicle internalization.

**Paper IV**

*Helicobacter pylori* vesicles and host cell responses

Host cells that come in contact with bacterial vesicles are exposed to the majority of components that makes up the bacterial OM. Thus, shedding of vesicles is suggested to be an additional secretion system for delivering host effector molecules and virulence factors. We have previously found that the CagA oncoprotein, which is normally translocated via the T4SS to host cells, is associated with the surface of *H. pylori* vesicles (Paper I). The aim of this study was to characterize the host cellular responses that correspond to vesicle contact, with a special focus on the CagA protein.

When we incubated *H. pylori* vesicles with gastric epithelial cells, we saw a change in morphology that resulted in apoptosis over time. Such cellular effects have previously been described to be independent of VacA-positive vesicles (Ayala et al., 2006). We did not see the typical “hummingbird” phenotype, *i.e.* cell scattering and elongation, that is usually associated with T4SS-mediated translocation of CagA into the host cell and subsequent phosphorylation of CagA (Segal et al., 1999). Nor were we able to detect any phosphorylation of vesicle-associated CagA. Thus, the morphological change that the vesicles induce is probably not associated with vesicle-associated CagA being phosphorylated upon delivery. However, non-phosphorylated CagA is also associated with a variety of host cellular responses (Tegtmeyer et al., 2011).

We saw that *H. pylori* vesicles activate the PKC kinase and the MAP kinases and that this was dependent on the time of incubation but independent of the delivery of CagA. The *virB*11 mutant of *H. pylori* does not have a functional T4SS, but the vesicles isolated from this strain still carry CagA on their surface. Interestingly, activation of the PKC, Erk1/2, and JNK kinases all required vesicles isolated from a strain with a functional T4SS. It is known that *H. pylori* vesicles induce a NOD-1 response via the delivery of peptidoglycan to host cells. This results in activation of MAP kinases, AP-1, and NFκB followed by a subsequent release of IL-8 (Allison et al., 2009; Kaparakis et al., 2010). We found that the activation of the PKC, Erk1/2, and JNK kinases is partially dependent on a functional T4SS in the parent bacteria in addition to the delivery of vesicle-associated peptidoglycan. Thus, we suggest that other components of either the T4SS or proteins that are
transported by the T4SS to the bacterial surface (and thus become associated with the vesicle surface) could be involved in cell signal activation.

*H. pylori* vesicles induced pro-inflammatory responses independently of CagA and a functional T4SS in the parent bacteria. However, our results did not show a significant difference compared to the control, and the results in this manuscript should be interpreted as an indication that there is no difference in the pro-inflammatory response depending on the vesicle type. Others have shown that *H. pylori* vesicles induce IL-8 release from gastric epithelial cells (Ismail et al., 2003; Mullaney et al., 2009). We argue that our lack of significance could be dependent on the experimental setup, such as the time of incubation, the amount of vesicles used, and the fact that there is a large variation in cellular response, *e.g.* the release of IL-8 in different gastric epithelial cell lines (Schneider et al., 2011). There was, however, a significant release of TNF from PMN upon incubation with vesicles from a wild-type and cagA mutant strain compared to the control, but this was not dependent on the delivery of CagA. This effect has not been described previously. These experiments need to be repeated with additional epithelial cell lines and different cell types such as a macrophage-like cell line. In addition, we plan to analyze whether *H. pylori* vesicles activate the transcription factors NFκB and AP-1 in the presence or absence of CagA and a functional T4SS in the parental bacterial strain.

Besides the results presented in the manuscript, we have also analyzed whether the *H. pylori* vesicles activate TLR4, mediate any effect on B-cell activation, or cause the release of IP-10 and IFN-β. No effects on any of these processes were observed. *H. pylori* has been shown to induce shedding of CD46, a regulator of complement activation, via the UreA urease subunit protein and the AhpC protein (Basmarke-Wehelie et al., 2011). In the MS analysis, we found that the UreA subunit was associated with the vesicles. This encouraged us to test if vesicles also caused shedding of CD46, but they did not.

Similarly to *H. pylori* bacterial cells (Amieva et al., 2003), we found that vesicles with CagA localized in close contact with junction areas in the epithelial cells. This was in contrast to vesicles devoid of CagA. Non-phosphorylated CagA delivered to host cells by *H. pylori* is known to affect the integrity of both the tight and adherence junctions and to disrupt cell polarity (Amieva et al., 2003; Murata-Kamiya et al., 2007; Saadat et al., 2007). We saw a slight modification in the distribution of the adherence and tight junction proteins E-cadherin, β-catenin, ZO-1, and occludin. We have initiated an MS approach to identify proteins in the cell junction complexes that the vesicles interact with.
In conclusion, we showed in Paper IV that the CagA delivered by *H. pylori* vesicles was not phosphorylated by the host cell, thus the typical cell scattering and cell elongation in response to CagA did not occur. Vesicles carrying the non-phosphorylated form of CagA localized close to junction areas and induced a slight modification of the distribution of the apical junction proteins. In addition, we found that additional factors other than peptidoglycan delivered from the vesicles can induce cell signaling and that these factors are dependent on a functional bacterial T4SS. Thus, we believe that vesicles constitute an important contributor of the bacterial-host cell interactions that occur during the persistent life-long *H. pylori* infection.
CONCLUDING REMARKS

Shedding of bacterial membrane vesicles occurs in Gram-negative and Gram-positive bacteria as well as in archaea, which suggests that it is a conserved and essential process. The presence of vesicles affect the host cells and surrounding microbes in different ways. To date, much attention has been given to vesicles from pathogenic bacteria and the role of these vesicles as vehicles for delivery of virulence factors and host cell effector molecules and the impact that these vesicles have on host cells and pathogenicity. However, several studies have shown that vesicles also play important roles in intra- and interbacterial communication and in protecting the intact bacteria from immune cells as well as transfer of genetic material.

My thesis concerns vesicles released from *H. pylori* and their interactions with host cells. The studies in this thesis have contributed to a better understanding of the biological roles of vesicles by characterizing their composition. *H. pylori* vesicles contain a number of virulence factors with diverse functions associated with the parental bacteria, thus vesicles are highly likely to play multiple roles during infection. In addition, virulence factors delivered by vesicles could possibly have biological activities that are different from those of the freely soluble proteins, as is suggested for the VacA cytotoxin in *H. pylori* vesicles (Ricci et al., 2005). In Paper I we found that the *H. pylori*, oncoprotein CagA, is associated with vesicles, in addition to several other virulence and host effector molecules that were found to be associated with the vesicles, *e.g.* the OipA and HtrA proteins were enriched in the vesicles compared to other proteins and to the parent bacteria.

We found both the BabA adhesin and the SabA adhesin on *H. pylori* vesicles and showed that they both mediate adhesion to human gastric mucosa. The BabA adhesin was recently shown to potentiate T4SS-dependent pathogenesis and the release of pro-inflammatory cytokines (Ishijima et al., 2011). We showed that multiple pathways are involved in the uptake of vesicles and it would, of course, be interesting to determine if BabA-Leb binding or SabA-sLex binding guides vesicles towards a specific uptake pathway and particular host cellular responses.

CagL and CagY on the T4S needle binds to β1-integrin receptors located basolaterally, and how *H. pylori* come into contact with these receptors is not fully understood. The high-affinity interaction of the BabA and SabA adhesins might contribute to the initial delivery of CagA to the apical side of the host cells and hence contribute to the CagA-mediated disruption of tight junctions and loss of cell polarity. In Paper III we followed the internalization of vesicles into gastric epithelial cells and in paper IV we explored host cellular effects associated with the presence of *H. pylori*.
vesicles and discussed the possibility of *H. pylori* vesicles having a role in disrupting the epithelial barrier and increasing the ability of bacteria to gain access to the basolateral side of the epithelial cells.

The human stomach is an ever-changing environment and the constant shedding of *H. pylori* vesicles might contribute to the persistence of the bacteria by acting as decoys against immune responses and by contributing to biofilm formation. Thus the vesicles could be directly contributing to ongoing gastritis in the human stomach. Electron micrographs revealed more than 10 years ago that *H. pylori* shed vesicles during infection (Fiocca et al., 1999). Since then research has suggested that *H. pylori* vesicles contribute to pathogenesis. The amount of vesicles shed during infection is not known, neither what triggers vesicle release *in vivo*. Research should try to better mimic the *in vivo* situation and perhaps micro-dissection of biopsy material in combination with mass spectrometry could be used to identify important players. A critical point is to continue to analyze the pathways that operate to internalize vesicles and to follow the uptake of individual proteins and their subsequent route within the cell. Live-cell experiments over time could contribute to further understanding the host-pathogen interactions and the biological roles of *H. pylori* vesicles during infection.
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