HcpE, a potential immuno-modulatory protein from Helicobacter pylori that is dependent on the Disulfide bond protein DsbHP

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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HcpE, a potential immuno-modulatory protein from *Helicobacter pylori* that is dependent on the Disulfide bond protein Dsb\textsubscript{HP}

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Graduate program

in

Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of

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The University of Western Ontario

London, Ontario, Canada

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ABSTRACT

*H. pylori* is a human gastric pathogen that colonizes ~50% of the world’s population. It can cause gastritis, gastric or duodenal ulcers and also gastric cancer. *H. pylori* produces *Helicobacter* cysteine rich protein HcpE, a secreted protein which may play a role in virulence. In this study we show that HcpE is secreted in the culture supernatant both as a soluble protein and in association with outer membrane vesicles, and may play a role in the modulation of *H. pylori* inflammatory responses. We identified that DsbHP is necessary for HcpE production and secretion in *H. pylori*, and demonstrated DsbHP has DiSulfide Bond (Dsb) forming activity on reduced lysozyme. Furthermore, we demonstrated that DsbHP has a DsbA-type of activity when expressed in *E. coli*, despite its similarity with DsbG, and that DsbHP is involved in maintaining redox homeostasis in *H. pylori*.

**Key words:** *Helicobacter pylori*. Disulfide bonds. Dsb proteins. *Helicobacter* Cysteine-rich proteins. Protein secretion.
DEDICATION

This thesis is dedicated to my entire family, specifically Jeff, Lorrie, Rachelle and Justin, who have supported me along the way.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-chloride-potassium</td>
</tr>
<tr>
<td>BD</td>
<td>Binding domain</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CagA</td>
<td>Cytotoxin associated gene A</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cRPMI</td>
<td>Complete Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>Dsb</td>
<td>Disulfide bond</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Hcp</td>
<td>Helicobacter cysteine rich protein</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicle</td>
</tr>
<tr>
<td>O/N</td>
<td>Over night</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAI</td>
<td>Pathogenicity Island</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease inhibitor cocktail</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPI</td>
<td>Peptidyl-propyl isomerases</td>
</tr>
<tr>
<td>PPR</td>
<td>Pentatricopeptide repeat</td>
</tr>
<tr>
<td>PR</td>
<td>Ponceau red</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLR</td>
<td>Sel1-like repeat</td>
</tr>
<tr>
<td>SS1</td>
<td>Sydney Strain</td>
</tr>
<tr>
<td>T2SS</td>
<td>Type II secretion system</td>
</tr>
<tr>
<td>T4SS</td>
<td>Type IV secretion system</td>
</tr>
<tr>
<td>Tat</td>
<td>Twin arginine transport</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeats</td>
</tr>
</tbody>
</table>
WB  Western blot
VacA  Vacuolating cytotoxin
CHAPTER 1: INTRODUCTION

1.1 *Helicobacter pylori*: a human gastric pathogen

*Helicobacter pylori* is a gram negative, spiral shaped microaerophillic bacterium that chronically infects human gastric mucosa (Kusters *et al.*, 2006). Over 50% of the world’s population is infected with *H. pylori*, with approximately 30% and 80% infection rates in industrialized and non-industrialized countries respectively (Carroll *et al.*, 2004). *H. pylori* causes infections in young and old, as well as healthy and immune compromised individuals equally. However, infection with *H. pylori* is thought to be acquired early on in childhood with the onset of disease usually occurring in adults (Goodman and Correa, 1995). Most acute infections remain asymptomatic, although some nausea, halitosis, and epigastric discomfort can occur. Long term chronic infections with *H. pylori* have been associated with peptic ulcers, gastric lymphoma, and gastric adenocarcinoma (Montecucco and Rappuoli, 2001). Transmission is believed to occur from person-to-person, via the oral-oral, or oral-fecal route (Goodman and Correa, 1995). *H. pylori* transmission is most prevalent in communities with poor sanitary conditions, overcrowding and unclean water, however host genetics, as well as the infecting strain are thought to also play a large role (Vakil and Go, 2000; Sinha *et al.*, 2004).

The bacterium was first discovered in 1982 by Barry Marshall and Robin Warren, for which they were awarded the Nobel Prize in 2005. By performing a self-ingestion experiment, they were able to establish the link between *H. pylori* and peptic ulcers, disproving the original theory that eating spicy foods was the primary cause (Marshall and Warren, 1984).
With approximately a million new cases every year, gastric cancer is the fourth most prevalent cancer worldwide, and has the second highest mortality of any cancer (CDC, 2012). About 65-80% of all gastric cancers are linked to *H. pylori* infection, and patients diagnosed with *H. pylori* infection are 6 times more likely to develop gastric cancers, with approximately 2% of infected patients eventually developing severe disease (Parkin et al, 2002). These findings led the World Health Organization to recognize *H. pylori* as a Group-1 carcinogen in 1995. *H. pylori* infections can persist over the entire life span of patients if gone untreated. Treatment usually include antibiotics and proton pump inhibitors which are administered simultaneously. They are poorly tolerated due to their numerous side effects, which can lead to a high rate of patient non-compliance and treatment failures (Vakil and Go, 2000). This has led to the emergence of antibiotic resistance in *H. pylori* (Gisbert and Pajares, 2002). Thus, a search for novel therapeutic targets will help us further understand the mechanisms involved in *H. pylori* disease progression, and help prevent chronic infections that lead to the development of gastric ulcers and cancers.

### 1.2 *H. pylori* virulence mechanisms

For successful colonization, *H. pylori* must be able to overcome the harsh environment of the stomach. This is achieved through the expression of various bacterial virulence factors that include structural features like bacterial flagella and lipopolysaccharide (LPS), the synthesis of urease to protect against the acidity of the stomach, and the production and secretion of toxins and other secretory proteins, which alter epithelial integrity and induce inflammatory responses. Together, all these factors
contribute to successful chronic infections which can ultimately lead to gastric disease (Montecucco and Rappuoli, 2001).

1.2.1 Urease

The acidic pH of the stomach makes it a very harsh and uninhabitable environment that usually prevents growth of most microorganisms. *H. pylori* has developed a unique strategy to combat this acidity, which allows for its survival in an environment to which it would otherwise be susceptible. In the genome of *H. pylori*, a gene cluster of seven genes named *ureA-G* exists which encode the enzyme urease, and all its accessory proteins (Hu and Mobley, 1990; Labigne et al, 1991). Urease is an intracellular nickel-containing metalloenzyme found in the cytosol which converts urea into carbon dioxide and ammonia. Urease is composed of two subunits, α and β, and has been shown to form a supra-molecular decameric complex of repeating α-β subunits. Each coupled pair of subunits has an active site, for a total of 12 active sites (Ge et al, 2013). The ammonia generated following hydrolysis of urea plays an essential role in the survival of *H. pylori*, neutralizing the gastric acid within close proximity. For this process to occur, *H. pylori* must be able to uptake urea from the external environment. Urea enters the periplasm via a proton-gated urea channel which is encoded by the gene *ureI* (Montecucco and Rappuoli, 2001). This channel remains closed and is only opened when *H. pylori* detects low pH within its environment (Weeks et al, 2000). Urease activity is essential for colonization by *H. pylori*, as mutants unable to express urease are unable to colonize the stomachs of gnotobiotic piglets and mice (Eaton et al, 1992).

Urease activity produces ammonia. Ammonia is toxic to cells at high concentrations, and thus may be considered a “double-edged” sword for the bacteria.
concentrations of ammonia that *H. pylori* produces during an *in vivo* infection are currently not known. However, *H. pylori* may have some strategic way to control the concentration of ammonia produced or it would succumb to toxicity and be unable to maintain chronic infection (Montecucco and Rappuoli, 2001).

### 1.2.2 Flagella

Once in the stomach, *H. pylori* needs to localize to the gastric epithelium to establish a successful infection. To accomplish this, *H. pylori* uses 2 to 6 unipolar flagella to propel itself through the mucosal lining until it reaches the underlying epithelium (Goodwin *et al*., 1985). *H. pylori* flagella are composed of two types of flagellins, flagellin A (FlaA) and flagellin B (FlaB). FlaA is the most abundant flagellin, and is essential for successful colonization as disruption of *flaA* results in the attenuation of motility and the inability of *H. pylori* to reach the gastric epithelium. Alternatively, disruption of *flaB* has no effect on the motility of the bacterium (Suerbaum *et al*., 1993). The flagella are covered by a sheath, which is a continuation of the outer membrane, containing proteins, lipids, and lipopolysaccharide that protects the flagellum from the acidic environment of the gastric lumen (Goodwin *et al*., 1985).

The flagellum is a complex multiprotein organelle that consists of the basal body, the hook, and the filament (Goodwin *et al*., 1985; Geis *et al*., 1989). The basal body spans from the cytoplasm to the outer membrane and is a complex assembly of proteins responsible for rotation of the flagellum. The hook serves as a joint connecting the basal body to the filament (Fraser *et al*., 1999). Due to the fact most of the flagellar assembly is located beyond the cytoplasmic membrane, many of the flagellar proteins must cross the membrane to reach their destination. The partially folded flagellin A and B proteins pass
through the central pore in the basal body, the hook and the growing filament, until they reach the distal end where they polymerize until the process is complete (Macnab, 2003).

1.2.3 LPS

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria (Raetz and Whitfield, 2002). Bacterial LPS plays an important role in the structural integrity of the outer membrane as well as protecting the cell from external chemical attack. LPS is also an endotoxin, capable of inducing strong immuno-stimulation. The LPS of many different Gram negative species share a common structure consisting of three conserved regions (Raetz and Whitfield, 2002). Lipid-A is a phosphorylated glucosamine disaccharide to which are esterified with fatty acid chains that anchor the LPS into the outer membrane. The lipid-A portion of the LPS is typically the source of the toxicity that is associated with Gram-negative bacteria (Moran, 2008). The oligosaccharide is bound directly to the lipid-A core, and is comprised of 10-15 sugars. The outermost domain of the LPS is the O-polysaccharide. This region consists of oligosaccharide repeat units that can vary from one strain to the next. Due to the fact that the O-polysaccharide is exposed at the outer-most surface of the bacterial cell, it is usually one of the primary targets of the host immune system via antibody recognition (Moran, 2008).

The structure of *H. pylori* LPS has been well studied and characterized (Moran, 2008). Unlike most other Gram negative species, the lipid-A component of *H. pylori* LPS has low immunogenic properties and low lethal toxicity toward host gastric epithelia (Muotiala *et al*, 1992). Several studies have proposed that the phosphorylation and acetylation patterns that are unique to *H. pylori* lipid-A result in under-phosphorylation and
under-acylation compared to other enterobacteria, which result in low immunological stimulatory properties (Stead et al, 2008).

Another unique characteristic feature that H. pylori O-antigen possesses is the presence of extended chains of fucosylated N-acetyllactosamine units. These side chains have been shown to mimic human type 2 Lewis blood group antigens Le^x and Le^y in a process known as molecular mimicry. A typical H. pylori O-antigen chain consists of a poly(N-acetyl-β-lactosamine) chain which is heavily fucosylated to form an inner Le^x region with a terminal Le^x or Le^y (Moran, 2008). The expression of Le^x or Le^y on the surface is very common in H. pylori and occurs in 80-90% of known strains (Marshall et al, 1998). Molecular mimicry allows H. pylori to evade the host humoral immune system by decorating itself with structures that the host does not recognize as foreign, and thus prevents clearance and promotes chronic infection (Moran et al, 2001).

H. pylori LPS has also been shown to be important in colonization and pathogenesis. The Le^x antigen has been shown to play an important role in adhesion to gastric tissue. Both O-antigen and Le^x expression have been shown to be important for in vivo colonization, as strains deficient in Le^x or expressing truncated LPS colonized mice less efficiently (Edwards et al, 2000; Moran et al, 2000).

1.2.4 Toxins

H. pylori encodes two main toxins that are implicated in the development of gastro duodenal disease; Vacuolating cytotoxin (VacA) and Cytotoxin associated gene A (CagA) (Argent et al, 2006). Strains expressing CagA and VacA are classified as type I strains, associated with a poor prognosis characterized by peptic ulceration (Covacci et al, 1993).
All strains of *H. pylori* contain a copy of the *vacA* gene, but there is a vast genetic diversity causing alleles from various strains to be categorized into multiple different families (Atherton, 1995). The allele s1/m1 is the most highly studied form of VacA due to the fact that it is associated with a high level of vacuolation compared to most other alleles (Atherton, 1995). VacA is a high-molecular weight oligomeric multi-functional toxin. VacA is known for its ability to produce vacuoles within the gastric epithelial cells, which eventually lead to cell death. However, more recently VacA has also been implicated in cellular apoptosis by localizing to and disrupting the mitochondria, preventing T-cell activation and proliferation, as well as disruption of endosomal and lysosomal activity (Kusters et al, 2006).

CagA is the most studied and characterized *H. pylori* virulence factor. The 120–145 kDa protein is encoded within the cytotoxin-associated gene pathogenicity island (*cag*PAI), a 40kb DNA segment that has been acquired via horizontal gene transfer. The *cag*PAI is composed of 27-31 genes (Censini et al, 1996), which encodes a *Helicobacter* specific type IV secretion system. The type IV secretion apparatus forms a syringe-like structure that penetrates the membrane of gastric epithelial cells and allows for the direct transport of CagA (reviewed in Blaser and Atherton, 2004). Translocation of CagA into gastric epithelial cells results in the induction of a phosphorylation cascade by Src kinases to phosphorylate CagA. Phosphorylation of CagA results in actin polymerization and rearrangement causing cellular elongation, known as the hummingbird phenotype (Tummuru et al, 1995; Segal et al, 1997; Segal et al, 1999). CagA can also have diverse effects on the gastric mucosa in the absence of phosphorylation. Unphosphorylated CagA can induce inflammation due to activation of NF-κB pathways, can disrupt tight junctions,
as well as cause cellular differentiation. These changes eventually lead to the secretion of cytokines and chemokines such as interleukin 8 (IL-8) (Tummuru et al, 1995).

1.2.5 Adhesins

For colonization to be successful, 4 steps must occur: transmission to the host, adherence to host cells within a specific niche, avoidance of host defenses, as well as nutrient acquisition. Due to the fact that the human gastric mucosa regularly experiences cellular turnover and peristalsis, strong adherence to epithelial cells by H. pylori is one of the most important steps and is essential to prevent displacement from the stomach (Ilver et al, 1998). Adhesins are bacterial proteins that facilitate interactions between a bacterium and host cells during initial stages of infection. H. pylori expresses numerous adhesins that can vary among strains as well as growth conditions. Blood group antigen-binding adhesin BabA, is one of the most studied and well characterized adhesin proteins of H. pylori. BabA is a member of the Hop family of outer membrane proteins (Ilver et al, 1998) that mediates binding to fucosylated Lewis b (Le\textsuperscript{b}) blood group antigens expressed on human cells (Odenbreit, 2005). The presence of BabA expression is highly correlated to strains responsible for duodenal ulcers and adenocarcinoma. The absence of BabA from the outer membrane of H. pylori results in decreased adhesion and pathogenicity in vivo, suggesting a crucial role in colonization and disease progression (Pohl et al, 2009). BabA is also thought to aid in the delivery of other H. pylori virulence factors, such as CagA, upon binding to Le\textsuperscript{b} on the surface of the gastric epithelia (Pohl et al, 2009).

Another well characterized adhesin is the sialic acid-binding adhesin (SabA). SabA binds to glycosphingolipids that display sialylated Lewis x (Le\textsuperscript{x}) antigens that are up regulated during states of inflammation. H. pylori induced gastric inflammation promotes
the replacement of non-sialylated Lewis antigens by sialylated Le\textsuperscript{x} and sialylated Le\textsuperscript{a}, implicating SabA as a virulence factor involved in long term chronic inflammation (Mahdaui \textit{et al}, 2002). Clinical isolates demonstrate heterogeneity in their ability to bind sialylated Le\textsuperscript{x}, and this variability depends on both the host and the changing epithelial receptors during inflammation. This adaptation during inflammation demonstrates the ability of \textit{H. pylori} to acclimate to different conditions during infection. (Pohl \textit{et al}, 2009).

\textit{H. pylori} expresses several other adhesins that have been well characterized and found important for colonization. AlpA and AlpB are two outer membrane proteins both belonging to the Hop family of proteins, which have been shown to be necessary for Lewis antigen independent adhesion (Dhar \textit{et al}, 2003). Without efficient adhesion, \textit{H. pylori} would be unable to inject toxins and other virulence effector proteins into host cells, as well as resist constant peristalsis, resulting in an inability to colonize the harsh environment of the stomach (Dhar \textit{et al}, 2003).

\textbf{1.3 Protein secretion systems}

\textbf{1.3.1 Protein secretion in \textit{Helicobacter pylori}}

The secretion of proteins is required for common bacterial functions such as the release of virulence factors into the extracellular matrix. In \textit{H. pylori}, the secretome has been difficult to analyze due to its preferred growth conditions (Kim \textit{et al}, 2002). \textit{H. pylori} is commonly cultivated in rich media supplemented with serum, and the proteins within the serum make it difficult to analyze any of the secreted proteins (Bumann \textit{et al}, 2002). However, growth in serum free culture conditions have been optimized to facilitate the study of \textit{H. pylori}
secreted proteins (Bumann et al., 2002).

Five major studies have attempted to analyze the secretome of \textit{H. pylori}. Using two-dimensional gel electrophoresis and mass spectrometry, 26 secreted proteins were identified using optimized growth conditions (Bumann et al., 2002). Five different secretory pathways have been well characterized in Gram-negative bacteria (Figure 1), however only the systems that have functional relevance to \textit{H. pylori} pathogenesis will be described below.

1.3.2 Sec secretion for the transport of proteins across the inner membrane

The Sec secretion system is responsible for transporting proteins from the bacterial cytoplasm to the periplasm for post-translational folding. This system has been best characterized in \textit{Escherichia coli}. The Sec system within \textit{E. coli} consists of nine proteins that are divided into 3 stages of the secretion process (Manting and Driessen, 2000). The first stage is targeting, whereby the proteins to be secreted into the periplasm are targeted to the cytoplasmic membrane. This is facilitated though a Sec signal peptide located at the N-terminus end of the protein. A chaperone protein SecB recognizes this signal sequence that is 20-30 amino acids in length (Economou, 1999) and facilitates its translocation to the cytoplasmic membrane. Due to the fact that the Sec secretion system can facilitate the transport of a wide array of different proteins and is found in many bacteria, it must remain highly conserved. The Sec secretion signal peptides are highly conserved and contain three domains. The first domain is a basic N-terminus with a net positive charge. The second is
Figure 1: Summary of the main secretion systems found in Gram-negative bacteria.

The secretion system relevant to this study is the Sec secretion system and the general secretory (type II) pathway.

Panel A: Sec-dependent secretion systems.

Panel B: Sec-independent secretion systems.

OM: outer membrane, IM: inner membrane. Adapted from Fronzes et al., 2009.
Sec-dependent
Type V secretion  Type II secretion

Sec-independent
Type IV secretion  Type III secretion  Type I secretion

OM
IM
OM
IM
a hydrophobic core, and the final domain is a hydrophilic C-terminus with a consensus
signal and cleavage site. Once the target protein is recognized by SecB, the second stage
involving the process of translocation is initiated. This second stage requires a cytoplasmic
membrane protein capable of translocating the targeted protein from the cytoplasm into the
periplasm. SecA is a peripheral membrane protein which has ATPase activity, and
functions as a molecular motor to force the unfolded protein through the protein conducting
channel (PCC). SecA accepts the target protein from SecB, and through the catalysis of
ATP, forces the protein through the PCC into the periplasm (Manting and Driessen, 2000).
This PCC is a translocase that is composed of heterodimers of membrane proteins SecY,
SecE and SecG. In the third and final stage, the translocated protein now within the
periplasmic space is released from the membrane via cleavage of the signal sequence by
signal-peptidases. From here, the protein undergoes proper folding with the aid of
periplasmic folding proteins. Once properly folded, the protein may reside in the periplasm,
or may be targeted to the outer membrane for final secretion, or may integrate into the
membrane (Economou, 1999). To date, homologues of proteins involved in Sec secretion
have been identified in the *H. pylori* genome (Fitchen et al, 2003) implying that this system
is intact and fully functional in *H. pylori* (Atherton et al, 1995).

### 1.3.3 Tat-secretion for the transport of proteins across the inner membrane

Twin-arginine translocation (Tat) systems are conserved transport systems found
in many different forms of life including bacteria, archaea, and plant plastids. Unlike the
Sec secretion system, Tat systems use a proton gradient to transport folded proteins
across the cytoplasmic membrane (Stanley et al, 2000; Saier, 2006). Tat substrates
possess N-terminal signal sequences that resemble the overall organizational structure of
the Sec secretion signal due to the fact that they contain a positively charged N-region, a hydrophobic h-region as well as a short polar C-region that contains the cleavage site. The Tat signal sequence is recognized by a conserved sequence containing two arginines. In bacteria this motif has been described as Z-R-R-x-Φ-Φ, where the Z refers to any polar residue and the Φ refers to a hydrophobic residue (Stanley et al., 2000). In *E. coli*, the Tat system is composed of five proteins, TatA, TatB, TatC, TatD and TatE. TatB and TatC associate together to form a stable TatBC complex which is responsible for recognizing the Tat signal sequence and bringing the protein into close proximity to the Tat pore apparatus encoded by TatA. TatD and TatE are involved in Tat secretion, however their functions are not yet understood. The Tat secretion system has been shown to be very important in the secretion of virulence factors such as phospholipases, proteins involved in pyoverdine-mediated iron uptake, anaerobic respiration, osmotic stress defense, motility, and biofilm formation (Ochsner et al., 2002; Natale et al., 2008). Analysis of all the sequenced *H. pylori* genomes reveals the presence of *tatA*, *tatB*, and *tatC* which are required to form a fully functional Tat secretion system. The Tat system is required for *H. pylori* virulence as TatC is required for viability and successful colonization of *H. pylori* (Benoit and Maier, 2014).

### 1.3.4 Protein folding in the periplasm

Proteins that are translocated into the periplasm via the Sec system and released into the periplasmic space by cleavage of their N-terminal signal sequence need to undergo proper folding and maturation. The stability of proteins within the periplasm is very important to maintain homeostasis as well as their potential secretion via the general secretory pathway. A large number of periplasmic enzymes that are able to catalyze the folding and turnover of these proteins have been identified and studied extensively in *E.*
coli. A common mechanism by which proteins containing more than one thiol group are folded, is the formation of disulfide bonds between 2 cysteine residues. These bonds can be vital for the stability of proteins within the periplasm. In E. coli, the formation of disulfide bonds in the periplasm is catalyzed by the Dsb protein system, which will be discussed in detail in section 1.4. Another way periplasmic proteins can be folded is by the isomerization of peptidyl-proline bonds (Economou, 1999). Proteins that contain proline residues can undergo catalysis facilitated by the peptidyl-proline isomerases or PPIs (Stathopoulos et al, 2000).

The periplasm also contains factors that assist proteins in folding correctly. This is typically mediated by molecular chaperones and is essential for the secretion of extracellular proteins which must acquire a properly folded state before transit across the outer membrane. Chaperones recognize hydrophobic regions of proteins that are exposed to the surface in the unfolded or mis-folded protein and assist in the prevention of protein aggregation or proteolysis. Chaperones can also function as shuttles by targeting secreted proteins from the inner membrane to their secretion apparatus in the outer membrane and facilitate movement across the periplasm (Economou, 1999).

1.3.5 **General secretory pathway for the transport of proteins from the periplasm to the extracellular matrix**

The general secretory pathway or type II secretion system (T2SS) of Gram-negative bacteria is a secretion system that translocates proteins from the periplasmic space across the bacterial outer membrane to the external milieu. The T2SS and all of its components have been well studied and characterized (Clanciotto, 2005). T2SS are encoded by a set of 12-16 general secretory pathway genes that are organized into large operons. A large
translocation pore that spans the outer membrane is responsible for translocating the properly folded proteins within the periplasm across the OM into the extracellular environment (Cao and Saier, 2003). This pore is composed of 12-15 secretin subunits that form a large ring structure with a central pore ranging from 50–80 Å in diameter. This pore is large enough to accommodate fully folded proteins. The remaining 11-14 conserved components are likely involved in anchoring of the pore and regulating its opening and closing (Clanciotto, 2005). This process by which proteins are transported across the periplasm to the outer membrane pore is still not fully understood. The type II secretion in *H. pylori* has not been well characterized, however, it is thought that the bacterium uses this pathway for the general secretion of proteins as no other general secretion systems specific to *H. pylori* have been identified.

1.3.6 Direct transport from the cytoplasm to the extracellular environment

In terms of secretion systems involved in the direct transport of proteins from the cytoplasm to the extracellular environment, one has been well characterized in *H. pylori*, the type IV secretion system (T4SS). The T4SS is found in both Gram-positive and Gram-negative bacteria and is homologous to the conjugation machinery. Thus, the T4SS is capable of translocating both DNA and proteins that alter various eukaryotic cell processes. The T4SS is composed of approximately 12 proteins that form an inner membrane complex, a core complex that spans the periplasm, and a central pilus (Cascales and Christie, 2003). The central pilus is composed of VirB2 and VirB5 subunits and serves as the secretion tube through which translocated proteins or DNA travel. Three ATPases located within the cytoplasm provide power to the secretion apparatus and are essential for its function. The T4SS facilitates direct secretion of complex proteins and genetic material
across both membranes and into the extracellular milieu or directly into the eukaryotic host cell cytosol. In *H. pylori*, a T4SS is encoded within the *cag* pathogenicity island and has been shown to be highly associated with virulent *H. pylori* strains. The *H. pylori* T4SS has been implicated in the uptake and secretion of genetic material to promote genetic exchange, but is most well-known for its involvement in the secretion of the virulence factor CagA into gastric epithelial cells following attachment (Backert and Meyer, 2006).

### 1.3.7 Outer membrane vesicle (OMV) mediated secretion

Outer membrane vesicles (OMVs) are spherical structures produced by Gram-negative bacteria that are composed of a bilayer membrane derived from the outer membrane of the cell (Beveridge *et al.*, 1999). They were first discovered in *E. coli* when it was observed that LPS was present within the cell-free culture supernatant (Bishop and Work, 1965). Analysis of the supernatant via electron microscopy revealed spherical structures composed of outer membrane lipids (Work *et al.*, 1966). Soon after this discovery, OMVs were being identified within the cell-free supernatants of many other bacteria such as *Pseudomonas* and *Neisseria*, revealing that this is a wide spread phenomena (Rothfield *et al.*, 1969). When OMVs are extruded from the cell they package some of the periplasmic contents within them, and these packaged proteins have been shown to be biologically active (Kesty and Kuehn, 2004). Thus, it has been proposed that OMVs may act as a secretion system to deliver bacterial proteins into the extracellular environment to interact with other bacteria or a host (Gankema *et al.*, 1980; McBroom and Kuehn, 2007).
Two delivery mechanisms by which OMVs can mediate the secretion of bacterial components into the extracellular environment have been proposed. The first mechanism hypothesizes that after OMV budding the OMV lyses and the contents within are released to diffuse and interact with the potential targets. However, little evidence for this mechanism exists. The second mechanism describes the ability of OMVs to fuse to the membrane of target cells and the bacterial contents enter via internalization or fusion (Kadurugamuwa and Beveridge, 1995).

OMVs have been shown to have important roles in virulence by trafficking toxins and other virulence factors to their targets. Enterotoxigenic E. coli (ETEC) produces heat-labile enterotoxin that has been found within OMVs in the culture supernatant. Purified ETEC OMVs have been shown to specifically bind, enter, and deliver its active toxin into both epithelial and adrenal cells (Hortsman et al, 2000). The toxin NarE, which is produced by Neisseria meningitidis has also been observed to be associated with OMV secretion (Massignani et al, 2003). Related to this study, H. pylori virulence factors such as VacA have been identified within OMVs and have been shown to target host cells through the OMV intermediate (Fiocca et al, 1999; Ilver et al, 2004; Ayala et al, 2006). Taken together, this clearly demonstrates that many virulence factors are associated and secreted outside of the cell through OMVs. Due to the fact that VacA has already been identified to be secreted through OMVs, other H. pylori virulence factors may be secreted through OMVs as well.

1.4 Disulfide bond proteins

Disulfide bond (Dsb) proteins are oxidoreductases that are involved in the formation of disulfide bonds (S-S) in the process of protein folding and maturation. In Gram-negative bacteria disulfide bond formation occurs in the periplasm. Dsbs have been
extensively studied in *E. coli*, and used as a model system when comparing homologous proteins within other organisms. The Dsb protein system in *E. coli* is composed of several proteins localized within the bacterial periplasm and inner membrane (Raina and Missiakas, 1997). Each of these proteins contains a pair of catalytic cysteine residues that are involved in the formation of mixed disulfides either with the unfolded protein or with a partner Dsb protein. Two separate pathways of S-S formation have been described.

### 1.4.1 Pathways of disulfide bond formation

The oxidative pathway of disulfide bond formation involves the proteins DsbA and DsbB. DsbA is an oxido-reductase that contains a thioredoxin domain with a catalytic CXXC motif which is responsible for the transfer of its disulfide to a protein with 2 reduced thiols by the formation of a disulfide-linked complex. To be active, the two cysteines within the CXXC motif of DsbA need to be in the oxidized state (Bardwell, 1994). Upon contact with a reduced thiol, a transient mixed disulfide forms between the substrate and the first cysteine of the CXXC motif, the disulfide bond is transferred onto the folding protein and DsbA is released. This enzymatic reaction results in DsbA being reduced, thus, re-oxidation must occur to be able to repeat another catalytic cycle. DsbB is a transmembrane protein responsible for this process, which obtains its oxidation potential via the Electron Transport Chain (Collet and Bardwell, 2002) (Figure 2). DsbA and DsbB interact directly (Guilhot *et al.*, 1995) and re-oxidation of DsbA involves two pairs of cysteines presented by DsbB in the characteristic CXXC motif.

In the absence of DsbA, impaired motility, as well as increased sensitivity to metal ions have been described, demonstrating the importance of this pathway (Hayashi *et al.*, 2000). For proteins with only two cysteines, only one disulfide bond is possible. However,
**Figure 2: Dsb pathways of disulfide bond formation in *E. coli*.** The paradigm for disulfide bond formation in the periplasm is best characterized in *E. coli*. The **oxidation pathway:** The oxidative pathway involves the proteins DsbA and DsbB. DsbA reacts with free thiol groups of newly translocated proteins to form a disulfide bond between the cysteine residues. DsbA then itself becomes reduced as it accepts electrons from the translocated protein. To start a new catalytic oxidation cycle, DsbA must be re-oxidized by its partner enzyme DsbB. Electrons flow from DsbB to ubiquinones and to terminal oxidases of the electron transport chain. The **isomerization pathway:** When incorrect disulfide bonds are formed, rearrangement is needed. This reaction is catalyzed by the thiol-disulfide oxidoreductases DsbC and/or DsbG. These isomerasers are maintained in a reduced state by the membrane protein DsbD, which is a cytoplasmic thioredoxin. DsbD is recycled by thioredoxin reductase in a NADPH-dependent manner. *Adapted from Kadokura et al., 2009.*
if a protein contains more than two S-S then mis-matched bonds can occur that are not present in the native protein. This can be corrected by the isomerization pathway.

The isomerization pathway is crucial for proteins that contain more than two cysteines. As the number of cysteine residues increases in a protein, the possibility that incorrect disulfide bonds will form also increases. In *E. coli*, the isomerization pathway is responsible for correcting these misfolded proteins and involves the isomerase DsbC, which rearranges improper S-S into their native configurations (Segatori *et al.*, 2004). Similar to the oxidative pathway, the catalytic activity of DsbC is sustained by a transmembrane protein, DsbD. However, this protein must maintain DsbC in a reduced state to preserve activity (Collet and Bardwell, 2002; Messens and Collet, 2006).

### 1.4.2 Importance of Dsb proteins in bacterial virulence

Dsb proteins of many pathogenic bacteria have been shown to be important for their virulence. When the genes encoding these proteins become disrupted or impaired, vast effects on virulence factor secretion or direct folding or function can be affected. Defects in the DsbA-B oxidation pathway have been shown to be important in bacterial adhesion through fimbriae or pili. For example, in *Salmonella enterica*, the DsbA homologue SeSrgA is absolutely essential for the production of fimbriae that are required for intestinal adhesion. In *S. enterica* mutants lacking SeSrgA, intestinal colonization within a murine infection model is significantly decreased (Miki *et al.*, 2004). In addition, proteins involved in the adhesion to host cells through type IV fimbriae have also been shown to be severely affected by DsbA mutations as enteropathogenic *E. coli* (EPEC) require disulfide bond formation within the major sub-unit involved in fimbriae production which the cells use to attach to human epithelial cells (Donnenberg *et al.*, 1997).
Dsb proteins have been shown to affect the secretion of virulence related proteins secreted by the general secretory pathway (type II) (Lasica and Jaguszyn-Krynicka, 2007). A hallmark of this pathway is that many of the secreted proteins adopt their active conformation in the periplasm following the formation of disulfide bonds, which are absolutely essential for their final secretion (Rahme et al, 1997). Dsb proteins promote maturation of a number of toxins in the periplasm belonging to the ABs toxin group, including cholera toxin in *V. cholerae*, pertussis toxin of *B. pertussis*, as well as the heat labile enterotoxin of enterotoxigenic *E. coli* (Yu et al, 1992).

Motility is essential for the colonization and or virulence of many bacterial species. Many bacteria with mutations in either *dsbA* or *dsbB* are non-motile due to their inability to produce flagella (Dailey and Berg, 1993). This phenotype is best characterized in *E. coli*. DsbA catalyzes the formation of disulfide bonds within the protein FlgI, which is a structural protein within the P-ring of the flagellar motor. The improperly folded FlgI protein prevents the integration of FlgC, thus making the cell non-motile (Dailey and Berg, 1993). DsbA has also shown to be important for the type IV pili mediated twitching motility of *P. aeruginosa* (Ha et al, 2003). Thus, Dsb proteins and the Dsb pathway of disulfide bond formation are important for many aspects of bacterial virulence and may serve as potential targets for novel therapeutics.

### 1.4.3 Dsb proteins in *H. pylori*

The mechanisms of action of Dsb proteins have been studied extensively in *E. coli*. However, this paradigm does not hold true for *H. pylori* as extensive bioinformatics studies have been unable to identify a DsbA homologue. Only 3 Dsb family members (DsbB, DsbC, DsbI) have been identified and studied (Tomb et al, 1997; Godlewska et al, 2006;
Dutton et al, 2008; Heras et al, 2009). DsbI is believed to function similar to DsbB in *Campylobacter* species, but due to the fact that a DsbA homologue is missing in *Helicobacter* species, the process of oxidative folding is thought to occur via different mechanisms. Nonetheless, DsbI has been implicated in playing an important role in oxidative folding within the periplasm, as it was recently shown that *dsbi* mutants display reduced colonization of the stomach within a mouse infection model (Godlewska et al, 2006).

Recently, there has been controversy surrounding the identification of a fourth Dsb protein. The solved structure of the protein encoded by *hp0231* (Yoon et al, 2011), which for this study will be referred to as DsbHP, reveals homology similar to that of the *E. coli* DsbG protein (Figure 3). Yoon et al proposed that DsbHP had function similar to *E. coli* DsbG by its ability to correct disulfide bonds and act as a reductase to the substrate HP0518. However, conflicting evidence demonstrates that DsbHP also demonstrates characteristics that resemble *E. coli* DsbA oxidase activity due to the act that it was capable of refolding denatured and reduced insulin (Roszczenko et al, 2012). Thus, more studies need to be conducted to elucidate its role in the Dsb pathway.

1.5 *Helicobacter* cysteine-rich proteins (Hcps)

Although *H. pylori* has a small genome, encoding approximately 1600 proteins, the function of many of these proteins remains unknown. Within the past decade, a family of proteins containing a high percentage of cysteine residues have been discovered, named *Helicobacter* cysteine rich proteins (Hcps). This family contains seven members (HcpA-F) which are specific to *Helicobacter* species. It is one of the largest protein families
Figure 3: *H. pylori* DsbH is an *E. coli* DsbG homologue. Comparison of the monomeric structure of *H. pylori* DsbHP to *E. coli* DsbG.

**Panel A:** Known structure of the *H. pylori* DsbG homologue.

**Panel B:** Known structure of the *E. coli* DsbG protein. *Adapted from Yoon et al., 2011.*
A.  

H. pylori DsbHP

B.  

E. coli DsbG
specific for proteobacteria from the delta/epsilon subgroup (Mittl et al, 2003). First identified and named for their high cysteine content (Cao et al, 1998), these proteins are highly conserved among all H. pylori strains that have been sequenced to date (Tomb et al, 1997; Alm et al, 1999). High antibody titers to HcpA, HcpC, and HcpE have been demonstrated within patients infected with H. pylori (Mittl et al, 2003) demonstrating that they are produced in vivo and that they are recognized by the host immune system. This suggests that Hcps may potentially play an important role in the host response as well as the interaction between the bacterium and the host. Understanding the function of Hcp family members will be important in determining their role in the host response as well as their potential role in H. pylori pathogenesis.

1.5.1 Sel1-Like Repeats (SLR), the structural components of Hcp proteins

Hcps are solenoid proteins, which are highly modular proteins that consist of repeating intra-molecular sequences. Most repeat units consist of 5-40 amino acids that fold into 2-4 secondary structural elements (Ogura et al, 2007).

SLR units have been divided into three groups based on the number of amino acids within each repeat unit. Tetratricopeptide repeats (TPR) were identified in the yeast cdc23 gene product and were designated tetratricopeptides because they have 34 amino acids (AA) in each repeat (Sikorski et al, 1990). Another repeat category is the pentatricopeptide repeats (PPR) group, which were identified following analysis of the Arabidopsis genome (Aubourg et al, 2000; Small and Peeters, 2000), and were designated penta- as each repeat contains of 35 AA. The third and final structural motif was identified following a genome analysis representing archaea, bacteria and eukaryotes. The study revealed a motif consisting of an α/α-repeat 36-44 AA in length, and was designated Sel1-like repeat (SLR)
(Shultz et al., 1998; Ponting et al., 1999), due to the fact that the motif was discovered in the *C. elegans* sel-1 gene (Grant and Greenwald, 1996).

The three-dimensional architecture of the SLR motif has been solved via crystallography (Das et al., 1998). These studies established that each SLR motif consists of a pair of anti-parallel α-helices which are equivalent in length and separated by a loop of amino acids. A single SLR repeat unit consists of the first α-helix (helix A) attached to the second α-helix (helix B) followed by the second loop of amino acids connecting helix B to helix A of the following repeat unit. Structurally, PPRs differ from TPRs by a single amino acid insertion in loop 2, and SLRs differ from TPRs by the presence of a 4-12 amino acid insertion in loop 1 and a deletion of 2 amino acids in loop 2 (Mittl and Schneider-Brachert, 2007).

SLRs are abundant across all forms of life and have been identified in over 858 different proteins (Ponting et al., 1999). SLRs are characteristic to proteins involved in forming macromolecular complexes (Mittl and Schneider-Brachert, 2007), and also can play a vital role in cellular processes such as mitosis and transcription within eukaryotic cells. However, SLRs have also been shown to be important within prokaryotes as bacteria commonly utilize SLR proteins for gene regulation and virulence (Das et al., 1998; Oguru et al., 2007). Due to the fact that these motifs are found within both eukaryotes and prokaryotes, it has been proposed that SLR motifs are a potential link between signal transduction pathways from the two branches of life, suggesting that proteins containing these motifs are functionally important in host pathogen interactions. The structure of the SLR motifs found within Hcp proteins of *H. pylori* was accomplished through the crystallization of HcpB and HcpC (Luthy et al., 2002; Luthy et al. 2004). These structural
studies conducted on HcpB discovered that the SLR motif within these proteins are unique due to the fact that these Hcp proteins contain a repetitive pattern of paired cysteine residues in which two cysteine residues are spaced by 7 AA, with 36 AA between adjacent pairs. Additionally, these two cysteine residues stabilize the two α-helices of each motif by cross-linking and forming disulfide bonds which provide an extra form of stability to the structure of the protein (Figure 4). Hcp family members are the only known proteins in *H. pylori* to contain these unique SLR motifs, and contain between 4 and 9 SLRs depending on the family member (Luthy *et al*, 2004).

1.5.2 *Helicobacter*-cysteine rich protein E (HcpE)

*Helicobacter*-cysteine rich protein E (HcpE) is a 355 amino acid protein encoded by the open reading frame (ORF) HP0235 of the *H. pylori* strain 26695. The protein is annotated in the *H. pylori* genome as a hypothetical secreted protein, with no functional designation to date. Work by Mittl *et al.* established that patients infected with *H. pylori* have high antibody titers to HcpE, indicating that it is well expressed *in vivo* and induces an immune response in the host (Mittl *et al*, 2003). The Creuzenet lab has also demonstrated this by analyzing the serum of over 200 patients (Unpublished). HcpE contains a total of 19 cysteine residues, one within its signal sequence, and the 18 others occurring in the mature protein within each of the 9 SLR motifs. With 19 cysteine residues, HcpE has the highest cysteine content (5.4%) of all Hcp family members. In the predicted model of HcpE, the 9 SLRs are believed to come together to form a super helical structure characteristic of the Hcp family of proteins (Figure 5).
Figure 4: **Structural model of a single SLR unit.** Structural modeling of a single SLR unit done using SwissProt. The diagram illustrates that the two helices are held together in a V-shape in the 3D structure of the protein. The cysteines from each helix (highlighted in red) are involved in stabilizing the position of the two helices of the SLR via formation of a disulfide bond. *Figure courtesy of Dr. Carole Creuzenet.*
Figure 5: Structural model of HcpE.

Panel A: HcpE contains 19 cysteine residues. Their position along the peptide sequence is depicted by a C. One of the cysteine residues is within the predicted 23 AA long signal peptide that is cleaved upon secretion of HcpE into the periplasm. The mature portion of the protein is predicted to contain 9 SLR repeats (depicted as red and black cylinders). Each SLR repeat is made of 2 α-helices (labeled a and b for each SLR) containing a pair of cysteine residues.


Panel C: Model of the reconstituted full-length HcpE. Modeling and figure courtesy of Dr. Creuzenet.
### A

<table>
<thead>
<tr>
<th>Signal peptide</th>
<th>Mature protein</th>
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<td>SLR: 1</td>
</tr>
<tr>
<td>23 44 80 117 152 188 214 261 297 332 355</td>
<td>2 3 4 5 6 7 8 9</td>
</tr>
</tbody>
</table>

### B

![Image of a molecular structure](image1)

### C

![Image of a molecular structure](image2)
The acquisition of an organized array of disulfide bonds within the \textit{HcpE} protein is anticipated to require proteins of the Disulfide bond protein (Dsb) family (Devi \textit{et al}, 2006). The Creuzenet lab recently discovered that the Dsb homologue Dsb$_{HP}$ interacts with, and is capable of refolding HcpE (Kichler, 2010). A modified affinity blot with purified Dsb$_{HP}$ demonstrated that HcpE is capable of interacting with the Dsb homologue (Figure 6). A refolding assay was also performed in which purified Dsb$_{HP}$ was incubated with insoluble HcpE, and its ability to solubilize HcpE was detected by anti-HcpE Western blotting. When increasing concentrations of Dsb$_{HP}$ were added to insoluble HcpE, the amount of soluble HcpE detected in the supernatants increased (Figure 7). This demonstrates that HcpE, and presumably the other Hcp family members require Dsb proteins to aid in their folding and maturation within the periplasm.

\subsection*{1.5.3 Putative function of Hcp proteins}

Even though Hcp proteins were discovered over a decade ago, very little has been elucidated about their function. Recombinant HcpA, B, and C have been the most studied family members, but a consensus on their functions has still not been obtained. Recombinant HcpA has been shown to alter the morphology of Thp1 monocytes from round to star-like morphologies \textit{in vitro}. The ability of HcpA to initiate change in morphological characteristics of Thp1 cells was shown to be equivalent to that of the tumor promoter phorbol 12-myristate 13-acetate (PMA). Star-like morphology is attributed to the protrusion of needle-like filopodia as seen by scanning electron microscopy (Dumrese \textit{et al}, 2009). A dose dependent increase in adherence of up to 90\% of Thp1 cells within cell
**Figure 6: HcpE and Dsb\textsubscript{HP} interact.**

Dsb\textsubscript{HP} was shown to interact with denatured HcpE when transferred onto a nitrocellulose membrane. Membranes were incubated with enriched Dsb\textsubscript{HP} (Panel A), or buffer only (Panel B), and subjected to both anti-Flag as well as anti-HcpE Western blots to visualize the presence of Dsb\textsubscript{HP} at 700nm and HcpE at 800nm respectively. Dsb\textsubscript{HP} binds to full length HcpE as seen in both Panel A. Full length and truncated HcpE are marked by a black arrow; Dsb\textsubscript{HP} -Flag is marked by a red asterix; non-specific *E. coli* proteins reacting with the anti-HcpE antibody marked by grey arrow. *Figure from Kichler (2010).*
A. Enriched DsbHP

B. Buffer
Figure 7: DsbHP catalyzes the solubilization of insoluble HcpE in vitro. Enriched DsbHP was incubated with insoluble HcpE that had been over-expressed and purified from E. coli. Anti-HcpE Western blot demonstrates a dose-dependent response of HcpE to DsbHP, resulting in increased soluble full length HcpE. HcpE was present as a doublet which is thought to reflect its cytoplasmic form with its signal peptide (+SP) and its periplasmic form without its signal peptide (-SP). Figure from Kichler (2010).
culture flasks was also demonstrated with addition of HcpA, which is thought to be a consequence of filopodia formation (Dumrese et al, 2009).

An increase in cellular adherence and onset of morphological changes are signature characteristics for cellular differentiation into macrophages. Dumrese et al investigated the uptake of fluorescent microspheres by the altered Thp1 cells. Results showed that Thp1 cells stimulated with HcpA were capable of phagocytosing 30% of the labelled microspheres compared to 10% of the control (Dumrese et al, 2009).

Additionally, splenocyte cultures incubated with HcpA were shown to promote a Th-1 bias response with respect to cytokine production (Deml et al, 2005). An increase in secretion of IL-6, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) as well as IL-12 were observed after addition of HcpA (Deml et al, 2005). These findings suggest that HcpA is potentially involved in the differentiation of host immune cells and progression of the immune response against *H. pylori*. However, this functional ability would not help clear *H. pylori* infections, but rather it is thought to promote the persistence of the bacterium in the gastric mucosa (Dumrese et al, 2009).

1.6 Animal models for studying *H. pylori* colonization

When *H. pylori* was first discovered to be the causative agent of ulcers and gastric cancers, studies on pathogenesis was limited due to the absence of adequate animal models. Over the years, various animal models have been developed to better understand the mechanisms of *H. pylori* infection and persistence (reviewed in Algood and Cover, 2006). Non-human primates such as Japanese monkeys have been used to study *H. pylori* induced gastritis due to the fact that their stomachs are anatomically and physiologically similar to
humans. Also, because of their long life span they qualify for long term infection studies involving endoscopy and biopsies which resemble human infections (Mattapallil et al, 2000). However, new animal models needed to be developed due to the difficulties in handling of these animals.

Several rodent models have now been developed to study *H. pylori* such as rat, mice and Mongolian gerbil. These models were chosen due to their ease of treatment and their relatively economical stature. However, the first studies to implement this model were unsuccessful due to the fact that colonization of clinical isolates were poor, and the development of gastric cancers were insufficient (Karita et al, 1991; Marchetti et al, 1995). This was until Lee et al successfully adapted a clinical isolate called the Sydney strain (SS1), which is capable of efficiently colonizing C57BL/6 mice for long periods of infection (≤ 8 months). By successive rounds of colonization and re-inoculation into mice, they were able to isolate bacteria which were adequate at colonizing various strains of mice including C57BL/6 and BALB/c (Lee et al, 1997). Colonization with strain SS1 results in inflammation, increased neutrophil recruitment, and expression of Major Histocompatibility Complex II (MHC II) class antigens, which makes this model suitable for studies of gastritis. However, peptic ulceration and gastric cancers have not been observed in this model (Matz-Rensing et al, 2001). Thus, to study *H. pylori* induced gastric carcinomas, a different rodent model had to be developed.

Mongolian gerbils were first described as an infection model by Yokota et al. They observed that during the first 2 months of infection no significant inflammation or infiltration of lymphocytes occured. It was later observed by Hirayama et al that if infection of Mongolian gerbils with *H. pylori* was allowed to proceed longer than 6 months, they
would begin observe the formation of irregularly thickened gastric walls, erosion of the mucosal lining as well as the formation of hemorrhages (Hirayama et al., 1996). These pathological observations were soon followed by ulcers as well as gastric metaplasia (Matsumoto et al., 1997; Watanabe et al., 1998). Thus, this model is implemented when wanting to study the role and pathogenicity of *H. pylori* in the development of gastric ulcers and adenocarcinomas.

None of these models are a perfect representation of the pathology observed in humans. Intestinal metaplasia has never been observed in non-human primates or mice models, and Mongolian gerbils develop lymphoid follicular hyperplasia in the sub mucosal layer and gastritis cystica profunda, which have never been observed in humans. However, as in humans, gastric inflammation in conjunction with immune activation is ineffective at clearing *H. pylori* (reviewed in Masaaki et al., 2008), which results in severe general gastritis, peptic ulcers or gastric metaplasia, depending on which model is utilized. Hence, these animal models have been successfully developed to study infections *in vivo* which enable us to observe some of the most common pathological characteristics in humans during *H. pylori* infection, thus demonstrating the clinical relevance to studying *H. pylori* infection in animal models.

### 1.7 Hypotheses and objectives

*Dsbp* has the ability to bind to and solubilize HcpE. *Dsbp* has also been shown to be capable of refolding both HP0518 and insulin, however, controversy in terms of its activity needs to be resolved. High antibody titers to the HcpE protein in patients infected with *H. pylori* have been observed and other Hcp family members have also been established to
potentially play a role in immune modulation. Based on these observations and our current understanding of both Dsb_{HP} and HcpE, the following hypotheses were proposed:

- **Dsb_{HP}** is a Dsb related protein, which possesses DsbG-like activity.
- **HcpE** relies on Dsb_{HP} for its maturation and secretion.
- **HcpE** contributes to *H. pylori* mediated inflammatory response
- **HcpE** is secreted outside of the cell via outer membrane vesicles.

To address these hypotheses, the objectives of this thesis were to:

1) **Determine if Dsb_{HP} is capable of forming disulfide bonds**

2) **Determine the catalytic activity of Dsb_{HP} in terms of its ability to perform oxidation or reduction of disulfide bonds**

3) **Assess the ability of HcpE to evoke a pro-inflammatory response**

4) **Determine if HcpE is localized within the outer membrane vesicles**

**CHAPTER 2: MATERIALS AND METHODS**

**2.1 Bacterial strains, growth conditions, and reagents**

Bacterial strains and plasmids used in these studies are listed in Table 1. *H. pylori* strains NCTC11637 and SS1 were grown in micro-aerobic conditions of 5% O_{2}, 10% CO_{2}, and 85% N_{2} for 48 hours on either Brain Heart Infusion or Brucella agar plates containing 10% horse serum or on Columbia agar plates containing 10% blood. Plates were supplemented with 0.05 μg/ml sodium pyruvate, trimethoprim (5 μg/ml), vancomycin (10 μg/ml), and amphotericin B (5 μg/ml). Kanamycin was added to the media when selecting
for hcpE::kan and dsbHP::kan knockout mutants and chloramphenicol (8 μg/ml) was used to select for the dsbHP and hcpE complemented strains. Unless stated otherwise, all E. coli strains were grown at 37°C, in LB broth supplemented with chloramphenicol (34 μg/mL), or kanamycin (30 μg/ml) for selection of expression plasmids.

2.2 SDS-PAGE and Western blot analysis

Bacterial cells or proteins were denatured in sodium dodecyl sulfate (SDS) buffer (0.625M Tris pH 6.8, 2% SDS, 2% 2-β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue), and then boiled for 5 min at 100°C and separated on 12% SDS polyacrylamide gels. Proteins were visualized by either Coomassie blue staining (10% acetic acid, 25% ethanol, 0.01% (w/v) Brilliant Blue R-250), silver nitrate staining, or Western blotting. Western blotting was completed by transferring proteins to a nitrocellulose membrane (BioRad). Transfer occurred for 50-100 minutes at 180 mAmp, depending on the thickness of the SDS gel, in cold transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). The membrane was washed in distilled water and then stained with Ponceau Red stain [0.1% (w/v) Ponceau S in 1% (v/v) acetic acid] for 5 min, then the membrane was washed in distilled water and scanned. The membrane was subsequently washed in Tris Buffered Saline (TBS) buffer (50mM Tris-HCl pH 7.5, 150mM NaCl) for 10 min and then blocked overnight at 4°C in 2.5% skim milk. The membrane was then washed twice in TBS-Tween/Triton (0.05% Tween-20, 0.2% Triton-X 100) buffer and once in TBS buffer, for 10 min each. The membrane was incubated in primary antibody
### Table 1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Description</th>
<th>Reference</th>
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<td><strong>Strains</strong></td>
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<td><strong>H. pylori</strong></td>
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<tr>
<td>NCTC 11637</td>
<td>Wild-type strain</td>
<td>Lab stock (provided by Dr. D. E. Taylor)</td>
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<tr>
<td>NCTC 11637</td>
<td><em>dsb</em>&lt;sub&gt;H&lt;/sub&gt;::<em>kan</em></td>
<td>This study (Made by J. Lester)</td>
</tr>
<tr>
<td>NCTC 11637</td>
<td><em>hcpE</em>::<em>kan</em></td>
<td>This study (Made by A. Oberc)</td>
</tr>
<tr>
<td>NCTC 11637</td>
<td><em>dsb</em>&lt;sub&gt;H&lt;/sub&gt; Comp</td>
<td>This study (Made by B. Oickle)</td>
</tr>
<tr>
<td>NCTC 11637</td>
<td><em>hcpE</em> Comp</td>
<td>This study (Made by Jeff Lester)</td>
</tr>
<tr>
<td>Sydney strain (SS1)</td>
<td>Wild-type strain</td>
<td>Lab stock (provided by Dr. N. Jones)</td>
</tr>
<tr>
<td>Sydney strain (SS1)</td>
<td><em>dsb</em>&lt;sub&gt;H&lt;/sub&gt;::<em>kan</em></td>
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<tr>
<td>Sydney strain (SS1)</td>
<td><em>hcpE</em>::<em>kan</em></td>
<td>This study (Made by A. Oberc)</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>DH5α</td>
<td><em>supE</em> Δlac &lt;i&gt;U&lt;/i&gt;169 (Φ80lacZAM15)hsdR recA endA gyrA hri relA</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; <em>ompT</em> hsdSB&lt;sup&gt;−&lt;/sup&gt; (r&lt;sub&gt;B&lt;/sub&gt; &lt;sup&gt;−&lt;/sup&gt; m&lt;sub&gt;B&lt;/sub&gt; &lt;sup&gt;−&lt;/sup&gt;) gal dcm Δ(srl recA)306::Tn10(DE3) pLy</td>
<td>Novagen, USA</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pHel3</td>
<td>Shuttle vector containing kanamycin resistance cassette; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Heuermann and Haas, 1998)</td>
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<tr>
<td>pUC18</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Yanisch-Perron &lt;i&gt;et al&lt;/i&gt;, 1985)</td>
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</tbody>
</table>
### Table 1: continued

<table>
<thead>
<tr>
<th>Expression plasmids</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>pET30a-<em>dsb</em>HP-<em>hcpE</em></td>
<td>The <em>hp0235</em> gene (1085 bp) sub-cloned into pET30a-<em>dsb</em>HP-Flag vector expressing a C-terminal Histidine tagged HcpE protein</td>
<td>This study (made by J. Lester)</td>
</tr>
<tr>
<td>pET30a-<em>dsb</em>HP-Flag-His</td>
<td>The <em>hp0231</em> gene (856 bp) cloned into pET30a expressing a C-terminal Flag and histidine tagged DsbHP protein</td>
<td>This study (Made by J. Lester)</td>
</tr>
<tr>
<td>pET30a-<em>dsb</em>HP-Flag</td>
<td>The <em>hp0231</em> gene (856 bp) cloned into pET30a expressing a C-terminal Flag tagged DsbHP protein</td>
<td>Creuzenet lab (Made by S. Kichler)</td>
</tr>
<tr>
<td>pET30a-<em>dsbA</em></td>
<td>The <em>ECK3852</em> gene (627 bp) cloned into pET-30a expressing a C-terminal histidine tagged DsbA protein</td>
<td>This study (Made by J. Lester)</td>
</tr>
<tr>
<td>pET30a-<em>hcpE</em></td>
<td>The <em>hp0235</em> gene (1085 bp) cloned into pET30a expressing a C-terminal histidine tagged HcpE protein</td>
<td>Creuzenet lab (Made by J. Chahal)</td>
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<tr>
<td>pET30a-<em>dsbG</em></td>
<td>The <em>ECK0598</em> gene (747 bp) cloned into pET-30a expressing a C-terminal histidine tagged DsbG protein</td>
<td>This study (Made by J. Lester)</td>
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<td>pET30a-<em>gmd</em></td>
<td>The <em>Cj1319</em> gene (972 bp) cloned into pET-30a expressing a C-terminal histidine tagged GMD protein</td>
<td>Creuzenet lab (Made by Alexandra Merkx-Jaques)</td>
</tr>
<tr>
<td>pET30a-<em>wbpB</em></td>
<td>The <em>hp0679</em> gene (870 bp) cloned into pET-30a expressing a C-terminal histidine tagged WbpB protein</td>
<td>Creuzenet lab (Made by Alexandra Merkx-Jaques)</td>
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</tbody>
</table>
for 1 hr at room temperature then washed twice in TBS-Tween/Triton-X 100 and once in TBS at room temperature. The membrane was incubated in the secondary antibody for 30 minutes at room temperature in the dark and then washed three times as mentioned above. Proteins were visualized using the Licor Infrared Imaging System at a wavelength of 700 or 800 nm. For anti-His, primary antibody (Mandel Scientific) was diluted 1:5000 and the secondary antibody (Mandel Scientific) was diluted 1:3000. For anti-HcpE, primary antibody (polyclonal) was diluted 1:5000, and the secondary (Mandel Scientific) was diluted 1:3000.

2.3 Transformation of E. coli competent cells

To transform plasmid DNA into E. coli cells, 100 µl aliquots of CaCl2-competent cells were thawed on ice as previously described (Cohen et al, 1972). Then 1 µL of DNA or 15 µL of a ligation mixture was added to the cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and put back on ice for 1 minute. Afterward, 600 µL of LB was added to the cells and the cells were grown at 37°C while shaking for 1 hr and 30 minutes. Then 50 µL of the cell culture was plated on LB agar with the appropriate selection antibiotics. For the pET30 constructs kanamycin was used for transformation into E. coli DH5α cells and kanamycin + chloramphenicol were used for transformation into E. coli BL21(DE3)pLys cells. The plates were incubated overnight at 37°C.

2.4 Cloning DsbHP-Flag-His in a pET30a vector for over-expression

To allow for easy purification via nickel chromatography, a cleavable C-terminal histidine tag was sub-cloned into our pET30a-DsbHP-Flag construct downstream of the Flag tag. An inverse PCR was conducted on the pET30a-DsbHP-Flag construct using
primers HP0231P3 and HP0231P4 (Table 2). Both primers contained a BamHI cut site to be used for ligation purposes with T4 ligase after cleavage by BamHI.

2.5 Cloning an expression vector containing both DsbHP and HcpE

To increase the yield of soluble HcpE during expression, we constructed a vector that would express both HcpE and DsbHP. The hcpE gene was sub-cloned into our pET30-DsbHP-Flag construct by PCR amplification of hcpE-His from our pET30a-HcpE-His vector with primers T7ProX and CtermHis (Table 2) which contain XbaI and BamHI sites used for insertion into the vector. As NheI and XbaI sticky ends are compatible, the pET30a-DsbHP-Flag construct was cut with NheI and BamHI and ligated together with the HcpE-His PCR product that was cut with XbaI and BamHI to generate the pET30a-DsbHP-Flag-HcpE-His.

2.6 Construction of vectors expressing E. coli DsbA-Flag-His and DsbG-Flag-His

To compare the activity of DsbA and DsbG to that of DsbHP, we constructed expression vectors containing E. coli dsbA and dsbG genes. The dsbA and dsbG genes were PCR amplified from E. coli strain DH5α using primers DsbAEcP1/P2 and DsbGEcP1/P2. The PCR products were then cut with NdeI and BamHI and ligated together with our pET30a-DsbHP-Flag-His that was cut with the same enzymes to remove dsbHP from the construct. This resulted in the vectors pET30a-DsbA-Flag-His and pET30a-DsbG-Flag-His.

2.7 Over-expression and purification of proteins via nickel chromatography

Protein expression was carried out in E. coli BL21(DE3)pLysS (Novagen). The cultures were grown overnight with shaking at 37°C in 3mL of LB. The next morning,
cells were sub-cultured by a 1/50 dilution into 1L of fresh LB medium and grown at 37˚C until they reached an OD_{600nm} of 0.5. For optimal expression of soluble HcpE, cultures were equilibrated to room temperature for 1 hr before induction. All other expression strains were induced at 37°C. All proteins were over-expressed from our pET constructs via induction with 0.15 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, BioShop).

Cells expressing HcpE were grown O/N at room temperature, whereas all other proteins were expressed for 3 hours at 37˚C. Cells were harvested by centrifugation at 6,000 g for 10 minutes at 4°C and stored at -20°C until needed. For purification of over-expressed HcpE, cell pellets were re-suspended in 30 mL cold binding buffer (5mM imidazole, 0.1M NaCl, 20mM Tris pH 8.3), whereas cells expressing all other recombinant proteins were re-suspended in binding buffer (5mM imidazole, 0.1M NaCl, 20mM Tris pH 7.5). To prevent proteolysis, 1 mL of a 1M protease inhibitor cocktail (PIC; Invitrogen) was added to the re-suspended cells. The cells were lysed by passing twice through a cell disruptor (Constant Systems LTD IS6/40/BA/AA model) at 25,000 psi. The cellular debris and insoluble proteins were removed by centrifugation at 12 000 g for 30 minutes at 4˚C, followed by the removal of membranes by ultracentrifugation (Beckman) at 100 000 g for 1 hr at 4˚C.

For purification, the supernatant was filtered sequentially through 0.8 μm and 0.45 μm pore size filters (Millipore Millex-HV) and stored on ice until purification. The histidine-tagged proteins were purified by nickel chelation chromatography using Fast Protein Liquid Chromatography (FPLC) system with a POROS 1.6 mL column (Applied Biosciences). The column was washed initially with 5 column volumes (CV) of water
Table 2: List of nucleotide sequences for primers

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<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
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<td>HP0231P1</td>
<td>GGAAGACCATATGATATTAAGAGCGAGTGTG</td>
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<td>HP0231P2</td>
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<tr>
<td>HP0231P3</td>
<td>GCGGGATCCGGACTGAAAATACAAATTCCTTGTCGTCGTTG</td>
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<td>HP0231P4</td>
<td>CGCGGATCCCACCACCACCACCACCACCTG</td>
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<td>HP0231P5</td>
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<td>HP0231P6</td>
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<td>CatHelP2</td>
<td>GAGGATCCGGGTACC</td>
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* Restriction sites are in **bold** and *underlined*
before being charged with 30 CV of 0.1M nickel sulphate. After the column was charged, the column was washed with 7.5 CV of 0.1M NaCl and 5 CV of water. The column was equilibrated with 10 CV binding buffer that was adjusted to pH 8.3 for HcpE and pH 7.5 for DsbHP and E. coli DsbG and DsbA. After loading the sample, the column was washed with 10 CV of 1X binding buffer to remove proteins that were non-specifically bound to the column. Bound proteins were eluted with an imidazole gradient from 50mM to 1.0M of elution buffer that was adjusted to the same pH as the 1X binding buffer. Elution fractions were collected and run on SDS-PAGE gel to determine which fraction contained the protein of interest. The fractions that contained the over-expressed pure proteins were pooled and dialyzed O/N in dialysis tubing (VWR) with a 12-14 kDa molecular weight cut off in 50mM ammonium bicarbonate buffer pH 8.0 to remove all remaining imidazole. Following dialysis the purified proteins were concentrated using polyethylene glycol (PEG-8000), then stored in 15% glycerol at -20°C.

2.8 Protein quantification using the Bio-Rad Bradford protein assay

To quantify the concentration of purified proteins, bovine serum albumin (BSA; BioRad) that was diluted to 1.36 g/l was used to generate a standard curve. The BSA protein standard was serially diluted (0-0.40 g/L) in 50mM ammonium bicarbonate buffer pH 8.0 in a 96-well plate. The pure protein sample was tested at 3 different concentrations. Then 10 μL of protein or standard was added to the 96-well plate in triplicate. The Protein Assay Dye Reagent Concentrate (BioRad) was diluted 1:4 in water, and 200 μL of the reagent was added to each well. Samples were read at 595nm on an Eon microplate reader (BioTek).
2.9 Solubility assay for expression of HcpE

To assess optimal conditions for soluble HcpE expression, *E. coli* BL21(DE3)pLys containing either pET30a-HcpE-His or pET30-Dsb<sub>HP</sub>-Flag-HcpE-His was inoculated into 3 mL of LB broth supplemented with kanamycin (30 µg/mL) and chloramphenicol (34 µg/mL) and grown overnight at 37°C. The cultures were then sub-cultured into 3 mL of LB broth supplemented with the same antibiotics as described above. Cultures were grown at either 37°C or were equilibrated for 1 hour at room temperature before being induced with 0.15mM IPTG. All cultures were incubated overnight, then the cells were pelleted by centrifugation at 6,000 g for 10 minutes. The supernatants were discarded and the cell pellets were re-suspended in buffer (50mM Tris pH 8). Fifty µl of glass beads (Sigma, diameter < 106 µm) that were washed 3 times in the same buffer were added. Cells were lysed by vortexing with glass beads, with a beads /cell suspension ratio of 1/3 (v/v) for 1 minute with an additional minute of incubation on ice in between repeats. The resulting lysate consisted of the total proteins, of which 100 µl was saved for protein analysis. The remaining cell lysate was centrifuged at 12,000 g for 15 minutes to pellet the insoluble proteins. The supernatant containing the soluble proteins as well as membrane proteins was removed and the pellet containing the insoluble protein fraction was re-suspended in buffer (50mM Tris pH 8). HcpE expression levels in the total, soluble and insoluble protein fractions were analyzed by SDS-PAGE analysis and Western blotting with anti-HcpE antibodies that were isolated from rabbits (Kichler, 2010).

2.10 Construction of the *H. pylori* dsb<sub>HP</sub> knockout mutant

Primers HP0231P5 and HP0231P2 were used to subclone dsb<sub>HP</sub> from the pET-dsb<sub>HP</sub>-Flag construct into a pUC18 vector. Both the PCR amplicon and pUC18 were cut
with BamHI and EcoRI and ligated together using T4 ligase. Inverse PCR was then conducted with primers HP0231P6 and HP0231P7 (Table 2), which introduced ClaI and KpnI cut sites to allow for gene disruption with a kanamycin cassette. The kanamycin resistance cassette was amplified from the vector pHel3 with Aph3P2 and Aph3P4 and both products were cut with ClaI and KpnI and ligated together. The resulting plasmid was transformed into *E. coli* DH5α cells using both ampicillin and kanamycin for selection. To generate the *H. pylori* knockout mutants, the constructs were transformed into *H. pylori* NCTC11637 or SS1 using natural transformation (Ge and Taylor, 1997). *H. pylori* NCTC11637 or SS1 were grown on agar plates as described above. Cells were harvested and diluted to an OD₆₀₀nm of 0.2 in a final volume of 100µl. Then, 15-20 ng of plasmid DNA was added to the cells, and the entire suspension was plated onto Columbia agar supplemented with blood and allowed to recover for 8 hours in micro-aerobic conditions at 37°C. Recovered cells were then transferred to Columbia agar containing 4 μg/ml kanamycin to allow for selection.

### 2.11 PCR screening of the dsb₅₆::kan knockout mutagenesis clones

Transformants were analyzed for proper gene integration by PCR using genomic DNA isolated with phenol/chloroform extraction. A small pellet of bacteria isolated from agar was re-suspended in 500 µl of Cetyltrimethyl ammonium bromide (CTAB) buffer to lyse the cells. An additional 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) was added to the suspension and vortexed for 30 seconds and then centrifuged for 5 minutes at 13,000 g. The supernatant was removed and put into a fresh Eppendorf tube where 500 µl of chloroform/isoamyl (24:1) was added. Vortexing and centrifugation were repeated as described above. The supernatant was removed and 48% (v/v) isopropanol and
10% (w/v) 3M sodium acetate was added and the suspension was kept on ice for 30 minutes to precipitate the chromosomal DNA. To pellet the DNA, the suspension was centrifuged at 13,000 g for 15 minutes. Then, 1 mL of ethanol was added to remove any salts and centrifugation was repeated. The ethanol was removed and the DNA was kept at 37°C for 10 minutes to evaporate any leftover ethanol. The DNA pellet was then re-suspended in 50 µl of distilled water and the chromosomal DNA was used in PCR screening reactions. The primer pairs used were HP0231P1/P2 and HP0230 P2/HP0232 P3 (Table 2).

### 2.12 Analyzing H. pylori for detection of HcpE

Wild-type, dsbH::kan, and hcpE::kan H. pylori strains were grown on Columbia Agar medium with background and selective antibiotics for 48 hrs. After 48 hours, the cells were sub-cultured into 100 mL of Brucella broth at an OD$_{600nm}$ that was adjusted to 0.2. The Brucella broth was supplemented with 1% β-cyclodextrin and the required antibiotics. Cell suspensions were grown at 37°C with gentle shaking under micro-aerobic conditions using sealed anaerobic jars and gas packs (CampyGen; Oxoid) for 16 hours. The cell suspensions were pelleted via centrifugation at 4000 g for 30 minutes at 4°C. Aliquots of each total culture, as well as culture supernatant and OMVs were set aside for quantification of cell lysis. OMVs were obtained via ultra-centrifugation of culture supernatants at 150,000 g for 3 hrs, and resuspended in 200 µl of Tris-HCl pH 8.0. The total cell pellets were re-suspended in 500 µL of 1X SDS-PAGE protein loading buffer, whereas the ultra-centrifuged supernatants were then concentrated 10 fold by centrifugation with a centricon with a 10 kDA cut off (Millipore), and 50 µl of 1X SDS-PAGE protein loading buffer was added. The total cells, concentrated supernatants, and OMVs were analyzed by SDS-PAGE (12%) and HcpE was detected by Western blotting.
with the anti-HcpE primary antibody diluted 1:5000, and goat anti-rabbit IgG secondary antibody detected at 800nm.

2.13 OMV preparation and analysis

The bacteria were inoculated into cyclodextrin-containing broth as described above. The supernatants were collected after 16 hour incubation by centrifugation of the culture at 2,057 g for 10 minutes, followed by filter-sterilization (0.45 µm size). The filter-sterilized supernatant was then ultra-centrifuged at 150,000 g for 3 h at 4 °C to pellet the OMVs. The OMVs were re-suspended in phosphate buffered saline (PBS, 9 mM NaPO4, 27 mM NaCl, pH 7.4).

2.14 Urease activity assay to assess cellular lysis of H. pylori cultures

To compare the urease activity of the total cells, cell supernatants, and OMVs, aliquots obtained from H. pylori Brucella broth cultures were recovered. Then, 20 µL of each sample, repeated in triplicate, was added to 200 µL of urease reagent (50mM sodium phosphate pH 7.5, 0.15M NaCl, 1M Urea, 0.001% phenol red). Urease activity was assessed by monitoring OD$_{565n m}$ over 2 hours in a 96-well plate using an Eon microplate Reader (BioTek). The colour change of the phenol red reagent from yellow to pink reflects the change of pH occurring upon the conversion of urea to ammonia and carbon dioxide by urease in a slightly buffered solution.

2.15 Cell fractionation of H. pylori

H. pylori cells were grown in cyclodextrin-supplemented Brucella broth for 16 hours (Marchini et al 1995). The cell pellet from 100 ml of culture was washed 3 times with PBS and re-suspended in 3 mL of ice cold 0.5 mM MgCl$_2$ and 30% (w/v) sucrose to
form spheroplasts. Spheroplast formation was checked by phase contrast microscopy (Leitz Laborlux K) every 20 minutes while cells were maintained on ice. The spheroplasts were pelleted by centrifugation at 4,000 g for 10 minutes at 4°C. The supernatant containing periplasmic proteins was reserved on ice. The spheroplasts were lysed by re-suspension in 1 mL of water, addition of acid-washed (and neutralized) glass beads (Sigma, diameter < 106 μm), with a beads /cell suspension ratio of 1/3 (v/v) with 4 cycles of vortexing for 30 seconds and cooling on ice for 30 seconds. The cytoplasmic protein fraction was obtained by centrifugation at 12,000 g for 30 minutes at 4°C followed by ultracentrifugation (Beckman) at 100,000 g for 1 h. The membranes recovered in the ultracentrifugation pellet were further separated into inner and outer membrane fractions by differential solubilization with lauryl sarcosyl (Filip et al., 1973). The pellet was re-suspended in 500 μl of 50 mM Tris-HCl pH 7.5 containing 1% (w/v) N-lauroyl-sarcosine sodium salt (Sigma) and incubated at room temperature for 1 h. The inner membranes were recovered in the supernatant following ultracentrifugation at 100,000 g for 1 h at 4°C. The outer membrane proteins recovered were re-suspended in 500 μl of 50 mM Tris-HCl pH 7.5. Then 20 μl of all fractions were analyzed by SDS-PAGE (12%), with Coomassie blue staining and Western blotting with anti-HcpE antibodies.

2.16 Electron microscopy of outer membrane vesicles

Electron microscopy images were obtained by Ryan Chanyi at the Electron Microscopy unit in the Molecular and Cellular Imaging Facility at the University of Guelph. The OMV samples were negatively stained with 2% uranyl acetate and visualized using an FEI Tecnai G2 F20 field emission electron microscope operating at 120 kV.
Images were obtained using a bottom mount Gatan 4k CCD camera using Tecnai Imaging and Analysis software.

### 2.17 Lysozyme refolding assay

This assay was performed following previously reported methods in the literature (Puig and Gilbert, 1994; Puig et al, 1997). Lysozyme was denatured and reduced with denaturing buffer (8M urea, 130 mM β-mercaptoethanol, 25 mM Tris-HCl pH 8.6) for 1 hour at 37°C to a final concentration of 0.7 mM to 6.7 mM. The sample was then diluted 35 fold in 0.1 M acetic acid to a final concentration of 20-200 μM of lysozyme. Refolding reactions were set up by first diluting the sample further to 1.0 to 10 μM of lysozyme in refolding buffer (5 mM reduced glutathione GSH, 0.5 mM oxidized glutathione GSSG, 100 mM HEPES, pH 7.0, 20 mM NaCl, 2 mM EDTA, 5 mM MgCl₂), then adding 5 μM of purified DsbHP-Flag-His, DsbA-Flag-His or DsbG-Flag-His to the reaction. Refolding was performed in 100 μl reactions and was left to proceed for 1h at 37°C. The reactions were then quenched by addition of an equal volume of 0.1 M acetic acid.

Lysozyme refolding was then assessed by measuring the ability of lysozyme to lyse *Micrococcus lysodeikticus* (Sigma) as described previously (120). Refolded lysozyme at a final concentration of 1.0 and 1.5 μM was added to a 0.5 g/l cell suspension of *Micrococcus lysodeikticus* (BioShop) in reaction buffer (60 mM potassium phosphate, 0.1% NaCl, pH 6.2). The decrease in turbidity was monitored spectrophotometrically at OD₆₅₀nm for a duration of 1 hour at 25°C. The assays were performed in triplicate in 96-well plates using an Eon (Bioteck) plate reader. Control reactions were done using the same molarities of native lysozyme that was diluted in the
same solutions (0.1 M acetic acid, refolding buffer and lysis reaction buffer) as well as no lysozyme.

2.18 Dithiothreitol exposure assays in \textit{E. coli}

\textit{E. coli} BL21(DE3)pLys containing expression vectors for either Dsb\textsubscript{HP}-Flag-His, DsbA-Flag-His, DsbG-Flag-His and GmD-His were grown O/N at 37°C in 3 mL of LB broth supplemented with kanamycin (30 µg/mL) and chloramphenicol (34 µg/mL). The following morning all cultures were sub-cultured (1/50 dilution) into 10 mL of LB broth with the same antibiotics and then induced with 0.1 mM IPTG when the OD\textsubscript{600nm} reached 0.5. Protein expression was allowed to proceed for 3 h at 37°C. Cultures not induced were grown in parallel to be used as controls. Cultures were then serial diluted on to LB agar containing either 0, 5, 7.5 or 10 mM dithiothreitol (DTT) in duplicate. The plates also contained kanamycin at 30 µg/ml and chloramphenicol at 34 µg/ml to maintain the expression of the pET30a- Dsb\textsubscript{HP}-Flag-His, DsbA-Flag-His, DsbG-Flag-His and GmD-His plasmids. Growth was assessed after overnight incubation at 37°C. Protein expression was assessed by anti-His Western blotting on the pre-induced cultures before spot plating as well as on cells that were recovered from the agar plates after overnight growth.

2.19 Dithiothreitol exposure in liquid broth

Similarly to the assay described above, \textit{E. coli} cultures were grown and pre-induced in the same manner except they were inoculated into LB broth at a 1/4000 dilution (100 µl total) in the wells of a Bioscreen plate instead of spotted onto LB agar. \textit{E. coli} was grown in the presence of 0, 7.5 or 10 mM DTT supplemented with 0.1 mM IPTG or not and growth was monitored automatically over 24 hours at 37°C under agitation. Protein expression was assessed similarly as in the plate spotting assay by anti-His Western
blotting on the pre-induced cultures before and after inoculation. Then 10 µl of each *E. coli* strain was withdrawn from the bioscreen plate at 6 hours and 24 hours post inoculation.

### 2.20 *H. pylori* exposure to dithiothreitol and hydrogen peroxide

*H. pylori* NCTC11637 wild-type, *dsb*<sub>HP</sub> and *hcpE* mutants were grown on Columbia agar containing background antibiotics and appropriate selection antibiotics (refer to section 2.1). After 48 hours of incubation, the cells were sub-cultured onto a full plate. After another 48 hours of incubation, the cells were harvested and re-suspended into Brucella broth and cell densities were normalized according to OD<sub>600nm</sub>.

For the dithiothreitol assay, the cell density was adjusted to 0.5. A 10 fold serial dilution was made (10<sup>0</sup>-10<sup>-5</sup>) and cells were spot plated (10 µl) onto agar containing 0, 8, 10 or 12 mM dithiothreitol.

For the hydrogen peroxide assay, the OD<sub>600nm</sub> was adjusted to 1 in a total volume of 100 µl of Brucella broth within a 96-well plate containing 75, 100 or 125 mM H<sub>2</sub>O<sub>2</sub>. After 30 min incubation in micro-aerobic conditions, a 10 fold serial dilution was made (10<sup>0</sup>-10<sup>-5</sup>) and cells were spotted on Columbia blood agar.

### 2.21 LPS extraction and analysis

LPS was extracted from the wild-type, *dsb*<sub>HP</sub> and *hcpE* knockout mutants (Hitchcock and Brown, 1983). Cell pellets from each strain were washed in 0.85% saline and re-suspended in 200 µL lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol, 1M Tris pH 6.8, and bromophenol blue). The samples were boiled for 30 minutes at 100°C. Proteinase K (BioShop) was added to a final concentration of 0.5 µg/mL and incubated for
1 hour at 60°C to digest protein within the sample. The LPS was analyzed by SDS-PAGE (12%) and stained with silver nitrate.

### 2.22 Silver nitrate staining of LPS samples

LPS preparations of wild-type, dsb\textsubscript{HP} and hcp\textsubscript{E} knockout mutants were stained using the modified Ultra-Fast silver staining protocol (Tsai and Frasch, 1982; Fomsgaard \textit{et al}, 1990). Following SDS-PAGE gel electrophoresis, LPS within the gel was oxidized with oxidizing buffer (0.7% periodic acid, 40% ethanol, 5% acetic acid) for 20 minutes, at room temperature. The oxidizing solution was removed using a vacuum filter and the gel was washed 5 times in distilled water for 3 minutes. The gel was stained for ten minutes with staining solution (20% (w/v) AgNO\textsubscript{3}, 0.002% (v/v) NaOH, 0.4% (v/v) NH\textsubscript{4}OH). The stain was removed and the gel was washed again 5 times with distilled water for 3 minutes. The gel was developed via reduction in water containing 0.00005% (w/v) citric acid and 0.00054% (v/v) formaldehyde. After colouration was resolved enough, the reaction was stopped via addition of 10% acetic acid for 1 min followed by thorough washing with distilled water.

### 2.23 Separation of inner and outer membranes

\textit{H. pylori} cell pellets were re-suspended in 0.85% saline and lysed via vortexing with glass beads (refer to section 2.9). Membranes were isolated from the lysate by ultracentrifugation (Beckman) at 100 000g for 1 hour at 4°C. After centrifugation the supernatant was removed and stored at -20 °C. The inner membrane proteins were then separated from outer membrane proteins by sodium lauroyl-sarcosine treatment of the total membrane preperation. The pellet was re-suspended in 50mM Tris-HCl pH 7.5 containing 1% (w/v) N-lauroyl-sarcosine sodium salt (Sigma) and incubated at room temperature for
1 hour with gentle shaking. After ultracentrifugation at 100 000 g for 1 hour at 4°C, the supernatant containing the soluble inner membrane proteins and peptidoglycan was removed and the pellet containing the outer membrane was re-suspended in 50mM Tris-HCl pH 7.5. All fractions were visualized following separation by SDS-PAGE (12%) and staining with Coomassie blue and silver nitrate.

2.24 Analysis of *H. pylori* clinical isolates

*H. pylori* strains isolated from gastric biopsies from patients diagnosed with various *H. pylori* related gastric diseases such as gastritis, ulceration and atrophy, were obtained from Dr. Hristo Atanassov. A total of 13 strains were analyzed for the presence of HcpE production. The clinical isolates were grown on Columbia blood agar plates for 48 hours in micro-aerobic conditions. After 48 hours, cells were sub-cultured onto 1 entire Columbia blood agar plate and grown for 48 hours at micro-aerobic conditions. Cells were then sub-cultured into Brucella broth supplemented with 1% β-cyclodextrin, sodium pyruvate (0.05 μg/ml), trimethoprim (5 μg/ml), vancomycin (10 μg/ml), and amphotericin B (5 μg/ml), and normalized to OD$_{600nm}$ 0.2 and grown for 16 hours at 37ºC. Liquid cultures were collected and the culture was centrifuged at 2,057 g for 10 minutes. The culture supernatant was removed and the cell pellets were stored at -20ºC until needed. The supernatant was then filter-sterilized (0.45 μm size). The cell pellets (total cells) and the culture supernatant were analyzed via SDS-PAGE electrophoresis and anti-HcpE Western blot.

2.25 Murine infection model and analysis with *H. pylori*
2.25.1 *H. pylori* colonization in C57BL/6 mice

All experiments were done according to approved protocol AUP 2012-10. *H. pylori* wild-type, *dsbHP* and *hcpE* knockout mutants were grown on Columbia blood agar plates for 48 hours in micro-aerobic conditions. After 48 hours, cells were sub-cultured onto 3 Columbia blood agar plates and grown for 48 hours at micro-aerobic conditions. Cells were then resuspended in Brucella broth and normalized to OD$_{600nm}$ 2 which was equivalent to 1.0x$10^9$ cells per 100 µl. C57BL/6 mice (Charles River) were then orally gavaged with 100 µl of either wild-type, *dsbHP* or *hcpE* mutant until a total of 12 mice had been gavaged with each strain. To establish infection, mice were orally gavaged every other day within a week totaling 3 oral gavages. During the first oral gavage, 50-100 µl of blood was also collected from the tail vein for anti-HcpE ELISA. Infection was allowed to proceed for 4 weeks post gavage until the first 6 mice from each group were sacrificed. Mice were sacrificed by peritoneal injection with 100 µl of pentobarbital (Ceva). Up to 500 µl of blood was taken by intracardial collection and the stomach as well as the spleens were harvested to analyze colonization, inflammation, and immune activation. The stomachs were separated into 2 halves, and 1 half was sent for histological analysis and the other half was used for homogenization to assess colonization. The other half of the mice were sacrificed 6 weeks post gavage and the blood and organs harvested as described above. Mice that were gavaged with Brucella broth only were used as controls.

2.25.2 Homogenization of stomach tissue to assess colonization

To assess colonization, half of the murine stomachs that were harvested from mice gavaged with either wild-type, *dsbHP* mutant, *hcpE* mutant or Brucella alone were homogenized and plated onto blood agar plates. Briefly, after being harvested and divided
into 2 halves, the murine stomach tissue was washed 3 times in 1X PBS supplemented with amphotericin B (5 µg/mL), vancomycin (10 µg/mL), trimethoprim (5 µg/mL), and nalidixic acid (2 µg/mL) to remove stomach contents. After washing, tissue samples were placed into 3 mL of the same PBS buffer with antibiotics and homogenized for 1-2 minutes with an electric homogenizer (Fisher Scientific). After homogenization, the gastric homogenate was diluted 10 and 100 fold, and 100 µl of each dilution was plated onto Columbia blood agar containing the same antibiotics as the PBS buffer. Plates were incubated in micro-aerobic conditions for 5-7 days. The remaining homogenate was stored at -20°C.

**2.25.3 Preparation and analysis of gastric tissue for histology**

Stomach tissue that was harvested from mice gavaged with either wild-type, \(dsb_{HP}\) mutant, \(hcpE\) mutant or Brucella alone was fixed in buffered formalin (VWR) for 72 hours. The fixed tissue was washed in PBS once every 8 hours for a total of 5 washes. Tissue was then stored in 70% ethanol until dehydration and paraffin wax infusion in an automatic tissue processor (Leica ASP200 S) at Robarts Research Institute was performed. Processed samples were then embedded into paraffin wax, and when hardened were cut into 5 µm thick sections using a microtome. Sections were processed in duplicate: half were sent to the pathology department in University Hospital for Warthin-Starry staining for \(H. pylori\) detection and visualization and the other half were stained with hematoxylin and eosin stain (H&E) to visualize and assess inflammation.

**2.25.4 Isolation and preparation of splenocytes**

Murine spleens were harvested from C57BL/6 mice that were infected with either wild-type, \(dsb_{HP}\::\text{kan}\), \(hcpE\::\text{kan}\) mutants, or naïve mice. Splenocytes were isolated by
manual homogenization with a glass homogenizer in 5 mL of sterile endotoxin free PBS (Corning) that contained trimethoprim (5 µg/mL) and vancomycin (10 µg/mL). The homogenization mixture was then centrifuged at 500 g for 7 minutes at room temperature. The cell pellet was re-suspended in 4 mL of ammonium-chloride-potassium (ACK) buffer (NH₄Cl 0.15M, KHCO₃ 10 mM, EDTA 0.1 mM) to lyse the red blood cells. Then 10 mL of 1XPBS was added to dilute the ACK buffer to prevent death of the splenocytes, and centrifuged at 500 g for 7 minutes. The supernatant was discarded, and the cellular pellet containing splenocytes was washed with 7 mL of 1XPBS and centrifuged again at 500 g for 7 minutes. The cell pellet was re-suspended in 10 mL of RPMI (Thermo Scientific) supplemented with 10% FBS (Gibco) and 2.5% Hepes (Gibco). Splenocytes were then counted using a haemocytometer and Trypan blue to determine total cell counts, as well as the number of live and dead cells.

2.26 Interaction assays with splenocytes

Splenocytes isolated as described above were plated into 96-well tissue culture plates (VWR) and equilibrated for 2 hours at 37°C in 5% CO₂ before cells were infected. For each experiment, the concentration of cells per milliliter was calculated by counting the cells with a haemacytometer and 0.5-2 x 10⁶ cells were plated in each well. Cells were then infected with H. pylori wild-type, dsbHP::kan or hcpE::kan which were grown for 48 hours on Columbia blood agar with background and selective antibiotics. The cells from plates were resuspended in serum-free Brucella broth, supplemented with β-cyclodextrin for 12 hours, as previously described (refer to section 2.12). The bacterial cultures were centrifuged at 2057 g for 30 minutes at room temperature to pellet the cells. The cell pellet, representing the total cells, was re-suspended in complete Roswell Park Memorial Institute
(cRPMI) medium such that the splenocytes were infected with \textit{H. pylori} at a multiplicity of infection (MOI) of 1, 0.5, and 0.1 bacteria per splenocyte. \textit{H. pylori} cell supernatants were concentrated via lyophilization and then re-suspended in 1/10 of the original volume of cRPMI medium and further diluted to final concentrations of 5, 3, and 1X. Splenocytes were also infected with pure HcpE and DdahB that were either treated or not with Proteinase K (at concentrations of 75, 125, and 200 mM). The splenocytes were infected for 12 or 24 hours. At each time point, the supernatants were recovered and was kept for cytokine analysis (stored at -20°C).

2.27 Quantification of IFN-\(\gamma\) production by ELISA

The IFN-\(\gamma\) ELISA was carried out using the READY-SET-GO! Mouse Interferon gamma kit (eBioscience) according from the manufacturer's recommendations. A 96-well plate was coated with 100 \(\mu\)L/well of capture antibody (purified anti-mouse IFN-\(\gamma\)) in Coating buffer. The coating buffer was diluted 10 fold and 1 \(\mu\)l of antibody was added for every 1 mL of coating buffer. The plate was covered and incubated O/N at 4°C. The next morning, the wells were aspirated and washed 3 times with 200 \(\mu\)L Wash Buffer (1X PBS, 0.05% Tween-20), allowing the buffer to soak for one minute between washes. The plate was blotted dry on absorbent paper after the final wash to remove residual buffer. To block the plate, Assay Diluent (provided in kit) was diluted 1 in 5 in MilliQ, to make a 1X solution, and 100 \(\mu\)L were added to each well and incubated for 1 hour at room temperature. After incubation, the plate was aspirated and washed 3 times, as before. To generate a standard curve, the standard recombinant mouse IFN-\(\gamma\) (provided in kit) was diluted into 1X assay diluent. The stock solution, 2000 pg/mL, was prepared by adding 20 \(\mu\)L of the standard to 10 mL of assay diluent. The stock was then serially diluted yielding solutions
of 1000, 750, 565.2, 421.9, 316.4, 237.3, 178, 133.5, 100, 75, and 0 pg/mL. The standard and splenocyte samples were added to the plate, 100 μL/well in triplicate. The plate was sealed and incubated at room temperature for 2 hours. The wells were aspirated and washed 3 times with wash buffer as previously described. Detection antibody diluted in 1X assay diluent was added, 100 μL/well, and incubated at room temperature for 1 hour. The detection antibody (Biotin-conjugated anti-IFN-γ, polyclonal) was prepared by adding 12 μL of antibody solution to 12 mL 1X assay diluent. The plate was washed 3 times with wash buffer and then the enzyme Avidin-HRP* diluted in 1X assay diluent was added, 100 μL/well and incubated as room temperature for 30 minutes. The Avidin-HRP enzyme was prepared by adding 48 μL of the enzyme solution to 12 mL assay diluent. The plate was washed 3 times with wash buffer, then again with 1X PBS to remove traces of Tween. After thorough washing and blotting the plate dry, 100 μL of Substrate solution (1X tetramethylbenzidine) was added to each well and incubated at room temperature for 15 minutes, or until sufficient blue colour developed. To end the reaction, 50 μL/well Stop solution (1M H₂SO₄) was added. The plate was read immediately after adding the stop solution using the EON plate reader (Biotec) at 450 and 570 nm. To analyze the data, the 570 nm readings (background) were subtracted from the 450 nm readings.

CHAPTER 3: RESULTS

3.1 Molecular tools developed to study DsbHP and HcpE

To facilitate the study of DsbHP and its effect on the secretion of HcpE, a number of molecular tools had to be developed. This included generating recombinant proteins by
over-expression and purification, as well as developing *H. pylori* *dsb*$_{HP}$ knockout mutant and *hcpE* complement strains.

### 3.1.1 *dsb*$_{HP}$::*kan* knockout mutagenesis

The Creuzenet lab has previously demonstrated that Dsb$_{HP}$ is a potential folding factor capable of binding to and refolding insoluble HcpE (Kichler, 2010). Thus, Dsb$_{HP}$ may be important for the secretion of HcpE into the extracellular matrix. To investigate the role that Dsb$_{HP}$ plays on the secretion of HcpE, the *dsb*$_{HP}$ gene was knocked out in the laboratory strain, NCTC 11637. The *dsb*$_{HP}$ mutant was constructed by disrupting the chromosomal copy of the *dsb*$_{HP}$ gene with a kanamycin resistance cassette via homologous recombination. The construct was designed as to orientate the kanamycin resistance cassette in the same transcriptional orientation as *dsb*$_{HP}$ to reduce the potential risk of polarity caused by altering gene expression (Figure 8A). All potential clones were isolated from agar plates containing kanamycin for selection, then screened for the presence of the disrupted *dsb*$_{HP}$ gene containing the kanamycin resistance cassette, as well as correct insertion and orientation into the chromosome via PCR. The *dsb*$_{HP}$ disrupted construct was amplified using the primers HP0231P1 and HP0231P2, whereas primers HP0230P3 and HP0232P2 (Table 2) were used to determine correct orientation and insertion into the chromosome. A PCR amplicon of 1773 bp within the *dsb*$_{HP}$ mutant indicated that the gene was disrupted by the Kan cassette and was successfully integrated in the genome (Figure 8B). Likewise the presence of a 3228 bp band in the mutant confirmed that the location and orientation were correct (Figure 8C).
**Figure 8: PCR screening of **$dsb_{HP}$** knockout mutagenesis clones.** For all panels, diagrams are not to scale.

**Panel A:** Diagram of the $dsb_{HP}$ coding region ($hp0231$) in wild-type and knockout mutant showing all primer locations used in the PCR screening and the expected PCR amplicon sizes.

**Panel B:** PCR amplification using knockout mutant chromosome with primers HP0231 P1/P2 indicate that the $dsb_{HP}$ gene was successfully disrupted in all clones tested. Amplification of $dsb_{HP}$ from wild-type chromosomal DNA results in a 798 bp product, whereas clones with the kanamycin cassette insertion result in a larger fragment at its predicted size of 1773 bp.

**Panel C:** To test whether insertion of the disruption construct occurred in the correct location, primers HP0230 P3 and HP0232 P2 were used to amplify *H. pylori* chromosomal DNA. A product of 2253 bp in the wild-type and 3228 bp in the knockout demonstrated that the kanamycin cassette was inserted in the correct location.
A.

**Wild-type**

![Diagram of HP0230, HP0231, and HP0232 in Wild-type configuration with distances 798 bp and 2253 bp.]

**dsb\text{HP}::\text{kan}**

![Diagram of HP0230, HP0231, and HP0232 in dsb\text{HP}::\text{kan} configuration with distances 1773 bp and 3228 bp.]

B.

![Image of gel electrophoresis for Wild-type and Clone 1 to Clone 5, with markers at 4, 3, 2, 1, and 1.5 Kb.]

C.

![Image of gel electrophoresis for Wild-type and Clone 1 to Clone 5, with markers at 4, 3, 2, 1, and 1.5 Kb.]

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The diagram illustrates the genetic relationships and distances between the HP0230, HP0231, and HP0232 genes in both Wild-type and dsb\text{HP}::\text{kan} configurations. The distances are measured in base pairs (bp) and kilobases (Kb). The gel electrophoresis images show the band patterns for different clones, indicating the presence or absence of certain genetic markers.
3.1.2 Complementing \textit{hcpE::kan} knockout mutant

To study HcpE and its potential function in \textit{H. pylori} pathogenicity, an \textit{hcpE::kan} knockout mutant was previously constructed by A. Oberc. However, to determine whether the loss of the \textit{hcpE} gene was the cause of the phenotype(s) observed and not due to downstream effects, an \textit{hcpE} complement had to be constructed. The construct was designed to replace the disrupted \textit{hcpE} gene within the \textit{H. pylori} NCTC 11637 \textit{hcpE} knockout mutant with a wild-type copy of \textit{hcpE}. The construct also contained a chloramphenicol (CAT) cassette downstream of \textit{hcpE} for selection purposes. All potential clones isolated from agar plates containing CAT were screened using PCR with primers HP0235P1 and HP0235P2 to determine whether the disrupted \textit{hcpE} gene was replaced with the wild-type copy, as well as primers HP0235P1 and HP0232P1 to ensure that the entire construct recombined into the correct location within the chromosome. All clones screened contained the wild-type copy of \textit{hcpE} (Figure 9B), however only clones 2 and 3 contained the entire complement construct in the correct orientation and location (Figure 9C).

To determine if our complement construct was capable of actually producing HcpE, total cell lysates from wild-type, \textit{hcpE} knockout and \textit{hcpE} complement were analyzed via SDS-PAGE separation and anti-HcpE Western blot (Figure 10). The \textit{hcpE} complement clones 1 and 2 are able to produce HcpE at similar levels compared to wild-type, whereas clone 3 is not as prominent. It is important to note that the non-specific reactivity in the anti-HcpE Western blot is due to the anti-rabbit secondary antibody binding non-specifically to proteins within the fraction. The size differences of HcpE observed in the complement clones compared to the wild-type is due to the fact that HcpE in the \textit{hcpE} complement clones contain a C-terminal histidine tag for purification via nickel affinity
**Figure 9: PCR screening of hcpE complement.** For all panels, diagrams are not to scale.

**Panel A:** Diagram of the *hcpE* coding region (*hp0235*) in the knockout mutant and the complement showing all primer locations used in the PCR screening and the expected PCR amplicon sizes.

**Panel B:** PCR amplification of potential *hcpE* complement clones using primers HP0235 P1/P2. The *hcpE* knockout mutant was used as a control. Amplification indicates that the wild-type form of *hcpE* was successfully re-introduced into the chromosome in all clones tested. Amplification of *hcpE* from the knockout mutant chromosomal DNA results in a 2273 bp product, whereas clones result in a smaller fragment at 1211 bp.

**Panel C:** To test whether insertion of the complementation construct occurred in the correct location, primers HP0232 P1 and HP0235 P1 were used to amplify *H. pylori* chromosomal DNA. A product of 5505 bp in the knockout and 5303 bp in the complement demonstrated that the complement construct was inserted in the correct location within clones 2 and 3.
A.

hcpE::kan

B.

C.
Figure 10: *hcpE complement produces HcpE*. Total cell lysates from wild-type, *hcpE*:kan mutant and *hcpE* complement were analyzed via SDS-PAGE and anti-HcpE Western blot. The *hcpE* complement clones 1 and 2 produce HcpE at levels similar to wild-type, whereas clone 3 is not a prominent as wild-type. PR: Ponceau Red, WB: Western blot.
chromatography. The added polyhistidine tag increases the molecular weight of the HcpE protein, resulting in increased size when run on an SDS-PAGE gel.

3.1.3 DsbHP is soluble when over-expressed in E. coli

Pure DsbHP was needed to determine its role as a potential oxidoreductase involved in the formation of disulfide bonds. DsbHP protein expressed from a previous expression construct was difficult to purify due to the presence of a C-terminal Flag tag that could not be used for purification purposes. Purification was conducted via anion exchange chromatography, which was not specific enough for our DsbHP protein, so contamination from other proteins that would non-specifically bind to the anion exchange column was observed. Thus, to be able to obtain pure DsbHP via nickel chromatography, a cleavable C-terminal histidine tag was cloned onto the dsbHP gene after the Flag tag. The C-terminally histidine-tagged DsbHP was then transformed into E. coli BL21(DE3)pLys cells for expression via IPTG induction.

To purify DsbHP, cultures were grown in LB at 37°C and induced with IPTG. Cells were lysed with a cell disrupter, and the soluble and insoluble protein fractions were separated by centrifugation. The soluble proteins contained within the supernatant were passed through a nickel column for purification. Eluted fractions were analyzed by Western blotting with the anti-histidine antibody. Pure DsbHP was obtained and visualized at the expected size of 33 kDa (Figure 11A and B).

3.1.4 E. coli DsbA and DsbG can be purified via nickel chromatography

For the purpose of these studies, E. coli DsbA and DsbG were used as controls for comparative analysis with DsbHP to help determine whether DsbHP is an oxidase or
**Figure 11: Dsb<sub>HP</sub> can be expressed in *E. coli*.** *E. coli* BL21(DE3)pLys cells were transformed with a pET vector containing Dsb<sub>HP</sub>-Flag-His which is inducible via IPTG.

**Panel A:** Expression of Dsb<sub>HP</sub> was performed at 37°C for 3 hours and was assessed using SDS-PAGE separation of total cell lysate and detected by anti-Histidine Western blot. Dsb<sub>HP</sub> is observed at 33 kDa (asterisk). PR: Ponceau Red, WB: Western blot.

**Panel B:** Dsb<sub>HP</sub> was purified using nickel affinity chromatography and the purity of the protein fractions was assessed using SDS-PAGE and Coomassie staining. Dsb<sub>HP</sub> is observed at its expected size of 33 kDa.
A.

Protein fraction 1
Protein fraction 2
Protein fraction 3
Protein fraction 4

MW - + IPTG

kDa
50
34.5
28.8
20.7

PR

WB

MW

kDa
50
34.5
28.8

B.

Protein fraction 1
Protein fraction 2
Protein fraction 3
Protein fraction 4

MW

kDa
50
34.5
28.8
isomerase in terms of the formation of disulfide bonds. The \textit{E. coli} \textit{dsbA} and \textit{dsbG} genes were cloned into a \textit{pET30} vector with the same construct configuration as our new Dsb\textsubscript{HP} to prevent any potential differences in activity, and then transformed into \textit{E. coli} BL21(DE3)pLys cells for expression via IPTG induction. Cultures were grown in LB at 37°C, induced with IPTG and proteins purified as for Dsb\textsubscript{HP} as described above. Eluted fractions were analyzed by Western blotting with the anti-histidine antibody (Figure 12A and B). Pure DsbA and DsbG were obtained and visualized at the expected size of 28 kDa and 31 kDa, respectively. Both DsbA and DsbG were visualized as 2 bands in both the total cell lysate and purified protein fractions, most likely representing the processed form of the proteins that made it through the Sec secretion system into the periplasm, and the un-processed form still containing its N-terminal signal sequence in the cytoplasm.

\subsection*{3.1.5 Co-expressing Dsb\textsubscript{HP} and HcpE simultaneously produces more soluble HcpE}

Expression of HcpE under previously optimized conditions resulted in the formation of mostly insoluble protein, with only a small amount of HcpE soluble. Due to the fact that pure soluble HcpE was required for this study, a vector encoding both \textit{dsbHP} and \textit{hcpE} was constructed. The \textit{hcpE} gene was sub cloned into our \textit{pET30-Dsb\textsubscript{HP}-Flag} construct by PCR. The construct was designed so that both genes would be transcribed into the same RNA sequence but translated separately. HcpE and Dsb\textsubscript{HP} were both over-expressed in the \textit{E. coli} \textit{pET-30} plasmid. Following induction via IPTG, the total cell extracts as well as the soluble and insoluble fractions were analyzed for HcpE expression via anti-HcpE Western blot. Expression of HcpE at 37°C resulted in the formation of insoluble HcpE in both expression strains (Figure 13A). However, the \textit{E. coli} containing the co-expression vector was able to produce soluble HcpE when IPTG induction occurred at room temperature,
Figure 12: *DsbA and DsbG can be expressed via IPTG induction.* *E. coli* BL21(DE3)pLys cells were transformed with a pET vector containing either *dsbA* or *dsbG* which is inducible via IPTG.

**Panel A:** Expression of both DsbA and DsbG were performed at 37°C for 3 hours and total cell lysate was assessed using SDS-PAGE and anti-Histidine Western blot.

**Panel B:** DsbA and DsbG were purified using nickel chromatography and purity was assessed using SDS-PAGE and Coomassie staining. Pure proteins were serial diluted to optimize the signal to noise ratio. DsbA was observed at its expected size of 28 kDa, and DsbG was observed at its expected size of 31 kDa.
A.

![Diagram A](image1)

B.

![Diagram B](image2)
whereas the original HcpE-His expression strain was still incapable of producing large amounts of soluble HcpE (Figure 13B).

3.2 Investigation into the activity of Dsb_{HP}

3.2.1 Dsb_{HP} is involved in the formation of disulfide bonds

The function of Dsb_{HP} is predicted to be a putative \textit{E. coli} DsbG based on structural homology, however, the function of Dsb_{HP} is still up for debate. To determine if Dsb_{HP} has DsbG activity, we investigated the capability of Dsb_{HP} to refold denatured and reduced lysozyme. HcpE has no known function, which makes it impossible to quantify Dsb_{HP} activity using reduced HcpE. Thus, lysozyme was chosen as a substrate due to the fact that lysozyme relies on four disulfide bonds for proper folding and stability (Eyles \textit{et al}, 1994). Also, the use of denatured and reduced lysozyme as a substrate to test disulfide bond forming enzymes has been previously established in the literature (Puig and Gilbert, 1994; Puig \textit{et al}, 1997).

Lysozyme was denatured and reduced using urea and β-mercaptoethanol respectively, and the reaction was quenched by dilution using acetic acid until a final concentration range of 1-10\(\mu\)M of lysozyme was obtained. Refolding reactions containing denatured and reduced lysozyme as well as Dsb_{HP}, \textit{E. coli} DsbA , \textit{E. coli} DsbG, or no enzyme were allowed to proceed for 1 hour before the reaction was quenched with acetic acid. \textit{E. coli} DsbA and DsbG were used as comparative controls to help establish whether Dsb_{HP} activity is characteristic of DsbA oxidase, or DsbG isomerase activity. Lysozyme refolding activity was assessed via measurement of \textit{Micrococcus lysodeikticus} lysis and
**Figure 13: Co-expression of both HcpE and DsbHP results in more soluble HcpE.** SDS-PAGE analysis and Western blotting with anti-HcpE antibodies was performed on *E. coli* cell pellets containing either our co-expression or HcpE-His vector.

**Panel A:** Expression was carried out at 37°C for 3 hours. Before induction half of the liquid culture was separated out to be used as a non-induction control. The anti-HcpE Western blot indicates that 37°C expression results in most of the HcpE remaining insoluble even in the co-expression strain.

**Panel B:** Expression was carried out at room temperature for 3 hours to compare HcpE production to that done at 37°C. HcpE production at room temperature resulted in a decrease of insoluble HcpE, even more so in the co-expression strain, suggesting that the lower temperature as well as presence of DsbHP aided the folding and solubilization of HcpE.
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**IPTG**

### B.

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**IPTG**
was monitored spectrophotometrically at OD$_{650\text{nm}}$. In the absence of lysozyme, the *Micrococcus* cells began to aggregate to the bottom of the well, which resulted in an increase in OD$_{650\text{nm}}$ (Figure 14A). When native lysozyme was added in the absence of any Dsb protein, rapid lysis of *Micrococcus* occurred (Figure 14A), which was to be expected. A slow linear decrease of OD$_{650\text{nm}}$ was observed when *Micrococcus* was in the presence of denatured and reduced lysozyme alone, which indicated that low levels of spontaneous refolding of lysozyme was occurring in the absence of Dsb protein (Figure 14, all panels). However, when either Dsb$_{\text{HP}}$, DsbA, or DsbG was incubated with the denatured and reduced lysozyme, an enhanced lysis of *Micrococcus* was observed similar to that seen with native lysozyme alone. This demonstrates that Dsb$_{\text{HP}}$ is able to regenerate the disulfide bonds that are essential for lysozyme activity similar to that of both *E. coli* DsbA and DsbG, indicating that Dsb$_{\text{HP}}$ has Dsb activity.

### 3.2.2 Dsb$_{\text{HP}}$ expression within *E. coli* is toxic

Dsb proteins play a crucial role in the periplasm. They are responsible for protein folding and maturation via disulfide bond formation, as well as maintaining the periplasmic oxidative status. We have demonstrated that Dsb$_{\text{HP}}$ behaves like a Dsb protein, however, were unable to conclude whether its catalytic activity is similar to an oxidase or isomerase. Therefore, we wanted to determine whether Dsb$_{\text{HP}}$ activity resembles DsbA or DsbG type of activity. To explore this, all 3 proteins were overexpressed separately. It was hypothesized that overexpression of a DsbA type protein would result in a more oxidative periplasmic environment resulting in a larger proportion of misfolded proteins that could
**Figure 14: DsbHP is able to form disulfide bonds on reduced lysozyme.** The ability of lysozyme to lyse *Micrococcus* was assessed by measuring the decrease in turbidity at OD650nm.

**Panel A:** Optimized assay for DsbHP whereby spontaneous refolding of lysozyme was minimized and addition of DsbHP restored activity to reduced and denatured lysozyme (Lys) to levels similar to those obtained with native lysozyme.

**Panels B-C:** Common reaction conditions show that addition of *E. coli* DsbG, and *E. coli* DsbA restored partial activity to reduced and denatured lysozyme.
A.

Optical Density (OD600nm)

[Graph showing optical density over time for different conditions: No Lys, Dsb+, No Lys, No Dsb, Lys+, No Dsb, Lys+, Dsb+, Native Lys]

B.

Optical Density (OD600nm)

C.

Optical Density (OD600nm)

[Graph showing optical density over time for different conditions: No Lys, Dsb+, No Lys, No Dsb, Lys+, No Dsb, Lys+, Dsb+, Native Lys]
potentially lead to cellular death. Overexpression of a DsbG type protein would most likely have no effect due to its nature of correcting improper disulfide bonds.

The proteins DsbHP, DsbA, and DsbG were expressed in *E. coli* BL21(DE3)pLys via IPTG for 3 hours at 37°C. GDP-mannose 4,6-dehydratase (GMD), which is a protein involved in sugar biosynthesis, was used as a negative control to show that our observations were not solely due to induction alone. To determine if all of the proteins were expressed during induction, an anti-histidine Western blot was performed. All proteins expressed at the 3 hour time point were detected by Western blot, indicating successful overexpression (Figure 15). All cultures were then diluted and spot plated onto agar plates containing either various concentrations of IPTG or no IPTG at all. When grown on agar in the presence of IPTG, no growth for either *E. coli* expressing DsbHP or DsbA was observed. However, growth of *E. coli* expressing DsbG was observed for cells not pre-induced before plating (Figure 16). However, no growth for any *E. coli* strain that was pre-induced and grown in the presence of IPTG was observed, indicating that constant induction results in additional cellular stress leading to growth inhibition. Alternatively, all *E. coli* that was spotted onto agar with no IPTG had no difficulty growing. This indicated that the *E. coli* expressing either DsbHP or DsbA was more sensitive to IPTG exposure than DsbG or the control, causing the increased toxicity during protein expression.

### 3.2.3 During reductive stress DsbHP exhibits DsbA-like behavior in *E. coli*

Dsb proteins that are involved in oxidation pathways have previously been shown to be important in protecting against reductive stress due to the fact that *dsbA* mutants have been shown to be sensitive to dithiothreitol (DTT) *in vitro* (Kamitani *et al*, 1992; Roszczenko *et al*, 2012). It was hypothesized that when DTT is added to the growth
Figure 15: DsbHP, DsbA, and DsbG were all expressed in E. coli before being plated onto agar plates containing IPTG. The proteins DsbHP, E. coli DsbA, E. coli DsbG, and a control protein GMD that were pre-induced with IPTG before serial dilution and spot plating were analyzed via SDS-PAGE and anti-histidine Western blot. All proteins were expressed when pre-induced with IPTG, although GMD expression was below the detection level of anti-histidine Western blot. All Dsb proteins were observed at their expected sizes of 33 kDa (DsbHP), 31 kDa (DsbG), and 28 kDa (DsbA). The control protein GMD was not expressed at detectable levels.
Figure 16: DsbHP has DsbA-like behavior when expressed in *E. coli*. The proteins DsbHP, *E. coli* DsbA, *E. coli* DsbG, and a control protein GMD were over-expressed under the same conditions and the cells were serial diluted before being spot plated. Dilutions were spotted onto plates containing either none or 0.15mM IPTG in decreasing order, as indicated by the triangles. DsbHP demonstrates the same toxic effects as DsbA due to the fact that pre-induction of protein expression resulted in less colonies compared with DsbG and the control protein.
Non Induced

IPTG

Dilution

Dsbur

DsbG

DsbA

Control

No IPTG

Non Induced

Pre-induced

Dilution

Dsbur

DsbG

DsbA

Control

Pre-induced
medium, the periplasmic space would change from an oxidizing environment to a reducing one, resulting in many unfolded proteins. Over-expression of a DsbA like protein should be able to compensate for this change due to an increase in the potential to form disulfide bonds, whereas a DsbG like protein would have no effect because little to no spontaneous disulfide bonds would form that would require correction.

\textit{E. coli} expressing Dsb\textsubscript{HP}, DsbA, and DsbG was grown and induced in the same conditions and time as described above. After induction, strains were diluted and spot plated onto agar containing 0, 5, 7.5, or 10mM DTT. Surprisingly, when \textit{E. coli} cells were pre-induced with IPTG, no colonies were observed on plates containing 10 mM DTT, and the growth of \textit{E. coli} expressing either Dsb\textsubscript{HP} or DsbA were severely impaired compared to DsbG or the control when plated onto lower DTT concentrations (Figure 17). This indicated that the presence of Dsb\textsubscript{HP} during exposure to DTT was not beneficial and actually resulted in a disadvantage. \textit{E. coli} cells that were not pre-induced with IPTG before being plated onto the various concentration of DTT had no noticeable defects in growth other than no colonies were recovered on 10 mM DTT. This indicates that the endogenous expression of \textit{E. coli} Dsb proteins was sufficient to counteract the effects of DTT up to 10 mM of DTT.

An anti-histidine Western blot was performed on cells recovered after 16 hours post induction to determine if proteins expressed during pre-induction were still present within the cells (Figure 18). No reactivity was observed in the cells recovered from plates inoculated with pre-induced bacteria, indicating that all protein expression had stopped. Thus, as observed above, the toxic effects observed were due to the expression of the
Figure 17: Treatment with the reducing agent DTT results in DsbHP toxicity. The proteins DsbHP, *E. coli* DsbA, *E. coli* DsbG, and a control protein GMD (Cj1319) were over-expressed as described above. Dilutions were spotted onto agar containing either 0, 5 or 7.5mM DTT in decreasing order, as indicated by the triangles. DsbHP demonstrates the same toxic effects as when pre-induced only except that the toxicity is more severe when DTT is in the media. DsbHP and DsbA displayed similar levels of toxicity when exposed to DTT, demonstrating a similar phenotype.
Figure 18: Dsb protein expression diminishes quickly after removal of IPTG. The proteins Dsb_{HP}, *E. coli* DsbA, *E. coli* DsbG, and a control protein GMD were analyzed via SDS-PAGE and anti-histidine Western blot 16hrs after spot plating.

**Panel A:** SDS-PAGE analysis of *E. coli* expressing Dsb_{HP}, DsbA, DsbG, or GMD that were pre-induced with IPTG before serial dilution and spot plating.

**Panel B:** SDS-PAGE analysis of *E. coli* expressing Dsb_{HP}, DsbA, DsbG, or GMD 16 hours after IPTG was removed from the medium. No histidine tagged proteins were observed in any of the cells 16 hours after the removal of IPTG from the media, indicating that protein expression had ceased and proteins from pre-induction had been turned over.
A.

B.
proteins during pre-induction. We currently do not understand the reason or mechanism behind the observed toxic effects of DsbHP and DsbA. Nevertheless, the results still clearly demonstrate that DsbHP has the same phenotype as DsbA, suggesting they may have similar enzymatic activity.

3.2.4 The toxicity observed during DsbHP expression results in bacteriostasis

The toxic effects observed while expressing DsbHP and DsbA demonstrated that these two enzymes may share similar catalytic activity. However, this phenotype had only been characterized during bacterial growth on agar plates and for only 1 time point. Thus, we repeated this experiment in liquid media to determine if this phenotype holds true over time.

Culture and induction conditions were the same as described above. Once the 3 hour induction phase finished, growth of the cultures was monitored continuously at OD$_{600nm}$ every 20 minutes using the bioscreen apparatus. We observed, as when grown on agar plates, that pre-induction with IPTG while grown in the absence of DTT results in toxic effects that inhibits growth of E. coli with DsbHP and DsbA (Figure 19). Both DsbHP and DsbA had an extended lag phase compared to DsbG, 3 and 4 hours respectively. However, both DsbHP and DsbA were still able to eventually reach stationary phase. When DTT was added to the liquid medium after the pre-induction with IPTG the lag phase for E. coli expressing either DsbHP or DsbA was even more severe, whereas the lag phase for DsbG was only slightly longer than compared to growth in the absence of DTT (Figure 19). Again, this demonstrates that DsbHP behaves similarly to DsbA rather than DsbG when expressed in E. coli. Also, expression of DsbHP and DsbA in liquid media suggests that the
Figure 19: Toxicity of DsbHP in liquid culture is bacteriostatic. The proteins DsbHP, *E. coli* DsbA, *E. coli* DsbG, and a control protein GMD were over-expressed in *E. coli* under the same conditions and the growth was monitored over a 20 hour period at OD_{600nm}. DsbHP shows a DsbA-like toxic effect when pre-induced with IPTG as demonstrated by a lengthened lag phase. This effect was bacteriostatic since growth rates and final culture densities were similar for all strains. When DTT was added to the growth medium, the toxic effect was exaggerated.
No Induction

Pre-induced

No DTT

7.5mM DTT

10mM DTT

Time (Hours)
toxicity generated by expressing these two proteins is bacteriostatic instead of bacteriocidal, due to the fact that in the presence or absence of DTT, the cells always reached stationary phase to the same degree as \textit{E. coli} not expressing protein and not exposed to DTT.

An anti-histidine Western blot was conducted to determine if expressed proteins were still present after induction had ceased (Figure 20). Western blot analysis indicated that proteins that were expressed during the pre-induction phase had already been turned over by 6 hours post induction. This strongly correlates with the duration of the extended lag phase observed in the \textit{E. coli} expressing Dsb$_{HP}$ and DsbA. Once expression has stopped and all proteins are eliminated from the periplasm, the cells are able to resume standard growth.

3.2.5 Dsb$_{HP}$ helps resist against oxidative stress within \textit{H. pylori}

Thus far we have established that Dsb$_{HP}$ is capable of forming disulfide bonds and is responsible for oxidizing these bonds similarly to the \textit{E. coli} oxidase DsbA. However, these experiments were conducted using \textit{E. coli} and therefore the physiological activity or importance of Dsb$_{HP}$ within \textit{H. pylori} is still unknown. To help determine the activity of this enzyme within \textit{H. pylori}, we constructed a mutant where we disrupted \texttt{dsb}$_{HP}$ with a kanamycin resistance cassette. This in itself demonstrated that \texttt{dsb}$_{HP}$ is not essential for survival as we were able to obtain knockout mutant clones.

When grown in micro-aerobic conditions, the \texttt{dsb}$_{HP}$ knockout mutant was capable of retaining growth similarly to that of wild-type (Figure 21A). However, when exposed to atmospheric concentrations of oxygen a 10 fold decrease in cellular viability was routinely
Figure 20: Expression of Dsb proteins does not persist past 6 hours when IPTG is removed from the media. The proteins Dsb$_{HP}$, *E. coli* DsbA, *E. coli* DsbG, and a control protein WbpB were analyzed via SDS-PAGE and anti-histidine Western blot for protein expression after IPTG was removed from the medium.

**Panel A:** SDS-PAGE analysis of *E. coli* expressing Dsb$_{HP}$, DsbA, DsbG, or WbpB that were pre-induced with IPTG before serial dilution.

**Panel B:** SDS-PAGE analysis of *E. coli* expressing Dsb$_{HP}$, DsbA, DsbG, or WbpB 6 and 16 hours after IPTG was removed from the media. No histidine tagged proteins were observed in any of the cells 6 hours after the removal of IPTG from the medium, indicating that protein expression had ceased and proteins from pre-induction are turned over quickly.

**Panel C:** SDS-PAGE analysis of *E. coli* expressing Dsb$_{HP}$, DsbA, DsbG, or WbpB 6 hours after IPTG was removed and grown in the presence of DTT. It was speculated that the protein turnover within the periplasm would be reduced if DTT was present in the medium, however no histidine tagged proteins were visualized 6 hours post induction.
**Figure 21: H. pylori dsbHP::kan mutant is sensitive to oxygen.** The growth of *H. pylori* wild-type, *dsbHP::kan* and *hcpE::kan* mutants was assessed in both micro-aerobic and atmospheric conditions.

**Panel A:** *H. pylori* wild-type, *dsbHP::kan* and *hcpE::kan* mutants were grown in micro-aerobic conditions and growth was assessed via OD$_{600\text{nm}}$. The data averaged from 9 independent cultures per strain show that all strains grew to similar cell densities under micro-aerobic conditions.

**Panel B:** The cultures obtained after 24h incubation in panel A were exposed to environmental atmosphere for 30 min followed by spot plating and further incubation in microaerobic conditions. The data are a representative of 6 independent experiments, each comprising 3 serial dilutions of each culture spot-plated in triplicate. The data show a 0.5-1 log reduction in viability for the *dsbHP* mutant compared to both wild-type and *hcpE* mutant, suggesting that it was sensitive to oxygen exposure.
A.

OD 600nm

![Graph showing OD 600nm for different strains and time points.]

- Wild-type
- dsbHP::kan
- hcpE::kan

12 hours
24 hours

B.

Cfu/mL

![Graph showing Cfu/mL for different strains and dilutions.]

- wild-type
- dsbHP::kan
- hcpE::kan

Dilution

0.1 0.5 1.0
observed (Figure 21B). We hypothesize that this phenotype is the result of Dsb_{HP} having a DsbA-like activity and being involved in the oxidative protein folding under thiol-dependent redox conditions within the periplasm of *H. pylori*. The absence of Dsb_{HP} may result in increased susceptibility to oxidative stress.

To further test whether Dsb_{HP} is involved in the protection against oxidative stress, we exposed wild-type *H. pylori*, *dsb_{HP}::kan* and *hcpE::kan* knockout mutants to various concentrations of hydrogen peroxide (H\(_2\)O\(_2\)). We hypothesized that if Dsb_{HP} is involved in the protection against oxidative stress by maintaining periplasmic homeostasis, then the absence of Dsb_{HP} while in the presence of H\(_2\)O\(_2\) would result in less oxidative tolerance leading to more cellular death. *H. pylori* wild-type and knockout mutants were exposed to various concentrations of H\(_2\)O\(_2\) for 30 minutes in micro-aerobic conditions. Cells were then diluted and spot plated onto agar and allowed to grow in micro-aerobic conditions for 5-7 days, or until colonies were observed. Results indicated that *H. pylori* is highly sensitive to oxidative stress when *dsb_{HP}* is disrupted due to the fact that growth of the *dsb_{HP}* knockout mutant was completely absent when exposed to 125mM H\(_2\)O\(_2\), as well as significantly reduced when exposed to 100mM H\(_2\)O\(_2\). Whereas the *H. pylori* wild-type was recovered readily and displayed no real defect in growth when exposed to H\(_2\)O\(_2\) (Figure 22). The *hcpE* knockout mutant was used as a control to determine if the phenotype we observed was solely due to the lack of Dsb_{HP}, or the reduction of HcpE due to *dsb_{HP}* disruption. The fact that we were able to recover our *hcpE* knockout mutant at similar levels compared to wild-type demonstrated that this increased susceptibility to H\(_2\)O\(_2\) was only due to the lack of Dsb_{HP}.
Figure 22: *H. pylori* is more sensitive to $\text{H}_2\text{O}_2$ in the absence of DsbHP. *H. pylori* wild-type, $\text{dsb}_{\text{HP}}$ knockout mutant and $\text{hcpE}$ knockout mutant were exposed to 75, 100, and 125mM $\text{H}_2\text{O}_2$. The $\text{dsb}_{\text{HP}}$ knockout mutant displayed enhanced sensitivity compared to the wild-type and $\text{hcpE}$ mutant based on number of colonies observed. This demonstrates that Dsb$_{\text{HP}}$ is important in the resistance to oxidative stress and in maintaining oxidative homeostasis within the periplasm.
3.2.6 Complementation of \(Dsb_{HP}\) restores \(H_2O_2\) resistance

To determine whether the phenotypes we observed when \(H. pylori\) devoid of \(Dsb_{HP}\) during \(H_2O_2\) exposure were specific to the \(Dsb_{HP}\) protein and not polarization or downstream effects due to \(dsb_{HP}\) disruption, we repeated the same experiment with our \(dsb_{HP}\) complemented strain. The \(dsb_{HP}\) complement was constructed by Brandon Oickle with the same strategy as the \(hcpE\) complement (refer to section 3.1.2).

As expected, the \(dsb_{HP}\) complement was able to resist the effects of \(H_2O_2\) better than the \(dsb_{HP}:kan\) knockout mutant, and at similar levels compared to WT (Figure 23). However, it is important to note that the recovery of \(dsb_{HP}\) complement was always less than that of the wild-type, demonstrating a partial restoration of the wild-type phenotype. This further supports that \(Dsb_{HP}\) has catalytic activity similar to that of \(DsbA\), and that the phenotypes observed in the \(dsb_{HP}:kan\) knockout mutant were due specifically to the absence of \(Dsb_{HP}\) production within the cell.

3.2.7 \(H. pylori\) devoid of \(Dsb_{HP}\) is more sensitive to DTT exposure

We have previously established an enhanced reduction in viability when \(E. coli\) expressing \(Dsb_{HP}\) was exposed to various concentrations of DTT. To determine if this phenotype would persist in \(H. pylori\). It was hypothesized that the \(dsb_{HP}:kan\) knockout mutant would be able to survive better in the presence of DTT.

\(H. pylori\) wild-type, \(dsb_{HP}:kan\), and the \(dsb_{HP}\) complement were grown on agar plates for 48 hours until growth was confluent. Cells were then diluted and plated onto agar containing 0, 8, 10, or 12mM DTT and allowed to grow in micro-aerobic conditions until
**Figure 23: H2O2 resistance is restored upon DsbHP complementation.** *H. pylori* wild-type, *dsb*<sub>HP</sub> knockout mutant and *dsb*<sub>HP</sub> complement were exposed to 75, 100, and 125mM H₂O₂. The *dsb*<sub>HP</sub> knockout mutant displayed enhanced sensitivity compared to the wild-type based on number of colonies observed. Restoration of *dsb*<sub>HP</sub> restored resistance as the number of colonies observed was similar to wild-type. This demonstrates the importance of Dsb<sub>HP</sub> in maintaining oxidative homeostasis within the periplasm.
No H$_2$O$_2$

100mM

125mM

Dilution
Wild-type

$dsb_{HP}$ mutant

$dsb_{HP}$ Comp

Dilution
Wild-type

$dsb_{HP}$ mutant

$dsb_{HP}$ Comp
colonies were visible. None of the strains were sensitive to the DTT up to 8mM concentration, however, all strains displayed sensitivity to DTT at 12mM concentration (Figure 24). Surprisingly, the \( \text{dsb}_{HP} \) knockout mutant displayed enhanced sensitivity to DTT compared to the WT. Similarly to when \( H. \text{pylori} \) was exposed to \( \text{H}_{2}\text{O}_{2} \), this severe loss in viability was specific to \( \text{Dsb}_{HP} \) as the \( \text{hcpE} \) knockout mutant did not experience such a drastic loss in viable cells. This interaction demonstrates that \( \text{Dsb}_{HP} \) is important for the resistance to reductive stress, and that the toxicity witnessed in \( E. \text{coli} \) was most likely due to the additional stress and burden of protein expression than the actual DTT itself.

### 3.2.8 Complementation of \( \text{Dsb}_{HP} \) reduces cellular death by reductive stress

As with the \( \text{H}_{2}\text{O}_{2} \) experiments, we wanted to determine if restoration of \( \text{Dsb}_{HP} \) would restore resistance to reductive stress comparable to WT. The same experiment was repeated as described above other than the fact that the \( \text{dsb}_{HP} \) complement was used instead of the \( \text{hcpE} \) knockout mutant. As expected, the \( \text{dsb}_{HP} \) complement resisted the effects of DTT better than the \( \text{dsb}_{HP}::\text{kan} \) knockout mutant, and at similar levels compared to wild-type (Figure 25). This result further supports the conclusion that the phenotypes observed in the \( \text{dsb}_{HP}::\text{kan} \) knockout mutant were due specifically to the absence of \( \text{Dsb}_{HP} \) production within the cell.

### 3.3 Intrinsic activity of \( \text{Dsb}_{HP} \)

#### 3.3.1 \( \text{HcpE} \) is secreted into the extracellular environment via outer membrane vesicles

Outer membrane vesicles (OMVs) are spherical structures composed of a bilayer membrane derived from the outer membrane of the cell. When they are extruded from the
**Figure 24: Dsb$_{HP}$ is important for survival of *H. pylori* when exposed to reducing stress.** To determine the importance of Dsb$_{HP}$ during reductive stress, *H. pylori* wild-type, $dsb_{HP}$ and $hcpE$ knockout mutants were grown to the same cell densities, then spot plated onto agar containing 0, 8, 10, or 12mM DTT to assess viability. All growth was performed in micro-aerobic conditions. The $dsb_{HP}$ mutant displayed less viability compared to the wild-type and $hcpE$ mutant, demonstrating that the increased susceptibility was Dsb$_{HP}$ specific.
**Figure 25: DsbHP complementation nullifies DTT sensitivity.** *H. pylori* wild-type, *dsb*$_{HP}$ knockout mutant and *dsb*$_{HP}$ complement were all grown to the same densities, then spot plated onto agar containing 0, 8, 10, or 12mM DTT to assess viability. All growth was performed in micro-aerobic conditions. Complementation of *dsb*$_{HP}$ back into the bacterial chromosome restored resistance to DTT comparable to wild-type levels.
cell they package some of the periplasmic contents within. OMVs have been shown to have important roles in virulence by trafficking toxins and other virulence factors to their targets. In *H. pylori*, virulence factors such as VacA have been shown to target host cells through OMVs (Ilver et al., 2004). Thus, due to the fact that HcpE has been observed in *H. pylori* culture supernatant, we also wanted to determine whether it is also packaged into OMVs.

To test this, supernatants from *H. pylori* cultures grown in liquid medium were ultra-centrifuged to collect membrane fragments and potential OMVs. Once recovered, membrane fragments were analyzed by anti-HcpE Western blot and indicated the presence of HcpE within the sample (Figure 26). The membrane fragments consisting of potential OMVs were also treated with proteinase K to be able to distinguish between proteins and the O-antigen, as well as compare the protein profiles to that of inner membranes (IM) and outer membranes (OM) to help ascertain whether the membrane fragments isolated were derived from either IM or OM (Figure 27). Analysis via silver staining revealed that the potential OMVs were composed of both LPS and O-antigen (Figure 27D).

Results demonstrated that HcpE was present in the isolated membrane fragments of wild-type and the *dsbHP::kan* mutant, however, the amount of HcpE in the *dsbHP* mutant was drastically reduced. We also demonstrated that the membrane fragments were most likely derived from the OM of *H. pylori*, as two distinct protein bands that are present...
Figure 26: HcpE is found within outer membrane vesicles. Outer membrane vesicles were analyzed to determine whether HcpE could be packaged and secreted outside the cell. SDS-PAGE analysis of the membrane pellets obtained by ultracentrifugation of wild-type, dsb$_{HP}$ and hcpE mutant culture supernatants. Detection of HcpE was done by Western blotting with anti-HcpE antibodies. HcpE is marked with an asterisk (*).
Wild-type  

dsbA::kan  
hcpE::kan  

Wild-type  

dsbA::kan  
hcpE::kan

*
Figure 27: Outer membrane vesicle fraction is derived from the outer membrane.

Panel A: SDS-PAGE analysis of inner and outer membrane fractions of wild-type, *hcpE* and *dsb*<sub>HP</sub> mutants stained with Coomassie brilliant blue to detect protein profile. Total membranes were obtained by ultracentrifugation of whole cell lysate, and the inner and outer membranes were separated using lauryl sarcosyl followed by ultracentrifugation to pellet the outer membranes.

Panel B: Silver stain analysis of the same samples as described in panel A.

Panel C: Silver stain analysis of the same samples as described in panel A treated with proteinase K (PK).

Panel D: SDS-PAGE analysis of the pellets obtained by ultracentrifugation of culture supernatants of wild-type, *hcpE::kan* and *dsb*<sub>HP::kan</sub> mutants that were either treated with proteinase K or left untreated.
A.

<table>
<thead>
<tr>
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</tr>
</thead>
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<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>dsb\textsuperscript{\textast}::kan</td>
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<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>hcp\textsuperscript{E}::kan</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

No PK

B.

No PK

C.

PK

D.

PK
at 26 and 32 kDa within the IM fraction are not present in either OM or membrane fragments. In addition, membrane samples obtained by ultracentrifugation of culture supernatants from wild-type, \textit{dsbHP} and \textit{hcpE} mutants were analyzed by electron microscopy after uranyl acetate staining. Spherical membrane-bound structures of 75 to 150 nm in width that are characteristic of outer membrane vesicles were observed in all strains (Figure 28). This demonstrates that the isolated membrane fragments containing HcpE are in fact OMV particles, and that the presence of HcpE in the OMVs relies to a large extent on DsbHP.

3.3.2 \textbf{DsbHP is required for secretion of HcpE}

The Creuzenet lab previously demonstrated that DsbHP is an interacting partner for HcpE, and that this interaction leads to the folding and solubilization of HcpE. Due to the fact that HcpE is a protein that contains 9 disulfide bonds that are important for its structure, and it is secreted into the outer matrix, we hypothesized that this interaction between DsbHP and HcpE would be required for proper secretion. To test this, anti-HcpE Western blot analysis was conducted using our \textit{dsbHP} knockout mutant. Various fractions were analyzed to determine if there were any differences in the abundance of HcpE within different locations within the cell.

Cellular fractionation was performed by subjecting \textit{H. pylori} to lysozyme treatment in 0.5 mM MgCl$_2$ and 30\% sucrose to form spheroplasts. The spheroplasts were pelleted by centrifugation at 4,000 g for 10 minutes and the supernatant containing the periplasmic proteins was retained for analysis. The spheroplasts were lysed by re-suspension in water and vortexing using glass beads. The cytoplasmic protein fraction was obtained by
Figure 28: Electron micrographs of outer membrane vesicles. Samples were negatively stained with uranyl acetate.


Panel B: Wild-type. Bar = 10 nm.

Panel C: $dsb_{HP}::kan$. Bar = 50 nm.

Panel D: $hcpE::kan$. Bar = 50 nm.
centrifugation at 12,000 g for 30 minutes. The outer membrane proteins were recovered by ultracentrifugation of the culture supernatant. All fractions were analyzed by SDS-PAGE and anti-HcpE Western blotting.

HcpE was in very low abundance in the total cellular fraction of our dsbHP knockout mutant compared to H. pylori wild-type (Figure 29A). HcpE was observed within the periplasmic fraction of the dsbHP knockout mutant, but again in lower amounts compared to wild-type. The fractions containing outer membrane vesicles were also analyzed to determine if the absence of DsbHP would result in less secretion of HcpE through OMVs. As expected, much less HcpE was present in the OMVs obtained from the dsbHP knockout mutant than compared to the wild-type. Due to cellular lysis during spheroplast formation, a pure periplasmic fraction could never be obtained. Nonetheless, the presence of HcpE within the periplasmic fraction and absence in the cytoplasmic fraction of the dsbHP knockout mutant indicates that the periplasmic fraction was enriched, and was contaminated with very few cytoplasmic proteins. This is also further supported by the fact that very little urease activity was observed in the periplasmic fraction (Figure 29B); urease is a cytoplasmic protein responsible for the hydrolysis of urea into ammonium and carbon dioxide. The presence of urea within the periplasmic fraction would indicate cellular lysis had occurred during spheroplasting.

This same phenotype was observed in the dsbHP knockout mutant of the H. pylori Sydney strain (SS1). This strain is used for in vivo animal studies as it has been adapted over time to colonize mice. The dsbHP knockout mutant produced and secreted very little HcpE compared to the wild-type (Figure 30). Taken together, these data indicate that DsbHP is involved in the production and secretion of HcpE. HcpE appears to be targeted to the
Figure 29: DsbHP is involved in the production and secretion of HcpE in H. pylori.

Wild-type and dsbHP knockout mutant whole cells were subjected to lysozyme digestion in a sucrose gradient to form spheroplasts, allowing for the extraction of periplasmic proteins. Spheroplasting was monitored every 20 minutes by bright field microscopy to prevent total cell lysis.

Panel A: Ponceau S red staining and Western blotting for the presence of HcpE using anti-HcpE antibodies. Abbreviations: Cyto: cytoplasmic proteins. Peri: periplasmic proteins. The disruption of dsbHP leads to reduction in the total production of HcpE. Most of the HcpE produced appears within the periplasmic fraction, with very little observed within the OMVs. PR: Ponceau Red, WB: anti-HcpE Western blot.

Panel B: Urease activity of the various fractions was tested to assess cellular lysis. A small amount of urease activity was observed in the periplasmic fraction, indicating potential contamination by cytoplasmic proteins during spheroplasting. However, due to the fact that HcpE was only found in the periplasmic fraction of the mutant and not in the cytoplasm, indicates that the periplasmic fraction had been enriched and that the protein translocates to the periplasm as expected.
A. | Total | Cyto. | Peri. | OMV |
<table>
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<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>Wild-type</td>
<td>dsb_HP::kan</td>
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PR

WB

B. **Wild-type**

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</tr>
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</tr>
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</tr>
<tr>
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<td>0.3</td>
</tr>
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</tbody>
</table>

**dsb_HP::kan**

<table>
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<th>OMVs</th>
<th>Periplasm</th>
<th>Control</th>
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Figure 30: Secretion of HcpE is also impaired in the absence of DsbHP in strain SS1. SDS-PAGE analysis and anti-HcpE Western blotting of total cells (top panels) or culture supernatants (bottom panels) of wild-type or dsbHP or hcpE knockout mutants shows decreased production and secretion of HcpE in the dsbHP mutant. HcpE-His is purified recombinant protein that served as a positive control for Western blotting. PR: Ponceau red. WB: Anti-HcpE Western blot.
periplasmic space, however, in the absence of DsbHP remains trapped there and is most likely targeted for degradation. To ensure that the absence of HcpE in the OMVs of the dsbHP knockout mutant was specific to DsbHP itself and not due to potential pleotropic effects which could affect OMV secretion, analysis of LPS profiles of the OMVs was conducted using silver nitrate (Figure 27D). The dsbHP knockout mutant displayed both normal LPS and protein profiles compared to wild-type demonstrating that OMV packaging and membrane composition had not changed.

3.3.3 HcpE is produced and secreted from H. pylori clinical isolates

We have demonstrated that HcpE is secreted into the supernatant as well as secreted through OMVs from H. pylori cultured in liquid media. The fact that antibodies to HcpE and other Hcp family members are present in the serum of patients infected with H. pylori suggests that Hcp family members are produced within the host. Thus, we wanted to determine if strains isolated from infected patients produced and secreted HcpE, as well as determine if there is any differences in HcpE expression among different clinical strains isolated from patients with various onset of H. pylori associated disease. To test this, anti-HcpE Western blot analysis was conducted on 13 different clinical isolates. Total cell lysate, culture supernatant as well as OMVs were analyzed to determine if there were any differences in the abundance of HcpE among various strains.

Clinical isolates were grown on Columbia agar in micro-aerobic conditions until growth was confluent. Strains were then sub-cultured into Brucella broth supplemented with β-cyclodextrin and antibiotics and grown for 16 hours in micro-aerobic conditions. Cells were harvested and supernatants were collected. Total cell lysate and culture supernatants were analyzed via SDS-PAGE and anti-HcpE Western blot to determine the
presence of HcpE. All clinical isolates tested produced and secreted HcpE (Figure 31). HcpE was observed in all fractions tested including cell lysate and supernatants. However, the clinical isolates obtained from patients diagnosed with ulcer disease seemed to produce more HcpE compared to the other strains analyzed. This result is further supported by the fact that the Creuzenet lab has demonstrated that patients with high antibody titers to HcpE had a greater chance of developing duodenal ulcer disease than those who displayed low HcpE titers (Unpublished).

3.4 Influence of HcpE on the production of inflammatory mediators

3.4.1 H. pylori impaired in HcpE production is deficient in colonization

*H. pylori* has been studied extensively, however, its ability to persist and interact with the host is still not fully understood. The discovery of SLR motifs within HcpE suggests they are interacting with host cells and/or proteins (39). However, the process or cells with which they interact has not been studied. Thus, to determine the effect of HcpE on the host, in terms of both colonization and recruitment of inflammatory mediators, *H. pylori* wild-type as well as *hcpE* and *dsbHP* knockout mutants need to be studied *in vivo*. *H. pylori* Sydney strain (SS1), is a mouse adapted strain which was repeatedly orally passaged through C57BL/6 mice until it was capable of efficiently colonizing mice for long periods of infection (≤ 8 months) (Lee *et al.*, 1997). Colonization results in inflammation of the gastric mucosa within 4-5 weeks, increased neutrophil recruitment, and expression of Major Histocompatibility Complex II (MHC II) class antigens. However, peptic ulceration and gastric cancers have not been observed (Fan *et al.*, 1998). As in humans, the gastric inflammation in conjunction with immune activation is ineffective at clearing
Figure 31: HcpE produced and secreted by *H. pylori* isolated from infected patients.

The presence and abundance of HcpE was analyzed in clinical isolates obtained from patients diagnosed with various gastric disorders.

**Panel A:** Ponceau S red staining and Western blotting for the presence of HcpE using anti-HcpE antibodies in total cell lysate of 16 clinical isolates. HcpE is present in the cell lysate of all clinical strains tested, with the strains obtained from ulcer patients displaying the highest abundance of HcpE.

**Panel B:** Ponceau S red staining and Western blotting for the presence of HcpE using anti-HcpE antibodies in culture supernatants of 12 clinical isolates. HcpE is present in the supernatant of all clinical strains tested, with the strains obtained from ulcer patients displaying the highest abundance of HcpE.

WT: wild-type, KO: *hcpE::kan* knockout mutant, GU: Gastric ulcer, DU: Duodenal ulcer, G: Gastritis, A: Atrophy, PR: Ponceau Red, WB: anti-HcpE Western blot.
A.

<table>
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<th>KO</th>
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<th>2</th>
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<th>6</th>
<th>7</th>
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<th>11</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GU</td>
<td>GU</td>
<td>GU</td>
<td>DU</td>
<td>DU</td>
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<td>A</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>G</td>
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Patient Disease

B.

<table>
<thead>
<tr>
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<th>KO</th>
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<th>2</th>
<th>3</th>
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Patient Disease
*H. pylori*. Thus, this mouse model has been successfully developed to study infections *in vivo* which show similar characteristics to infection in humans.

Hcps are highly conserved within *H. pylori* strains, thus must be beneficial to their perseverance. Other Hcp proteins such as HcpA and HcpB have been implicated in the production of pro-inflammatory cytokines, suggesting they may play a role in *H. pylori* induced gastritis. To determine whether HcpE impacts colonization or inflammation, C57BL/6 mice were intra-gastrically inoculated with strain SS1 wild-type and knockout mutants as described by Ferrero *et al.* Briefly, mice were orally gavaged with $10^9$ bacteria 3 times, once every other day for the course of a week. Infection was allowed to proceed for 4 and 6 weeks post gavage before mice were sacrificed. To assess infection, plating of stomach homogenate for cfu counting was conducted. After being harvested and divided into 2 halves, the murine stomach tissue was placed into PBS buffer with antibiotics and homogenized with an electric homogenizer. After homogenization, the gastric homogenate was diluted 10 and 100 fold, and each dilution was plated onto Columbia blood agar. Plates were incubated in micro-aerobic conditions for 5-7 days. Colonization was also assessed via histological sections of stomach tissue that were prepared, and observed using light microscopy. The *dsbHP* and *hcpE* knockout mutants in SS1 strain had not been previously established, thus knockout mutants were constructed using the same constructs and technique described in section 2.10.

The presence of *H. pylori* within the gastric mucosa of mice gavaged with either wild-type, *dsbHP::kan*, or *hcpE::kan* knockout mutants was not detected via plating of the gastric homogenate. No colonies from mice gavaged with either strain were recovered. However, Warthin-Starry staining, which is a silver nitrate based stain originally used to
detect spirochetes, established the presence of \textit{H. pylori} within the gastric mucosa of infected mice (Figure 32). Both $dsb_{HP}$ and $hcpE$ knockout mutants had impaired colonization compared to wild-type based on total cells visualized and counted within the processed tissue, with the $dsb_{HP}$ knockout mutant being the most attenuated (Figure 33). This trend was observed for mice sacrificed at both 4 and 6 weeks post gavage, indicating that \textit{H. pylori} is still capable of colonization over long periods when devoid of either Dsb$_{HP}$ or HcpE, but colonization is significantly impaired compared to wild-type. We currently do not fully understand why this defect in colonization is occurring, but we hypothesize that motility may be a factor due to the fact that both knockout mutants have impaired motility compared to wild-type (Figure 34). The $dsb_{HP}$ knockout mutant is less capable of colonizing than the $hcpE$ mutant, however retains more motility. Thus there is another factor(s) that is contributing to the decreased colonization observed in the $dsb_{HP}$ knockout mutant.

3.4.2 Effects of HcpE on interaction of \textit{H. pylori} with splenocytes

3.4.2.1 Pro-inflammatory cytokine IFN-$\gamma$ is secreted from splenocytes exposed to \textit{H. pylori} cells

To determine the effects of HcpE on the inflammatory response, splenocytes were isolated from mice colonized with \textit{H. pylori} or not, and subjected to incubation with bacteria. Splenocytes were inoculated with wild-type, $dsb_{HP}$ and $hcpE$ knockout mutants at a multiplicity of infection of either 1, 0.5, or 0.1. Low MOIs were chosen due to the fact
Figure 32: *H. pylori* wild-type, *dsb*<sub>HP</sub> and *hcp*<sub>E</sub> knockout mutants are able to infect mice. Comparison of the ability of *H. pylori* wild-type, *dsb*<sub>HP</sub> and *hcp*<sub>E</sub> knockout mutants to colonize mice. Sections of murine gastric tissue analyzed by bright field microscopy for the presence of *H. pylori*. *H. pylori* was observed in all mice infected with either wild-type, *dsb*<sub>HP</sub> or *hcp*<sub>E</sub> knockout mutants. Sections were stained with Warthin-Starry stain to visualize *H. pylori*. *H. pylori* cells are highlighted by the red arrow.
**Figure 33:** *H. pylori* wild-type colonizes better than both *dsbHP* and *hcpE* knockout mutants. To compare the colonization ability of our wild-type as well as *dsbHP* and *hcpE* knockout mutants, total cell counts observed within gastric tissue samples of mice that were sacrificed 4 and 6 weeks post gavage were averaged between mice, and multiplied by the length of an average murine stomach to produce a calculated estimate of total cells within the gastric mucosa. A total of 2 sections per mouse from a total of 6 mice for each time point post gavage were used in the calculations. *P<0.05, **P<0.01, ***P<0.001, N.S.:* Not significant.
CFU /g tissue

4 weeks

6 Weeks

Wild-type

dsb mutants:kan

hcpE mutants:kan

Wild-type

dsb mutants:kan

hcpE mutants:kan

N.S.

**

***

10.00E+05

8.00E+04

6.00E+04

4.00E+04

2.00E+04

1.00E+05

0.00E+00

4 weeks

6 Weeks
Figure 34: *H. pylori* devoid of HcpE is less motile than wild-type. Comparison of the ability of *H. pylori* strain SS1 wild-type, *dsb*$_{HP}$ and *hcpE* knockout mutants.

Panel A: *H. pylori* strain SS1 wild-type, *dsb*$_{HP}$ and *hcpE* knockout mutants were inoculated into agar plates containing 0.1% Bacto Agar to test how motile they were by measuring their ability to move through the agar. Differences in motility were measured by the diameter of the outward growth from the initial site of inoculation. Both *dsb*$_{HP}$ and *hcpE* knockout mutants displayed impaired growth compared to wild-type.

Panel B: Quantification of differences in motility. Results are representative of 3 independent experiments. *P*<0.05, N.D.: Not detected.
A.  
Wild-type  \hspace{1cm} \textit{dsb}_{HP}:\text{kan} \hspace{1cm} \textit{hcpE}:\text{kan} \\

B.  
\begin{center}
\begin{tabular}{c|c|c|c}
 & Wild-type & \textit{dsb}_{HP}:\text{kan} & \textit{hcpE}:\text{kan} \\
\hline
Diameter (mm) & 15 & * & N.D. \\
\end{tabular}
\end{center}
that *H. pylori* has been shown to induce apoptosis of splenocytes at high MOIs (Bussiere et al., 2006). After 2 hours of incubation, splenocytes were stimulated with *E. coli* LPS and supernatants were collected after 12 and 24 hours. Stimulation with *E. coli* LPS was needed as *H. pylori* LPS has low immunogenic activities, making it difficult to measure differences in inflammatory responses (Muotiala et al., 1992; Nielson et al., 1994). Splenocytes were stimulated with 5 different concentrations of LPS (Q1-Q5) that were diluted 3 fold for each subsequent concentration. This was done to obtain a range of LPS concentrations to ensure we could obtain the best dose to response ratio possible. Based on experiments comparing production of IFN-γ after exposure to *H. pylori* cells or *E. coli* LPS, activation of splenocytes was lower with *H. pylori* cells than when *E. coli* LPS was added (data not shown). When the splenocytes are exposed to *H. pylori* before being exposed to LPS, there is a dose dependent reponse to *H. pylori* cells in relation to LPS, in the splenocytes isolated from naïve mice, but lower MOIs resulted in less LPS response compared to high MOIs (Figure 35A). This dose dependence was not observed in the splenocytes obtained from mice previously exposed to *H. pylori* dsbHP::kan mutant (Figure 35B). However, cells from *H. pylori* wild-type, dsbHP or hcpE knockout mutants did not show any significant difference in their abilities to modulate the capacity of *E. coli* LPS to initiate an IFN-γ response when exposed to splenocytes from naïve mice (Figure 35A) or mice previously exposed to *H. pylori* (Figure 35B). The same was observed using splenocytes pre exposed to *H. pylori* wild-type, dsbHP::kan, and hcpE::kan mutants (data not shown). This suggests that the lack of HcpE has no effect on the ability of *H. pylori* cells to induce an IFN-γ response. However, it should be noted that *H. pylori* encodes many virulence factors responsible for initiating inflammatory responses such as CagA and VacA. Thus,
**Figure 35:** *H. pylori* knockout mutant cells activate splenocytes similar to wild-type.

Exposure of splenocytes for 12 or 24 hours with wild-type, *dsb*<sub>HP</sub>::*kan* and *hcp*<sub>EP</sub>::*kan* *H. pylori* modulates the ability of splenocytes to respond to LPS exposure via release of pro-inflammatory cytokine IFN-γ.

**Panel A:** Infection of naïve splenocytes for 12 or 24 hours with wild-type, *dsb*<sub>HP</sub>::*kan* and *hcp*<sub>EP</sub>::*kan* *H. pylori*. There was no difference between the wild-type or knockout mutants.  
*Calculations done by Dr. Creuzenet.*

**Panel B:** Infection of splenocytes from mice previously exposed to *dsb*<sub>HP</sub>::*kan* knockout mutant for 12 or 24 hours with wild-type, *dsb*<sub>HP</sub>::*kan* and *hcp*<sub>EP</sub>::*kan* *H. pylori*. *ELISA by N. Bronnec and calculations done by Dr. Creuzenet.*
A. Dilution of LPS with different concentrations of *H. pylori* cells

B. Dilution of LPS with different concentrations of *H. pylori* cells
expression of these virulence factors may over-shadow the effects of HcpE resulting in the inability to see differences. To address this, we decided to investigate the effects of pure HcpE as well as culture supernatants of wild-type and both mutants on IFN-γ production.

**3.4.2.2 HcpE induces production of IFN-γ when secreted outside the cell**

The experiment described above was repeated using culture supernatants obtained from *H. pylori* grown in liquid medium as well as pure HcpE protein. Culture supernatants were obtained by growing *H. pylori* wild-type, *dsb*<sub>HP</sub> and *hcpE* knockout mutants in liquid medium as described in section 3.3.2. Splenocytes were inoculated with culture supernatants at 1, 3, and 5X concentrations.

Exposure of splenocytes with culture supernatants was carried out in the same manner as described above. Splenocyte supernatants that were collected 12 and 24 hours post infection were analyzed for the production of IFN-γ by ELISA. The IFN-γ response was lower in the splenocytes exposed to the *dsb*<sub>HP</sub> and *hcpE* knockout mutant culture supernatants compared to wild-type. This trend was true for splenocytes isolated from both naïve mice (Figure 36A) and mice previously exposed to the *dsb*<sub>HP</sub>::*kan* knockout mutant (Figure 36B). Both knockout mutants displayed a decreased response in IFN-γ in all concentrations of both culture supernatants as well as LPS tested, with the *hcpE* knockout mutant showing the most prominent decrease in IFN-γ production in the splenocytes previously exposed to *H. pylori* (Figure 36B), however the *dsb*<sub>HP</sub> knockout mutant displayed the most prominent decrease in the naïve splenocytes (Figure 36A). This suggests that HcpE is able to modulate the response of splenocytes to LPS as the reduction or absence of HcpE within the culture supernatant resulted in less IFN-γ production.
**Figure 36:** *H. pylori* knockout mutant supernatants reduce the response of splenocytes to LPS compared to wild-type. Exposure of splenocytes for 12 or 24 hrs to LPS in the presence of *H. pylori* wild-type, dsb$_{HP}$::kan and hcpE::kan *H. pylori* culture supernatants lead to the release of pro-inflammatory cytokine IFN-γ. This effect was lower in both mutants compared to the wild-type, suggesting that HcpE is involved with modulation of the response of splenocytes to LPS.

**Panel A:** Exposure of naïve splenocytes for 12 or 24 hrs with wild-type, dsb$_{HP}$::kan and hcpE::kan *H. pylori* culture supernatants. *Calculations done by Dr. Creuzenet.*

**Panel B:** Exposure of splenocytes (taken from mice previously exposed to dsb$_{HP}$::kan mutant) for 12 or 24 hrs with wild-type, dsb$_{HP}$::kan and hcpE::kan *H. pylori* culture supernatants. *ELISA by N. Bronnec and calculations done by Dr. Creuzenet.*
### A.

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Dilution of LPS with different concentrations of supernatants

### B.

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Dilution of LPS with different concentrations of supernatants
For exposure to pure HcpE protein, HcpE was expressed in *E. coli* BL21(DE3)pLys via induction with 0.15mM IPTG and purified using nickel affinity chromatography. The protein DdahB was provided by H. Barnawi and used as a control. Both proteins were added to splenocytes at concentrations of 75, 125, and 200 ng, and production of IFN-γ was measured directly without further addition of LPS. *E. coli* LPS is able to easily activate the innate and adaptive immune system. Thus, to prevent accidental contamination from *E. coli* LPS during lysis and protein purification, the purified HcpE as well as the control protein were run through a polymyxin B column to remove any LPS. The purified proteins were then split into 2 equal fractions and one fraction was treated with Proteinase K for 2 hours to degrade the proteins into peptides. This was done as an additional control to demonstrate that the HcpE protein and not an additional contaminant within the sample would be the cause for activation of splenocytes. To ensure full proteolysis and removal of LPS was successful, protein fractions of both HcpE and control protein DdahB were analyzed via separation by SDS-PAGE and stained with silver nitrate (Figure 37). Proteins treated with Proteinase K were digested into smaller peptides and the absence of Lipid A and O-antigen within the samples indicated that no LPS contamination was present.

Splenocytes that were exposed to purified HcpE produced significantly more IFN-γ than when exposed to the control protein DdahB (Figure 38). Little to no IFN-γ production was observed in the control protein as well as in the samples that were degraded with proteinase K in both the naïve splenocytes (Figure 38A) as well as the splenocytes from mice previously exposed to *dsbH::kan* knockout mutant (Figure 38B). This suggests that the purified HcpE protein alone was responsible for the direct induction of a
Figure 37: HcpE purified via nickel affinity chromatography is not contaminated with trace amount of LPS. To test whether our purified HcpE as well as control protein DdahB were contaminated with *E. coli* LPS after treatment with the polymyxin B column, various dilutions of each sample were analyzed by SDS-PAGE and stained with either Coomassie brilliant blue or silver nitrate. No LPS was observed in any samples indicating any potential LPS contamination had been removed by the polymyxin B column. Both HcpE and DdahB were not fully digested but all proteins within the sample had been partially digested into smaller peptides. HcpE and DdahB were observed at 40 kDa and 38 kDa respectively.
<table>
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<tr>
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**Silver nitrate**

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**Figure 38: Pure HcpE activates splenocytes.** Exposure of splenocytes for 12 or 24 hrs with pure HcpE or DdahB at 25, 75, 125, and 200 ng that was treated with either proteinase K (PK) or non-treated lead to the release of pro-inflammatory cytokine IFN-γ. IFN-γ release was observed for pure HcpE that was not treated with proteinase K, whereas little to no IFN-γ response was observed for the control protein or HcpE that was treated with proteinase K. This effect suggests that only native HcpE is involved with activation of splenocytes, as degraded HcpE as well as the control protein were unable to initiate an IFN-γ response.

**Panel A:** Infection of naïve splenocytes for 12 or 24 hrs with pure HcpE or DdahB at 75, 125, and 200 ng that was treated with either proteinase K (PK) or non-treated lead to the release of pro-inflammatory cytokine IFN-γ. *Calculations done by Dr. Creuzenet.*

**Panel B:** Infection of splenocytes (previously exposed to dsbHP::kan mutant) for 12 or 24 hrs with pure HcpE or DdahB at 25, 75, 125, and 200 ng that was treated with either proteinase K (PK) or non-treated lead to the release of pro-inflammatory cytokine IFN-γ. *ELISA by N. Bronnec and calculations done by Dr. Creuzenet.*
A. Concentration of IFN-γ (pg/mL) vs. Amount of protein and incubation time

B. Concentration of IFN-γ (pg/mL) vs. Amount of protein and incubation time
pro-inflammatory IFN-γ response.

These results suggest that HcpE is involved in the direct induction of IFN-γ production, is capable in inducing a pro-inflammatory response and further modulate the splenocytes response to other virulence factors such as LPS as the reduction or absence of HcpE results in less IFN-γ is released by splenocytes.

**DISCUSSION**

**4.1 The activity of DsbHP and its role in disulfide bond formation**

Dsb proteins are oxidoreductases that are involved in the formation of disulfide bonds in the process of protein folding and maturation. Dsbs have been extensively studied in *E. coli*, and two separate pathways of S-S formation have been described. This established paradigm does not hold true for *H. pylori* as only 3 Dsb family members (DsbB/I, DsbC, and DsbD) have been thoroughly investigated (Tomb *et al*, 1997; Godlewska *et al*, 2006). Recently, two separate studies with conflicting results attempted to elucidate the function of DsbHP in regards to its ability to form disulfide bonds. Based on structural modelling and the ability of DsbHP to act as a reductase on HP0518, a putative L,D-transpeptidase, Yoon *et al* proposed the function of DsbHP to be similar to that of an *E. coli* DsbG. Roszczenko *et al* proposed the activity of DsbHP to be a DsbA-like oxidase based on the ability of DsbHP to refold insulin. Our work on DsbHP has provided additional evidence that DsbHP has Dsb-like activity by its ability to refold the surrogate substrate lysozyme and restore its activity. Its behavior is similar to that of the *E. coli* DsbA oxidase when expressed in *E. coli*. Although these results were unexpected based on our original hypothesis that DsbHP would behave like an isomerase due to structural similarities to *E.
coli DsbG, we provide further support for Dsb$_{\text{HP}}$ having oxidase catalytic activity similar to that of DsbA.

It was hypothesized that if Dsb$_{\text{HP}}$ had activity similar to that of DsbA, that an abundance of Dsb$_{\text{HP}}$ within the periplasm would have a protective effect when exposed to the reducing agent dithiothreitol. Previous studies have demonstrated that DsbA is important during DTT exposure as either E. coli DsbA or DsbC knockout mutants are sensitive to DTT (Kamitani et al., 1992; Roszczenko et al., 2012). This phenotype is most likely associated with a defect in the proper folding of proteins containing disulfide bonds that are translocated into the periplasm. We unexpectedly observed the opposite, where E. coli expressing Dsb$_{\text{HP}}$ or DsbA showed increased toxicity when exposed to dithiothreitol. We cannot currently explain the enhanced toxicity of DsbA expression during exposure to dithiothreitol. However, the purpose of this study was to demonstrate that Dsb$_{\text{HP}}$ has Dsb catalytic activity, and in particular to distinguish between a DsbA or DsbG-like activity.

In addition to the experiments in E. coli demonstrating Dsb$_{\text{HP}}$ activity, we examined Dsb$_{\text{HP}}$ activity in H. pylori as well. To accomplish this we constructed a dsb$_{\text{HP}}$ knockout mutant. The fact that we were able to isolate clones containing this disrupted gene further supports that Dsb$_{\text{HP}}$ does not contain DsbG-like activity. A dsbG mutant in E. coli is lethal and mutants are unable to be recovered unless the medium is supplemented with cysteine or another oxidant (Anderson et al., 1997). When the dsb$_{\text{HP}}$ mutant was exposed to an oxidant in the form of H$_2$O$_2$, an increased sensitivity was observed, clearly demonstrating a difference in phenotypes compared to a dsbG mutant in E. coli. Similar to what was observed when expressed in E. coli, the exposure of the dsb$_{\text{HP}}$ mutant to a reducing stress such as dithiothreitol resulted in sensitivity and lethality. These data, in addition to that
obtained from the oxidative stress experiments, suggest that DsbHP may have both DsbA and DsbG activities. Thus, this would explain why a DsbA homologue (based on structure) has not yet been identified in H. pylori because DsbHP comprises both DsbA and DsbG roles within the periplasm.

DsbHP also appears to have features that E. coli DsbA and DsbG do not possess. When HcpE is expressed in E. coli in the absence of DsbHP, insoluble proteins are produced. Only when HcpE was co-expressed on the same vector as DsbHP were we able to obtain increased amounts of soluble HcpE. This demonstrates that the endogenous Dsb proteins were unable to successfully fold and process HcpE even though they contain similar activity and homology to DsbHP.

4.2 HcpE secretion requires DsbHP

Within the past decade, a family of proteins containing a high percentage of cysteine residues has been discovered, named Helicobacter cysteine rich proteins (Hcps). HcpE is the largest and most complex member of the Hcp family. HcpE contains the greatest number of cysteines (Mittl et al., 2003), and acquisition of an organized array of disulfide bonds in the periplasm is anticipated to require proteins of the Disulfide bond protein (Dsb) family.

Previous work from the Creuzenet lab demonstrated that the insoluble unprocessed form of HcpE was a substrate for DsbHP. This finding itself was important due to the fact that HcpE is the first natural substrate for DsbHP. This was reinforced by the fact that the dsbHP knockout mutant was deficient in both HcpE production and secretion, suggesting
that HcpE is dependent on Dsb_{HP} for proper folding and its secretion into the extracellular matrix.

Due to the fact that Hcp proteins share homologous sequences (Mittl et al, 2003), Dsb_{HP} could also serve as an important folding factor for the other family members as well. This may not be limited to just Hcp family members, as to date Dsb_{HP} has been the only Dsb protein of *H. pylori* to demonstrate a DsbA-like activity. Thus, many other *H. pylori* proteins containing cysteine residues could be potential substrates for Dsb_{HP}.

While investigating the relationship between Dsb_{HP} and secretion of HcpE, we also found that some of HcpE is secreted outside of the cell through OMVs. OMVs have been well documented and have been shown to be important delivery mechanisms for many enzymes and virulence factors, as well as quorum sensing. OMV production within *H. pylori* has been studied extensively, and implicated in the delivery mechanism for virulence factors such as VacA, as well as activation of phagocytes and gastric epithelial cells (Mullaney et al, 2009; Parker and Keenan, 2012). Interestingly, previous studies that investigated the general proteome of *H. pylori* OMVs found that other Hcp family members, such as HcpC, D, E, and F were contained within the OMVs (Olofsson et al, 2010). This provides further evidence that HcpE, in addition to other Hcps, are actively secreted via OMVs and not just a result of the random packaging of periplasmic contents into the OMVs.

Analysis of clinical strains obtained from biopsies of patients infected with *H. pylori* via anti-HcpE Western blot demonstrated that HcpE is produced and secreted by these strains. Taken together, we have provided evidence that HcpE is expressed from *H. pylori*.
*H. pylori* isolated from infected patients suggesting HcpE is produced *in vivo*. We have also provided novel insight to the fact that HcpE may be differentially expressed during periods of infection and disease progression as clinical isolates obtained from patients diagnosed with ulcer disease seem to produce more HcpE than the other strains tested. However, this needs to be investigated further.

We demonstrated that the OMVs isolated from our *dsb*<sub>HP</sub> knockout mutant were almost devoid of HcpE compared to wild-type. Electron microscopy of both *dsb*<sub>HP</sub> and *hcpE* knockout mutants provided evidence that our mutants were still capable of producing OMVs and that the absence of HcpE was not due to defects in OMV secretion. However, we cannot conclude whether the decrease in HcpE in the OMVs of the *dsb*<sub>HP</sub> mutant was due to a defect in HcpE packaging due to the absence of Dsb<sub>HP</sub>, or just the result of an overall decrease in HcpE production inside the cell. We were also not able to determine if the disruption of *dsb*<sub>HP</sub> resulted in a substantial decrease in other Hcp proteins due to the lack of specific antibodies. Nevertheless, this study further demonstrates the importance of Dsb<sub>HP</sub> to HcpE production and secretion.

**4.3 Difficulties associated with cloning and expression within *H. pylori***

Cloning and the expression of tagged-proteins is difficult to achieve in *H. pylori*, as the bacterium is not hospitable to the uptake of foreign DNA (Ando *et al.*, 2000). This is due to the many DNA restriction and modification systems present in *H. pylori* which help prevent transformation by degrading any foreign DNA (Donahue *et al.*, 2000). The DNA of *H. pylori* is methylated, and when DNA is transformed that is either non-methylated or the methylation pattern is different from its own, the bacterial restriction modification systems become active. To address this challenge, we optimized transformation conditions
as well as DNA concentrations. When transforming suicide vectors into *H. pylori*, as in the case with our knockout mutants as well as our complements, the plasmid DNA was either treated with *H. pylori* cell-free extracts (CFE) in the presence of a methyl donor, or was transformed without methylation. Incubation with *H. pylori* CFE would allow the plasmid DNA to acquire the same methylation pattern as host genomic DNA, resulting in less restriction attack on the plasmid and increase the transformation efficiency in *H. pylori* (Donahue *et al.*, 2000). However, we also tested if transforming non-methylated plasmid DNA, but at a much higher concentration, would also increase the transformation efficiency in *H. pylori*. When using non-methylated plasmid DNA, we performed natural transformation with 15-20 µg/µl of plasmid. Within this range, clones were usually obtained, indicating that when that much foreign DNA is introduced to *H. pylori* the restriction machinery gets overwhelmed, leading to homologous recombination and successful transformation.

*E. coli* is generally used as a model system for the production and over-expression of recombinant *H. pylori* proteins. For this study, *E. coli* was used to over-express all recombinant proteins as the expression of tagged proteins within *H. pylori* is difficult due to the fact that their endogenous expression levels are too low to purify the quantity of proteins needed. Hence, the *H. pylori* proteins Dsb_{HP} and HcpE, as well as *E. coli* DsbA and DsbG were expressed using the pET expression system with a C-terminal hexahistidine tag. The pET expression system is a widely used system to mass produce proteins that allows for manipulation of the desired protein as well as control of when that expression will occur (Studier *et al.*, 1990). The system is highly specific as it uses the T7 promoter which only binds T7 RNA polymerase, and is controlled through the *lac* promoter and
operator. The T7 polymerase must be present before the recombinant gene can be transcribed, as well as regulated to ensure expression can be manipulated and controlled. T7 expression is under the control of an inducible promoter that is activated by IPTG. IPTG is a sugar that mimics the lactose metabolite allactose, and when present, displaces the repressor from the lac operator allowing T7 polymerase to be expressed. The recombinant protein is thus transcribed. This system usually works for most proteins, resulting in the production of substantial amounts of the recombinant protein. However, this was not the case for HcpE. The expression of HcpE within this system was less efficient, producing low amounts of the HcpE protein and always resulting in the production of insoluble protein. This made it very difficult to manipulate not only the expression of HcpE, but purification needed to be done in denaturing conditions. Thus, we designed a pET expression vector which contained both dsbHP and hcpE allowing for simultaneous expression of both proteins. Due to the fact that DsbHP is able to interact with and solubilize HcpE in vitro, we proposed that expressing both proteins simultaneously would result in a larger proportion of soluble HcpE. This proved to be correct as HcpE was observed in the soluble protein fraction when expressed in *E. coli* BL21(DE3)pLys at room temperature. Even though this vector still only produced nominal amounts of soluble HcpE, it allowed for easier purification as denaturing conditions no longer had to be used.

The difficulties associated with purifying HcpE was not an isolated incident, as difficulties expressing other Hcp family members have also been documented. Expression of both HcpC and HcpA in *E. coli* produces insoluble proteins, thus both proteins need to be re-folded before they can be used in experiments (Mittl *et al*, 2000; Luthy *et al*, 2004). Similarly, before the construction of the co-expression vector, HcpE had to be purified
from inclusion bodies in the presence of 6M guanidine-hydrochloride. Considering how structurally complex HcpE as well as the other Hcp family members are, it is not surprising that they are unable to be folded properly within the periplasm of *E. coli*. Without their proper folding partners/factors to facilitate the formation of the essential disulfide bonds between their various cysteine residues, it is not unreasonable that these proteins would become insoluble and aggregate into inclusion bodies. However, in the presence of their corresponding periplasmic folding factors, as observed within *E. coli* containing the co-expression vector, these proteins (HcpE) are able to achieve their proper conformation and remain soluble, facilitating their purification from the soluble protein fraction.

### 4.4 Implications of HcpE and DsbHP on *H. pylori* colonization

Various animal models have been developed to study the colonization as well as the pathogenesis of *H. pylori* within the gastric mucosa. Early studies using larger animals such as gnotobiotic piglets, non-human primates and cats were the only animal models that provided successful colonization as well as comparable pathology that is associated with human infection (reviewed in Masaaki *et al.*, 2005). These models proved to be cumbersome due to the difficulties in handling such large animals as well as housing them in the large numbers required for study. However, in 1991 Karita and colleagues were the first to demonstrate that colonization of *H. pylori* within mice was possible, as they were able to colonize immune-deficient BALB/c mice. This observation led Lee and colleagues to screen clinical isolates for their ability to colonize mice. They identified the Sydney strain (SS1), and after adaptation in mice were able to sustain colonization in mice over long periods of time. They also reported that different mouse strains such as C57BL/6 and BALB/c, could be infected to a high degree with the mouse adapted SS1 strain (Lee *et al,*
In our hands, infection and colonization of C57BL/6 mice with the SS1 wild-type strain resulted in a bacterial loads ranging from $10^4$-$10^5$ cells per gram of stomach tissue when calculations were made of numbers of bacteria observed within histological slides. Our colonization numbers are lower than that obtained in other studies, as it has been shown that mice infected with strain SS1 usually harbor $10^5$-$10^7$ CFU per gram of tissue when gavaged with as little as $10^3$ CFU (Lee et al., 1997; Ferrero et al., 1997). However, we were able to obtain colony counts up to six weeks within the low end of that range with our wild-type strain. Even though our calculated CFUs were relatively low, we were still able to see significant differences among the wild-type, dsbHP and hcpE knockout mutants, demonstrating that colonization is reduced when HcpE production is absent. The dsbHP knockout mutant was even less capable of maintaining colonization, which may contribute to its inability to express any of the Hcp family members. Because Hcp family members are homologous in structure (Mittl et al., 2003) and all require disulfide bonds between adjacent cysteine residues, DsbHP would probably be required for their maturation and secretion similarly to HcpE. We also have to consider that the reduction in colonization of both the dsbHP and hcpE knockout mutants may be the result of impaired motility. Motility is essential for the successful colonization of H. pylori (Ottemann and Miller, 1997; Ottemann and Lowenthal, 2002). H. pylori made non-motile by disruption of motB is unable to colonize mice as efficiently as the wild-type strain (Nguyen and Saier, 1996). Motility allows the bacterium to locate to its preferred region within the gastric mucosa, and enter into the neutral mucosal lining to avoid the acidic environment and expulsion from the stomach due to peristalsis (Eaton et al., 1992; Ottemann and Miller, 1997; Ottemann and Lowenthal, 2002). Thus, the differences in colonization observed may be irrelevant to the
absence of HcpE production and secretion, and may be due to poor localization into the gastric mucosa leading to expulsion from the stomach. However, this needs to be investigated further.

We currently do not understand why bacterial counts were unobtainable via colony counts on agar plates or why colonization numbers of the wild-type strain were low, as all preliminary tests with various antibiotics and the homogenization process revealed no signs of cellular death in the wild-type SS1 strain. However, it is important to note that even though high colonization rates within mice have been obtained in other studies, *H. pylori* infections within a murine model can be complicated. Previous studies from Marchetti *et al* reported poor colonization of several strains of *H. pylori* within their SPF conditioned BALB/c mice. In addition, *H. pylori* colonization as well as inflammation and disease progression are dependent on both the host and strain of *H. pylori* (van Doorn *et al*, 1995; Nathalie *et al*, 1999), indicating that many different factors can have drastic effects on the colonization success of *H. pylori*.

### 4.5 Influence of HcpE on murine splenocytes

Dominant T-helper-1 (Th1) cellular responses are characteristic of *H. pylori* infection, and are thought to play a role in the ability of *H. pylori* to evade host detection and persist over the course of the host’s life time (Mohammadi *et al*, 1996). Th1 cells are the subtype of T helper cells that are responsible for secreting cytokines such as IFN-γ in response to pathogens. These are used to communicate to other immune cells to enhance phagocytosis as well as iNOS killing. In a mouse infection model using *Helicobacter felis*, it was demonstrated that Th1 cells were responsible for *Helicobacter* induced disease,
whereas Th2 cells had a protective effect due to the fact that they were capable of reducing the bacterial load within the gastric mucosa of mice (Mohammadi et al, 1997). It has been proposed that the protective effect observed from a Th2-type response in mice may result from the induction of local IgA antibody production (Xu-Amano et al, 1993). Infection with *H. pylori* and its ability to promote a Th1 response is thought to counteract this effect by blocking the development of a protective (Th2) immune response, and inducing chronic inflammation by the production of the pro-inflammatory cytokine IFN-\(\gamma\). It is thought that the detrimental effect of IFN-\(\gamma\) produced by Th1 cells during *H. pylori* infection is due to the induction and activation of major histocompatibility complex class II molecules (MHCII), which may result in the enhanced adherence of *H. pylori* to the gastric epithelial cells as well as induce host cell apoptosis (Fan et al, 1998).

Our study highly suggests that the secreted protein HcpE produced by *H. pylori* is a virulence factor that may be associated and involved with inducing a polarized Th1 immune response via the production of IFN-\(\gamma\). When splenocytes were exposed to supernatants obtained from *H. pylori* grown in liquid medium in the presence of LPS, IFN-\(\gamma\) was produced. This effect was significantly reduced with supernatants from both *dsb*\(_{HP}\) and *hcpE* knockout mutants, suggesting that HcpE was the antigen responsible for the IFN-\(\gamma\) modulation to LPS. Purified HcpE was also capable of eliciting an IFN-\(\gamma\) response. IFN-\(\gamma\) secreted from splenocytes that were incubated with pure HcpE were significantly higher than the IFN-\(\gamma\) produced when DdahB was present. Also, the IFN-\(\gamma\) response was negligible when HcpE was treated with proteinase K, further supporting that HcpE was the causative agent of the IFN-\(\gamma\) response, and demonstrating its role as a pro-inflammatory mediator of the polarized Th1 immune response.
The observation that HcpE deficient mutants revealed no difference in their capacity to induce an IFN-γ response when whole cells were incubated with splenocytes indicates that HcpE is not the only IFN-γ inducing component that *H. pylori* produces. This is not unexpected as *H. pylori* produces several non-secreted virulence factors thought to initiate cytokine production that could compensate or mask the differences that would normally be seen in response to HcpE (Censini *et al.*, 1996; Dhar *et al.*, 2003; Argent *et al.*, 2008).

The Hcp family member HcpA has also been shown to contain immuno-stimulatory properties with its ability to induce both IFN-γ and IL-6 secretion (Deml *et al.*, 2005). The activation and maturation of immature dendritic cells was observed upon co-culture with HcpA, resulting in the induction of pro-inflammatory cytokines TNF-α and IL-6 as well as the Th1 polarizing mediators IL-12 and IFN-γ. This was also observed upon co-culture with splenocytes from naïve mice, indicating that HcpA is a secreted virulence factor capable of inducing pro-inflammatory responses that result in the activation of Th1 lymphocytes (Deml *et al.*, 2005). Taken together, there is clear evidence that HcpE, as well as HcpA, and potentially other Hcp family members, are bacterial components that *H. pylori* employs to trigger the release of pro-inflammatory cytokines to instruct the adaptive immune response to initiate a Th1 cellular response. Th1 cellular responses have the potential to stimulate and activate the innate immune system causing cellular recruitment of phagocytes to the site of infection leading to inflammation and eventually tissue damage. However, the mechanisms by which HcpE induces this response, and the balance between the Th1/Th2 response needs to be studied in more detail.
4.6 Summary and significance

Through this work, Dsb_{HP} was identified as a Dsb protein homologue that is involved in the process of disulfide bond formation. Through exposing both *E. coli* and *H. pylori* to oxidative and reducing stress we demonstrated that Dsb_{HP} is functionally similar to *E. coli* DsbA when expressed in *E. coli*, and may possess both oxidase and isomerase properties within *H. pylori*. This also supports the fact that the paradigm for disulfide bond formation established in *E. coli* is not conserved in *H. pylori*.

This work also established that HcpE secreted by *H. pylori* is largely dependent on the presence of functional Dsb_{HP}, as HcpE production and secretion was significantly reduced in the *dsb_{HP}:kan* mutant. We demonstrated a potential role for outer membrane vesicles in the secretion of HcpE, which could be used as a delivery system to interact with host cells. We also established a role for HcpE in the pro-inflammatory Th1 response observed during *H. pylori* infection, demonstrating that HcpE is responsible for inducing IFN-γ production during infection of murine splenocytes cells.

Overall, this work provides novel insights into disulfide bond formation in *H. pylori*, and specifically about the role of Dsb_{HP} in *H. pylori*’s physiology. We have enhanced our understanding of interactions between HcpE and Dsb_{HP}, as well as laid the groundwork to identify a function for HcpE during *H. pylori* infections. This knowledge will contribute to our understanding of *H. pylori* pathogenicity, and thus provide further insight on how we can prevent, or control *H. pylori* infections in the future.
REFERENCES


Characterization of the *Helicobacter pylori* cysteine-rich protein a as a T-helper cell type 1 polarizing agent. Infect Immun **73**:4732-4742.


CURRICULUM VITAE

Jeffrey Lester

EDUCATION:

09/2010 - Present
Masters Candidate: Department of Microbiology and Immunology, University of Western Ontario. Thesis: HcpE, a potential immuno-modulatory protein from Helicobacter pylori that is dependent on the Disulfide bond protein DsbHP
Supervisor: Dr. Carole Creuzenet

09/2004 – 04/2010
Bachelor of Science
Honors Double Major in Microbiology and Immunology and Medical Sciences
Western University, London, Ontario

SCHOLARSHIPS/AWARDS:

Western Graduate Research Scholarship, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario. September 2010 - Present

Deans Honour List, Western University, London, Ontario.
September 2009 – April 2010
RELATED FIELD EXPERIENCE:

Oral Presentations:

Jeff Lester, Sari Kichler and Carole Creuzenet: Deciphering The Role of CRPs, Novel Proteins From Helicobacter pylori. Research Topics in GI Disease, Victoria Convention Center, Victoria, BC. February 2013.

Poster Presentations:


Jeff Lester and Carole Creuzenet: Determining the Role of CRPs in Helicobacter pylori Disease Progression. Canadian Digestive Diseases Week, Royal York Hotel, Toronto, ON. February 2014.

Jeff Lester, Sari Kichler and Carole Creuzenet: Deciphering The Role of CRPs, Novel Proteins From Helicobacter pylori. Research Topics in GI Disease, Victoria Convention Center, Victoria, BC. February 2013.


Manuscripts in Progress:

Lester J.; Kichler S.; Oickle B; Fairweather S.; Oberc A.; Chahal J.; Ratnayake D.; and Creuzenet C. *Helicobacter pylori* disulfide bond protein DsbHP: role in disulfide bond formation, redox stress resistance and production of *Helicobacter* cystein-rich protein HcpE. (Submitted)

Research Experience:

- Microaerophilic growth techniques (liquid and plates) and media development
- Molecular cloning techniques, including design and generation of knockout constructs, transformation and the development of strategies for the disruption of *H. pylori* specific genes.
- Protein expression including Western blotting, Coomassie Blue staining, Ponceau staining, Bradford Protein Assays
- SDS-PAGE analysis of protein and LPS
- Preparation of sterile cultures, aseptic technique and fundamental bacteriology
- Growth Curves
- Animal training involving mouse injection, blood collection, anesthetization, and mouse dissection
- Presentation of findings in the form of posters and oral presentation at conferences, research days and departmental seminars
- Maintaining an up to date and thorough lab book, lab upkeep, WHIMIS, Biohazard Level 2 safety training.
Supervisory Experience:

09/2013 – 12/2013

Teaching Assistant:

Microbiology & Immunology 3810W: Microbiology and Immunology for Nursing Students, Department of Microbiology and Immunology, Western University, London, Ontario

Responsibilities include:

- Instruction of students
- Answered various questions relating to course material online
- Interacted with individuals from varying backgrounds and lead them to reach a positive goal

Volunteer and Community Experience

04/2012 – Present

Infection and Immunity Research Forum Committee Member

Department of Microbiology and Immunology, Western University, London, Ontario

09/2001 – 05/2004

Crime Stoppers Formal Committee

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