UNIVERSITY OF CALIFORNIA

SANTA CRUZ

# The *Helicobacter pylori* protein ImaA modulates the immune response and promotes chronic infection

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

**William E. Sause**

September 2014

The Dissertation of William E. Sause is approved:

_____
Professor Karen Ottemann, chair

_____
Professor Fitnat Yildiz

_____
Assistant Professor Victoria Auerbuch Stone

_____
Professor Jay Solnick, UC Davis

_____
Tyrus Miller
Vice Provost and Dean of Graduate Studies

# Table of Contents

# List of Tables and Figures

## Abstract

**William Sause**

**The *Helicobacter pylori* protein ImaA modulates the immune response and promotes chronic infection**

The primary agent responsible for both gastric ulcers and cancers is the human pathogen *Helicobacter pylori*. The aim of this dissertation is to enhance our understanding of how this bacterium can cause disease in humans and persist in the host over many years. We have identified a novel *H. pylori* virulence factor, called ImaA, which dampens *H. pylori* caused inflammation and promotes long-term colonization in the host. ImaA is an outer membrane protein that *H. pylori* expresses at exceptionally high levels in the stomach and is critical for the bacterium's ability to survive.

The first part of this dissertation expands on the discovery that *imaA* is expressed at higher levels in the mouse stomach, when compared to laboratory conditions. We went on to demonstrate that an *imaA* isogenic mutant exhibits a colonization defect in the mouse and is greatly outcompeted for colonization in a co-infection with wild-type *H. pylori*. We furthermore performed localization assays using an ImaA-targeted antibody to verify that the ImaA protein resides in the outer membrane. We identified low pH to be the signal for *imaA's* upregulation in the stomach and demonstrated that this upregulation is

dependent on the ArsRS two-component regulatory system. Concluding the first part of this dissertation, we revealed that the *imaA* mutant evokes a stronger immune response in gastric epithelial cells, compared to that of wild-type *H. pylori*.

The second part of this dissertation focuses on ImaA's interactions with the host. The primary mechanism for *H. pylori* caused inflammation is through the secretion of the effector molecule, CagA, into host cells. We show here that in an *imaA* mutant infection, there are more CagA molecules delivered into gastric epithelial cells, than in a wild-type infection. We went on to show that the *imaA* mutant induces elevated levels of inflammation, independently of CagA, as an *imaA cagA* double mutant causes more inflammation than a *cagA* single mutant. This result directed us to the *cag*PAI T4SS, the surface-exposed unit that delivers CagA into the host cell. Through flow cytometry experiments, we showed that *imaA* mutants bind higher levels of the host receptor for the *cag*PAI T4SS, α5β1 integrin. Finally, using the gerbil mode for infection, we discovered that ImaA is dispensable for the very initial stages of host colonization, but that the protein becomes essential very rapidly, as *imaA* mutants are cleared from the stomach within a week, post-infection.

Taken together, this dissertation characterizes the novel *H. pylori* virulence factor, ImaA, and demonstrates that this protein is critical for the bacterium's ability to control inflammation and persist in the host stomach.

## Acknowledgments

I am extremely grateful for all of the excellent guidance and support I have received from many, many, many people over the course of this adventure.

I would first like to thank my family. My parents are exceptional role models and have supported me through all of my journeys. Matt, Andy, and Maggie have been the best older brothers and sister a person could ask for. Their unique personalities and individual successes have created an amazing resource for me and their advice is invaluable.

I would like to acknowledge my amazing mentor, Karen Ottemann. Karen has had a profound impact on both my development as a scientist and as a person, she truly has been the perfect mentor. It will certainly be a difficult transition leaving her lab, but I know she will always be available for a conversation.

I would like to thank the Ottemann lab, past and present. Susan Williams and Andrea Castillo were instrumental in introducing me to microbiology and I am grateful for their mentorship. Annah Rolig was a great lab mate and is a great friend; additionally she played a huge role in introducing me to many of the techniques that I now use on a daily basis. My undergraduate researcher, Soufiane Aboulhouda, was an incredibly bright student and contributed excellent work. I've had the pleasure of working with many terrific people in the Ottemann lab and I am very grateful for that experience.

Lastly, I'd like to thank my wonderful group of friends, both in the sciences and out of the sciences. I am tremendously grateful for the balanced network of friends I have maintained through the years.

Chapter 2 of this dissertation is a multi-author publication. Andrea Castillo generated the *imaA* isogenic mutant. I performed all of the experiments that generated the figures. Karen Ottemann is the principal investigator.

# Chapter 1: Autotransporter secretion systems (TVSS) in Gram-negative bacteria

## 1.1 Background

Of the various pathways that exist for protein secretion in Gram-negative bacteria, the type V secretion system (T5SS) offers the most simplistic mechanism for outer membrane protein translocation (Dautin and Bernstein, 2007). It was originally thought that all of the machinery necessary to direct subcellular movement of T5SS family proteins was contained within each protein's polypeptide sequence, thus proteins in this family were named autotransporters (Pohlner et al., 1987). While this concept has since been proven to be partially inaccurate, as a small group of supplemental proteins have been found to contribute to the T5SS pathway, the mechanism still serves as a simple model for secretion (Desvaux et al., 2004). Autotransporters are extracellular proteins that often promote pathogen colonization and persistence within mammalian hosts, thus autotransporters serve critical roles in microbial fitness. Autotransporter proteins can act as toxins, proteases, adhesins, and other factors that help resist host-generated antimicrobial defenses (Henderson and Nataro, 2001). In accordance with their relevance during the infection process, it is not surprising that many bacteria upregulate transcription of genes encoding autotransporters upon entry into the mammalian host (Alamuri and

Mobley, 2008; Henderson and Nataro, 2001; Maurer et al., 1998; Porter and Dorman, 1997; van Amsterdam et al., 2003).

The first model for autotransporter secretion was proposed in *Neisseria gonorrhoeae*. This autotransporter, termed IgA1 protease, contained domains that were similar to a protease and were subsequently shown to produce the mature extracellular peptide (Pohlner et al., 1987). This work generated the idea that autotransporters have three regions: an N-terminal leader sequence, a middle domain that enacts the function of the protein, e.g. the mature IgA1 protease, and a C-terminal region called the "helper domain". This research generated a model in which the C-terminal helper domain facilitates secretion of the protease domain by forming a pore in the outer membrane. The middle domain then can undergo autoproteolysis from the helper domain and is released into the extracellular environment, where the protease is then active (Halter et al., 1984; Klauser et al., 1990) (Figure 1 (Cotter et al., 2005)). Many autotransporters have been identified in other Gram-negative bacteria since this seminal work was published and in each case, the fundamental three-domain structure of the autotransporter protein resembles that of the IgAI protease (Henderson and Nataro, 2001; Jose et al., 1995). The three distinct autotransporter domains are now commonly referred to as the signal sequence, the passenger domain, and the C-terminal β-domain. It is the passenger domain that confers a multitude of virulence related phenotypes, including

autoaggregation, biofilm formation, host protein digestion, and cytotoxicity (Henderson et al., 2004).

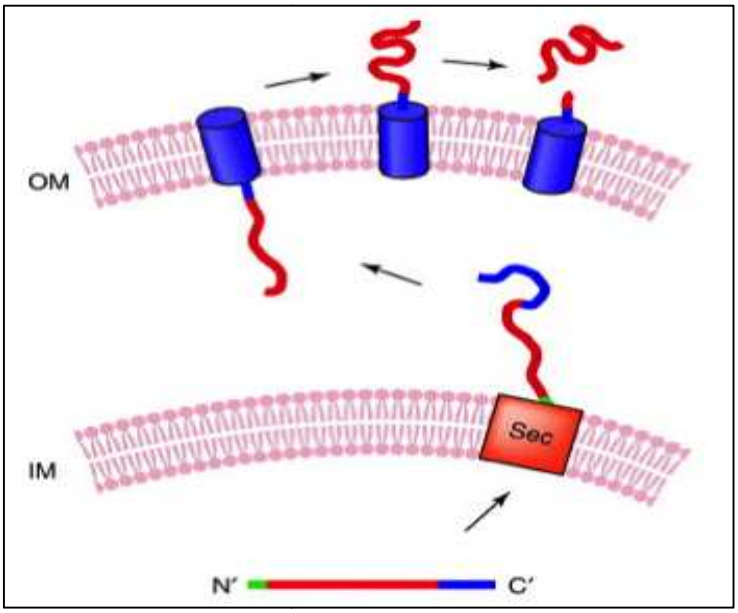



**Figure 1. The autotransporter secretion pathway**
Autotransporter proteins are transported through the inner membrane in a Sec dependent manner, the protein is then translocated through the periplasm in an unfolded state. Once at the outer membrane, the C-terminal β-barrel domain forms a pore and threads the N-terminal passenger domain up to the cell surface (Cotter et al., 2005).

1.1.2 The signal sequence

The N-terminus of all autotransporters contains a Sec-dependent signal sequence that directs movement of the protein to the inner membrane after translation (Brandon et al., 2003; Henderson et al., 1998). Brandon *et al.* showed that the *Shigella flexneri* IcsA autotransporter is reliant on the entire SecYEG

translocation complex for protein movement through the inner membrane (Brandon et al., 2003). This work revealed that while autotransporters might be capable of independently directing their passage through the periplasm, they do not represent an entirely autonomous pathway.

Most autotransporter signal sequences have a conserved pattern that is similar/dissimilar as compared to other secreted proteins. This signal sequence consists of (i) an n-domain containing positively charged amino acids, (ii) an h-domain with hydrophobic amino acids, and (iii) a c-domain consisting of uncharged and short lateral-chain residues at positions -3 and -1, relative to the mature N-terminus, that dictate the position of signal sequence cleavage. The c-domain additionally possesses helix-breaking proline and glycine residues, which make up the actual consensus signal peptidase recognition site (Martoglio and Dobberstein, 1998). Not all autotransporters strictly adhere to this pattern. However, two well-studied autotransporters, AIDA-1 and Hbp, possess signal sequences with h and c-domains that are characteristic of most Sec-dependent bacterial proteins but also display a conserved extension of the n-domain, which results in an abnormally long signal sequence (Henderson et al., 1998; Hiss et al., 2008). It has been shown with Hbp that the unusually long signal sequence facilitates the recruitment of SRP, an accessory protein different from the Sec pathway that functions by targeting proteins to the inner membrane through the formation of a cotranslational complex with the ribosome (Keenan et al., 2001; Sijbrandi et al., 2003). There is no evidence to support the utilization of other

common Gram-negative post-translational secretion systems with autotransporters, such as the Tat pathway (Henderson et al., 1998).

### 1.1.3 Periplasmic transport

Upon cleavage of the signal peptide in the inner membrane, the processed protein is transported through the periplasmic space to its eventual position in the outer membrane (Henderson et al., 1998; Veiga et al., 1999). Much controversy exists over the conformational state of the autotransporter as it traverses the periplasm. This is due in large part to the pore size generated in the outer membrane, which at less than 2 nm would likely be incapable of transporting a protein in its native state (Veiga et al., 2002). Yet studies have shown that autotransporters transiting the periplasm are resistant to degradation by proteases, suggesting that these proteins either exist in a partially folded state or are under the protection of a chaperone protein (Brandon and Goldberg, 2001; Oomen et al., 2004; Velarde and Nataro, 2004). Further discrediting the name "autotransporter", Ruiz-Perez *et al*. demonstrated that in the absence of the periplasmic chaperone, DegP, the *E. coli* autotransporter EspP develops a misfolded or prematurely folded passenger domain (Ruiz-Perez et al., 2009). This irregular folding causes a stalling in the outer membrane pore where the β-barrel forms, leading to eventual cell lysis. Under this model, periplasmic chaperones bind to the passenger and β-domain

to prevent aggregation and premature folding, while maintaining the protein in a partially folded translocation-competent state.

Recent work suggests that partial folding of autotransporters in the periplasm is unlikely and that these proteins likely translocate in an unfolded manner. Junker *et al*. demonstrated that the deliberate placement of cysteine residues at various locations within the passenger domain of the autotransporter pertactin, and subsequent disulphide bond formation in the oxidizing environment of the periplasm, was enough to stall secretion of pertactin to the outer membrane (Junker et al., 2009). The inability to secrete pertactin with such Cys-pairs in the passenger domain suggests that locking the passenger domain into a particular state prevents secretion. This finding further strengthens the notion that while the passenger domain crosses through the periplasm in an unfolded manner, it must be receiving some external protection from proteolytic digestion (Ieva et al., 2008).

### 1.1.4 The β-barrel domain

The highly conserved ~30-kDa β-barrel domain is responsible for forming a pore in the outer membrane that facilitates the threading of the passenger domain into the extracellular space (Loveless and Saier, 1997). The β-barrel domain is comprised of β-pleated sheets and includes 14 antiparallel strands consisting of 9-12 residues each (Loveless and Saier, 1997; Yen et al.,

2002). An area of strong conservation amongst autotransporters is present in the most C-terminal amino acids, which consist of alternating aromatic/hydrophobic and charged/hydrophilic residues, with the last residue being either a tryptophan or a phenylalanine (Jose et al., 1995; Struyvé et al., 1991). It has been shown that these C-terminal residues are of particular importance for stability and localization in the outer membrane (Hendrixson et al., 1997). Hendrixson *et al*. demonstrated that by deleting the three most C-terminal residues of the Hap autotransporter, the protein was no longer able to effectively insert itself into the outer membrane (Hendrixson et al., 1997). C-terminal β-strands are also thought to protect the passenger domain from proteolytic digestion and autoaggregation upon initial entry into the outer membrane (Oomen et al., 2004). Recently, work done by Sauri *et al*. downplayed the role of the β-barrel domain in the secretion of proteins though the outer membrane. This group found that through stalling the transport of the Hbp autotransporter across the outer membrane, they were able to bring down protein complexes with Hbp that included BamA, BamB, and SurA, which all serve an important role in the targeting and assembly of outer membrane proteins (Sauri et al., 2009). According to their model, BamA serves as a channel for transport of the passenger domain prior to the assembly of the β-barrel in the outer membrane, providing a larger pore that would allow that transport of folded protein structures (Sauri et al., 2009). When *bamA* expression was turned off through the use of a repressible promoter, there was a 95% decrease in the

production of Hbp compared to that of the parental strain grown under the same conditions (Sauri et al., 2009).

### 1.1.5 Passenger domain structure and transport

While much homology exists between autotransporter β-barrel domains, there are virtually no conserved sequences amongst passenger domains (Henderson and Nataro, 2001). Despite the lack of homology or disparity in overall function between passenger domains, greater than 97% are predicted to form right handed parallel β-helix structures (Gangwer et al., 2007; Junker et al., 2006). As described above, it is not known how the passenger domain might maintain an unfolded state in the periplasmic space (Thanassi et al., 2005). Junker *et al*. provided a possible explanation to this quandary when they revealed the unusually slow folding kinetics of the passenger domain in the periplasm. Slow folding would inhibit premature folding prior to outer membrane translocation, thus keeping the protein in a secretion competent state (Junker et al., 2006; Oliver et al., 2003). While there may be little overall sequence homology between passenger domains, there are motifs that can be seen amongst some autotransporters with adhesin properties (Hynes, 1987; Wells et al., 2007). In particular, the tripeptide Arg-Gly-Asp (RGD) motif, which is the attachment site for fibronectin, vitronectin and fibrinogen, is present in a few

characterized autotransporter adhesins, including pertactin, Ag43, and BrkA (Fernandez and Weiss, 1994; Klemm et al., 2004).

The direction of passenger domain secretion across the outer membrane is from C to N-terminus (Henderson et al., 1998; Junker et al., 2009). The pore created by the β-barrel domain is 1-2 nm in size, which allows for the unfolded passenger domain to be threaded to the cell-surface (Oomen et al., 2004). The formation of a folded β-helical structure in the outer membrane is thought to prevent backsliding of the passenger domain into the periplasm, as it is being secreted (Junker et al., 2006; Renn and Clark, 2008). As the passenger domain is threaded through the pore, it forms a hairpin structure and emerges from the β-domain channel in a vectorial manner (Junker et al., 2009). Upon exposure to the bacterial surface, the protein adopts its native structure where it then takes on a specialized function as dictated by the passenger domain (Henderson and Nataro, 2001; Henderson et al., 2004). The mature autotransporter can assume a diverse array of roles at this point. For one, the passenger domain can be cleaved and released into the extracellular environment, where it oftentimes functions as a cytotoxin, as is the case with the *H. pylori* autotransporter VacA (Brunder et al., 1997; Cover and Blaser, 1992; Eslava et al., 1998). Alternatively, the processed autotransporter can be cleaved from the body of the protein but still remain closely associated with the surface of the cell through noncovalent interactions with the β-domain (Leininger et al., 1991; Owen et al., 1996). This situation occurs often with proteins such as Ag43 from *E. coli* and BabA from *H. pylori*,

which are instrumental in the respective bacteria's ability to adhere to host cells (Ilver et al., 1998a; Owen et al., 1996).

Much controversy exists over the method by which the passenger domain is cleaved from the β-domain, particularly whether the domain is released by a membrane-bound protease or through autoproteolysis. A number of autotransporters, including IgA1, Hap, and App have been shown to be cleaved through an autoproteolytic event, particularly through the internal serine protease active sites on the autotransporter (Hendrixson et al., 1997; Serruto et al., 2003). In the case of the EspP autotransporter, two conserved residues within the β-barrel, an aspartate within the translocator domain and an asparagine within the cleavage junction, are crucial for processing and are predicted to assemble a unique catalytic dyad that mediates self-cleavage (Dautin et al., 2007). The *Shigella* autotransporter IcsA is cleaved by an external outer membrane protease called IcsP that is related to the omptin family of proteases, cleavage of autotransporters by omptin family proteases is common in many bacterial species (Shere et al., 1997). Further adding to the confusion of which form of proteolysis predominates, the passenger domains of multiple *N. meningitidis* autotransporters are cleaved by a serine protease called NalP, which is an autotransporter itself (van Ulsen et al., 2003).

### 1.1.6 Glycosylation of passenger domains

While the identification of glycosylated proteins in prokaryotes might be in its infancy stages, there is strong evidence that the *E. coli*-mammalian cell adhesins AIDA-1, Ag43, and TibA are glycoproteins (Klemm et al., 2006). The passenger domains of these proteins have decent homology to each other (~30% identical) and all share a pattern of ~19 residue repeats (Klemm et al., 2006). In addition to their responsibilities of interacting with human cells, these autotransporters also self-associate and induce bacterial aggregation (Klemm et al., 2006). Glycosylation of AIDA-1 greatly enhances its stability and both AIDA-1 and TibA contain numerous O-linked heptose moieties that are necessary for their binding to mammalian cells(Benz and Schmidt, 2001; Lindenthal and Elsinghorst, 2001). The glycosyltransferases that interact with these two proteins have strong homology with each other, which allows for interchangeable glycosylation between AIDA-1 and TIbA and their respective glycosyltransferases (Moormann et al., 2002). While the nature of the carbohydrate moieties that associate with Ag43 have not been determined, it has been shown that Ag43 has an enhanced ability to adhere human cell lines when the protein is glycosylated (Sherlock et al., 2006).

### 1.1.7 Autotransporter localization

Proteins in the autotransporter family display a diverse array of phenotypes on the bacterial surface but across most studied Gram-negative bacteria, they localize to the cell pole (Jain et al., 2006). Jain *et al*. demonstrated that autotransporters from an assorted collection of pathogens, including *Shigella*, *E. coli*, and *B. pertussis*, are all secreted to the outer membrane in a polar specific manner (Jain et al., 2006). A certain requirement for polar localization is a fully synthesized lipopolysaccharide (LPS) (Jain et al., 2006). In *E. coli* strains that express truncated LPS, the autotransporter AIDA-1 is distributed evenly on the cell surface (Benz and Schmidt, 1992; Henderson et al., 1997). The rates of diffusion in the outer membrane of strains expressing truncated LPS is much higher than in strains with fully intact LPS, which may account for surface-wide autotransporter distribution (Jain et al., 2006; Rajakumar et al., 1994). In bacteria that express complete LPS, LPS molecules form a helical arrangement on the cell surface and do not diffuse freely from across the membrane(Robbins et al., 2001). It is hypothesized that through interactions with LPS molecules at the cell pole, autotransporters are stabilized.

To demonstrate that polar localization is not restricted to rod shaped bacteria, a heterologous system was created in which the NalP autotransporter from spherically shaped *N. meningitidis* is expressed in *E. coli* with a fully intact LPS (Jain et al., 2006). NalP is efficiently transported to the bacterial pole in this construct, indicating that the protein contains all of the necessary components

for asymmetrical transport. Every autotransporter examined by Jean *et al*. was polar in the cytoplasm prior to secretion (Jain et al., 2006). To test which factors dictate the localization of IcsA in the cytoplasm, Janakiraman *et al*. performed a screen to identify proteins that share similar localization patterns to that of IcsA-GFP in cells lacking the cell division protein FtsZ (Janakiraman et al., 2009; Janakiraman and Goldberg, 2004). This group found that not only do *Shigella* autotransporters migrate to the pole independently of FtsZ but that they are dependent on the cytoplasmic chaperone and unfoldase DnaK for proper localization (Janakiraman et al., 2009). DnaK is likely responsible for preventing the formation of partially folded autotransporter intermediates that would be prone to aggregation and to maintain a protein confirmation that allows secretion through the Sec translocon (Janakiraman et al., 2009). Recently, it was demonstrated that the *H. pylori* autotransporter protein, FaaA, localizes to the bacterial flagella and contribute to flagellar synthesis and function (Radin et al., 2013)

### 1.1.8 Autotransporter host ligands

Autotransporters interact with the extracellular space and exert numerous effects on host cells, so it would make sense that these proteins often have dedicated mammalian receptors. Due to the great heterogeneity that exists across autotransporter passenger domains, there is no universal

autotransporter host-receptor. One autotransporter that contributes to virulence and has a dedicated receptor is the *Pseudomonas aeruginosa* autotransporter EprS. EprS activates the transcription factor, nuclear factor-κB (NF-κB), and drives the production of the pro-inflammatory chemokine, interleukin-8 (IL-8). Upstream of this activation, EprS cleaves the extracellular domain of the mammalian protein, protease-activated receptor-1 (PAR-1), at serine residues, leading to the initiation of NF-κB signaling (Kida et al., 2013). These results indicate that autotransporters can modulate immune responses through proteolytic activation of mammalian receptors. This is an interesting insight because many autotransporters are predicted to have proteolytic or lipolytic activity.

### 1.1.9 Autotransporter transcriptional regulation

In Gram-negative bacteria, most autotransporters serve as virulence factors that contribute to microbial pathogenesis (Henderson and Nataro, 2001). Not surprisingly then, the transcription of genes that encode autotransporters are often times upregulated in response to environmental cues, particularly when bacteria are introduced into mammalian systems (Alamuri and Mobley, 2008; Porter and Dorman, 1997; van Amsterdam et al., 2003). The *Yersinia pestis* Yap autotransporter proteins are expressed at very low levels in the lab, but in the bubonic form of plague, all 9 *yap* genes are significantly upregulated within

the lymph nodes (Lenz et al., 2011). A number of these proteins have been shown to be important for bacterial dissemination within the host, so a timely boost in their expression seems critical for *Yersinia* pathogenesis (Lenz et al., 2012).

### 1.1.10 General autotransporter conclusions

Autotransporters represent a crucial component of the Gram-negative secretome. Identifiable by their unique modular structure, these proteins facilitate their own transport through the bacterial membrane system in a seemingly independent fashion (Henderson et al., 1998). Despite the apparent simplicity of the secretion pathway, proteins from the autotransporter family include some of the most potent and useful extracellular virulence factors that have been characterized for Gram-negative bacteria(Henderson and Nataro, 2001). The advancement of research continues to reveal unforeseen complexities regarding the autotransporter mechanism, as the scientific community develops an enhanced understanding of this system, it will in turn obtain a better grasp of general microbial pathogenesis.

## 1.2 *Helicobacter pylori* autotransporters

*H. pylori* possesses four predicted autotransporters, and aside from the well-studied VacA protein, the other three autotransporters are still in the early

stages of being characterized (Cover and Blaser, 1992; Radin et al., 2013; Sause et al., 2012). The VacA autotransporter is one of *H. pylori*'s most well examined proteins and is central to pathogenesis. VacA is a multifunctional toxin that is best known for its ability to generate vacuoles in host cells and cause apoptosis. Upon secretion from the *H. pylori* cell surface, VacA becomes internalized by host cells through the protein-tyrosine phosphatase alpha (RPTP-α) receptor (Yahiro et al., 2003). After that, vacuolation is induced and VacA is directed to the mitochondria. Thus far, VacA is the only *H. pylori* autotransporter that has been shown to undergo proteolysis from the bacterial surface and be released into the extracellular space as a soluble protein (Radin et al., 2013). VacA also possesses the unique ability to suppress the proliferation of mature T-cells, thus promoting long-term infection through evasion of the adaptive immune system (Gebert et al., 2003). VacA's also works synergistically with the main *H. pylori* effector protein, CagA, to promote CagA's correct trafficking and breakdown within the host cell (Tsugawa et al., 2012).

Characterization of the three other *H. pylori* autotransporters is still in its infancy stages, but much progress in understanding their roles has been made in recent years. It was shown that all three remaining autotransporters, *imaA*, *faaA*, and *vlpC*, are upregulated in the host and that mutants for each autotransporter exhibit a deficiency in mouse colonization (Radin et al., 2013; Sause et al., 2012). Unlike ImaA and VlpC, which were shown to localize at the poles of the bacterial cell, FaaA localizes to the flagella and plays a critical role in maintaining flagella

stability (Radin et al., 2013). Mutants for *faaA* produce smaller quantities of FlaA, which is the major component of the flagella, and exhibit reduced motility (Radin et al., 2013). No obvious functional roles for VlpC have been identified yet. The role of ImaA in pathogenesis will be elaborated in detail in the remainder of this thesis.

## Chapter 2: The *Helicobacter pylori* autotransporter ImaA (HP0289) modulates the immune response and contributes to host colonization

### 2.1 Abstract

The human pathogen *Helicobacter pylori* employs a diverse collection of outer membrane proteins to colonize, persist and drive disease within the acidic gastric environment. In this study, we sought to elucidate the function of the host-induced gene, *HP0289*, which encodes for an uncharacterized outer membrane protein. We first generated an isogenic *H. pylori* mutant that lacks *HP0289* and found that the mutant has a colonization defect in single strain infections and is greatly outcompeted in mouse co-infection experiments with wild-type *H. pylori*. We furthermore used protease assays and biochemical fractionation coupled with an HP0289-targetted peptide antibody to verify that the HP0289 protein resides in the outer membrane. Our previous findings showed that the *HP0289* promoter is upregulated in the mouse stomach, and here we demonstrate that *HP0289* expression is induced under acidic conditions in an ArsRS-dependent manner. Finally, we have shown that the *HP0289* mutant induces greater expression of the chemokines IL-8 and the cytokine TNF-α in gastric carcinoma cells (AGS). Similarly, transcription of the IL-8 homolog KC is elevated in murine infections with the HP0289 mutant as compared to those with wild type. Based on this phenotype, we renamed HP0289 as ImaA, for ImmunoModulatory Autotransporter protein. Our work has revealed that *in vivo*

induced genes play an important role in *H. pylori* pathogenesis. Specifically, the outer membrane protein, ImaA, modulates a component of the host-inflammatory response, and thus may allow *H. pylori* to fine tune the host immune response based on ImaA expression.

## 2.2 Introduction.

The human pathogen *Helicobacter pylori* infects half of the world's population and causes chronic infection that elevates the risk of multiple gastric diseases, including gastric adenocarcinoma (Herrera and Parsonnet, 2009; Montecucco and Rappuoli, 2001; Suerbaum and Michetti, 2002). In an effort to better understand *H. pylori* pathogenesis, Castillo *et al*. identified a set of *H. pylori* genes that were expressed to higher levels when the bacterium was in the mouse stomach compared to the lab setting. Host-induced genes have been shown to be crucial for colonization and virulence in a number of bacterial species, including *H. pylori* (Bron et al., 2004; Camilli and Mekalanos, 1995; Castillo et al., 2008b; Janakiraman and Slauch, 2000; Lee et al., 2001; Lowe et al., 1998; Saviola et al., 2003; Veal-Carr and Stibitz, 2005). This previous work used recombination-based *in vivo* expression technology (RIVET) to identify host-induced *H. pylori* genes (Castillo et al., 2008b). The RIVET system utilizes fusions of transcriptional promoters to a promoter-less gene encoding a recombinase protein. If these promoters are transcribed, for example in the mouse stomach,

the recombinase is created and mediates site-specific recombination events that convert the strain from antibiotic resistance to antibiotic sensitivity (Slauch and Camilli, 2000). The previous study analyzed ~71% of the genome and found six *in vivo* induced promoters (Castillo et al., 2008b). Two of the promoters (P*ivi10* and P*ivi66*) regulated genes, *mobABD* and *cagZ*, respectively, that were important for mouse stomach colonization (Castillo et al., 2008b). Of the four remaining unstudied promoters from the RIVET screen, one regulated a gene, *HP0289*, that was previously annotated as a toxin-like outer membrane protein in the complete genome sequence of *H. pylori* strain 26695 (Schultz et al., 1998). Here we explore how *HP0289* contributes to *H. pylori* pathogenesis.

The *H. pylori* genome is predicted to encode more than 30 outer membrane proteins (OMP), or approximately 4% of the bacteria's coding potential (Alm et al., 2000; Dossumbekova et al., 2006a). This level of dedication to *omp* genes is not commonly seen in other bacterial species and only a small percentage of these OMPs have actually been characterized in *H. pylori* (Alm et al., 2000; Baik et al., 2004; Dossumbekova et al., 2006a; Henderson et al., 2004). The abundance of specialized OMPs in the *H. pylori* proteome has been proposed to allow the bacterium to persist within an environment that is demanding and often changing (Kuipers et al., 1995). Of the well-studied *H. pylori omp* genes, a number encode proteins that play a prominent role in *H. pylori* pathogenesis (Henderson et al., 2004). The most notable of these proteins include the vacuolating cytotoxin protein, VacA and the host-antigen specific adhesins BabA

and SabA. VacA belongs to a family of OMPs called autotransporters, a class of proteins that appear three other times in the *H. pylori* proteome (Finn et al., 2010). While a number of *H. pylori* OMPs have been characterized, VacA is the only OMP with an autotransporter domain that has been well described, thus a void exists in our knowledge of the remaining autotransporters.

Autotransporters are a family of Gram-negative secreted proteins that can be toxins, proteases, or adhesins (Dossumbekova et al., 2006a; Henderson and Nataro, 2001). These proteins are called autotransporters because originally they were thought to contain all of the machinery necessary for secretion to the outer membrane. Recent work, however, suggests many interact with an additional protein, the beta-barrel assembly machinery BAM (Henderson et al., 2004; Sauri et al., 2009). All autotransporters possess a conserved domain structure, which consists of (i) an N-terminal signal peptide that facilitates Sec-dependent secretion across the inner membrane, (ii) a generally non-conserved central region called the passenger domain, which confers the effector function of the protein, and (iii) a C-terminal β-barrel domain that is the hallmark of the autotransporter family and is critical for protein translocation across the outer membrane (Henderson et al., 2004). Passenger domains represent the surface-exposed component of the protein and typically adopt an extended right-handed beta helix structure (Benz and Schmidt, 2011; Williams et al., 2008). These domains are extremely diverse in both sequence and function, making it difficult to predict what a particular autotransporter does (Henderson et al., 2004).

Known autotransporter functions include (i) binding to host proteins to mediate adhesion, invasion, immunoglobulin binding or intracellular movement; (ii) interacting with other bacterial molecules to mediate agglutination and biofilm formation; (iii) acting as intracellular toxins and; (iv) behaving as proteases that target such proteins as host immunoglobulin. The autotransporter studied here, HP0289, was originally annotated as a toxin-like outer membrane protein, however there is no experimental evidence for such a function (Tomb et al., 1997).

*H. pylori* colonization causes chronic inflammation, a host response that is considered the primary risk factors for adenocarcinoma (Herrera and Parsonnet, 2009). *H. pylori* strains are highly variable, and are well documented to contain various combinations of genes that enhance inflammation, including genes of the cag pathogenicity island, *oipA*, *babA*, and *sabA* (Eaton et al., 2001; Ishijima et al., 2011; Mahdavi et al., 2002; Yamaoka et al., 2000). Recent reports suggest that *HP0289* might too vary between strains. Specifically, Kawai *et al.* analyzed 20 *H. pylori* genome sequences and found that the highly carcinogenic East Asian (*hspEAsia*) strains had several changes including a large deletion in *HP0289* that removed 83% of the protein (Kawai et al., 2011). Indeed, Lee *et al.* previously demonstrated that East Asian clinical isolates induce epithelial cells to produce significantly higher proinflammatory cytokine levels than do Western strains, however the strains in this specific study were not analyzed for the presence of

*HP0289* (Lee et al., 2004). These findings thus suggest that loss of *HP0289* may create *H. pylori* strains that are more proinflammatory.

In this study, we characterize the *H. pylori* autotransporter, HP0289, and show that it contributes to murine stomach colonization, and is under the control of the acid-sensing ArsRS two-component system. Additionally, we demonstrate that the protein decreases expression of inflammatory chemokines and cytokines in both cultured epithelial cells and infected stomachs.

## 2.3 Methods

**Bacterial strains and growth conditions.** All strains are described in Table 1. *H. pylori* strain LSH100, a mouse-adapted descendent of the clinical isolate G27 (Covacci et al., 1993; Lowenthal et al., 2009), was used for proteinase K digestions, all of the mouse colonization, gene expression, and AGS cell experiments. Strain 26695 was used for additional AGS cell inflammation experiments (Akopyants et al., 1995), and *H. pylori* strain SS1 (Lee et al., 1997) was used for murine infection and ImaA subcellular localization experiments. *H. pylori* strains were maintained on Columbia blood agar base (Difco, Detroit, MI.) supplemented with 5% defibrinated horse blood (Hemostat Laboratories, Dixon, CA), 0.2% (wt/vol) ß-cyclodextrin (Sigma) plus 5 μg/mL trimethoprim, 8 μg/mL amphotericin B, 50 μg/mL, cyclohexamide, 10 μg/mL vancomycin, 5 μg/mL cefsulodin, 2.5 Units/mL polymyxin B to inhibit the growth of unwanted

microbes (CHBA) under 10% $CO_2$, 7-10% $O_2$ and balance $N_2$, at 37°C. Liquid *H. pylori* cultures were grown in 1X Ham's F-12 medium (Gibco, Grand Island, NY) containing 10 % heat-inactivated fetal bovine serum (Gibco) or brucella broth supplemented with 10% fetal bovine serum (BB10). The antibiotic chloramphenicol (Cm) was used for selection at a concentration of 13 μg/mL. *H. pylori* strains were stored at –80°C in brain heart infusion media supplemented with 10% fetal bovine serum, 1% (wt/vol) β-cyclodextrin, 25% glycerol, and 5% dimethyl sulfoxide.

**Acid exposure**. *H. pylori* cultured for ~36 hours on CHBA were resuspended in sterile BB10 and concentrations were determined by optical density ($OD_{600}$). For the 2 hour acid treatment to examine *imaA* and *ureA* transcript levels, cell suspensions were diluted to an $OD_{600}$ of 1.75 in 1 mL of BB10 and then centrifuged at 2,500 x g for 8 min. The resulting pellet was then resuspended in 2 mL of BB10 at a pH of either 5 or 7, and then incubated at 37°C under *H. pylori* culture conditions for 2 hr. For the time course measuring ImaA protein levels, cells were prepared the same way, except that cultures were diluted to an $OD_{600}$ of 0.220 at the initiation of the incubation period. The cell densities of each sample taken at every time point were then normalized with the $OD_{600}$ to ensure that equal amounts of protein were being examined for each respective culture.

**Mammalian cell culture**. AGS (ATCC CRL 1739) human gastric epithelial cells were obtained directly from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) containing 10% fetal bovine serum (FBS) at 37°C with 10% $CO_2$. To assay IL-8 production, AGS cells were seeded at 1 x $10^5$ cells/mL in 24-well tissue culture dishes and incubated for 24 hours. After this period, *H. pylori*, cultured for ~36 hours on CHBA, were scraped from a plate and resuspended in sterile DMEM+FBS to a concentration of 1 x $10^7$ to create a multiplicity of infection (MOI) of 100. *H. pylori* concentrations were determined by $OD_{600}$, assuming 3x$10^8$ bacteria/ml/$OD_{600}$. AGS cells were infected for 2 hr under 10% $CO_2$. After 2 hr incubation, culture supernatant was removed and AGS monolayers were washed twice in 1X phosphate-buffered saline (PBS), and then the cells were resuspended in TRIzol for RNA isolation.

**Construction of *H. pylori* mutants**. *H. pylori* SS1 *ΔimaA::cat* was created using splicing by overlap extension (SOE) PCR with the primers D1 (5'-gcccttagttcaggtgtggcagtttaagg) and D2 (5'-caaggaggatcccggccgcggctaccttctcatttcctagatagtagcc), D3 (5'-atccacttttcaatctatatcacggttgccgggaatgtgggcatgcgagtggcg) and D4 (5'-gttttagcgtcaatgttggggttgattctaatgg) that amplified the *imaA* chromosomal region, and primers catF (Castillo et al., 2008b) and catR2 (Castillo et al., 2008b) that amplified the *cat* gene. This gene deletion extends from 7 base pairs upstream of

the *imaA* start codon to 31 base pairs upstream of the *imaA* stop codon and places a terminatorless *cat* gene in the same transcriptional orientation as *imaA*. To generate the deletion in *H. pylori* LSH100, genomic DNA from SS1 *ΔimaA::cat* was used to naturally transform wild-type LSH100, to create strain KO1370. The deletion in 26695 was produced by natural transformation of wild-type 26695 with genomic DNA from strain KO1370, to generate strain KO1374. Selection was done on Cm CHBA and proper integration was confirmed with PCR using primers D4 and catR2. To generate the LSH100 *ΔarsS::cat* mutant (KO1371), the chloramphenicol resistance cassette (*cat*) was inserted into the *arsS* gene (HP0165) by SOE-PCR. In brief, primers were generated that reside approximately 300 base pairs upstream, ArsS1.1 (5'-aaccctatgatcctaaggaatta) and ArsS3.1 (5'-atccacttttcaatctatatcaacgcaaaaccccttaactcc), and downstream, ArsS2.2 (5'-ggcttcctgtagcgtccttatg) and ArsS4.1 (5'-cccagtttgtcgcactgataagagaacatgttcaaacgattga) of *arsS*. The two *arsS* PCR products were spliced to third PCR product that contained the nonpolar *cat* allele generated from the primers catR2 and catF (Castillo et al., 2008b). The PCR product composed of the *cat* gene flanked by upstream and downstream regions of the *arsS* gene was then cloned into the TOPO-TA vector (Invitrogen) to generate plasmid p*cat-arsS* (Table 1). This plasmid was then used to naturally transform LSH100 to Cm resistance. Proper integration was confirmed by PCR using primers ArsS1.1 and ArsS2.2 and by sequencing of that PCR product. The original *cagE* mutant was a kind gift from David McGee and Kylie Nolan (Table

1). It consists of an insertion of the *aphA3* gene a unique *BglII* site in *cagE* (HP0544, cag23) that is ~600 bp from the start site of the 3000 bp gene. We used genomic DNA from this strain to naturally transform either wild-type LSH100 or KO1370 to kanamycin resistance.

**Mouse colonization experiments**. *H. pylori* strains used for colonization analyses were passaged minimally in the lab (two or three times) and then inoculated into either HAMS F12 culture media (Testerman et al., 2001) for LSH100 or BB10 for SS1 for ~18 hours, as described above. After this period, cells were analyzed to determine motility and cell concentration ($OD_{600}$) prior to infection. For all infections, 4-6-week-old male FVB/N mice (Charles River) were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care-accredited facility in microisolator cages with free access to standard food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee. Approximately 1 mL of *H. pylori* cells containing 9 x $10^7$ to 1 x$10^8$ CFU/mL was used to orally gavage the mice. For co-infections, wild-type and mutant cells were grown separately and then combined in equal concentrations. To determine the true CFU/mL of each culture, all cultures were serially diluted and plated on CHBA. Infections were allowed to persist for two-three weeks, after which time mouse stomachs were excised as described before (Ottemann and Lowenthal, 2002), homogenized using the Bullet Blender (Next Advance, Averill Park, NY), and then plated on

CHBA ± Cm (described above) supplemented with 10 μg of nalidixic acid/mL and 200 μg of bacitracin/mL For the co-infection, stomachs were plated on both nonselective and Cm supplemented CHBA as described previously (Terry et al., 2005). The cells counts obtained from the input and output data allowed us to calculate the competitive index, as follows: (CFU/g mutant strain output/ CFU/g wild-type strain output)/(CFU/g mutant strain input/CFU/g wild-type strain input).

For KC (mouse IL-8 homolog) detection in mouse tissue, infections persisted for three weeks (Algood et al., 2007). When excising the stomach, half of the tissue was placed into BB10 for plating and the other half was placed immediately into liquid $N_2$ and then stored at –80°C within an hour of extraction. To isolate RNA, tissue samples were suspended in TRIzol and then homogenized using the Polytron (Kinematica, Switzerland) automated tissue homogenizer.

**RNA preparation**. Total RNA was isolated from *H. pylori* strains *LSH100* and its isogenic mutants, *ΔimaA::cat* and *ΔarsS::cat*, using TRIzol reagent (Invitrogen, Carlsbad, CA) combined with RNeasy columns (Qiagen, Valencia, CA). Bacterial cells were pelleted and resuspended in 1 mL of TRIzol at room temperature for 5 min before 200 μL of chloroform was added. Samples were then centrifuged (12,000 x g, 15 min, 4˚C), and the aqueous layer was removed and placed into new tubes. RNA was precipitated by combining 500 μL of isopropanol with the aqueous layer and incubating at room temperature for 10 min, followed by a

centrifugation as above. The RNA pellet was washed with 75% ethanol was and then dried and resuspended in RNase-free water. To remove contaminating genomic DNA from purified RNA, samples were treated with 4U of RNase-free DNaseI (Ambion) for 3 hours at 37˚C, followed by further purification using the Qiagen RNeasy spin columns as described by the manufacturer's instructions. RNA was ultimately eluted in RNase-free water, concentrations were quantified on a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE) and the absence of contaminating genomic DNA was confirmed with PCR. RNA was immediately transcribed into cDNA (see below) and the remaining sample stored at -80˚C.

RNA was isolated from AGS cells in a similar manner. Briefly, 1 mL TRIzol reagent was added directly to cells in the culture dish per 10 $cm^2$ of culture dish surface. The cells, including infecting *H. pylori* cells, were lysed directly in the culture dish by pipetting the cells up and down several times. Homogenized samples then underwent the same preparation as described above, except the DNaseI treatment and secondary purification with the Qiagen RNeasy kit were omitted.

**cDNA synthesis and quantitative real-time PCR**. Total RNA served as a template for cDNA synthesis using the Tetro cDNA synthesis kit (Bioline, London, UK). Synthesis was carried out following manufacturer's protocol, starting with 0.5-1 µg total RNA, 50 ng random hexamers, and 10mM dNTPs per 20-µL reaction. The mixture was incubated at 65˚C for 10 min before being combined with 10 µL of master mix, which includes the reverse transcriptase

enzyme (200 U/μL). The reaction proceeded for 1 hour at 37˚C until the reverse transcriptase enzyme was inactivated at 70˚C for 15 min. Quantitative real-time PCR was performed using the Opticon 2 Real-Time Cycler (Bio-Rad, Hercules, CA) and SYBR Green supermix reagents (Bioline, London, UK). For relative expression of *imaA* and *ureA*, transcript levels were normalized to the levels of *groEL (Sharma et al., 2010)* in each sample. Transcripts were amplified with HP0289 For1.1 (5'-taacgatccaaaacgcttcc) and HP0289 Rev1.1 (5'-tcccttgaggcgagagtgatt*),* UreA F1 and UreA R1 (Janzon et al., 2009), and groEL F (JVO-529) and groEL R (JVO-5298) (Sharma et al., 2010). *Il8* (Nazarenko et al., 2002) and *TNF-*$\alpha$ (Yamaoka et al., 2002) expression levels from AGS cells were normalized to *18S* (Nazarenko et al., 2002) rRNA and KC levels from mouse tissues were normalized to *GAPDH (Eaton et al., 2006)*. All reactions were performed in triplicate and a melting curve analysis was used to ensure that a single product was amplified with each primer set. To validate RNA purity, no reverse transcriptase control reactions were also performed. *imaA* gene expression at low pH was determined from 4 independent experiments, statistics evaluated with the Mann-Whitney U-test. Relative expression of *Il8* was determined from 5 independent experiments, statistics evaluated by Student's t-test. All differences in gene expression were calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

**ELISA**. Enzyme-linked immunosorbent assays (ELISAs) for human IL-8 were performed using the Human IL-8 EASIA kit (Invitrogen). AGS cells were infected by either wild-type *H. pylori* or its isogenic mutant, *ΔimaA::cat*, at concentrations of 1 x $10^7$, 2 x $10^6$, and 1 x $10^5$ cells/mL and culture supernatant was preserved for ELISA at 4, 6, 12, and 24 hr post-infection.

**Proteinase K treatment of *H. pylori* cells**. Digestion of *H. pylori* outer membrane proteins with the extracellular protease, proteinase K, was conducted as described previously by Sabarth *et al*. (Sabarth et al., 2005). *H. pylori* cells grown for 48 hours on CHBA plates were collected with an inoculation loop and suspended in 1 mL phosphate buffered saline (PBS). Cells were centrifuged at 5,000 x g for 10 minutes, then resuspended in PBS at a concentration of 3 x $10^8$ cells/mL, based on the $OD_{600}$. Cells were treated with either 40 or 400 μg/mL proteinase K for 30 minutes at room temperature in 1X PBS. The reaction was halted with the addition of 5mM phenylmethylsulfonyl fluoride (PMSF); the cells were then washed twice in PBS. After a final centrifugation at 5,000 x g for 5 minutes, the cells were resuspended in PBS and then diluted into NuPAGE 4x sample buffer (Invitrogen, Carlsbad, CA) for subsequent Western analysis.

**Sarcosine preparation of *H. pylori* outer membrane.** The sarcosine-insoluble outer membrane fraction was prepared as described previously *(Baik et al., 2004)* with slight modifications. *H. pylori* wild-type strain SS1 and its isogenic

mutant, Δ*imaA::cat*, were grown on CHBA for 48 hours. To optimize the outer membrane yield, each respective strain was grown to confluent growth on two full CHBA plates, with all of the cells utilized for fractionation. Cells were collected using a sterile inoculation loop and suspended in 1 mL 20 mM Tris-HCL (pH 7.5) and collected by centrifugation (8,000 x g, 10 min, 4°C). The pellet was then resuspended in 1 mL 20 mM Tris-HCl containing a protease inhibitor (1mM PMSF) and a cell wall hydrolase (0.25 mg/mL lysozyme). The resuspended pellet was sonicated 9 times for 15 sec each time (Fisher sonicator, 80 amplitude) and unbroken bacteria were removed by centrifugation (6,000 x g, 10 min, 4°C). Total membranes were isolated by centrifugation for 45 min at 50,000 x g, 4°C. The supernatant containing the soluble fraction was removed and the total membrane pellet was washed once in PBS and resuspended in 1 mL sonication buffer containing 2.0% (w/v) sodium lauryl sarcosine, and incubated at room temperature for 30 min. The inner membrane fraction was separated by centrifugation (50,000 x g, 45 min, 4°C) and the pellet containing the outer membrane was resuspended in sarcosine for an additional treatment to optimize outer membrane purity. The resuspended pellet was incubated at room temperature for another 30 min and then centrifuged (50,000 x g, 45 min, 4°C). The final pellet was resuspended in 1 mL 20 mM Tris-HCl and stored at -20°C.

**Western blotting and ImaA antibody creation**. The α-ImaA-1 polyclonal antibody was prepared in rabbits using a 19 amino acid peptide (amino acids 2065-2084) from the passenger domain of the ImaA protein (Open Biosystems, Huntsville, AL). The antibody specifically recognizes ImaA, as well as several unidentified nonspecific proteins that were significantly different in size.

Proteins for Western analysis were resuspended in 4X NuPAGE sample buffer (Invitrogen, Carlsbad, CA) with 0.025% 2-mercaptoethanol and heated at 70˚C for 15 min. Samples were separated on 3-8% NuPAGE Tris-Acetate gels for 60 min at 150 volts. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) with the Bio-Rad Semi-Dry Transfer Cell for 35 min at 16 volts. Membranes were then incubated with a 1:300 dilution of α-ImaA-1 antibody or a 1:2000 dilution of α-GST-TlpA22 antibody (Williams et al., 2007) for ~18 hr at 4˚C. For visualization, blots were incubated with goat α-rabbit antibody conjugated to horseradish peroxidase (Santa Cruz Biotech) at a dilution of 1:2,000 for 1 hr, followed by incubation with luminol, *p*-coumoric acid, and hydrogen peroxide. Luminescent blots were visualized by exposure to Ultra Cruz autoradiography film (Santa Cruz Biotech).

## 2.4 Results

***H. pylori* ImaA is predicted to be an autotransporter**. To examine the functional significance of the uncharacterized *H. pylori* genes that were upregulated in the stomach (Castillo et al., 2008b), we employed *in silico*

sequence analyses of each protein to identify signature domains. The gene predicted to be regulated by promoter P*ivi77*, *HP0289*, is predicted to encode a protein with all of the typical characteristics of an autotransporter (Fig. 1). Based on experiments described below, we designate HP0289 as ImaA (ImmunoModulating Autotransporter). *imaA* is transcribed monocistronically (Sharma et al., 2010) and the encoded protein is 2,893 amino acids in length with a calculated molecular mass of 311 kiloDaltons. The SignalP algorithm predicts that ImaA bears an N-terminal signal peptide with a signal peptidase cleavage site between positions 42 and 43 (VYA-NN) (Bendtsen et al., 2004). ImaA also carries the highly conserved C-terminal beta-barrel autotransporter domain, readily identified by domain finding software such as the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998). The passenger domain of ImaA, which likely confers the effector function of the protein, is 2,581 amino acids in length. This region has little conservation with any other previously characterized protein, with the exception of three "VacA2" regions that are 59 amino acids long. In the initial sequencing of *H. pylori*, HP0289 was annotated as toxin-like protein, with small regions of similarity noted between VacA, HP0289 and two other *H. pylori* autotransporters (*HP0610* and *HP0922*) (Tomb et al., 1997). The main block of homology is at the C-terminal autotransporter domain. There were, however, additional regions of low similarity (26-31%) at several spots in the central passenger domain called VacA2 regions, although these regions do not correspond to a functional portion

of VacA. Based on this homology, ImaA has been annotated as a VacA paralog. Our analysis, however, suggests that ImaA is an autotransporter that is not specifically related to VacA.

**ImaA promotes mouse stomach colonization**. RIVET studies in several organisms have identified pathogen colonization and virulence factors (Bron et al., 2004; Camilli and Mekalanos, 1995; Castillo et al., 2008b; Lee et al., 2001; Lowe et al., 1998; Saviola et al., 2003; Veal-Carr and Stibitz, 2005). We thus first examined whether *imaA* was required for mouse stomach colonization, a model routinely utilized in *H. pylori* studies. We generated an *imaA* mutant, *ΔimaA::cat*, in which nearly the entire *imaA* open reading frame (ORF) is replaced with the chloramphenicol acetyltransferase (*cat*) gene, and thus is a null allele. This allele was used to replace the endogenous *imaA* locus in the *H. pylori* strain LSH100 (Lowenthal et al., 2009). LSH100 is a mouse-adapted derivative of *H. pylori* G27 (Covacci et al., 1993). LSH100 arose from mouse adaptation of strain G27 to create strain NSH57, followed by repair of a mutation in the *fliM* locus to the original wild-type sequence to yield LSH100 (Lowenthal et al., 2009). We used LSH100 both because it and the original RIVET strain, mG27, are derived from the same parent but LSH100 infects mice more consistently. Because of their high genetic relatedness, we felt that experiments with both mG27 and LSH100 would not have revealed significantly different conclusions. Unless noted otherwise, all subsequent *in vivo* and *in vitro H. pylori* infections, localization, and

gene expression experiments were done with strain LSH100 to maintain consistency with our murine infecting strain. Male FVB/N mice were infected with wild-type LSH100 or its isogenic *ΔimaA::cat* mutant for two to three weeks. These time points have been widely used in other *H. pylori* murine colonization studies and have been shown to accurately reflect colonization levels at longer infection time points (Baldwin et al., 2007; Chevalier et al., 1999; Harris et al., 2003; Marchetti and Rappuoli, 2002; Pappo et al., 1999; Styer et al., 2010; Terry et al., 2005; Watanabe et al., 2010). While the *imaA* mutant was able to sustain an infection for these durations, the output CFU/gram stomach was significantly lower than that obtained from wild-type infections (Fig. 2A). To address whether the colonization defect of the *ΔimaA::cat* strain would be altered by the presence of wild-type *H. pylori*, we carried out co-infection experiments with equal concentrations of wild-type and *ΔimaA::cat* strains. Two weeks post-infection, we determined the ratio of CFU/g stomach for mutant:wild-type bacteria and calculated a competitive index. In all infections, the *imaA* mutant was greatly outcompeted by the wild-type bacteria (Fig. 2B). These results demonstrate that *H. pylori* requires ImaA to reach wild-type gastric colonization levels. We did not complement the *imaA::cat* mutant, because *imaA* is over 8000 basepairs in length and therefore would be readily targeted by *H. pylori*'s extensively developed restriction modification system, which comprises over 4% of the genome (Lin et al., 2001). Despite advances in methods to circumvent the *H.*

*pylori* restriction modification system, gene complementation remains one of the most difficult endeavors in *H. pylori* molecular genetics (Donahue et al., 2000).

ImaA has been shown to be important in other *H. pylori* strains. Specifically, an *imaA* (HP0289) transposon mutant in *H. pylori* strain G1.1 was outcompeted by wild type for gerbil colonization (Kavermann et al., 2003). To expand this analysis, we checked whether a third strain, SS1, would similarly need *imaA* for stomach colonization. We found, surprisingly, that *H. pylori* SS1 Δ*imaA::cat* colonized as well as wild type in both single-strain and competition infections (Fig. 2C and D). This strain difference is not surprising given that others have observed that there is extensive variability in whether particular *H. pylori* proteins are essential for mouse colonization (Baldwin et al., 2007). Thus these results suggest that ImaA is needed by some strains and that ImaA's importance is possibly dependent on each strain's unique interactions with the host.

**ImaA is secreted to the outer membrane of the cell**. We next wanted to confirm the *in silico* prediction that ImaA is exported to the outer membrane of the cell. We first generated a peptide antibody directed at the passenger domain of the protein that accurately detects mature ImaA protein from whole cell lysates (Fig. 3). We then used proteinase K digestion to assess whether ImaA was surface localized in LSH100 and mG27. Proteinase K does not diffuse across the outer membrane of Gram-negative bacteria and thus cleaves only proteins

residing on the bacterial surface. This approach has been widely used to assess autotransporter surface localization (Dautin et al., 2007; Lenz et al., 2011; Van Gerven et al., 2009). As predicted for a surface-localized protein, ImaA is digested by the protease and is thus surface exposed in strain LSH100 (Fig. 3A) as well as mG27 (not shown). To demonstrate that proteinase K treatments were not breaching the membrane and degrading internal proteins, we determined that the inner-membrane localized TlpABC chemoreceptors were not digested (Fig. 3A). To further validate these findings and to assess whether ImaA localization to the outer membrane is conserved in strain SS1, we performed subcellular fractionation experiments with the detergent sarcosine, which selectively solubilizes the inner membrane and thus enables separation from the outer membrane. Western blot analysis demonstrated that ImaA is in the outer membrane fraction, as well as somewhat in the inner membrane (Fig. 3B). A control blot, using antibody that detects the inner membrane protein showed that the fractions were fairly pure (Fig. 3B). Inner membrane-localized ImaA may represent protein that is transiting to the outer membrane or an indication of incomplete membrane separation. Other studies have detected autotransporter proteins in both inner and outer membrane fractions (Ashgar et al., 2007). Of note, these analyses were performed in three different *H. pylori* strain backgrounds, providing strong evidence that ImaA is translocated to the outer membrane. In addition, we did not detect any ImaA in concentrated

supernatant from *H. pylori* cultures (data not shown), suggesting that ImaA stays associated with the outer membrane.

**ImaA is a member of the acid-responsive ArsRS regulon**. The RIVET studies showed that *imaA* transcription is upregulated within the host environment (Castillo et al., 2008b). Therefore, we wanted to identify the signal responsible for inducing *imaA in vivo*. In a recently published *H. pylori* whole transcriptome paper, Sharma *et al.* demonstrated that *imaA/hp0289* is induced 10-fold at low pH (Sharma et al., 2010). Acidic pH is the key environmental signal for activating the *H. pylori* two-component regulatory system ArsRS (acid-responsive signaling) (Pflock et al., 2006a; Pflock et al., 2006b). We thus examined whether *imaA* is a member of the ArsRS regulon, by creating a null mutant for the histidine kinase, ArsS, and observing *imaA* gene expression through quantitative real-time PCR (qRT-PCR) under neutral and acidic conditions. The response regulator, ArsR, is an essential gene so the ArsS mutant serves as the ArsRS representative (Beier and Frank, 2000). We employed the housekeeping gene, *groEL*, for normalization, as used in previous work (Sharma et al., 2010). After two hours of acidic pH exposure, *imaA* expression increased ~10-fold in the wild-type background but not in the *arsS* mutant (Fig. 4A). Furthermore, *imaA* expression is depressed in the *arsS* deletion strain, even at neutral pH (Fig. 4A). These results suggest that *imaA* is a member of the ArsRS regulon. We additionally compared the expression of *imaA* to that of a known ArsRS-

regulated acid-induced gene, *ureA* (Pflock et al., 2005). We found that *ureA* gene expression in wild-type *H. pylori* was induced ~14-fold in acid over expression at neutral pH in a partially *arsS*-dependent manner, similar to the findings of Pflock *et al.* (Fig. 4B) (Pflock et al., 2004). At neutral pH, *ureA* required ArsS for expression more so than *imaA* did. These results thus show that the experimental conditions affect *arsS*-regulon members as expected. Furthermore, our data support that *imaA* is a member of the ArsRS regulon, due to the ArsS-dependent increased expression in acid, but that it is not regulated identically to *ureA*.

We next examined whether ImaA protein levels were affected by pH. For these experiments, we grew *H. pylori* cultures at pH=5 and then sampled them after 2, 5, or 8 hours. Despite observing a 10-fold increase in *imaA* mRNA (Fig. 4A), we did not detect any elevation in ImaA protein levels at low pH (Fig. 4C). We did observe, however, that ImaA protein expression was *arsS*-dependent at low pH (Fig. 4D). This observation suggested that *H. pylori* relies on ArsRS to maintain ImaA expression under acidic conditions. All together, these results thus show that acid induces *imaA* transcription, and that ArsRS is needed to maintain both *imaA* transcript and ImaA protein levels at acidic pH.

**Loss of *imaA* creates *H. pylori* strains that induce elevated *Il8* transcription**. We next examined whether loss of *imaA* influenced levels of inflammatory mediators in mouse and *in vitro* cell culture models. Colonization of *H. pylori* in

the stomach results in the release of the chemoattractant IL-8 in humans or its analog, KC, in mice. IL-8 stimulates the infiltration of neutrophils into the gastric mucosa, leading to chronic inflammation (Huang et al., 1995; Obonyo et al., 2002), therefore *Il8* transcription levels are often used as a readout for a proinflammatory response (Lamb et al., 2009). To examine ImaA's influence on *Il8*/*KC* levels, we performed *KC* or *Il8* qRT-PCR on mouse tissue or AGS gastric epithelial cells infected with *H. pylori*. In mice infected with *H. pylori* LSH100 wild-type or *ΔimaA::cat* strains for three weeks, a time point used by others for similar analyses (Algood et al., 2007), we found very low levels of *KC* overall. There was, however, elevated *KC* in mice infected with *H. pylori ΔimaA::cat* as compared to those infected with wild-type, but not statistically significant (Fig. 5A). While the difference in *KC* levels between uninfected mice and mice infected with the *ΔimaA::cat* mutant was significant, there was minimal difference in KC levels between uninfected mice infected and mice infected with wild-type *H. pylori* (Fig. 5A). These experiments thus suggest that ImaA's normal function is to decrease *KC* levels. To confirm this finding, we employed the well-established AGS human gastric cell model to investigate *Il8* levels. AGS cells infected with wild-type *H. pylori* for two hours revealed a ~75-fold induction in *Il8* transcription in AGS cells compared to the uninfected, while the *ΔimaA::cat* mutant strain generated a significantly greater ~189-fold induction in *Il8* levels (Fig. 5B). To confirm that *Il8* transcript levels seen in *ΔimaA::cat* mutant infections translated to increased levels of the protein product, we next

measured secreted IL-8 levels with an ELISA. *Il8* transcript levels were measured from AGS cells that were infected with *H. pylori* at an multiplicity of infection (MOI) of 100, however, when we measured IL-8 protein levels at this MOI, we saw no difference in cytokine production between *ΔimaA::cat* mutant and wild-type infections (data not shown). We reasoned that the amount of *H. pylori* might be saturating the IL-8 protein production, so lowered the MOI. At an MOI of one, we witnessed elevated cytokine levels in the *ΔimaA::cat* mutant as compared to wild-type infections at 4, 6, and 12 hours post infection, with hour 12 providing a significant difference in IL-8 between wild-type and *ΔimaA::cat* mutant infections (Fig 5C). This data suggests that ImaA serves to modulate the amount of IL-8 that is generated during infection.

We next examined whether the inflammation phenotype associated with loss of *imaA* in LSH100 was common to other CagA+ *H. pylori* strains, so we created an *ΔimaA::cat* mutant in the widely used CagA+ strain, 26695, and performed AGS cell infections. Similar to the response we witnessed with LSH100, the 26695 *ΔimaA::cat* mutant infections induced higher levels of *Il8* transcription overall when compared to that of the wild-type infections (Fig 5D). Wild-type 26695 induced an ~106-fold increase in *Il8* transcription compared to uninfected AGS cells, while the 26695 *ΔimaA::cat* mutant generated a significantly greater ~274-fold increase in *Il8* transcription.

In addition to IL-8, *H. pylori* infection promotes the production of numerous proinflammatory cytokines. To test whether the *ΔimaA::cat* mutant induces elevated concentrations of other immune mediators, we measured transcript levels of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α). TNF-α is associated with an increased severity and distribution of gastritis in infected individuals (Shibata et al., 1999). We found that AGS cells infected with wild-type *H. pylori* produced an~7-fold increase in *TNF-α* transcript levels compared to uninfected cells, while AGS cells infected with the *ΔimaA::cat* mutant displayed a significantly greater 19-fold increase in *TNF-α* levels (Fig. 5E). Taken together, these results suggest that in the absence of ImaA, there is a stronger induction of the mammalian proinflammatory pathway. When mouse tissue was examined for *TNF-α*, there was no difference in the levels between the uninfected mice and either wild-type or *ΔimaA::cat* infected mice (data not shown). This outcome is not entirely unexpected, as *H. pylori* induced TNF-α levels appear to be much smaller than *H. pylori* induced IL-8 levels (Yamaoka et al., 1997).

**The *cag*PAI type four-secretion system underlies the enhanced IL-8 production seen in *imaA* mutant infections.** *H. pylori* is known to control IL-8 levels by action of the type IV secretion system (T4SS) encoded by the *cag*PAI. To establish whether the increase in inflammation we witnessed in the

*ΔimaA::cat* infections was dependent on the activity of the *cag*PAI type IV secretion system (cag-T4SS), we created the double mutant, *ΔimaA::cat ΔcagE::kan,* and examined *Il8* levels using the same *in vitro* infection model. *cagE* encodes a putative ATPase and is required for IL-8 induction (Day et al., 2000). *cagE* mutants had dramatically decreased levels of *Il8* transcription (Fig 5B), as predicted from other studies with G27-related strains (Bach et al., 2002). The *cagE* effect was dominant over the *imaA Il8*-upregulation, as both a *ΔcagE* single mutant and the *ΔcagE ΔimaA::cat* double mutant induced *Il8* levels that were not significantly above that of the uninfected AGS cells (Fig. 5B). This outcome suggests that the immunomodulatory activity of ImaA requires *cag*PAI function.

## 2.5 Discussion

*H. pylori* relies on multiple outer membrane proteins to chronically persist within the gastric environment (Ilver et al., 1998b; Mahdavi et al., 2002; Senkovich et al., 2011). In this study, we demonstrate that a previously uncharacterized *H. pylori* autotransporter, HP0289 or ImaA, is important for host colonization and dampens the inflammatory response. We furthermore show that *imaA* is under the control of the acid-responsive ArsRS two-component regulatory system. Our findings thus support the hypothesis that the *in vivo*-induced gene, *imaA*, contributes to *H. pylori* pathogenesis and that the

protein product normally decreases the inflammatory response brought about by the action of the *cag*PAI.

*H. pylori* must adapt to the changing landscape of the stomach during the course of a chronic infection (Blaser, 1997). One way the bacterium can accomplish this adaptation is through tailoring the expression of virulence genes to particular conditions. We found that *imaA* transcription is under the control of the ArsRS regulon. Whole-genome transcriptional profiling of *H. pylori* strains cultured at low pH identified more than 100 genes that were differentially expressed in an *ars*-dependent manner, although *imaA* was not one of them (Pflock et al., 2006b). Others similarly have found that there is some variability in *H. pylori* gene expression at low pH. For example, α-carbonic anhydrase (HP1186) expression has been shown to be repressed in some cases and upregulated in others at low pH (Merrell et al., 2003; Wen et al., 2007). We found that *imaA* expression is induced under acidic conditions, similar to the findings of Sharma and colleagues (Sharma et al., 2010) and furthermore that transcriptional control of *imaA* is mediated to a significant degree by the ArsRS two-component system. While *imaA* mRNA was greatly increased at low pH, we did not detect a corresponding increase in ImaA protein levels at low pH. This paradox of increased transcript levels not directly translating to increases in protein levels is not unprecedented in studies of the *H. pylori* ArsRS system. Loh *et al.* recently examined the proteomes of wild-type *H. pylori* and an isogenic *arsS* mutant under neutral and acidic conditions and compared them to the

previously established transcriptional profiles for each strain under these conditions (Loh et al., 2010). They found very few acid-responsive protein changes in either strain, as only 15 proteins were differentially expressed in total. *imaA* likely belongs to the group of more than 100 genes that show altered transcriptional profiles at low pH, but do not exhibit clear changes in protein levels. Loh *et al.* attributed the discrepancy between transcript and protein levels to posttranscriptional regulatory processes, which may dilute alterations in acid-induced protein expression (Loh et al., 2010). Despite discrepancies in protein levels, it is clear that ArsS is important for expression of *imaA* at low pH, demonstrating that *imaA* is under the control of the ArsRS regulon.

We show here that ImaA is important for mouse colonization. ImaA was previously found to be crucial for colonization in a different animal model, the gerbil, as part of a global transposon mutagenesis screen done in strain G1.1 that was evaluated in a competition model (Kavermann et al., 2003). Thus these two studies demonstrate that ImaA's presence is necessary to achieve wild-type gastric colonization in multiple animal models. Conversely, a third analyzed strain, SS1, tolerates the loss of *imaA* in murine infections. Of note, SS1 does express ImaA (Fig. 3). There are many differences between SS1 and LSH100/G27, the most notable of which is that SS1 has an inactive *cag*PAI T4SS while that of G27 and its mouse-selected variants is active (Baldwin et al., 2007). G1.1, like SS1 does not secrete CagA (Eaton et al., 2001; Israel et al., 2001). Thus ImaA appears to be important for colonization in both Cag+ and Cag- strains, and

furthermore ImaA may have roles in the host that are not limited to affecting the *cag*PAI, although those remain to be determined.

A rodent-colonization defect is unusual with *H. pylori* outer membrane proteins. The *H. pylori* adhesin proteins BabAB or SabA do not display any colonization defects, and AlpAB exhibits a defect that is statistically insignificant (Amieva and El-Omar, 2008; Senkovich et al., 2011). In fact, the only characterized *H. pylori* outer membrane protein to display a rodent-colonization defect is the autotransporter, VacA (Salama et al., 2001). VacA, like ImaA, possesses immunomodulatory activity albeit through suppression of T-cell activation (Boncristiano et al., 2003). However, unlike VacA, ImaA appears to act while cell-associated, as we were unable to identify an ImaA secreted peptide in cell culture supernatant. Interestingly, it was recently shown that mutants deficient for the laminin binding proteins AlpA and AlpB caused greater levels of inflammation in gerbil infections (Senkovich et al., 2011). This outcome, however, was not attributed to any inherent AlpAB immunomodulatory properties but rather the mutant's inability to intimately adhere to gastric epithelial cells and express other immunosuppressive proteins. We were unable to detect any *in vitro* adherence ability associated with ImaA (data not shown), so believe it operates in a different manner from AlpAB.

A central component of *H. pylori*-induced inflammation is delivery of proinflammatory molecules into host cells via the *cag*PAI T4SS. We have demonstrated that the Δ*imaA*::*cat* mutant evokes a significant increase in

expression of the proinflammatory cytokines, IL-8, and TNF-α, compared to AGS cell infections with wild-type *H. pylori*. While the bulk of the inflammation experiments were done with the *H. pylori* G27 derivative, LSH100, we also found that ImaA had a similar affect in strain 26695, suggesting ImaA function is conserved. We furthermore found that the *imaA*-mutant inflammation phenotype requires a functional *cag*PAI-T4SS. This outcome suggests that ImaA acts to diminish the normal *cag*PAI-mediated induction of proinflammatory cytokines. The *cag*PAI-T4SS aids the delivery of two effectors capable of inducing IL-8 expression in epithelial cells, CagA and peptidoglycan (Brandt et al., 2005; Viala et al., 2004). While we do not know how ImaA interacts with the *cag*PAI, it is not unprecedented for *H. pylori* outer membrane proteins to influence *cagPAI*-T4SS activity. The ABO/Lewis b ($Le^b$) blood group antigen binding protein, BabA, facilitates interactions between the *cag*PAI-T4SS machinery and the host cell. Strains null for *babA* induce reduced levels of IL-8 in infected host cells, opposite to what we see in *imaA* mutant infections, (Ishijima et al., 2011). Interestingly, the Protein Homology/analogY Recognition Engine (PHYRE) predicts that ImaA has homology to the bacterial integrin binding protein, invasin ($E = 1.8 \times 10^{-4}$). Components of the *cag*PAI-T4SS pilus bind directly to the $\alpha_5\beta_1$ intregrin receptor to facilitate secretion of CagA and peptidoglycan into the host cell cytoplasm (Kwok et al., 2007). Thus, it is possible that ImaA and the *cag*PAI-T4SS machinery compete for integrin binding and that in the absence of ImaA, there is

increased T4SS binding and therefore, enhanced effector molecule secretion into host cells.

In conclusion, we have determined that the *H. pylori* host-induced gene, *HP0289*, encodes a surface-localized autotransporter protein that we designate as ImaA. ImaA promotes colonization of the host stomach and diminishes the inflammatory response. Specifically, ImaA decreases the amount of *Il8* transcript generated by the *H. pylori cag*PAI. *imaA* expression is furthermore controlled by the acid-sensing two-component regulatory system, ArsRS in response to acid. These findings support the notion that *in vivo* induced genes play a central role in *H. pylori* pathogenesis. They furthermore suggest that *H. pylor*i has sophisticated mechanisms to modulate the host inflammatory response, by controlling expression of a protein that decreases bacterially-triggered inflammatory gene expression.

## 2.6 Acknowledgements

We thank David McGee and Kylie Nolan for providing the SS1 *cagE::kan* mutant. We are also grateful to Fitnat Yildiz and Annah Rolig for critical reading of the manuscript. This work was supported by a Research Scholar Grant (RSG-05-249-01-MBC) from the American Cancer Society (to K.M.O.) and also by Grant Number AI050000 (to K.M.O.) from the National Institutes of Allergy and Infectious Disease (NIAID) at the National Institutes of Health. Its contents are

solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

## 2.7 Figures

**Figure 1**. ImaA contains hallmarks of autotransporter proteins. Diagram shows the highly conserved domains in ImaA as predicted by SignalP 3.0 and SMART. Signal peptide probability = 1.00 and autotransporter E value = 5 x $10^{-7}$. Numbers below and above indicate amino acid positions of predicted domains within ImaA.

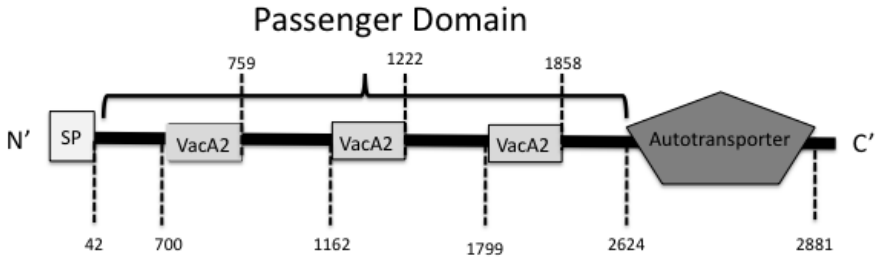



**Figure 2**. (A) The LSH100 *ΔimaA::cat H. pylori* mutant colonizes mice at levels significantly lower than that of the LSH100 wild-type *H. pylori* parent. Oral single-strain infection studies with wild-type *H. pylori* or *ΔimaA::cat* were carried out using male FVB/N mice. Infections persisted for 2 or 3-weeks. Single strain infections were conducted with an n=7 for the wild-type strain and an n=10 for *ΔimaA::cat*. Each circle represents one infected mouse, derived from independent two week (open circles) or three week (filled circles) infections; *P* =

0.01, Student's t test. (B) The LSH100 *ΔimaA::cat H. pylori* mutant is outcompeted by the wild-type strain in a co-infection colonization assay. Each point represents the competitive index for one mouse stomach, n = 8, two independent infections. The competitive index is a ratio of the (mutant output/wild-type output):(mutant input/wild-type input). $P < 0.001$, Student's t test when compared to a hypothetical strain with no defect (CI=1). (C) The SS1 *ΔimaA::cat H. pylori* mutant colonizes mice at levels that are comparable to that of SS1 wild-type *H. pylori*. These infections persisted for 2 weeks, with an n=4 for the wild-type strain and n=6 for *ΔimaA::cat*. (D) The SS1 *ΔimaA::cat* is not outcompeted for mouse colonization in a co-infection with wild-type *H. pylori*, n = 8, two independent infections.

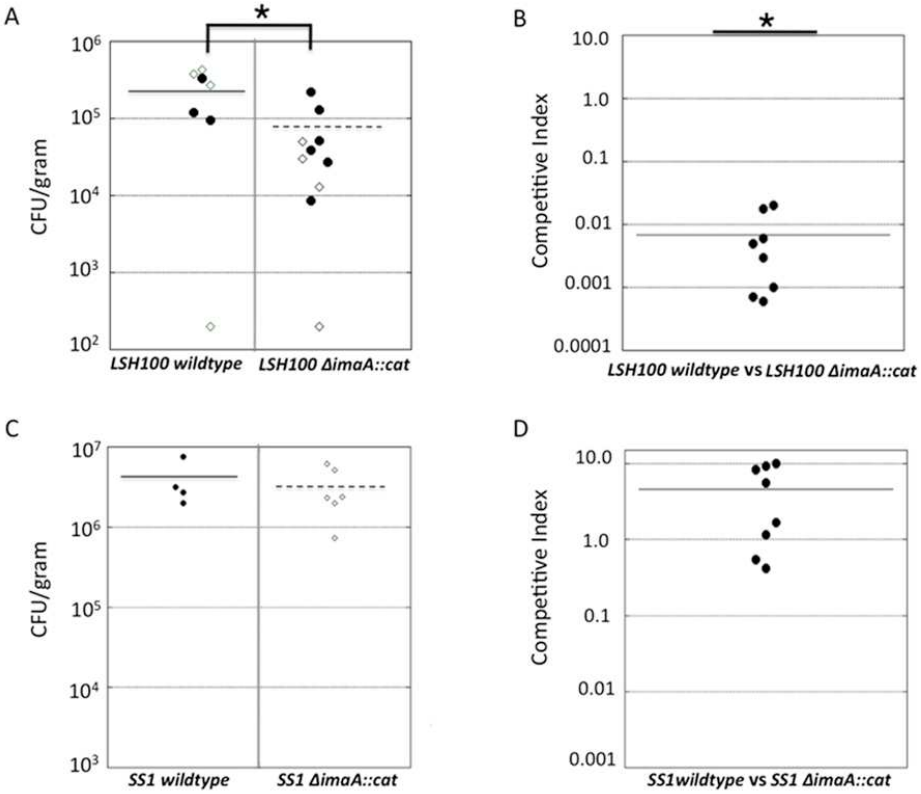


**Figure 3**. ImaA localizes to the outer membrane. (A) Whole cells from *H. pylori* strain LSH100 were treated with different concentrations of proteinase K (40 or 400 mg $ml^{-1}$) or a no proteinase K control. The top panels show blots probed with α-ImaA-1, while the bottom panels are probed with α-GST-TlpA22 antibody, which recognizes inner membrane chemoreceptors (Williams et al., 2007). Similar results were obtained with strain mG27 (not shown). (B) Sarcosine-insoluble outer membrane (OM) fractions and sarcosine-soluble inner membrane (IM) fractions were obtained from wild-type and *ΔimaA::cat H. pylori* SS1 cells, and then probed with α-ImaA-1. In both panels, arrows denote full length ImaA, and arrowheads denote nonspecific proteins recognized by the anti-ImaA serum.

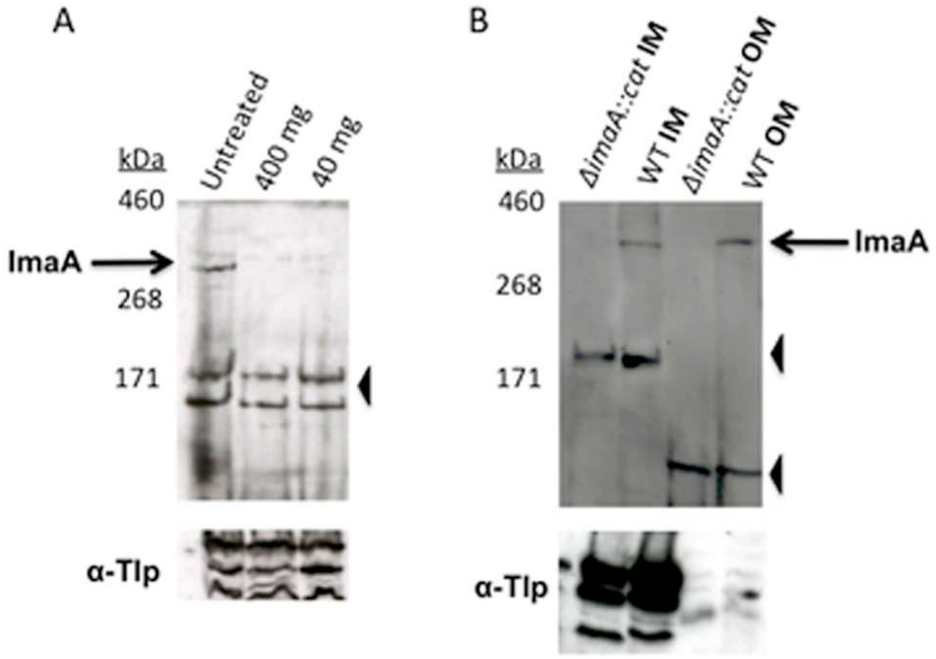


**Figure 4**. The ArsRS two-component regulatory system influences *imaA* transcription. (A) Quantitative real-time PCR (qRT-PCR) was performed using cDNA generated from *H. pylori* strains LSH100 or its isogenic mutant Δ*arsS*::*cat* that were exposed to either neutral or acidic BB10 for 2 hours. Expression levels of WT (pH=5) and Δ*arsS*::*cat* (pH=5) are relative to WT (pH=7) from four independent biological replicates, each performed in triplicate, and normalized to the housekeeping gene *groEL*. Asterisks (*) denote significantly higher or lower *imaA* expression ($P < 0.05$, Wilcoxon rank sum test). (B) The *ureA* gene responds to acid and depends on the ArsRS regulatory system for expression. qRT-PCR was performed with the same cDNA that was used in the *imaA* transcription analysis, four independent biological replicates, each performed in triplicate. $P < 0.05$, Wilcoxon rank sum test. (C) Western blots with the α-ImaA-1 antibody showing ImaA expression at multiple time points under both neutral and acidic conditions in strains LSH100 or in the mutant Δ*arsS*::*cat*. This data is representative of three independent time courses.

**Figure 4**.

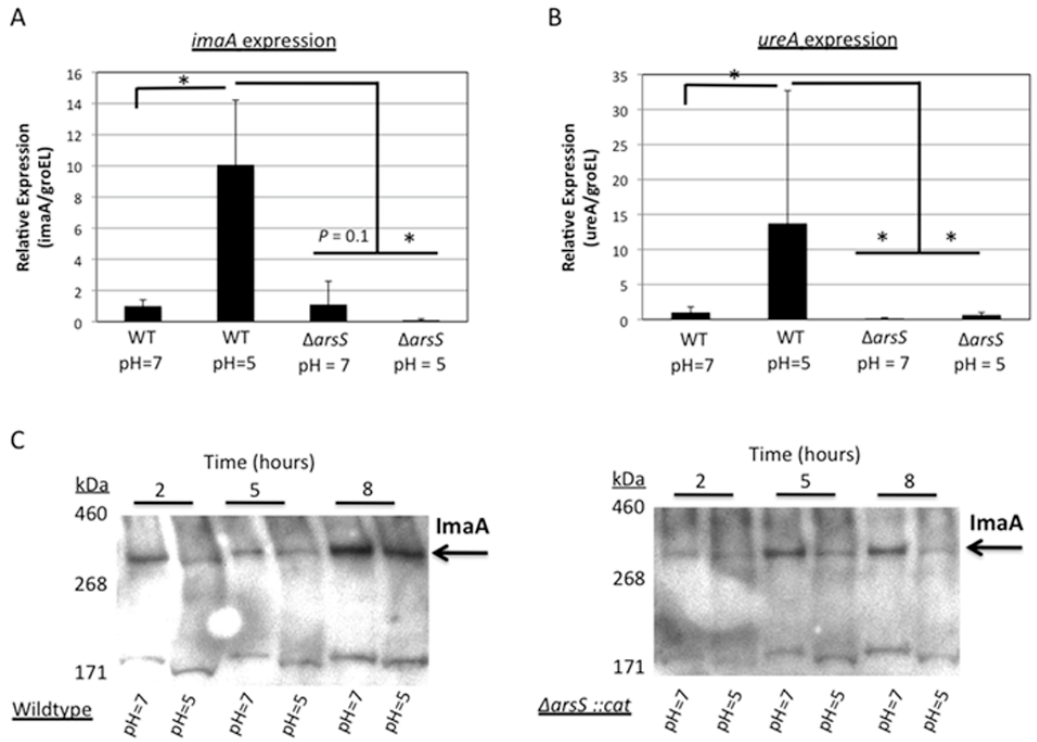
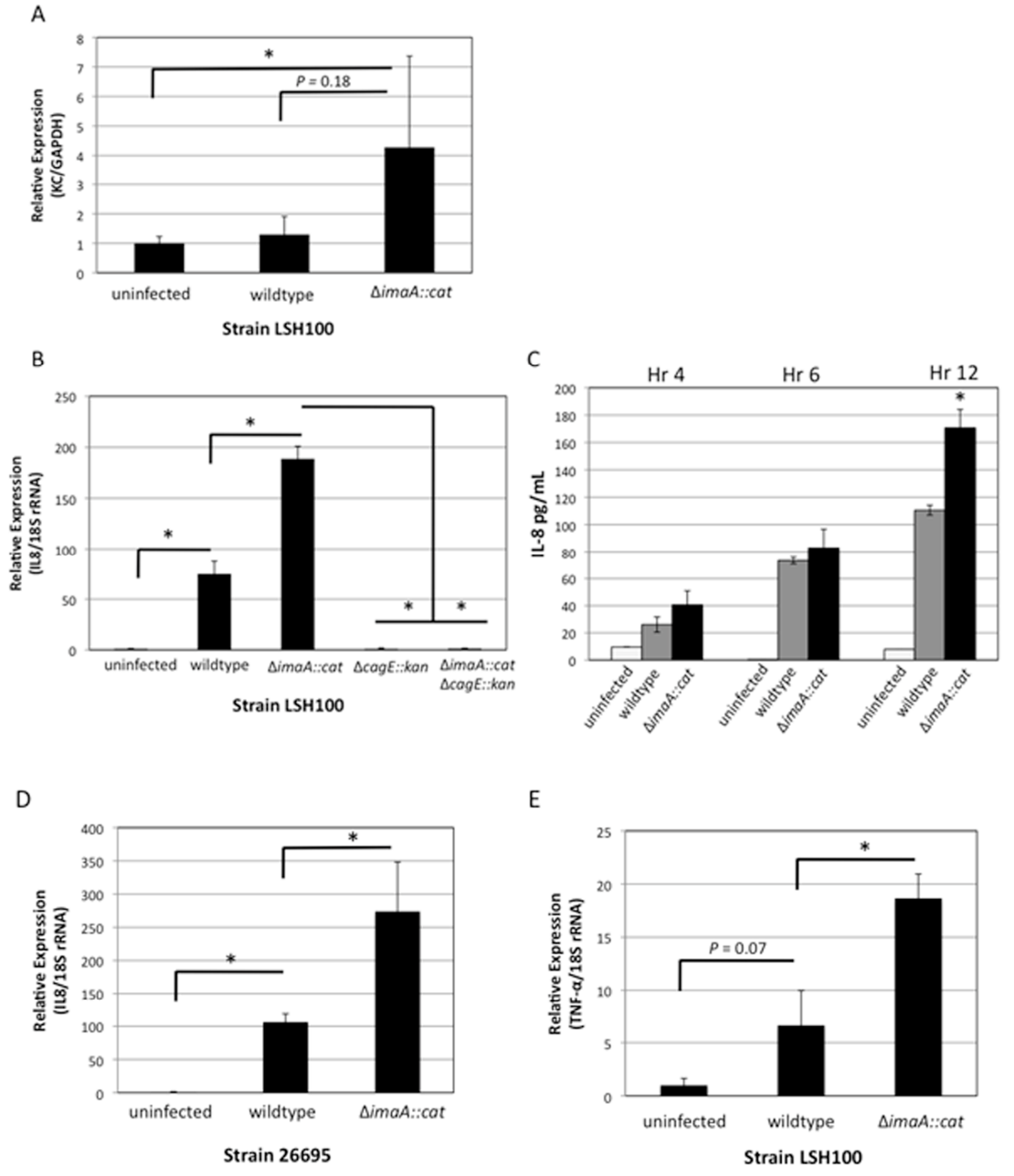



**Figure 5**. *KC*/*Il8* levels are significantly elevated in *Δima::cat* mouse and AGS cell infections, respectively. (A) Male FVB/N mice were infected with either wild-type LSH100 or its isogenic mutant, *ΔimaA::cat* for 3 weeks. Quantitative RT-PCR (qRT-PCR) was performed on whole gastric tissue to analyze the expression of *KC* using primers as in Yamaoka *et al*, (2002). Mouse samples are the same as in Fig. 2A, and include 5 mice infected with wild type, 8 with *ΔimaA::cat*, and 6 uninfected mice. There was a significant difference in *KC* between uninfected mice and *ΔimaA::cat* infected mice, $P < 0.05$, Student's t test. (B) *Il8* transcript

levels are elevated in AGS cells infected with the *ΔimaA::cat* mutant. For transcript analysis, AGS cells were infected with either wild-type LSH100 or its isogenic single mutants, *ΔimaA::cat*, *cagE::kan,* or the double mutant *ΔimaA::cat cagE::kan* at an MOI=100. qRT-PCR was performed to analyze the expression of interleukin-8 (*Il8*) using primers as in Nazarenko *et al.* (2002) after 2 hours of infection. This data represents 5 independent infections (biological replicates) with reactions done in triplicate. All expression differences were calculated with the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) $P < 0.01$, Student's t test, for AGS cell infections. (C) ELISAs for IL-8 levels were conducted on culture media taken from AGS cells infected with either wild-type or *ΔimaA::cat* mutant *H. pylori* (MOI=1) at the following time points: 4, 6, and 12 hours post-infection. Data shows two biological replicates, each done with two technical replicates $P < 0.03$, Wilcoxon rank sum test. (D) *Il8* expression levels in AGS cells infected with either wild-type 26695 or its isogenic mutant *ΔimaA::cat*. Data represents 5 independent infections with reactions done in triplicate, expression differences analyzed with $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). $P = 0.01$, Student's t test. (E) Analysis of *TNF-*$\alpha$ transcript levels in AGS cells infected with wild-type LSH100 or its isogenic mutant *ΔimaA::cat.* Data represents 5 independent infections with reactions done in triplicate, expression differences analyzed with $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). $P < 0.05$, Student's t test.

**Figure 5.**


A
Relative Expression (KC/GAPDH)
P = 0.18
uninfected
wildtype
ΔimaA::cat
Strain LSH100
B
Relative Expression (IL8/18S rRNA)
uninfected
wildtype
ΔimaA::cat
ΔcagE::kan
ΔimaA::cat ΔcagE::kan
Strain LSH100
C
Hr 4
Hr 6
Hr 12
IL-8 pg/mL
uninfected
wildtype
ΔimaA::cat
D
Relative Expression (IL8/18S rRNA)
uninfected
wildtype
ΔimaA::cat
Strain 26695
E
Relative Expression (TNF-α/18S rRNA)
P = 0.07
uninfected
wildtype
ΔimaA::cat
Strain LSH100

**2.8 Table 1. Strains used in this study.**

| *H. pylori* strain or plasmid | Description | Reference |
|---|---|---|
| G27 | Wild type (NSH57 parent strain) | (Covacci et al., 1993) |
| NSH57 | Mouse adapted isolate of G27 | (Baldwin et al., 2007) |
| LSH100 | NSH57 with repaired *fliM* allele | (Lowenthal et al., 2009) |
| SS1 | Wild type | (Lee et al., 1997) |
| 26695 | Wild type | (Akopyants et al., 1995) |
| KO954 | *SS1 ΔimaA::cat* | This study |
| KO1370 | *LSH100 ΔimaA::cat* | This study |
| KO1371 | *LSH100 ΔarsS::cat* | This study |
| KO1163 | *SS1 cagE::kan* | This study. From David McGee. |
| KO1372 | *LSH100 cagE::kan* | This study |
| KO1373 | *LSH100 cagE::kan ΔimaA::cat* | This study |
| KO1374 | *26695 ΔimaA::cat* | This study |

## Chapter 3: The *Helicobacter pylori* autotransporter ImaA tempers the bacterium's interaction with $\alpha_5\beta_1$ integrin and promotes chronic infection

### 3.1 Abstract

The primary mechanism for *Helicobacter pylori* caused inflammation is through the delivery of the effector molecule, CagA, into host cells. We show here that an *H. pylori* mutant for the immunomodulatory protein, *imaA*, delivers greater amounts of CagA into host cells, compared to that of wild-type *H. pylori*. We go on to show that the *imaA* mutant induces elevated levels of inflammation, independently of CagA, as an *imaA cagA* double mutant causes more inflammation than a *cagA* single mutant. This result directed us to the *cag*PAI T4SS, the surface-exposed unit that delivers CagA into the host cell. Through flow cytometry experiments, we showed that *imaA* mutants bind higher levels of the host receptor for the *cag*PAI T4SS, α5β1 integrin. Finally, using the gerbil mode for infection, we discovered that ImaA is dispensable for the very initial stages of host colonization, but that the protein becomes essential very rapidly, as *imaA* mutants are cleared from the stomach within a week, post-infection.

### 3.2 Introduction

The human pathogen *Helicobacter pylori* colonizes the gastric mucosa of approximately half of the world's population. Infection with *H. pylori* is the primary risk factor for multiple gastric diseases, including ulcers and gastric adenocarcinoma (Herrera and Parsonnet, 2009; Montecucco and Rappuoli,

2001; Suerbaum and Michetti, 2002). Gastric adenocarcinoma is a disease that killed over 700,00 people worldwide in 2012 (World Health Organization). In the United States, an expected 22,220 people will be diagnosed with gastric cancer in 2014 (National Cancer Institute).

While environmental factors and personal genetics influence disease outcome, infections with *H. pylori* strains that carry the cytotoxin associated gene pathogenicity island (*cag*PAI), which encodes a functional type IV secretion system (T4SS), is the strongest risk factor for disease (Blaser and Berg, 2001). The *cag*PAI T4SS is a large multiprotein secretion system that binds to the host receptor $\alpha_5\beta_1$ integrin (Jiménez-Soto et al., 2009; Kwok et al., 2007; Shaffer et al., 2011). The *cag*PAI T4SS injects the pro-inflammatory effector protein CagA, as well as the pro-inflammatory molecule peptidoglycan, into host cells. Upon entry, a population of CagA molecules are phosphorylated by the host kinases, Src and Abl, while another remains in an unphosphorylated state (Backert et al., 2001; Kwok et al., 2007; Poppe et al., 2007). Once intracellular, CagA exerts a number of effects on the host cell that are both phosphorylation-dependent and independent. The phosphorylated form of CagA is known to induce cytoskeletal perturbations that disrupt cell morphology, while the unphosphorylated form of CagA activates the pro-inflammatory response, primarily through the production of the chemokine interleukin-8 (IL-8) (Oldani et al., 2009).

The major virulence factor that contributes to *H. pylori* caused disease is the *cag*PAI, yet the *H. pylori* genome is predicted to encode more than 30 outer

membrane proteins (OMPs), or approximately 4% of the bacteria's coding potential (Alm et al., 2000; Dossumbekova et al., 2006a). There is limited knowledge of how cell surface proteins, outside of the pathogenicity island, influence the delivery of CagA at the interface between host and pathogen. While the majority of OMPs are uncharacterized, a handful of these proteins have been shown to impact the function of the *cag*PAI T4SS, primarily in a synergistic manner. One such OMP, the Lewis B ($Le^b$) antigen binding protein, BabA, has been demonstrated to potentiate the activity of the *cag*PAI T4SS through enhanced host-cell binding at $Le^b$ antigens (Ishijima et al., 2011). Recently, it was shown that mutants for the *H. pylori* OMP, *hopQ*, have a reduced ability to both translocate CagA and induce production of IL-8 in host cells (Belogolova et al., 2013). Another *H. pylori* OMP, *oipA*, has been shown to be important for bacterial adherence and in some cases, the promotion of inflammation, although its mechanism remains unclear (Dossumbekova et al., 2006b; Yamaoka et al., 2000). A commonality between all of these described OMPs and their interactions with the *cag*PAI T4SS, is that they work to enhance the delivery of CagA and stimulate pro-inflammatory signaling pathways. Thus, there are no known *H. pylori* OMPs that work to reduce the activity of the *cag*PAI T4SS, in a form of self-control that could modulate the secretion of CagA and discourage the generation of an overwhelming inflammatory response. Bacteria driven pathways that work to temper the immune response have been described in great detail across a

number of pathogens, and there is little known about *H. pylori* mechanisms that work in this manner (Finlay and McFadden, 2006).

We have previously shown that mutants for the *H. pylori* OMP, *imaA*, induce greater levels of IL-8 in infected gastric epithelial cells, as compared to wild-type *H. pylori* (Sause et al., 2012). While a mechanism for this phenotype was not demonstrated, this was the first case of an *H. pylori* OMP that countered the induction of inflammation (Sause et al., 2012). The *imaA* transcript was also shown to be upregulated in the host and in a pH dependent manner, indicating that appropriate expression of the gene is important for pathogenesis (Castillo et al., 2008a; Sause et al., 2012). Mutants for *imaA* also exhibit colonization defects in mice and are vastly outcompeted by wild-type *H. pylori* during competition infections (Sause et al., 2012). Thus, we had shown that ImaA is important for pathogenesis and controlling inflammation, but the mode of action for this activity remained elusive.

Multiple *cagPAI*-encoded proteins decorate the exterior of the T4SS pilus and have been shown to interact directly with $\alpha_5\beta_1$ integrin to facilitate the delivery of CagA (Jiménez-Soto et al., 2009; Kwok et al., 2007; Wiedemann et al., 2012). In this work, we demonstrate that in the absence of *imaA*, there is enhanced binding of *H. pylori* cells to $\alpha_5\beta_1$ integrin in a *cag*PAI-*independent* manner. This heightened binding facilitates the delivery of greater numbers of CagA molecules into the host cell and sets off an elevated inflammatory response.

## 3.2 Methods

**Bacterial strains and growth conditions.** *H. pylori* strain LSH100, a mouse-adapted descendent of the clinical isolate G27 (Covacci et al., 1993; Lowenthal et al., 2009), was used for AGS cell infections and $\alpha_5\beta_1$ integrin assays. Strain 7.13 (Franco et al., 2005), a gerbil passaged descendent of clinical isolate B-128 was used for Mongolian gerbil infections. *H. pylori* strains were maintained on Columbia blood agar base (Fisher) supplemented with 5% defibrinated horse blood (Hemostat Laboratories, Dixon, CA), 0.2% (wt/vol) β-cyclodextrin plus 5 μg/ml trimethoprim, 8 μg/ml amphotericin B, 50 μg/ml cycloheximide, 10 μg/ml vancomycin, 5 μg/ml cefsulodin, and 2.5 U/ml polymyxin B (CHBA) to inhibit the growth of unwanted microbes under 10% $CO_2$, 7 to 10% $O_2$ and balance $N_2$, at 37°C. Liquid *H. pylori* cultures were grown in brucella broth supplemented with 10% fetal bovine serum (BB10). The antibiotic chloramphenicol (Cm) was used for selection at a concentration of 13 μg/ml. *H. pylori* strains were stored at −80°C in brain heart infusion medium supplemented with 10% fetal bovine serum, 1% (wt/vol) β-cyclodextrin, 25% glycerol, and 5% dimethyl sulfoxide. All antibiotics and chemicals were obtained from Sigma, Gold Biosciences or Fisher.

**Generation of *H. pylori* mutants.** *H. pylori* mutants *ΔimaA*, *ΔcagE*, *ΔcagE imaA* were generated as previously described (Sause et al., 2012). The LSH100 mutants, *ΔcagPAI* and *ΔcagPAI imaA*, were generated by naturally transforming *H. pylori* with genomic DNA (gDNA) kindly provided by Nina Salama (Pinto-Santini and Salama, 2009). The LSH100 mutants, *ΔcagA and Δcag imaA,* were generated by naturally transforming *H. pylori* with gDNA kindly provided by Manuel Amieva (Amieva et al., 2003).

**Cell culture.** AGS (ATCC CRL 1739) human gastric epithelial cells were obtained directly from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) containing 10% FBS at 37°C under 10% $CO_2$. To assay interleukin-8 (IL-8) production, AGS cells were seeded at $1 \times 10^5$ cells/ml in 24-well tissue culture dishes and incubated for 24 h. After this period, *H. pylori*, cultured for ≥20 hours on CHBA, were scraped from a plate and resuspended in sterile DMEM plus FBS to a concentration of $1 \times 10^7$ to create a multiplicity of infection (MOI) of 100. *H. pylori* concentrations were determined by $OD_{600}$, assuming $3 \times 10^8$ bacteria/ml/$OD_{600}$ unit. AGS cells were infected for 2 hours under *H. pylori* incubation conditions. After 2-hour incubation, culture supernatant was removed, and AGS monolayers were washed twice in 1X phosphate-buffered saline (PBS), and then the cells were resuspended in TRIzol for RNA isolation.

For CagA translocation, AGS cells seeded at $1 \times 10^5$ cells/ml in 24-well dishes were infected at an MOI=100 for 3 hours with either wild-type *H. pylori* or the *imaA* isogenic mutant under the same conditions as described above. In the first assay, without antibiotics, the culture media containing microbes was aspirated after 3 hours of infection, the cells were then washed 3X with ice cold 10 mM sodium orthovanadate, and lysed in 100 µL sample buffer. In the infections using antibiotics, the same procedure was used as described above, except following the removal of the culture media, fresh media containing 300 mg/mL kanamycin was added to the AGS cells and allowed to incubate for 1.5 hours as described previously (Tsugawa et al., 2012). The cells were then washed in 3X with ice cold 10 mM sodium orthovanadate and lysed in sample buffer. For analysis of $\beta_1$ integrin protein levels, AGS cells were seeded in 6-well plates until ~90% confluency was obtained. Cells were then infected with $1 \times 10^7$ *H. pylori* for 3 hours. Following the infection, AGS cells were washed in PBS and then lysed and resuspended in 200 µL sample buffer.

**RNA preparation**. RNA was isolated from AGS cells using TRIzol reagent (Invitrogen). Briefly, 1 mL TRIzol reagent was added directly to cells in the culture dish per 10 $cm^2$ of culture dish surface. The cells were lysed directly in the culture dish by pipetting the cells up and down several times. For RNA isolation, 200 µL of chloroform was added to Trizol resuspensions. Samples were then centrifuged (12,000 x g, 15 min, 4˚C), and the aqueous layer was

removed and placed into new tubes. RNA was precipitated by combining 500 µL of isopropanol with the aqueous layer and incubating at room temperature for 10 min, followed by a centrifugation as above. The RNA pellet was washed with 75% ethanol and then dried and resuspended in RNase-free water. Concentrations were then quantified on a Nanodrop spectrophotometer (Nanodrop). RNA was immediately transcribed into cDNA (see below) and the remaining sample stored at -80˚C.

RNA from gerbil tissue was isolated in a similar manner as described above, except contaminating DNA was removed using Turbo DNase Treatment (Ambion). Briefly, following resuspension of the RNA pellet in RNase-free water, the sample was mixed with DNase treatment for 30 minutes at 37˚C, and then deactivated for 5 minutes at room temperature with the inactivating reagent. Samples were then centrifuged at 10,000 x g for 1.5 minutes, the supernatant was extracted and used for cDNA synthesis.

**cDNA synthesis and quantitative real-time PCR**. Total RNA served as a template for cDNA synthesis using the Tetro cDNA synthesis kit (Bioline, London, UK). Synthesis was carried out following manufacturer's protocol, starting with 0.5-1 µg total RNA, 50 ng random hexamers, and 10mM dNTPs per 20-µL reaction. The mixture was incubated at 65˚C for 10 min before being combined with 10 µL of master mix, which includes the reverse transcriptase enzyme (200 U/µL). The reaction proceeded for 1 hour at 42˚C until the reverse

transcriptase enzyme was inactivated at 70˚C for 15 min. Quantitative real-time PCR was performed using the CFX Connect Real-Time Cycler (Bio-Rad, Hercules, CA) and SYBR Green supermix reagents (Bioline, London, UK). *Il8* (Nazarenko et al., 2002) expression levels from AGS cells were normalized to *18S rRNA* (Nazarenko et al., 2002) rRNA. *KC* (Rieder et al., 2005) expression levels from gerbil tissues were normalized to *18S rRNA* (Rieder et al., 2005).

**FITC labeling of $\alpha_5\beta_1$ integrin.** Purified full-length human $\alpha_5\beta_1$ integrin, octyl-β-D-glucopyranoside formulation (0.418 mg/mL) obtained from Millipore was conjugated to FITC using the Calbiochem FITC labeling kit. Prior to labeling, the entire integrin sample (50 µL) was dialyzed overnight at 4°C with 1 L of 1X carbonate buffer (Calbiochem) containing 1% Triton-X (Fisher) in a Thermo 2,000 MWCO Slide-A-Lyzer Mini Dialyzer. The dialyzed protein sample was then incubated with 10 µL of the Calbiochem FITC mixture at room temperature for 2 hours in the dark. The freshly conjugated FITC-integrin sample was then dialyzed again overnight at 4°C with 300 µL of 1X PBS containing 1% Triton-X in the Thermo Mini Dialyzer unit. Finally, the dialyzed FITC- $\alpha_5\beta_1$ integrin sample (~45 µL) was removed from the unit and stored at -20°C until use.

**Flow cytometry.** *H. pylori* cells that had been growing for ~18 hours were scraped from plates and diluted to an $OD_{600}$ of 1.1 in 1X PBS with 2% BSA. 1 mL cell suspensions were rotated at room temperature in 1X PBS with 2% BSA for

30 minutes. Following the blocking incubation, individual *H. pylori* cultures were split into 5 tubes and 2 μL of the FITC-$\alpha_5\beta_1$ integrin conjugation mixture was added to the cell suspensions, generating a total of 5 replicates for each strain. The cells were then rotated in the dark at room temperature for 5 hours. Following the incubation, cells were spun down at 8,000 x g for 3 minutes, supernatant removed, cells washed in 1X PBS with 2% BSA, and then spun down again at 8,000 x g for 3 minutes. The cell pellets were then resuspended in 1 mL 4% formaldehyde and stored overnight at 4°C. Flow cytometry was conducted on a BD LSR II (BD Biosciences) with 10,000 cells counted per sample. Samples included *H. pylori* that had been incubated with FITC alone. A total of 5 FITC-$\alpha_5\beta_1$ integrin incubations were conducted for each strain and all analyses were done using FlowJo software (BD Biosciences). Results are presented as levels of FITC compared to wild type, statistical analysis was done using a student's t test.

**Western blotting.** Proteins for Western blot analysis were resuspended in 4× NuPAGE sample buffer (Invitrogen, Carlsbad, CA) with 0.025% 2-mercaptoethanol and heated at 70°C for 15 min. Samples were separated on 3-8% NuPAGE Tris-acetate gels for 60 min at 150 V. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) with the Bio-Rad semidry transfer cell for 35 min at 16 V. Antibodies for detection of phosphorylated CagA (anti-p-Tyr, PY99), total CagA (anti-CagA, B-300), and GAPDH (anti-GAPDH, G-9) were obtained from Santa

Cruz Biotech and used at dilutions of 1/200, 1/400, and 1/500, respectively. β1 integrin was detected with antibody anti-β1 integrin (#4706) from Cell Signaling at a 1/600 dilution. For visualization, blots were incubated with either goat anti-rabbit or goat-anti mouse antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech) at a dilution of 1:2,000 for 1 h, followed by incubation with luminol, p-coumaric acid, and hydrogen peroxide. Luminescent blots were visualized by exposure to Ultra Cruz autoradiography film (Santa Cruz Biotech).

**Animal infections**. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the UC Santa Cruz Institutional Animal Care and Use Committee. Minimally passaged *H. pylori* strains in the 7.13 background were inoculated into BB10, and cultured with shaking at 37ºC, under microaerobic conditions, for 24 hours and then centrifuged at 1,300 x g for 8 minutes to concentrate the cultures to ~1 x $10^9$ cells/mL. Prior to infection, 24- to 48-day old male Mongolian gerbils (Charles River Laboratories, Inc., Wilmington, MA) were fasted for 12 hours. Approximately 1 mL of 1 x $10^9$ cells/mL *H. pylori* were administered to the gerbils by oral gavage route. Following infections, the animals were killed via $CO_2$ narcosis, and the stomach was dissected, opened along the lesser curvature, and divided into longitudinal strips for preservation and analysis. Tissues were then (i) homogenized in BB10 using the Omni Tissue Homogenizer (Omni International) and plated on CHBA to determine the colony forming units per

gram of tissue (CFU/g); (ii) snap frozen in liquid nitrogen and stored at -80°C for qRT-PCR of cytokines; (iii) fixed in formalin and analyzed via immunohistochemistry.

## 3.3 Results

### Elevated unphosphorylated CagA during *imaA* mutant infection.

An earlier report from our lab showed that infections of gastric epithelial cells (AGS cells) with an *H. pylori* mutant for the outer membrane protein, ImaA, leads to elevated production of the proinflammatory chemokine IL-8 (Sause et al. 2012). To elucidate the mechanism underlying this phenotype, we monitored the activity of the *H. pylori* inflammation inducing protein, CagA. Upon secretion into the host cell, CagA can either be phosphorylated by host kinases or remain in an unphosphorylated state (Backert et al., 2001; Kwok et al., 2007; Poppe et al., 2007). We examined protein levels for each of CagA's intracellular states in the context of either a wild-type or *imaA* mutant infection. We found that three hours post-inoculation, levels of phosphorylated CagA are lower in AGS cells infected with the *imaA* mutant compared to that of wild-type (Fig. 1A). Surprisingly however, levels of total CagA were elevated in the mutant infection (Fig. 1A). Thus, it appears that loss of ImaA create *H. pylori* strains with abnormal CagA delivery. Our initial experimental design, however, did not robustly remove the *H. pylori* and thus some CagA may still be associated with

adherent bacteria, despite thorough washing. We therefore performed the same experiment, but following the 3-hour infection, we introduced the antibiotic kanamycin into the media as described by Tsugawa *et al* (Tsugawa et al., 2012). Following antibiotic treatment, and rounds of washing, we still found elevated levels of total CagA in the *imaA* mutant infections (Fig. 1B). Taken together, these results suggest that *imaA* mutants deliver elevated CagA to host cells, and that ImaA normally acts to temper CagA delivery.

**ImaA affects Cag delivery**

To determine whether the *imaA* mutant inherently produces more CagA or if the mutant delivers more CagA to the host cell, we examined levels of the pro-inflammatory chemokine, IL-8, from AGS cells infected with *H. pylori* strains in *cagA* mutant backgrounds. In this setting, mutants for *cagA* still generate a *cag*PAI T4SS and deliver the other known *H. pylori* pro-inflammatory effector, peptidoglycan, into host cells, but will not deliver CagA (Viala et al., 2004). Upon infection of AGS cells, we found that an *imaA cagA* double mutant generates greater levels of IL-8 than a *cagA* single mutant (Fig. 2A). These results indicate that the enhanced inflammation seen in *imaA* mutant infections is not due to overproduction of CagA, but rather increased interactions between the host and the *cag*PAI T4SS. To provide further support for this idea, we analyzed the amount of CagA produced by wild-type *H. pylori* and the *imaA* mutant grown to the same density that we use to infect AGS cells, and found no differences in

CagA levels (Fig. 2B). ImaA's effect on inflammation has already been shown to be *cag*PAI T4SS dependent, via analysis of an *imaA cagE* double mutant (Sause et al. 2012). Specifically, loss of the *cag*PAI T4SS via mutation of the ATPase, *cagE*, which is essential for T4SS assembly, abrogates ImaA's effect on inflammation. Taken together, these experimental results suggest that ImaA prevents normal *cag*PAI T4SS host cell interactions or substrate delivery.

### *imaA* mutants exhibit enhanced binding to $\alpha_5\beta_1$ integrin

The CagA independent inflammation phenotype for the *imaA* mutant suggests that the mutant may be associating with the host cell at higher levels than wild-type *H. pylori*. To study this possibility, we looked at the interactions occurring between *H. pylori* and the cognate receptor for the *cag*PAI T4SS, $\alpha_5\beta_1$ integrin (Jiménez-Soto et al., 2009; Kwok et al., 2007; Shaffer et al., 2011). We examined *H. pylori*-integrin interactions directly using flow cytometry experiments with various *H. pylori* strains and FITC-labeled $\alpha_5\beta_1$ integrin. We found that when both the *imaA* single mutant and an *imaA cagPAI* double mutant are incubated with the FITC-$\alpha_5\beta_1$ integrin conjugate, there was a unique population of cells in flow cytometry that exhibit elevated fluorescence, indicative of enhanced binding to $\alpha_5\beta_1$ integrin (Fig. 3A). This dramatic shift was not seen in either wild-type *H. pylori* or a *cagPAI* single mutant, indicating that a functional ImaA abrogates the binding effect (Figure 3B). In this assay, there was no binding due to the *cag*PAI T4SS, because the T4SS machinery is only assembled in the

presence of host cells (Fig. 3A). These results suggest that *imaA* mutants have an enhanced ability to bind integrin that is independent of the *cag*PAI T4SS. One possible explanation for the increased intracellular CagA levels, in light of these findings, is that *imaA* mutants have increased $\alpha_5\beta_1$ integrin interactions that in turn promote enhanced *cag*PAI T4SS interactions and effector delivery.

**Reduced $\beta_1$ integrin following wild-type infection**

The secondary structure prediction software (PHYRE) predicts ImaA to have regions that match proteases and proteins that bind $\alpha_5\beta_1$ integrin (Kelley and Sternberg, 2009). To examine the state of integrin in the context of an *imaA* mutant infection, we analyzed the quantity of the $\beta_1$ subunit of $\alpha_5\beta_1$ integrin following infection with either wild-type *H. pylori* or the *imaA* mutant. We found that compared to uninfected AGS cells, there was less $\beta_1$ integrin in lysates from both wild-type or *imaA* mutant infected AGS cells (Figure 4A). However, cell lysates from *imaA* mutant infections harbor more $\beta_1$ integrin than lysates from wild-type infected cells (Figure 4B). Given the relative high abundance of $\beta_1$ integrin in uninfected cells and the hastened disappearance of $\beta_1$ integrin in wildtype infections compared to *imaA* mutant infections, there appears to be an ImaA driven reduction in $\beta_1$ integrin.

**ImaA is critical for prolonged infection**

We next sought to examine how ImaA contributes to *H. pylori*'s ability to infect the Mongolian gerbil, which is the established *H. pylori* animal model for disease in humans. Gerbils were infected with either wild-type *H. pylori* or the *imaA* mutant and groups of animals were euthanized one, three, five, and seven days post-inoculation to enumerate *H. pylori* colonization. We found that by one day post-inoculation, the *imaA* mutant is able to establish colonization in the gerbil stomach, but that the number of viable bacteria extracted from the stomach steadily declines over the course of the week, until the mutant is cleared by day seven (Figure 5A). These results demonstrate that ImaA is dispensable for initial colonization but the protein becomes essential for maintaining colonization very early into infection. Our attempts to histologically examine inflammation in *imaA* mutant infected gerbil tissues were unsuccessful, as there was no gradable inflammation a week into infection (data not shown). In fact, tissues from wild-type infections that were carried out to a month showed very little histopathology, but by 3 months there were multiple cases of adenocarcinoma and ulceration (data not shown). This demonstrates that a chronically infected gerbil is needed to produce tissue that can be histologically examined for pathologies, as reported by others (Rieder et al., 2005) To address the early stages of inflammation, we studied transcript levels of the rodent IL-8 analog, keratinocyte chemoattractant (KC). We found that there was no elevation in KC levels between either wild-type or *imaA* mutant infected gerbils, and uninfected

gerbils (Figure 5B). In addition to KC, we screened for levels of TNF-α, IL-6, IFN-γ, IL-18, and IL-1β, and found no differences (data not shown). There is no literature on inflammation at time points this early into infection, but it appears to be premature for detection of noticeable differences in chemokine or cytokine levels.

## 3.4 Discussion

*H. pylori*'s primary mechanism for inducing inflammation in host cells is through the delivery of its effector molecule, CagA (Yamaoka et al., 1997). Here, we describe a novel role for the *H. pylori* outer membrane protein, ImaA, in which the bacterium utilizes the protein to temper the quantities of CagA it secretes into host cells, presumably to modulate inflammation and avoid host clearance. We have previously shown that the gene encoding *imaA* is upregulated within the first two weeks of infection (Castillo et al., 2008b). This timely upregulation coincides with *H. pylori*'s need to express factors that enable the bacterium to survive the initial stages of colonization and prepare for the chronic stage of infection. The precipitous drop seen in *H. pylori* colonization levels when ImaA is absent affirms that the first few days following infection are critical for the longevity of the bacterium in the stomach and that ImaA is indispensible for this process.

Upon initial delivery into host cells, CagA can either be phosphorylated by the host kinase Src or remain in an unphosphorylated state (Stein et al., 2002). We have shown that three hours into an *imaA* mutant infection, there are higher numbers of CagA molecules within AGS cells, compared to that of wild-type (Fig. 1A). The disparity between unphosphorylated and phosphorylated CagA seen in the first set of experiments, without antibiotics, could be due to a negative feedback loop that has been observed when CagA reaches a level of saturation inside the cell. In this situation, CagA directly sequesters Src and inhibits kinase activity (Tsutsumi et al., 2003). We believe that the high abundance of CagA secreted by the *imaA* mutant could hasten the progression of this feedback loop. Alternatively, it has been shown that when the *cag*PAI T4SS protein, CagL, engages with the $\beta_1$ subunit of $\alpha_5\beta_1$, it initiates a signal transduction to activate Src (Kwok et al., 2007). It is possible, that in the absence of ImaA, there is an over-engagement between $\alpha_5\beta_1$ and either CagL or the unknown *H. pylori* protein that binds $\alpha_5\beta_1$ when ImaA is gone, and this interaction dysregulates Src activity. Interestingly, it has been shown that the unphosphorylated form of CagA is responsible for activating nuclear factor-κB (NF-κB) and leading to the production of IL-8 (Oldani et al., 2009). This phenomenon coincides with the inflammation phenotype seen in *imaA* mutant infections, where AGS cells produce elevated levels of IL-8 compared with wild-type infections.

While there are known mechanisms that allow *H. pylori* to evade detection by the immune response, the majority of these pathways are passive

and inherent properties of the bacterium's structural physiology, not active processes. For example, a number of *H. pylori*'s pathogen associated molecular patterns (PAMPs), such as flagellin or lipopolysaccharide (LPS), have evolved to be poorly recognized by host pro-inflammatory Toll-like receptors (TLRs) (Gewirtz et al., 2004; Lepper et al., 2005). This allows the bacteria to persist by preventing pro-inflammatory signaling cascades. In addition to avoiding detection, *H. pylori*'s genetic makeup can also suppress the immune response, as the composition of the bacterium's DNA has been shown to activate TLR9, which initiates an anti-inflammatory response (Owyang et al., 2012). *H. pylori* is also capable of altering the makeup and abundance of its outer membrane proteins, through antigenic variation (Solnick et al., 2004). This may be done to minimize or stay ahead of the production of antibodies against the bacteria, however this has not been demonstrated. All of these pathways have one thing in common, they do not involve active effectors that suppress the immune response, as is seen in a number of pathogenic bacteria (Rüter and Hardwidge, 2014). In fact, only one secreted *H. pylori* protein, the autotransporter VacA, has been shown to actively thwart the mobilization of the immune response. VacA is a pore-forming toxin that promotes apoptosis and prevents the proliferation of T-cells (Gebert et al., 2003; Phadnis et al., 1994). Essentially, there is very little known of *H. pylori's* potential to generate enzymatic proteins that modulate the strength of the immune response, and we believe ImaA participates in that function.

ImaA is predicted to have proteolytic activity and the ability to bind $\alpha_5\beta_1$ integrin (Kelley and Sternberg, 2009; Löwer et al., 2008). Our results show decreased levels of $\beta_1$ integrin following infection with *H. pylori* harboring a functional ImaA, compared to mutants lacking ImaA, suggesting that ImaA plays a role in decreasing the number of available $\alpha_5\beta_1$ integrins. This finding suggests the novel idea that *H. pylori* controls the number of docking events that can occur between the *cag*PAI T4SS and $\alpha_5\beta_1$ integrin, thereby modulating the amount of CagA delivered into the host cell.

The *imaA* mutant has the ability to initially colonize the Mongolian gerbil stomach, but within a few days post-infection the mutant is cleared. Interestingly, ImaA is not necessary for initial establishment of colonization, as all gerbils inoculated with the *imaA* mutant show infection by 1-day post-inoculation. However, there is a linear drop in the number of *imaA* mutant bacteria recovered from the gerbils over a week, as ImaA becomes critical for persistence. Our previous work showed that the *imaA* mutant causes elevations in IL-8 and TNF-α levels (Sause et al., 2012). To address the idea that increased inflammation in the *imaA* infected gerbil stomach may be causing the abrupt removal of the bacteria, we examined tissue histology and chemokine transcript levels in the gerbil. Unfortunately, gerbils did not display any notable histopathological features until at least a month into infection, and the *imaA* mutant had been cleared from the gerbil well before then (data not shown). Also, results from qRT-PCR on stomach tissues, looking at chemokine and cytokine

levels, were extremely variable at these early times, and typically yielded no differences when compared to uninfected gerbils (Figure 5B).

While we did not observe any noticeable differences in inflammation between *imaA* mutant infected and wild-type infected gerbils, alternative explanations for rapid clearance exist. The *imaA* mutant's ability to colonize but not maintain infection could be attributed to cell turnover kinetics within the gastric epithelium. Host-mediated clearance of *H. pylori* is influenced by the timely removal and replenishment of epithelial cells in the epithelium, a process that *H. pylori* attempts to downregulate (Mimuro et al., 2007). It has been demonstrated that CagA secretion disrupts matrix adhesion of gastric epithelial cells, resulting in severe deficiencies in integrin mediated adhesion of gastric epithelial cells to the extracellular matrix (Moese et al., 2007). We propose that the hyper-secretion of CagA by the *imaA* mutant causes excessive damage to the adhesions that exist between epithelial cells and the extracellular matrix, impairing *H. pylori*'s ability to control cell proliferation and turnover. This uncontrolled breakdown of adhesion complexes within the gastric epithelium results in premature clearance from the stomach.

In summary, we have identified a novel mechanism utilized by *H. pylori* to regulate the amount of inflammation it induces during infection. These results suggest a model in which *H. pylori* utilizes ImaA to dilute the number of available $\alpha_5\beta_1$ integrin receptors that can be bound by the T4SS, which dampens the severity of the CagA driven immune response and promotes persistence.

## 3.5 Figures

**Figure 1.** Elevated CagA is present in *imaA* mutant infections as compared to wild-type. (A) AGS cells were infected for 3 hours with either wild-type *H. pylori* or *ΔimaA*, then washed 3 times to remove extracellular bacteria. Levels of CagA and phosphorylated-CagA were quantified using western blotting. (B) The same experiment was conducted as in (A), except following the three hour infection, culture media containing kanamycin was added to the AGS cells for 1.5 hr to kill extracellular bacteria, cells were then washed 3 times to remove extracellular debris. Levels of CagA and phosphorylated-CagA were quantified. Protein levels for each set of experiments are relative to GAPDH and all quantifications were done with Image J software, the mean values ± SEM of three independent infections for each set of experiments are shown. Statistical analyses were done with Kruskal-Wallis test. * = $p < 0.05$

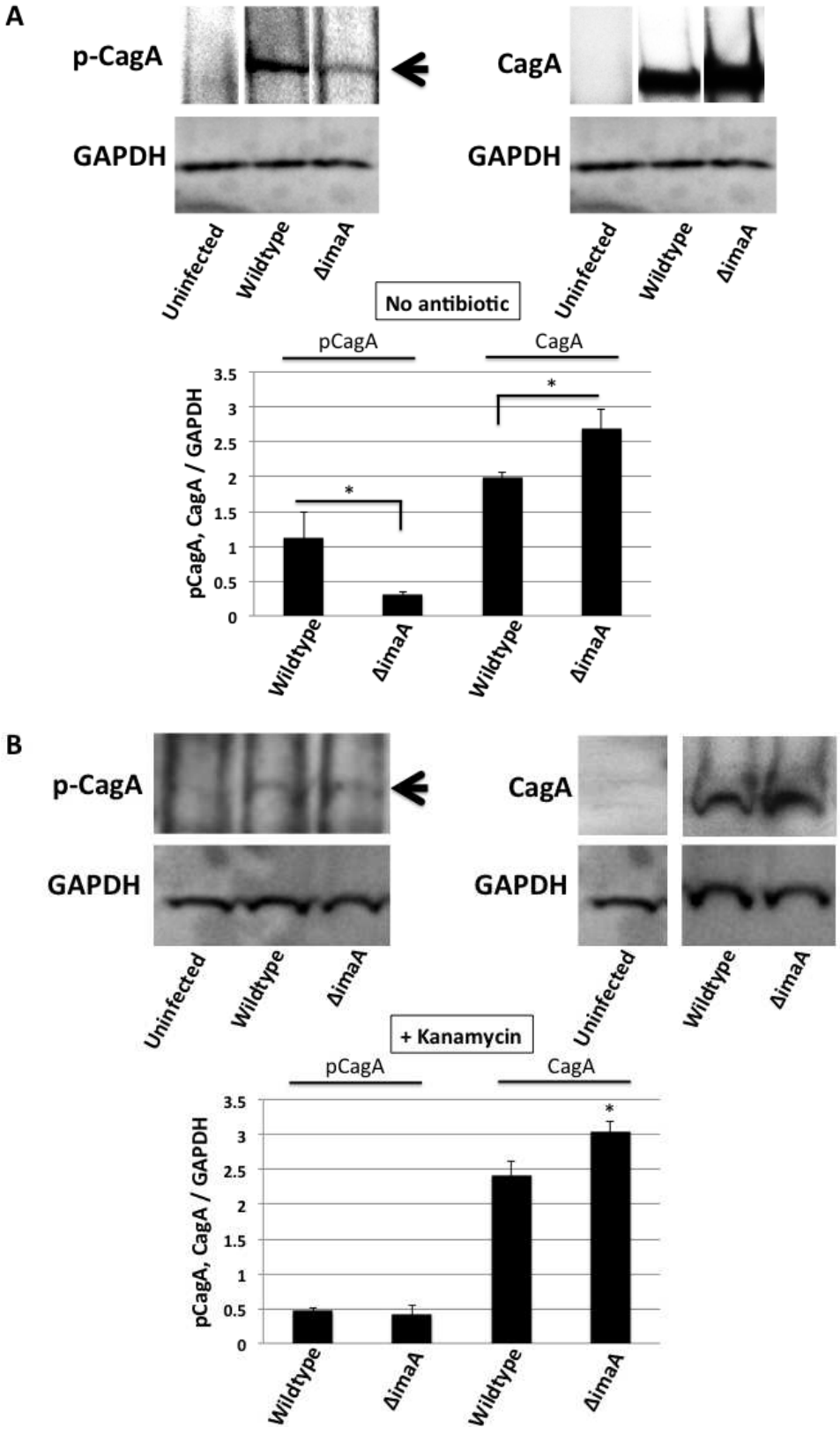

A
p-CagA
CagA
GAPDH
GAPDH
Uninfected
Wildtype
ΔimaA
Uninfected
Wildtype
ΔimaA
No antibiotic
pCagA
CagA
pCagA, CagA / GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Wildtype
ΔimaA
Wildtype
ΔimaA
B
p-CagA
CagA
GAPDH
GAPDH
Uninfected
Wildtype
ΔimaA
Uninfected
Wildtype
ΔimaA
+ Kanamycin
pCagA
CagA
pCagA, CagA / GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
*
Wildtype
ΔimaA
Wildtype
ΔimaA

**Figure 2**. *imaA* mutants create high amounts of IL-8 independent of CagA. (A) For *Il8* transcript analysis, AGS cells were infected for 2 hours with either wild-type *H. pylori* or its isogenic mutants (*ΔimaA*, *ΔcagA, ΔcagE, ΔcagA imaA* or *ΔcagE imaA)*. qRT-PCR was performed to analyze the expression of *Il8.* This data shows at least 5 independent infections (biological replicates) with reactions done in triplicate. All expression differences were calculated with the $\Delta\Delta C_T$ method (Nazarenko et al., 2002). $p < 0.05$, Student's *t* test, for AGS cell infections. (B) Western blot of CagA levels from wild-type and the *imaA* mutant grown to a concentration approximately = 1 x $10^7$ cells/mL.

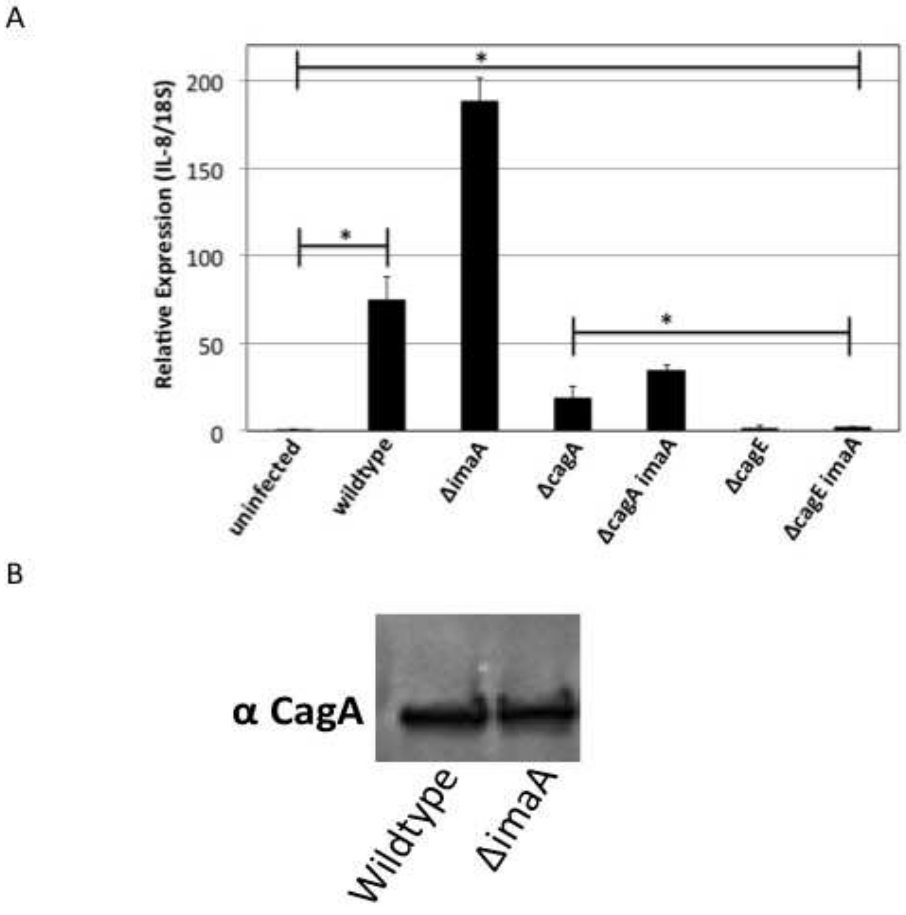
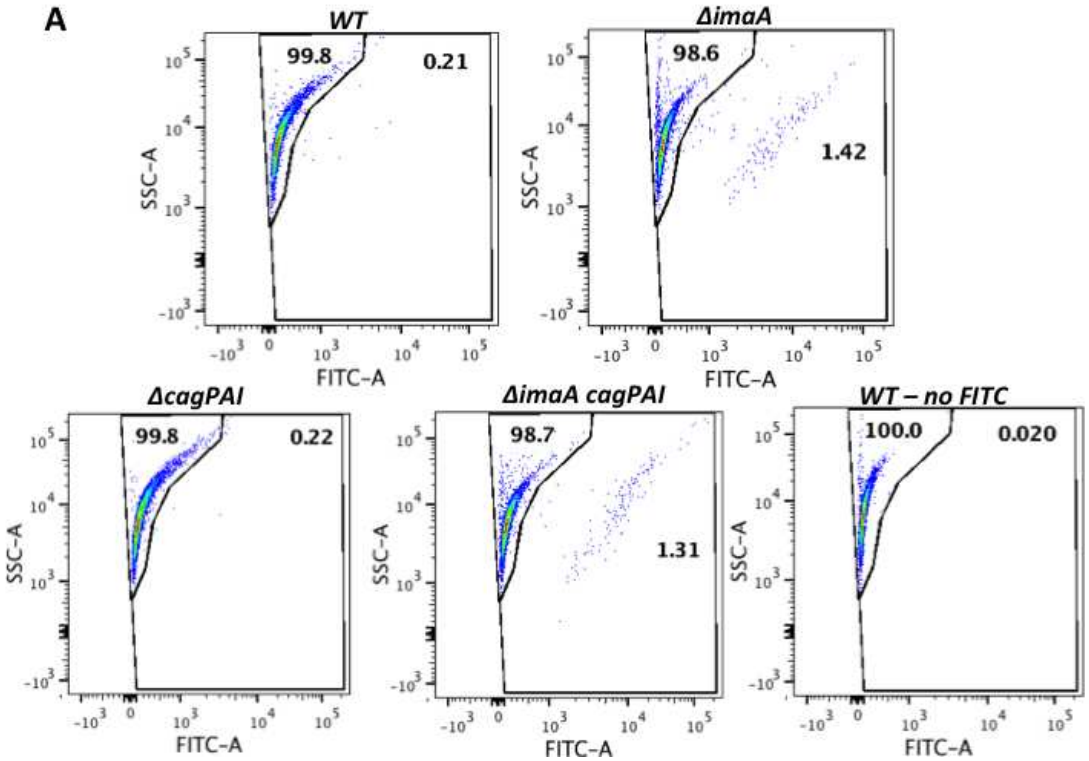


**Figure 3**. *imaA* mutants exhibit enhanced binding to $\alpha_5\beta_1$ integrin. (A) Wild-type *H. pylori* and its isogenic mutants, *ΔimaA*, *ΔcagPAI*, and *ΔcagPAI imaA*, were incubated with FITC-labeled $\alpha_5\beta_1$ integrin for 5 hours. Bacteria were washed and fixed in 4% formaldehyde prior to analysis by flow cytometry. 10,000 bacteria were counted per replicate, with a total of 5 replicates per strain. A no FITC-wild-type treatment serves as a negative control. (B) A gate was generated for the population of cells exhibiting exceptionally high fluorescence, indicative of high levels of bound $\alpha_5\beta_1$ integrin. Quantification of bound FITC- $\alpha_5\beta_1$ integrin is relative to the wild-type shift in fluorescence, which is set to 1.0. Statistical analyses were done using Student's *t* test, $p < 0.005$.



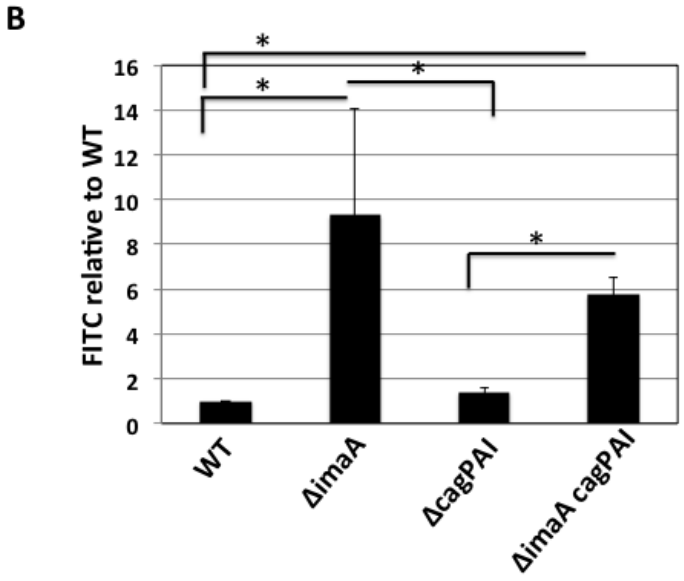



**Figure 4**. $\beta_1$-integrin levels reduced in the presence of ImaA. (A)AGS cells seeded in 6-well plates at high confluency were infected with either wild-type *H. pylori* or *ΔimaA* at an MOI = 100. Following 3 hours of infection, cells were lysed and probed for the presence and integrity of $\beta_1$-integrin with the antibody, $\alpha$-$\beta_1$-integrin (4706). (B) Quantification of $\beta_1$-integrin levels were done using Image J software. The data represents two individual wild-type infections and 3 individual *ΔimaA* infections.

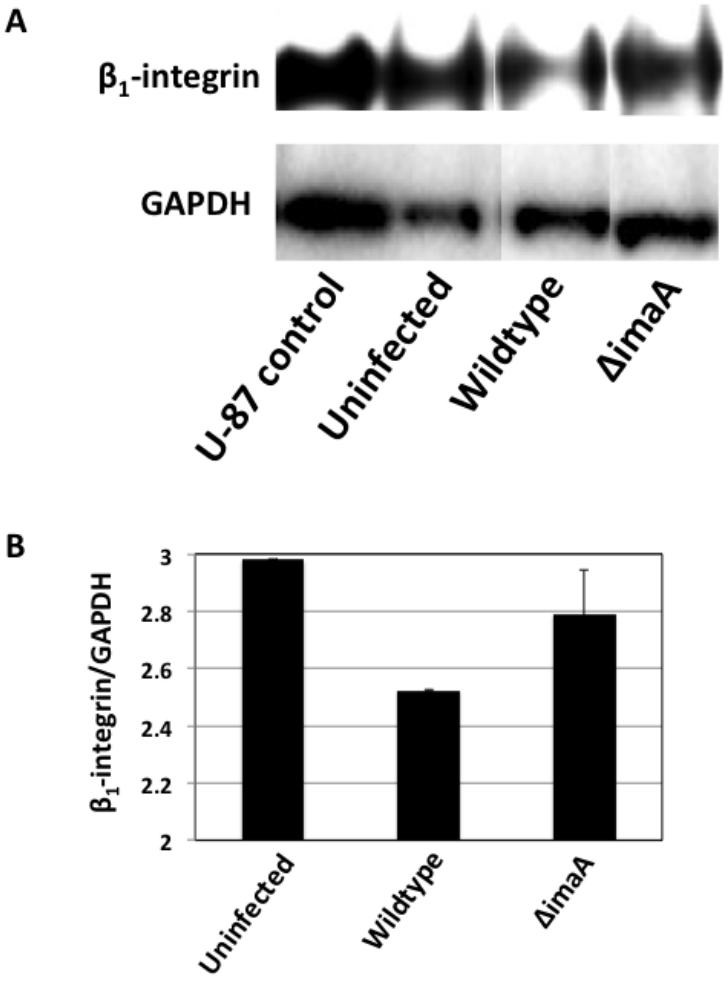
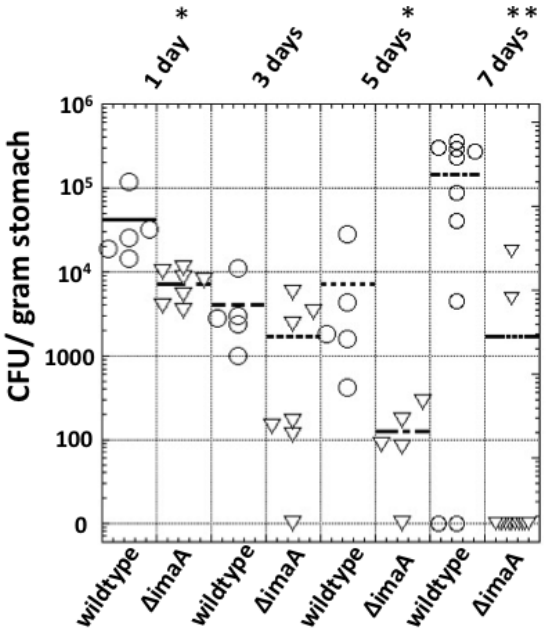
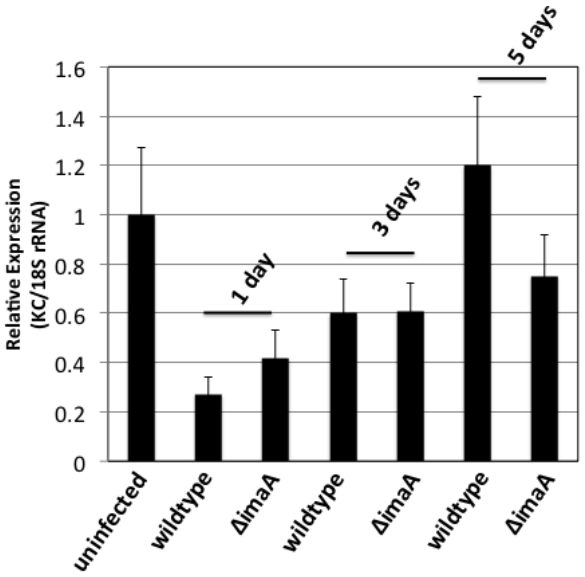



**Figure 5**. ImaA is required for chronic infection. (A) Male Mongolian gerbils were infected with either wild-type *H. pylori* or *ΔimaA.* Infections persisted for either 1, 3, 5, or 7 days. Following infection, stomachs were harvested and colonization was expressed as CFU/gram of stomach tissue. Statistical analysis done with the Mann-Whitney U-test. (*, $p \leq 0.008$), (**, $p \leq 0.0001$). (B) qRT-PCR for *KC* levels was performed on cDNA generated from gastric tissues taken from either wild-type, *ΔimaA,* or uninfected gerbils. Each column represents at least 5 independent infections.

A


1 day *
3 days
5 days *
7 days **
CFU/ gram stomach
10^6
10^5
10^4
1000
100
0
wildtype
ΔimaA
wildtype
ΔimaA
wildtype
ΔimaA
wildtype
ΔimaA

B


5 days
3 days
1 day
Relative Expression
(KC/18S rRNA)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
uninfected
wildtype
ΔimaA
wildtype
ΔimaA
wildtype
ΔimaA

**3.6 Table 1. Strains and plasmids used in this study**

| *H. pylori* strain or plasmid | Description | Reference |
|---|---|---|
| LSH100 | NSH57 with repaired *fliM* allele | (Lowenthal et al., 2009) |
| KO1370 | *LSH100 ΔimaA::cat* | This study |
| KO1163 | *SS1 ΔcagE::kan* | This study. From David McGee. |
| KO1372 | *LSH100 ΔcagE::kan* | This study |
| KO1373 | *LSH100 ΔcagE::kan imaA::cat* | This study |
| 7.13 | Gerbil passaged strain | (Franco et al., 2005) |
| KO1412 | *7.13 ΔimaA::cat* | This study |
| KO1413 | *LSH100 ΔcagPAI imaA* | This study |
| KO1414 | *LSH100 ΔcagPAI* | This study |
| K01415 | *LSH100 ΔcagA* | This study |
| KO1416 | *LSH100 ΔcagA imaA* | This study |

## Chapter 4: Conclusions and Future Directions

The aim of my dissertation was to investigate the importance of an *H. pylori* gene that we found to be expressed at exceptionally high levels in the mouse stomach, compared to laboratory conditions (Castillo et al., 2008b). This uncharacterized gene carried great intrigue because it was predicted to encode an outer membrane protein and these types of proteins have proven to be integral components of *H. pylori* pathogenesis (Ilver et al., 1998a; Mahdavi et al., 2002; Schultz et al., 1998). This project had humble beginnings, as the gene that we now call *imaA*, went simply by its gene number, *hp0289*, for the first two years of my graduate career. We have since gone on to show that ImaA is an important *H. pylori* virulence factor that modulates inflammation levels in host cells and is critical for the bacterium's progression to the chronic stage of infection within the host stomach. Thus, through time, *hp0289* graduated to its official name, *imaA* (ImmunoModulatoryAutotransporter A) and has become an important fixture of the Ottemann lab's research interests.

Initial ImaA characterization experiments were centered on validating bioinformatic predictions that the protein was an outer membrane protein and identifying the environmental signal that was responsible for inducing higher gene expression. Through a proteinase K digestion assay and biochemical fractionation coupled with an ImaA-targeted peptide antibody, we demonstrated

that ImaA is translocated to the outer membrane. Additionally, qRT-PCR studies examining *imaA* gene expression at low pH showed that the acidic conditions are responsible for inducing transcriptional upregulation and that this process is dependent on *H. pylori*'s acid-responsive two component regulatory system, ArsRS. We went on demonstrate that an isogenic *imaA* mutant displays a significant mouse colonization defect when compared to a wild-type strain and that when put into a competition assay in mice with wild-type *H. pylori*, the *imaA* mutant is vastly outcompeted for colonization. The concluding touch on this initial body of work was showing that *imaA* mutants evoke a greater immune response in infected host cells when compared to infections with wild-type *H. pylori*. This can be seen with two proinflammatory markers of the immune response, IL-8 and TNF-α.

Our follow-up work to the initial *imaA* characterization experiments focused on the mechanism that drives the heightened immune response observed in *imaA* mutant infections. To this end, we tracked levels of the *H. pylori* pro-inflammatory effector, CagA, within host cells and found that there are greater levels of CagA delivered during *imaA* mutant infections compared to wild-type infections. We went on to show that the *imaA* mutant induces elevated levels of inflammation, independently of CagA, as an *imaA cagA* double mutant causes more inflammation than a *cagA* single mutant. This result directed us to the *cag*PAI T4SS, the surface-exposed unit that delivers CagA into the host cell.

Through flow cytometry experiments, we showed that *imaA* mutants bind higher levels of the host receptor for the *cag*PAI T4SS, α5β1 integrin. Finally, we sought to determine how important *imaA* is to *H. pylori's* ability to colonize and cause disease in the gerbil model of infection. Interestingly, we discovered that ImaA is dispensable for the very initial stages of host colonization, but that the protein becomes essential very rapidly, as *imaA* mutants are cleared from the stomach within a week, post-infection.

The final piece of work on *imaA* was supplemental to our characterization experiments and sought to determine if there are any correlations between the severity of disease state in infected humans and the presence of *imaA* in the infecting *H. pylori* strain. This work was done in conjunction with an internship program for aspiring high school scientists and has provided some framework for future experiments. Briefly, we PCR screened for the presence of *imaA* in genomic DNA taken from close to 100 *H. pylori* isolates that were biopsied from infected individuals in a country with high rates of *H. pylori* infection (Mexico). While, it was difficult to make any correlations between disease state and the amplification patterns observed in these isolates, we did determine that there was substantial variation in the *imaA* locus. This work served as a platform for future experiments, where we will actually sequence *imaA* in these isolates and obtain a better idea of the makeup of these mutations and their prevalence within the population.

I believe this dissertation has provided a solid framework for future experiments on ImaA and understanding its importance for *H. pylori* pathogenesis. Firstly, this work provides the first piece of data that suggests that an *H. pylori* protein outside of the *cag*PAI can bind $\alpha_5\beta_1$ integrin, as we see enhanced integrin binding in an *imaA cagPAI* double mutant. It would be very interesting to identify this unknown protein and investigate its interplay with the *cag*PAI T4SS and CagA delivery. This dissertation also presents an *H. pylori* protein that is actively involved in controlling the strength of the immune response, unlike the majority of understood *H. pylori* immune evasion pathways, which are passive in nature (Gewirtz et al., 2004; Owyang et al., 2012). It would be of relevance to further explore whether *H. pylori* produces additional active proteins that suppress inflammation, in conjunction with the passive pathways that are already understood. We also found that the *imaA* mutant is cleared from the gerbil stomach in what appears to be an inflammation-independent manner. It would be of interest to explore the mechanism for this clearance. One possibility relates to epithelial cell exfoliation. It has been shown that multiple pathogens suppress exfoliation of epithelial cells during infection, thus preventing cell turnover and pathogen clearance (Hauck et al., 2012). *H. pylori* dampens the rate of death in mature epithelial cells that are normally shed every 2-3 days, this promotes colonization (Mimuro et al., 2007). We find that the *imaA* mutant can colonize the host, but becomes rapidly cleared from the stomach. Perhaps the *imaA* mutant is incapable of suppressing this exfoliation event. *imaA*

mutants could also be hastening cell proliferation in the epithelium through hyper-secretion of CagA, as it has been shown that CagA can induce cell proliferation in a CagA-phosphorylation independent manner (Neal et al., 2013). Assays examining cell proliferation within the gastric epithelium in the context of an *imaA* mutant infection could shed light on this clearance phenotype. Experiments should also be conducted in gerbils with a *cagPAI imaA* double mutant to demonstrate that the *imaA* mutant clearance phenotype is indeed driven by over-activity of the *cag*PAI T4SS, as a *cagPAI* single mutant and a *cagPAI imaA* double mutant should have similar colonization outputs. We also believe it is important to explore what regions of ImaA are necessary for function, and have already generated ImaA variants that have deletions within the N-terminus portion of the protein. Animal and integrin binding experiments should be conducted with these truncated proteins to determine how important these N-terminal regions are for ImaA-associated phenotypes. Lastly, given ImaA's size and enhanced expression in the host, we believe the protein could be quite immunogenic in nature. Thus, it would be interesting to perform experiments that investigate ImaA's potential as a vaccine candidate. This could involve purifications of ImaA peptides thought to have the most potential for immunogenicity and screening their ability to protect mice or gerbils from subsequent infection with *H. pylori*.

## Literature Cited

Akopyants, N.S., Eaton, K.A., and Berg, D.E. (1995). Adaptive mutation and cocolonization during Helicobacter pylori infection of gnotobiotic piglets. Infect Immun *63*, 116-121.

Alamuri, P., and Mobley, H. (2008). A novel autotransporter of uropathogenic Proteus mirabilis is both a cytotoxin and an agglutinin. Mol Microbiol *68*, 997-1017.

Algood, H.M., Gallo-Romero, J., Wilson, K.T., Peek, R.M., and Cover, T.L. (2007). Host response to Helicobacter pylori infection before initiation of the adaptive immune response. FEMS Immunol Med Microbiol *51*, 577-586.

Alm, R.A., Bina, J., Andrews, B.M., Doig, P., Hancock, R.E., and Trust, T.J. (2000). Comparative genomics of Helicobacter pylori: analysis of the outer membrane protein families. Infect Immun *68*, 4155-4168.

Amieva, M.R., and El-Omar, E.M. (2008). Host-bacterial interactions in Helicobacter pylori infection. Gastroenterology *134*, 306-323.

Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J., and Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science *300*, 1430-1434.

Ashgar, S.S., Oldfield, N.J., Wooldridge, K.G., Jones, M.A., Irving, G.J., Turner, D.P., and Ala'Aldeen, D.A. (2007). CapA, an autotransporter protein of Campylobacter

jejuni, mediates association with human epithelial cells and colonization of the chicken gut. J Bacteriol *189*, 1856-1865.

Bach, S., Makristathis, A., Rotter, M., and Hirschl, A.M. (2002). Gene expression profiling in AGS cells stimulated with Helicobacter pylori isogenic strains (cagA positive or cagA negative). Infect Immun *70*, 988-992.

Backert, S., Moese, S., Selbach, M., Brinkmann, V., and Meyer, T.F. (2001). Phosphorylation of tyrosine 972 of the Helicobacter pylori CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells. Mol Microbiol *42*, 631-644.

Baik, S.C., Kim, K.M., Song, S.M., Kim, D.S., Jun, J.S., Lee, S.G., Song, J.Y., Park, J.U., Kang, H.L., Lee, W.K.*, et al.* (2004). Proteomic analysis of the sarcosine-insoluble outer membrane fraction of Helicobacter pylori strain 26695. J Bacteriol *186*, 949-955.

Baldwin, D.N., Shepherd, B., Kraemer, P., Hall, M.K., Sycuro, L.K., Pinto-Santini, D.M., and Salama, N.R. (2007). Identification of Helicobacter pylori genes that contribute to stomach colonization. Infect Immun *75*, 1005-1016.

Beier, D., and Frank, R. (2000). Molecular characterization of two-component systems of Helicobacter pylori. J Bacteriol *182*, 2068-2076.

Belogolova, E., Bauer, B., Pompaiah, M., Asakura, H., Brinkman, V., Ertl, C., Bartfeld, S., Nechitaylo, T.Y., Haas, R., Machuy, N.*, et al.* (2013). Helicobacter pylori outer membrane protein HopQ identified as a novel T4SS-associated virulence factor. Cell Microbiol *15*, 1896-1912.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol *340*, 783-795.

Benz, I., and Schmidt, M. (1992). Isolation and serologic characterization of AIDA-I, the adhesin mediating the diffuse adherence phenotype of the diarrhea-associated Escherichia coli strain 2787 (O126:H27). Infect Immun *60*, 13-18.

Benz, I., and Schmidt, M. (2001). Glycosylation with heptose residues mediated by the aah gene product is essential for adherence of the AIDA-I adhesin. Mol Microbiol *40*, 1403-1413.

Benz, I., and Schmidt, M.A. (2011). Structures and functions of autotransporter proteins in microbial pathogens. Int J Med Microbiol *301*, 461-468.

Blaser, M.J. (1997). The versatility of Helicobacter pylori in the adaptation to the human stomach. J Physiol Pharmacol *48*, 307-314.

Blaser, M.J., and Berg, D.E. (2001). Helicobacter pylori genetic diversity and risk of human disease. J Clin Invest *107*, 767-773.

Boncristiano, M., Paccani, S.R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., Amedei, A., D'Elios, M.M., Telford, J.L., and Baldari, C.T. (2003). The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. J Exp Med *198*, 1887-1897.

Brandon, L., Goehring, N., Janakiraman, A., Yan, A., Wu, T., Beckwith, J., and Goldberg, M. (2003). IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. Mol Microbiol *50*, 45-60.

Brandon, L., and Goldberg, M. (2001). Periplasmic transit and disulfide bond formation of the autotransported Shigella protein IcsA. J Bacteriol *183*, 951-958.

Brandt, S., Kwok, T., Hartig, R., König, W., and Backert, S. (2005). NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. Proc Natl Acad Sci U S A *102*, 9300-9305.

Bron, P.A., Grangette, C., Mercenier, A., de Vos, W.M., and Kleerebezem, M. (2004). Identification of Lactobacillus plantarum genes that are induced in the gastrointestinal tract of mice. J Bacteriol *186*, 5721-5729.

Brunder, W., Schmidt, H., and Karch, H. (1997). EspP, a novel extracellular serine protease of enterohaemorrhagic Escherichia coli O157:H7 cleaves human coagulation factor V. Mol Microbiol *24*, 767-778.

Camilli, A., and Mekalanos, J.J. (1995). Use of recombinase gene fusions to identify Vibrio cholerae genes induced during infection. Mol Microbiol *18*, 671-683.

Castillo, A., Woodruff, A., Connolly, L., Sause, W., and Ottemann, K. (2008a). Recombination-based in vivo expression technology identifies Helicobacter pylori genes important for host colonization. Infect Immun *76*, 5632-5644.

Castillo, A.R., Woodruff, A.J., Connolly, L.E., Sause, W.E., and Ottemann, K.M. (2008b). Recombination-based in vivo expression technology identifies Helicobacter pylori genes important for host colonization. Infect Immun *76*, 5632-5644.

Chevalier, C., Thiberge, J.M., Ferrero, R.L., and Labigne, A. (1999). Essential role of Helicobacter pylori gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. Mol Microbiol *31*, 1359-1372.

Cotter, S.E., Surana, N.K., and St Geme, J.W. (2005). Trimeric autotransporters: a distinct subfamily of autotransporter proteins. Trends Microbiol *13*, 199-205.

Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., and Figura, N. (1993). Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A *90*, 5791-5795.

Cover, T., and Blaser, M. (1992). Purification and characterization of the vacuolating toxin from Helicobacter pylori. J Biol Chem *267*, 10570-10575.

Dautin, N., Barnard, T., Anderson, D., and Bernstein, H. (2007). Cleavage of a bacterial autotransporter by an evolutionarily convergent autocatalytic mechanism. EMBO J *26*, 1942-1952.

Dautin, N., and Bernstein, H. (2007). Protein secretion in gram-negative bacteria via the autotransporter pathway. Annu Rev Microbiol *61*, 89-112.

Day, A.S., Jones, N.L., Lynett, J.T., Jennings, H.A., Fallone, C.A., Beech, R., and Sherman, P.M. (2000). cagE is a virulence factor associated with Helicobacter pylori-induced duodenal ulceration in children. J Infect Dis *181*, 1370-1375.

Desvaux, M., Parham, N., and Henderson, I. (2004). Type V protein secretion: simplicity gone awry? Curr Issues Mol Biol *6*, 111-124.

Donahue, J.P., Israel, D.A., Peek, R.M., Blaser, M.J., and Miller, G.G. (2000). Overcoming the restriction barrier to plasmid transformation of Helicobacter pylori. Mol Microbiol *37*, 1066-1074.

Dossumbekova, A., Prinz, C., Gerhard, M., Brenner, L., Backert, S., Kusters, J.G., Schmid, R.M., and Rad, R. (2006a). Helicobacter pylori outer membrane proteins and gastric inflammation. Gut *55*, 1360-1361; author reply 1361.

Dossumbekova, A., Prinz, C., Mages, J., Lang, R., Kusters, J.G., Van Vliet, A.H., Reindl, W., Backert, S., Saur, D., Schmid, R.M.*, et al.* (2006b). Helicobacter pylori HopH (OipA) and bacterial pathogenicity: genetic and functional genomic analysis of hopH gene polymorphisms. J Infect Dis *194*, 1346-1355.

Eaton, K.A., Benson, L.H., Haeger, J., and Gray, B.M. (2006). Role of transcription factor T-bet expression by CD4+ cells in gastritis due to Helicobacter pylori in mice. Infect Immun *74*, 4673-4684.

Eaton, K.A., Kersulyte, D., Mefford, M., Danon, S.J., Krakowka, S., and Berg, D.E. (2001). Role of Helicobacter pylori cag region genes in colonization and gastritis in two animal models. Infect Immun *69*, 2902-2908.

Eslava, C., Navarro-García, F., Czeczulin, J., Henderson, I., Cravioto, A., and Nataro, J. (1998). Pet, an autotransporter enterotoxin from enteroaggregative Escherichia coli. Infect Immun *66*, 3155-3163.

Fernandez, R., and Weiss, A. (1994). Cloning and sequencing of a Bordetella pertussis serum resistance locus. Infect Immun *62*, 4727-4738.

Finlay, B.B., and McFadden, G. (2006). Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell *124*, 767-782.

Finn, R.D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J.E., Gavin, O.L., Gunasekaran, P., Ceric, G., Forslund, K.*, et al.* (2010). The Pfam protein families database. Nucleic Acids Res *38*, D211-222.

Franco, A.T., Israel, D.A., Washington, M.K., Krishna, U., Fox, J.G., Rogers, A.B., Neish, A.S., Collier-Hyams, L., Perez-Perez, G.I., Hatakeyama, M.*, et al.* (2005). Activation of beta-catenin by carcinogenic Helicobacter pylori. Proc Natl Acad Sci U S A *102*, 10646-10651.

Gangwer, K., Mushrush, D., Stauff, D., Spiller, B., McClain, M., Cover, T., and Lacy, D. (2007). Crystal structure of the Helicobacter pylori vacuolating toxin p55 domain. Proc Natl Acad Sci U S A *104*, 16293-16298.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R., and Haas, R. (2003). Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science *301*, 1099-1102.

Gewirtz, A.T., Yu, Y., Krishna, U.S., Israel, D.A., Lyons, S.L., and Peek, R.M. (2004). Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. J Infect Dis *189*, 1914-1920.

Halter, R., Pohlner, J., and Meyer, T. (1984). IgA protease of Neisseria gonorrhoeae: isolation and characterization of the gene and its extracellular product. EMBO J *3*, 1595-1601.

Harris, A.G., Wilson, J.E., Danon, S.J., Dixon, M.F., Donegan, K., and Hazell, S.L. (2003). Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the Helicobacter pylori SS1 mouse model. Microbiology *149*, 665-672.

Hauck, C.R., Borisova, M., and Muenzner, P. (2012). Exploitation of integrin function by pathogenic microbes. Curr Opin Cell Biol *24*, 637-644.

Henderson, I., Meehan, M., and Owen, P. (1997). Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in Escherichia coli K-12. FEMS Microbiol Lett *149*, 115-120.

Henderson, I., and Nataro, J. (2001). Virulence functions of autotransporter proteins. Infect Immun *69*, 1231-1243.

Henderson, I., Navarro-Garcia, F., Desvaux, M., Fernandez, R., and Ala'Aldeen, D. (2004). Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev *68*, 692-744.

Henderson, I., Navarro-Garcia, F., and Nataro, J. (1998). The great escape: structure and function of the autotransporter proteins. Trends Microbiol *6*, 370-378.

Hendrixson, D., de la Morena, M., Stathopoulos, C., and St Geme, J.r. (1997). Structural determinants of processing and secretion of the Haemophilus influenzae hap protein. Mol Microbiol *26*, 505-518.

Herrera, V., and Parsonnet, J. (2009). Helicobacter pylori and gastric adenocarcinoma. Clin Microbiol Infect *15*, 971-976.

Hiss, J., Resch, E., Schreiner, A., Meissner, M., Starzinski-Powitz, A., and Schneider, G. (2008). Domain organization of long signal peptides of single-pass integral membrane proteins reveals multiple functional capacity. PLoS One *3*, e2767.

Huang, J., O'Toole, P.W., Doig, P., and Trust, T.J. (1995). Stimulation of interleukin-8 production in epithelial cell lines by Helicobacter pylori. Infect Immun *63*, 1732-1738.

Hynes, R. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549-554.

Ieva, R., Skillman, K., and Bernstein, H. (2008). Incorporation of a polypeptide segment into the beta-domain pore during the assembly of a bacterial autotransporter. Mol Microbiol *67*, 188-201.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I., Kersulyte, D., Incecik, E., Berg, D., Covacci, A., Engstrand, L., and Borén, T. (1998a). Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science *279*, 373-377.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I.M., Kersulyte, D., Incecik, E.T., Berg, D.E., Covacci, A., Engstrand, L., and Borén, T. (1998b). Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science *279*, 373-377.

Ishijima, N., Suzuki, M., Ashida, H., Ichikawa, Y., Kanegae, Y., Saito, I., Borén, T., Haas, R., Sasakawa, C., and Mimuro, H. (2011). BabA-mediated adherence is a potentiator of the Helicobacter pylori type IV secretion system activity. J Biol Chem *286*, 25256-25264.

Israel, D.A., Salama, N., Arnold, C.N., Moss, S.F., Ando, T., Wirth, H.P., Tham, K.T., Camorlinga, M., Blaser, M.J., Falkow, S.*, et al.* (2001). Helicobacter pylori strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. J Clin Invest *107*, 611-620.

Jain, S., van Ulsen, P., Benz, I., Schmidt, M., Fernandez, R., Tommassen, J., and Goldberg, M. (2006). Polar localization of the autotransporter family of large bacterial virulence proteins. J Bacteriol *188*, 4841-4850.

Janakiraman, A., Fixen, K., Gray, A., Niki, H., and Goldberg, M. (2009). A genome-scale proteomic screen identifies a role for DnaK in chaperoning of polar autotransporters in Shigella. J Bacteriol *191*, 6300-6311.

Janakiraman, A., and Goldberg, M. (2004). Recent advances on the development of bacterial poles. Trends Microbiol *12*, 518-525.

Janakiraman, A., and Slauch, J.M. (2000). The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium. Mol Microbiol *35*, 1146-1155.

Janzon, A., Bhuiyan, T., Lundgren, A., Qadri, F., Svennerholm, A.M., and Sjöling, A. (2009). Presence of high numbers of transcriptionally active Helicobacter pylori in vomitus from Bangladeshi patients suffering from acute gastroenteritis. Helicobacter *14*, 237-247.

Jiménez-Soto, L.F., Kutter, S., Sewald, X., Ertl, C., Weiss, E., Kapp, U., Rohde, M., Pirch, T., Jung, K., Retta, S.F.*, et al.* (2009). Helicobacter pylori type IV secretion

apparatus exploits beta1 integrin in a novel RGD-independent manner. PLoS Pathog *5*, e1000684.

Jose, J., Jähnig, F., and Meyer, T. (1995). Common structural features of IgA1 protease-like outer membrane protein autotransporters. Mol Microbiol *18*, 378-380.

Junker, M., Besingi, R., and Clark, P. (2009). Vectorial transport and folding of an autotransporter virulence protein during outer membrane secretion. Mol Microbiol *71*, 1323-1332.

Junker, M., Schuster, C., McDonnell, A., Sorg, K., Finn, M., Berger, B., and Clark, P. (2006). Pertactin beta-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins. Proc Natl Acad Sci U S A *103*, 4918-4923.

Kavermann, H., Burns, B.P., Angermuller, K., Odenbreit, S., Fischer, W., Melchers, K., and Haas, R. (2003). Identification and characterization of Helicobacter pylori genes essential for gastric colonization. J Exp Med *197*, 813-822.

Kawai, M., Furuta, Y., Yahara, K., Tsuru, T., Oshima, K., Handa, N., Takahashi, N., Yoshida, M., Azuma, T., Hattori, M.*, et al.* (2011). Evolution in an oncogenic bacterial species with extreme genome plasticity: Helicobacter pylori East Asian genomes. BMC Microbiol *11*, 104.

Keenan, R., Freymann, D., Stroud, R., and Walter, P. (2001). The signal recognition particle. Annu Rev Biochem *70*, 755-775.

Kelley, L.A., and Sternberg, M.J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc *4*, 363-371.

Kida, Y., Taira, J., Yamamoto, T., Higashimoto, Y., and Kuwano, K. (2013). EprS, an autotransporter protein of Pseudomonas aeruginosa, possessing serine protease activity induces inflammatory responses through protease-activated receptors. Cell Microbiol *15*, 1168-1181.

Klauser, T., Pohlner, J., and Meyer, T. (1990). Extracellular transport of cholera toxin B subunit using Neisseria IgA protease beta-domain: conformation-dependent outer membrane translocation. EMBO J *9*, 1991-1999.

Klemm, P., Hjerrild, L., Gjermansen, M., and Schembri, M. (2004). Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from Escherichia coli. Mol Microbiol *51*, 283-296.

Klemm, P., Vejborg, R., and Sherlock, O. (2006). Self-associating autotransporters, SAATs: functional and structural similarities. Int J Med Microbiol *296*, 187-195.

Kuipers, E.J., Uyterlinde, A.M., Peña, A.S., Roosendaal, R., Pals, G., Nelis, G.F., Festen, H.P., and Meuwissen, S.G. (1995). Long-term sequelae of Helicobacter pylori gastritis. Lancet *345*, 1525-1528.

Kwok, T., Zabler, D., Urman, S., Rohde, M., Hartig, R., Wessler, S., Misselwitz, R., Berger, J., Sewald, N., König, W.*, et al.* (2007). Helicobacter exploits integrin for type IV secretion and kinase activation. Nature *449*, 862-866.

Lamb, A., Yang, X.D., Tsang, Y.H., Li, J.D., Higashi, H., Hatakeyama, M., Peek, R.M., Blanke, S.R., and Chen, L.F. (2009). Helicobacter pylori CagA activates NF-kappaB by targeting TAK1 for TRAF6-mediated Lys 63 ubiquitination. EMBO Rep *10*, 1242-1249.

Lee, A., O'Rourke, J., De Ungria, M.C., Robertson, B., Daskalopoulos, G., and Dixon, M.F. (1997). A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology *112*, 1386-1397.

Lee, K.H., Cho, M.J., Yamaoka, Y., Graham, D.Y., Yun, Y.J., Woo, S.Y., Lim, C.Y., Ko, K.S., Kim, B.J., Jung, H.C.*, et al.* (2004). Alanine-threonine polymorphism of Helicobacter pylori RpoB is correlated with differential induction of interleukin-8 in MKN45 cells. J Clin Microbiol *42*, 3518-3524.

Lee, S.H., Butler, S.M., and Camilli, A. (2001). Selection for in vivo regulators of bacterial virulence. Proc Natl Acad Sci U S A *98*, 6889-6894.

Leininger, E., Roberts, M., Kenimer, J., Charles, I., Fairweather, N., Novotny, P., and Brennan, M. (1991). Pertactin, an Arg-Gly-Asp-containing Bordetella pertussis surface protein that promotes adherence of mammalian cells. Proc Natl Acad Sci U S A *88*, 345-349.

Lenz, J.D., Lawrenz, M.B., Cotter, D.G., Lane, M.C., Gonzalez, R.J., Palacios, M., and Miller, V.L. (2011). Expression during host infection and localization of Yersinia pestis autotransporter proteins. J Bacteriol *193*, 5936-5949.

Lenz, J.D., Temple, B.R., and Miller, V.L. (2012). Evolution and virulence contributions of the autotransporter proteins YapJ and YapK of Yersinia pestis

CO92 and their homologs in Y. pseudotuberculosis IP32953. Infect Immun *80*, 3693-3705.

Lepper, P.M., Triantafilou, M., Schumann, C., Schneider, E.M., and Triantafilou, K. (2005). Lipopolysaccharides from Helicobacter pylori can act as antagonists for Toll-like receptor 4. Cell Microbiol *7*, 519-528.

Lin, L.F., Posfai, J., Roberts, R.J., and Kong, H. (2001). Comparative genomics of the restriction-modification systems in Helicobacter pylori. Proc Natl Acad Sci U S A *98*, 2740-2745.

Lindenthal, C., and Elsinghorst, E. (2001). Enterotoxigenic Escherichia coli TibA glycoprotein adheres to human intestine epithelial cells. Infect Immun *69*, 52-57.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Loh, J.T., Gupta, S.S., Friedman, D.B., Krezel, A.M., and Cover, T.L. (2010). Analysis of protein expression regulated by the Helicobacter pylori ArsRS two-component signal transduction system. J Bacteriol *192*, 2034-2043.

Loveless, B., and Saier, M.J. (1997). A novel family of channel-forming, autotransporting, bacterial virulence factors. Mol Membr Biol *14*, 113-123.

Lowe, A.M., Beattie, D.T., and Deresiewicz, R.L. (1998). Identification of novel staphylococcal virulence genes by in vivo expression technology. Mol Microbiol *27*, 967-976.

Lowenthal, A.C., Hill, M., Sycuro, L.K., Mehmood, K., Salama, N.R., and Ottemann, K.M. (2009). Functional analysis of the Helicobacter pylori flagellar switch proteins. J Bacteriol *191*, 7147-7156.

Löwer, M., Weydig, C., Metzler, D., Reuter, A., Starzinski-Powitz, A., Wessler, S., and Schneider, G. (2008). Prediction of extracellular proteases of the human pathogen Helicobacter pylori reveals proteolytic activity of the Hp1018/19 protein HtrA. PLoS One *3*, e3510.

Mahdavi, J., Sondén, B., Hurtig, M., Olfat, F.O., Forsberg, L., Roche, N., Angstrom, J., Larsson, T., Teneberg, S., Karlsson, K.A*., et al.* (2002). Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. Science *297*, 573-578.

Marchetti, M., and Rappuoli, R. (2002). Isogenic mutants of the cag pathogenicity island of Helicobacter pylori in the mouse model of infection: effects on colonization efficiency. Microbiology *148*, 1447-1456.

Martoglio, B., and Dobberstein, B. (1998). Signal sequences: more than just greasy peptides. Trends Cell Biol *8*, 410-415.

Maurer, J., Brown, T., Steffens, W., and Thayer, S. (1998). The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin tsh among avian Escherichia coli. Avian Dis *42*, 106-118.

Merrell, D.S., Goodrich, M.L., Otto, G., Tompkins, L.S., and Falkow, S. (2003). pH-regulated gene expression of the gastric pathogen Helicobacter pylori. Infect Immun *71*, 3529-3539.

Mimuro, H., Suzuki, T., Nagai, S., Rieder, G., Suzuki, M., Nagai, T., Fujita, Y., Nagamatsu, K., Ishijima, N., Koyasu, S.*, et al.* (2007). Helicobacter pylori dampens gut epithelial self-renewal by inhibiting apoptosis, a bacterial strategy to enhance colonization of the stomach. Cell Host Microbe *2*, 250-263.

Moese, S., Selbach, M., Brinkmann, V., Karlas, A., Haimovich, B., Backert, S., and Meyer, T.F. (2007). The Helicobacter pylori CagA protein disrupts matrix adhesion of gastric epithelial cells by dephosphorylation of vinculin. Cell Microbiol *9*, 1148-1161.

Montecucco, C., and Rappuoli, R. (2001). Living dangerously: how Helicobacter pylori survives in the human stomach. Nat Rev Mol Cell Biol *2*, 457-466.

Moormann, C., Benz, I., and Schmidt, M. (2002). Functional substitution of the TibC protein of enterotoxigenic Escherichia coli strains for the autotransporter adhesin heptosyltransferase of the AIDA system. Infect Immun *70*, 2264-2270.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., and Rashtchian, A. (2002). Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res *30*, e37.

Neal, J.T., Peterson, T.S., Kent, M.L., and Guillemin, K. (2013). H. pylori virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model. Dis Model Mech *6*, 802-810.

Obonyo, M., Guiney, D.G., Harwood, J., Fierer, J., and Cole, S.P. (2002). Role of gamma interferon in Helicobacter pylori induction of inflammatory mediators during murine infection. Infect Immun *70*, 3295-3299.

Oldani, A., Cormont, M., Hofman, V., Chiozzi, V., Oregioni, O., Canonici, A., Sciullo, A., Sommi, P., Fabbri, A., Ricci, V*., et al.* (2009). Helicobacter pylori counteracts the apoptotic action of its VacA toxin by injecting the CagA protein into gastric epithelial cells. PLoS Pathog *5*, e1000603.

Oliver, D., Huang, G., Nodel, E., Pleasance, S., and Fernandez, R. (2003). A conserved region within the Bordetella pertussis autotransporter BrkA is necessary for folding of its passenger domain. Mol Microbiol *47*, 1367-1383.

Oomen, C., van Ulsen, P., van Gelder, P., Feijen, M., Tommassen, J., and Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. EMBO J *23*, 1257-1266.

Ottemann, K.M., and Lowenthal, A.C. (2002). Helicobacter pylori uses motility for initial colonization and to attain robust infection. Infect Immun *70*, 1984-1990.

Owen, P., Meehan, M., de Loughry-Doherty, H., and Henderson, I. (1996). Phase-variable outer membrane proteins in Escherichia coli. FEMS Immunol Med Microbiol *16*, 63-76.

Owyang, S.Y., Luther, J., Owyang, C.C., Zhang, M., and Kao, J.Y. (2012). Helicobacter pylori DNA's anti-inflammatory effect on experimental colitis. Gut Microbes *3*, 168-171.

Pappo, J., Torrey, D., Castriotta, L., Savinainen, A., Kabok, Z., and Ibraghimov, A. (1999). Helicobacter pylori infection in immunized mice lacking major histocompatibility complex class I and class II functions. Infect Immun *67*, 337-341.

Pflock, M., Dietz, P., Schär, J., and Beier, D. (2004). Genetic evidence for histidine kinase HP165 being an acid sensor of Helicobacter pylori. FEMS Microbiol Lett *234*, 51-61.

Pflock, M., Finsterer, N., Joseph, B., Mollenkopf, H., Meyer, T.F., and Beier, D. (2006a). Characterization of the ArsRS regulon of Helicobacter pylori, involved in acid adaptation. J Bacteriol *188*, 3449-3462.

Pflock, M., Kennard, S., Delany, I., Scarlato, V., and Beier, D. (2005). Acid-induced activation of the urease promoters is mediated directly by the ArsRS two-component system of Helicobacter pylori. Infect Immun *73*, 6437-6445.

Pflock, M., Kennard, S., Finsterer, N., and Beier, D. (2006b). Acid-responsive gene regulation in the human pathogen Helicobacter pylori. J Biotechnol *126*, 52-60.

Phadnis, S.H., Ilver, D., Janzon, L., Normark, S., and Westblom, T.U. (1994). Pathological significance and molecular characterization of the vacuolating toxin gene of Helicobacter pylori. Infect Immun *62*, 1557-1565.

Pinto-Santini, D.M., and Salama, N.R. (2009). Cag3 is a novel essential component of the Helicobacter pylori Cag type IV secretion system outer membrane subcomplex. J Bacteriol *191*, 7343-7352.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T. (1987). Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease. Nature *325*, 458-462.

Poppe, M., Feller, S.M., Römer, G., and Wessler, S. (2007). Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. Oncogene *26*, 3462-3472.

Porter, M., and Dorman, C. (1997). Differential regulation of the plasmid-encoded genes in the Shigella flexneri virulence regulon. Mol Gen Genet *256*, 93-103.

Radin, J.N., Gaddy, J.A., González-Rivera, C., Loh, J.T., Algood, H.M., and Cover, T.L. (2013). Flagellar localization of a Helicobacter pylori autotransporter protein. MBio *4*, e00613-00612.

Rajakumar, K., Jost, B., Sasakawa, C., Okada, N., Yoshikawa, M., and Adler, B. (1994). Nucleotide sequence of the rhamnose biosynthetic operon of Shigella flexneri 2a and role of lipopolysaccharide in virulence. J Bacteriol *176*, 2362-2373.

Renn, J., and Clark, P. (2008). A conserved stable core structure in the passenger domain beta-helix of autotransporter virulence proteins. Biopolymers *89*, 420-427.

Rieder, G., Merchant, J.L., and Haas, R. (2005). Helicobacter pylori cag-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. Gastroenterology *128*, 1229-1242.

Robbins, J., Monack, D., McCallum, S., Vegas, A., Pham, E., Goldberg, M., and Theriot, J. (2001). The making of a gradient: IcsA (VirG) polarity in Shigella flexneri. Mol Microbiol *41*, 861-872.

Ruiz-Perez, F., Henderson, I., Leyton, D., Rossiter, A., Zhang, Y., and Nataro, J. (2009). Roles of periplasmic chaperone proteins in the biogenesis of serine protease autotransporters of Enterobacteriaceae. J Bacteriol *191*, 6571-6583.

Rüter, C., and Hardwidge, P.R. (2014). 'Drugs from bugs': bacterial effector proteins as promising biological (immune-) therapeutics. FEMS Microbiol Lett *351*, 126-132.

Sabarth, N., Hurvitz, R., Schmidt, M., Zimny-Arndt, U., Jungblut, P.R., Meyer, T.F., and Bumann, D. (2005). Identification of Helicobacter pylori surface proteins by selective proteinase K digestion and antibody phage display. J Microbiol Methods *62*, 345-349.

Salama, N.R., Otto, G., Tompkins, L., and Falkow, S. (2001). Vacuolating cytotoxin of Helicobacter pylori plays a role during colonization in a mouse model of infection. Infect Immun *69*, 730-736.

Sauri, A., Soprova, Z., Wickström, D., de Gier, J., Van der Schors, R., Smit, A., Jong, W., and Luirink, J. (2009). The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease. Microbiology *155*, 3982-3991.

Sause, W.E., Castillo, A.R., and Ottemann, K.M. (2012). The Helicobacter pylori autotransporter ImaA (HP0289) modulates the immune response and contributes to host colonization. Infect Immun *80*, 2286-2296.

Saviola, B., Woolwine, S.C., and Bishai, W.R. (2003). Isolation of acid-inducible genes of Mycobacterium tuberculosis with the use of recombinase-based in vivo expression technology. Infect Immun *71*, 1379-1388.

Schultz, J., Milpetz, F., Bork, P., and Ponting, C.P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A *95*, 5857-5864.

Senkovich, O.A., Yin, J., Ekshyyan, V., Conant, C., Traylor, J., Adegboyega, P., McGee, D.J., Rhoads, R.E., Slepenkov, S., and Testerman, T.L. (2011). Helicobacter pylori AlpA and AlpB bind host laminin and influence gastric inflammation in gerbils. Infect Immun *79*, 3106-3116.

Serruto, D., Adu-Bobie, J., Scarselli, M., Veggi, D., Pizza, M., Rappuoli, R., and Aricò, B. (2003). Neisseria meningitidis App, a new adhesin with autocatalytic serine protease activity. Mol Microbiol *48*, 323-334.

Shaffer, C.L., Gaddy, J.A., Loh, J.T., Johnson, E.M., Hill, S., Hennig, E.E., McClain, M.S., McDonald, W.H., and Cover, T.L. (2011). Helicobacter pylori exploits a unique repertoire of type IV secretion system components for pilus assembly at the bacteria-host cell interface. PLoS Pathog *7*, e1002237.

Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermüller, J., Reinhardt, R.*, et al.* (2010). The primary transcriptome of the major human pathogen Helicobacter pylori. Nature *464*, 250-255.

Shere, K., Sallustio, S., Manessis, A., D'Aversa, T., and Goldberg, M. (1997). Disruption of IcsP, the major Shigella protease that cleaves IcsA, accelerates actin-based motility. Mol Microbiol *25*, 451-462.

Sherlock, O., Dobrindt, U., Jensen, J., Munk Vejborg, R., and Klemm, P. (2006). Glycosylation of the self-recognizing Escherichia coli Ag43 autotransporter protein. J Bacteriol *188*, 1798-1807.

Shibata, J., Goto, H., Arisawa, T., Niwa, Y., Hayakawa, T., Nakayama, A., and Mori, N. (1999). Regulation of tumour necrosis factor (TNF) induced apoptosis by soluble TNF receptors in Helicobacter pylori infection. Gut *45*, 24-31.

Sijbrandi, R., Urbanus, M., ten Hagen-Jongman, C., Bernstein, H., Oudega, B., Otto, B., and Luirink, J. (2003). Signal recognition particle (SRP)-mediated targeting and Sec-dependent translocation of an extracellular Escherichia coli protein. J Biol Chem *278*, 4654-4659.

Slauch, J.M., and Camilli, A. (2000). IVET and RIVET: use of gene fusions to identify bacterial virulence factors specifically induced in host tissues. Methods Enzymol *326*, 73-96.

Solnick, J.V., Hansen, L.M., Salama, N.R., Boonjakuakul, J.K., and Syvanen, M. (2004). Modification of Helicobacter pylori outer membrane protein expression during experimental infection of rhesus macaques. Proc Natl Acad Sci U S A *101*, 2106-2111.

Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W.J., and Covacci, A. (2002). c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol *43*, 971-980.

Struyvé, M., Moons, M., and Tommassen, J. (1991). Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J Mol Biol *218*, 141-148.

Styer, C.M., Hansen, L.M., Cooke, C.L., Gundersen, A.M., Choi, S.S., Berg, D.E., Benghezal, M., Marshall, B.J., Peek, R.M., Borén, T.*, et al.* (2010). Expression of the

BabA adhesin during experimental infection with Helicobacter pylori. Infect Immun *78*, 1593-1600.

Suerbaum, S., and Michetti, P. (2002). Helicobacter pylori infection. N Engl J Med *347*, 1175-1186.

Terry, K., Williams, S.M., Connolly, L., and Ottemann, K.M. (2005). Chemotaxis plays multiple roles during Helicobacter pylori animal infection. Infect Immun *73*, 803-811.

Testerman, T.L., McGee, D.J., and Mobley, H.L. (2001). Helicobacter pylori growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. J Clin Microbiol *39*, 3842-3850.

Thanassi, D., Stathopoulos, C., Karkal, A., and Li, H. (2005). Protein secretion in the absence of ATP: the autotransporter, two-partner secretion and chaperone/usher pathways of gram-negative bacteria (review). Mol Membr Biol *22*, 63-72.

Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A.*, et al.* (1997). The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature *388*, 539-547.

Tsugawa, H., Suzuki, H., Saya, H., Hatakeyama, M., Hirayama, T., Hirata, K., Nagano, O., Matsuzaki, J., and Hibi, T. (2012). Reactive oxygen species-induced autophagic degradation of Helicobacter pylori CagA is specifically suppressed in cancer stem-like cells. Cell Host Microbe *12*, 764-777.

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M., and Hatakeyama, M. (2003). Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. J Biol Chem *278*, 3664-3670.

van Amsterdam, K., van Vliet, A., Kusters, J., Feller, M., Dankert, J., and van der Ende, A. (2003). Induced Helicobacter pylori vacuolating cytotoxin VacA expression after initial colonisation of human gastric epithelial cells. FEMS Immunol Med Microbiol *39*, 251-256.

Van Gerven, N., Sleutel, M., Deboeck, F., De Greve, H., and Hernalsteens, J.P. (2009). Surface display of the receptor-binding domain of the F17a-G fimbrial adhesin through the autotransporter AIDA-I leads to permeability of bacterial cells. Microbiology *155*, 468-476.

van Ulsen, P., van Alphen, L., ten Hove, J., Fransen, F., van der Ley, P., and Tommassen, J. (2003). A Neisserial autotransporter NalP modulating the processing of other autotransporters. Mol Microbiol *50*, 1017-1030.

Veal-Carr, W.L., and Stibitz, S. (2005). Demonstration of differential virulence gene promoter activation in vivo in Bordetella pertussis using RIVET. Mol Microbiol *55*, 788-798.

Veiga, E., de Lorenzo, V., and Fernández, L. (1999). Probing secretion and translocation of a beta-autotransporter using a reporter single-chain Fv as a cognate passenger domain. Mol Microbiol *33*, 1232-1243.

Veiga, E., Sugawara, E., Nikaido, H., de Lorenzo, V., and Fernández, L. (2002). Export of autotransported proteins proceeds through an oligomeric ring shaped by C-terminal domains. EMBO J *21*, 2122-2131.

Velarde, J., and Nataro, J. (2004). Hydrophobic residues of the autotransporter EspP linker domain are important for outer membrane translocation of its passenger. J Biol Chem *279*, 31495-31504.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Mémet, S., Huerre, M.R., Coyle, A.J.*, et al.* (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol *5*, 1166-1174.

Watanabe, T., Asano, N., Fichtner-Feigl, S., Gorelick, P.L., Tsuji, Y., Matsumoto, Y., Chiba, T., Fuss, I.J., Kitani, A., and Strober, W. (2010). NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest *120*, 1645-1662.

Wells, T., Tree, J., Ulett, G., and Schembri, M. (2007). Autotransporter proteins: novel targets at the bacterial cell surface. FEMS Microbiol Lett *274*, 163-172.

Wen, Y., Feng, J., Scott, D.R., Marcus, E.A., and Sachs, G. (2007). The HP0165-HP0166 two-component system (ArsRS) regulates acid-induced expression of HP1186 alpha-carbonic anhydrase in Helicobacter pylori by activating the pH-dependent promoter. J Bacteriol *189*, 2426-2434.

Wiedemann, T., Hofbaur, S., Tegtmeyer, N., Huber, S., Sewald, N., Wessler, S., Backert, S., and Rieder, G. (2012). Helicobacter pylori CagL dependent induction

of gastrin expression via a novel αvβ5-integrin-integrin linked kinase signalling complex. Gut *61*, 986-996.

Williams, C.L., Haines, R., and Cotter, P.A. (2008). Serendipitous discovery of an immunoglobulin-binding autotransporter in Bordetella species. Infect Immun *76*, 2966-2977.

Williams, S.M., Chen, Y.T., Andermann, T.M., Carter, J.E., McGee, D.J., and Ottemann, K.M. (2007). Helicobacter pylori chemotaxis modulates inflammation and bacterium-gastric epithelium interactions in infected mice. Infect Immun *75*, 3747-3757.

Yahiro, K., Wada, A., Nakayama, M., Kimura, T., Ogushi, K., Niidome, T., Aoyagi, H., Yoshino, K., Yonezawa, K., Moss, J.*, et al.* (2003). Protein-tyrosine phosphatase alpha, RPTP alpha, is a Helicobacter pylori VacA receptor. J Biol Chem *278*, 19183-19189.

Yamaoka, Y., Kita, M., Kodama, T., Imamura, S., Ohno, T., Sawai, N., Ishimaru, A., Imanishi, J., and Graham, D.Y. (2002). Helicobacter pylori infection in mice: Role of outer membrane proteins in colonization and inflammation. Gastroenterology *123*, 1992-2004.

Yamaoka, Y., Kita, M., Kodama, T., Sawai, N., Kashima, K., and Imanishi, J. (1997). Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. Gut *41*, 442-451.

Yamaoka, Y., Kwon, D.H., and Graham, D.Y. (2000). A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. Proc Natl Acad Sci U S A *97*, 7533-7538.

Yen, M., Peabody, C., Partovi, S., Zhai, Y., Tseng, Y., and Saier, M. (2002). Protein-translocating outer membrane porins of Gram-negative bacteria. Biochim Biophys Acta *1562*, 6-31.