REGULATION OF THE *HELICOBACTER PYLORI* RpoN REGULON BY THE FLAGELLAR PROTEIN EXPORT APPARATUS

by

TODD GARETH SMITH

(Under the Direction of Timothy R. Hoover)

ABSTRACT

Helicobacter pylori is a significant human pathogen that infects a large percentage of the worldwide population with infections potentially resulting in acute gastritis, peptic ulcers, gastric carcinoma and non-Hodgkin lymphoma. Many essential *H. pylori* colonization factors have been identified including flagellar motility. Flagellar biosynthesis requires over 40 proteins and all three sigma factors (RpoD, RpoN and FliA) in the cell. Transcription of the *H. pylori* RpoN regulon is controlled by the FlgS/FlgR two-component regulatory system which responds to undefined cellular cues. Previous studies showed that expression of the RpoN- and FliA-dependent flagellar genes is linked to a functional flagellar protein export apparatus. FlhB, a membrane-bound component of the export apparatus, has a large cytoplasmic domain ($FlhB_C$) which is processed by a site-specific autocleavage. $FlhB_C$ processing accompanies a switch in substrate specificity of the export apparatus. To determine if processing of FlhB influenced flagellar gene expression, two mutations at the cleavage site in FlhB were constructed. Both substitutions inhibited autocleavage of FlhB as well as motility. The mutants were able to export rod-/hook-type substrates but not filament-type substrates. The FlhB variant strains expressed RpoN- and FliA-dependent reporter genes at wild-type levels. Disruption of the hook length

control protein FliK in the FlhB variant strains had different consequences for expression of the reporter genes suggesting that FliK has different effects on the export apparatus depending on the conformation of FlhB. Disruption of *fliK* in a Δ*flhB* mutant did not restore expression of RpoN-dependent reporter genes indicating that failure to export FliK does not account for inhibition of the RpoN regulon in export apparatus mutants. FlhA, another membrane-bound component of the export apparatus, also has a large cytoplasmic domain. Specific *flhA* insertion mutations stimulated expression of RpoN-dependent reporter genes. These *flhA* mutants were deficient in motility and export of both rod-/hook-type and filament-type substrates confirming that the export apparatus is not required for export of an inhibitor of the RpoN regulon. Taken together, these results support a model in which FlgS directly or indirectly senses the conformation of the export apparatus.

INDEX WORDS: *Helicobacter pylori*, flagella, regulation, flagellar export apparatus, FlhB, FliK, FlhA, RpoN

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DEDICATION

This work is dedicated to all the teachers in my life from my first and greatest teachers Mom and Dad, to my elementary school teacher Betty Spradlin who let me "experiment" by making paper airplanes, to Scott Kegley who first opened my eyes to the microbial world, to my high school science teachers Rick Alwood and Randy McClay who taught me more than just basic chemistry and biology, to Rick Clark my high school soccer coach, to my pastors Dr. Harley Roston and Rev. Troy Diersing, to my undergraduate mentors Dr. Neil Baker, Dr. Stephanie Scott and Dr. Bob Tabita who gave me my first opportunities to "do" science, through my graduate advisor Dr. Tim Hoover who has led by example and shown abundant patience in teaching me how to think about science and life. Last but not least, this work is dedicated to my loving and supportive wife Sarah.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Background and Significance

Helicobacter pylori, the etiological agent of acute gastritis leading to peptic ulcers, infects a large percentage of the worldwide population (Dooley *et al.*, 1989; Graham *et al.*, 1992; Marshall *et al.*, 1985; Megraud *et al.*, 1989). In the United States approximately 50% of the population over the age of 60 is infected with *H. pylori* , and diseases associated with *H pylori* infection result in an estimated one million hospitalizations and 6,500 deaths each year (N.D.D.I.C., 2004; Sonnenberg & Everhart, 1997). In developing countries the infection rate is predicted to be as high as 90% of the population (Blaser, 1990). *H. pylori* is the only bacterium designated as a Class 1 carcinogen by the World Health Organization for its role in gastric carcinoma and B-cell mucosa-associated lymphoma (Anonymous, 1994).

In addition to being involved in morbidity and mortality, *H. pylori* infections also have a significant monetary impact. In the United States in 1997 (the most recent year for which the impact *H. pylori* infection was assessed) the financial burden associated with diseases caused by *H. pylori* was estimated to be $6 billion (Sonnenberg & Everhart, 1997). Five years later the cost associated with just peptic ulcer disease was estimated to be $3.1 billion (Sandler *et al.*, 2002). Treatment of *H. pylori* infections generally involves a triple therapy consisting of two antibiotics and an acid inhibitor. Although the triple therapy is greater than 90% effective in eradicating *H. pylori* (Lind *et al.*, 1996), compliance can be difficult for patients who must take as many as 20

pills per day for up to 14 days. This is particularly problematic for patients in developing countries who have limited access to adequate health care and where it is difficult for medical professionals to monitor patients' compliance with the therapy. In addition, general side effects including nausea, vomiting, diarrhea, dizziness, headaches, and yeast infections in women are associated with the triple therapy. As with other bacterial pathogens antibiotic resistance is also a growing risk to effective treatment (Megraud *et al.*, 1999). The continued impact of *H. pylori* infection on society highlights the need for better strategies for treatment and prevention.

Marshall and Warren first cultured *H. pylori* in 1982 from human stomach biopsy samples from patients with peptic ulcers and then subsequently demonstrated its pathology (Marshall & Warren, 1984). In recognition of their pioneering work, they shared the 2005 Nobel Prize in Medicine. *H. pylori* is a Gram type-negative, spiral shaped, ε-Proteobacteria closely related to other pathogenic bacteria. These include members of the genus *Campylobacter* and *Arcobacter* including: *Campylobacter jejuni* and *Campylobacter coli* which are the leading causes of bacterial, food-borne gastroenteritis in humans (Mead *et al.*, 1999) and are also responsible for the autoimmune disease Guillain-Barre Syndrome (Mishu & Blaser, 1993); *Campylobacter upsaliensis* and *Campylobacter lari* which also cause gastroenteritis in humans but are not as common as *C. jejuni, C. coli* or *Arcobacter butzleri* (Prouzet-Mauleon *et al.*, 2006); *Campylobacter rectus* which is associated with periodontal diseases (Macuch & Tanner, 2000); *Campylobacter fetus* which mainly infects livestock resulting in infertility, aborted pregnancy, meningitis, and septicemia , but also opportunistically infects humans (Blaser, 1998); and *Arcobacter butzleri* which causes gastroenteritis in humans (Kiehlbauch *et al.*, 1991). The genus *Helicobacter* has expanded with new species being added from different hosts such as

Helicobacter acinonychis isolated from cheetahs (Eaton *et al.*, 1993) and different colonization sites such as *Helicobacter hepaticus* isolated from the liver of mice (Fox *et al.*, 1994).

Many tools are available for the study of *H. pylori* including six completed and seven in-progress *H. pylori* genome sequences (Alm *et al.*, 1999; Oh *et al.*, 2006; Tomb *et al.*, 1997) and a protein-protein interaction map from the yeast two-hybrid system (Rain *et al.*, 2001). In addition, several different animal models have been established. Currently mice infected with mouse-adapted *H. pylori* strains are the principal animal model (Lee *et al.*, 1997). Other animal models include gnotobiotic piglets, Mongolian gerbils, cats infected with *Helicobacter felis* (Esteves *et al.*, 2000) and ferrets infected with *Helicobacter mustelae* (Fox *et al.*, 1990). All of the animals experimentally infected display gastritis, most develop ulceration, and Mongolian gerbils proceed to develop adenocarcinoma similar to humans (Watanabe *et al.*, 1998). Using the available animal models, many factors essential for colonization have been identified including but not limited to urease (Eaton *et al.*, 1991), hydrogenase (Olson & Maier, 2002), oxidative stress response enzymes (Alamuri & Maier, 2004; Harris *et al.*, 2003; Seyler *et al.*, 2001), adhesins (Hessey *et al.*, 1990), and motility (Eaton *et al.*, 1992).

Colonization and Virulence Factors

One of the first *H. pylori* colonization factors described was urease (Eaton *et al.*, 1991). Urease is one of the most abundant proteins in *H. pylori* and catalyses the breakdown of urea into ammonia and carbamate. The carbamate spontaneously decomposes to ammonia and carbonic acid, which increase the pH in the microenvironment around *H. pylori* (Marshall *et al.*, 1990). Despite the lack of a clear secretion signal peptide, urease localizes both to the cytoplasm and outer membrane, probably attaching to the cell surface after the lysis of neighboring cells

(Bauerfeind *et al.*, 1997). Urease is a large complex made up of two subunits that contain nickel cofactors in the active site (Hawtin *et al.*, 1991; Labigne *et al.*, 1991). Nickel acquisition is a vital function in *H. pylori* (Bauerfeind *et al.*, 1996) as hydrogenase also requires nickel cofactors (Maier *et al.*, 1996).

The membrane-bound, hydrogen-uptake hydrogenase is important for respiratory energy and redox generation (Olson & Maier, 2002) since *H. pylori* utilizes very few carbohydrates as energy sources (Doig *et al.*, 1999). Moreover, the gastric epithelium is relative poor in carbohydrates that *H. pylori* can utilize, but molecular hydrogen is readily available in these tissues (Maier, 2005). Consistent with a role in energy maintenance, hydrogenase mutants are not able to colonize and persist in the mouse stomach as well as wild type (Olson & Maier, 2002).

Catalase, like urease, is expressed at very high levels in the cytoplasm and attaches to the cell surface possibly by a similar mechanism as urease. Oxidative stress response enzymes such as catalase, superoxide dismutase and methionine sulphoxide reductase are required for *H. pylori* colonization (Alamuri & Maier, 2004; Harris *et al.*, 2003; Seyler *et al.*, 2001). To establish a persistent infection, *H. pylori* must combat reactive oxygen species produced from the harsh microaerophilic environment and from the host immune response. Superoxide dismutase detoxifies the superoxide anion to hydrogen peroxide which is further detoxified by catalase to water and molecular oxygen, while methionine sulphoxide reductase repairs methionine-rich proteins such as catalase that are damaged by reactive oxygen species (Alamuri & Maier, 2006).

In addition to its primary function in oxidative stress, catalase also participates in adherence by binding phosphatidylethanolamine and other lipids (Lingwood *et al.*, 1993). Several outer membrane proteins and lipopolysaccharide (LPS) have been identified as *H. pylori*

adhesins. The major adhesins binding to host receptors are BabA to Lewis B antigen (Ilver *et al.*, 1998), HpaA to sialic acid (Evans *et al.*, 1993), Hsp60 to sulfatides (Huesca *et al.*, 1996) and Nap to mucin (Namavar *et al.*, 1998). Adherence is initiated by the interaction of LPS with mucin, followed by the other adhesins in an arbitrary fashion (Piotrowski *et al.*, 1994). LPS also initiates laminin-binding with the host cell (Slomiany *et al.*, 1991). Outer membrane proteins (OMPs) AlpA, AlpB, HopZ and HorB participate in adherence to host cells, but their cognate host receptors have not been identified(Odenbreit *et al.*, 2002; Peck *et al.*, 1999; Snelling *et al.*, 2007). HpaA, in addition to adhering to sialic acid residues, is the major protein component of the flagellar sheath. The flagellar sheath, which surrounds the flagellum and is contiguous with the outer membrane (Goodwin *et al.*, 1985), may protect the flagella from the highly acidic environment in the gastric lumen (Jones *et al.*, 1997). The flagellum itself does not appear to participate directly in host cell adherence (Foynes *et al.*, 1999).

Approximately 15% of those infected with *H. pylori* develop advanced pathology. Environmental factors such as the host immune response, genetic variability of the host and genetic variability of the colonizing *H. pylori* strain may influence disease outcome (Atherton, 2006). The primary virulence factors are the cytotoxin-associated genes (*cag*) and vacuolating cytotoxin (VacA). The cytoxin-associated genes are located on the *cag* pathogenicity island which encodes components of a type IV secretion system (Censini *et al.*, 1996). The first gene of the pathogenicity island, *cagA* encodes the effector (Odenbreit *et al.*, 2000; Stein *et al.*, 2000). When CagA is translocated into host epithelial cells it can be phosphorylated by Src kinase allowing it to disrupt MAP kinase signaling (Selbach *et al.*, 2002; Stein *et al.*, 2002). This results in changes in host cell morphology (Moese *et al.*, 2004; Segal *et al.*, 1999) including induction of the hummingbird (elongated cell shape) phenotype, cell proliferation (Peek *et al.*,

1997) and altered apoptosis (Moss *et al.*, 2001). Unphosphorylated CagA disrupts tight junctions possibly causing nutrient leakage around the infection site (Amieva *et al.*, 2003; Atherton, 2006). Clearly CagA-mediated pathogenesis would be predicted to increase occurrence and severity of pathology. Consistent with this hypothesis, the majority of *H. pylori* strains isolated from patients with peptic ulcer or gastic cancers express *cagA* while *H. pylori* strains that do not express *cagA* or lack part or all of the *cag* pathogenicity island are rarely associated with disease (Blaser *et al.*, 1995; Crabtree *et al.*, 1991; Nomura *et al.*, 2002). In addition, the number of reactive tyrosine phosphorylation sites in CagA can vary , and more phosphorylation sites are associated with increased risk of gastric cancer (Argent *et al.*, 2004; Azuma *et al.*, 2002).

Genetic variation in *vacA* is also associated with disparities in virulence between strains (Atherton *et al.*, 1995; Cover *et al.*, 1994). VacA is exported from the cell by an autotransport pathway. Both the autotransporter and a signal peptide are cleaved to produce the mature toxin which is subdivided into two subunits (Telford *et al.*, 1994). The C-terminal subunit is responsible for host cell binding, while the N-terminal subunit forms a hexameric pore (Atherton, 2006; Czajkowsky *et al.*, 1999). These pores cause osmotic stress and large acid vacuoles in host cells (Szabo *et al.*, 1999). However, secondary effects such as induction of apoptosis (Kuck *et al.*, 2001), nutrient leakage (Papini *et al.*, 1998) and interference in host cell signaling by sequesting proteins in the acid vacuole (Nakayama *et al.*, 2004) may be the primary functions of VacA that result in pathology.

H. pylori motility is driven by two to six polar, sheathed flagella. Flagellar motility is essential for colonization of the gastric mucosa (Eaton *et al.*, 1992) as *H. pylori* is unable to persist in the extremely low pH of the gastric lumen and must penetrate the gastric mucosa to survive in the stomach (Schreiber *et al.*, 2005). Similar to other Gram type-negative bacteria,

the *H. pylori* flagellum structure consists of a basal body, hook, and filament. The basal body anchors the flagellum in the bacterial cell envelope and contains a type III secretion flagellar protein export apparatus, a reversible rotary motor and a rod that acts as the driveshaft. The hook, which is a flexible linker, acts as a universal joint between the rod and the filament. Finally, the filament acts as the propeller pushing against the extracellular milieu to move the bacterium forward (Berg, 2003; Macnab, 1996). Assembly of the flagellum structure requires the coordinated expression of over 40 genes scattered though out the chromosome as single open reading frames or short operons (Tomb *et al.*, 1997). All three sigma factors found in the cell are required for flagellar gene expression (Spohn & Scarlato, 2001). The flagellar protein export apparatus is responsible for translocating most of the flagellar proteins localized outside the cell membrane (Macnab, 2003) and plays a key role in flagellar gene regulation as described in later chapters.

Flagellar Gene Regulation and Flagellum Assembly in *H. pylori*

As mentioned above σ^{80} (RpoD), σ^{54} (RpoN) and σ^{28} (FliA) are required for transcription of flagellar genes in *H. pylori*. These are the only sigma factors found in *H. pylori* and both σ^{54} and σ^{28} are dedicated to transcription of flagellar genes. The first gene products assembled form the basal body and are encoded by genes that are σ^{80}-dependent. The RpoD regulon is by far the largest of the flagellar regulons. This regulon contains the genes for most of the basal body structural proteins, all of the export apparatus components, the motor, many of the associated accessory proteins, and all of the chemotaxis proteins. Little is known about transcriptional control of the RpoD regulon. When other flagellar regulatory proteins such as RpoN and FlgM are inactivated the majority of flagellar genes are unaffected and thus appear to be σ^{80}-dependent

(Niehus *et al.*, 2004). Neither the signal that induces expression of the RpoD flagellar regulon nor a master regulator that initiates a flagellar gene transcriptional hierarchy have been identified. Since σ^{80} is also the housekeeping sigma factor in *H. pylori* low level expression of the RpoD regulon may be constitutive.

The MS-ring is the first flagellar substructure to be assembled. The MS-ring consists of subunits of the FliM protein and is assembled within the inner membrane (Homma *et al.*, 1987a). The MS-ring forms a central pore into which the flagellar protein export apparatus is assembled (Katayama *et al.*, 1996). The flagellar protein export apparatus is a type III secretion system and transports most of the flagellar proteins that are localized outside the cell membrane (Hirano *et al.*, 2003). The export apparatus is made up of six integral membrane proteins FlhA, FlhB, FliO, FliP, FliQ and FliR, and three cytoplasmic proteins, FliH, FliI and FliJ (Minamino & Macnab, 1999). The H. pylori export apparatus is made up of the same components except no FliJ homolog has been identified. The membrane-bound components of the export apparatus form a specialized pore through which flagellar proteins are translocated in an unfolded state (Aizawa, 1996). FlhA and FlhB have large cytoplasmic domains referred to as $FlhA_C$ and $FlhB_C$ (McMurry *et al.*, 2004; Minamino & Macnab, 2000a). These domains along with other proteins control which substrates are exported by the export apparatus.

The export apparatus transports rod-/hook-type substrates initially. One of the first flagellar proteins exported is FliE, which forms a junction between the MS-ring and rod (Minamino *et al.*, 2000b). The rod is a hollow structure, and once rod subunits are transported across the membrane by the export apparatus they diffuse along the channel and assemble spontaneously at the growing tip of the rod (Asakura, 1970). As the rod is assembled it passes through the peptidoglycan layer via the P-ring. The P-ring is composed of FlgI subunits, which

are exported by the type I secretion system, rather than the flagellar protein export apparatus (Homma *et al.*, 1987b). Expression of *flgI* is σ^{80}-dependent, and we have successfully captured the *flgI* promoter to fuse to different reporter genes (Brahmachary *et al.*, 2004). As the rod is elongated it continues through the L-ring which forms a pore in the outer membrane.

Following completion of the rod, the hook cap, FlgD, and hook protein, FlgE, are exported and assembled. Expression of *flgD* is dependent on σ^{80}, while *flgE* depends on σ^{54} for its expression (Niehus *et al.*, 2004; Spohn & Scarlato, 1999). We have constructed a FlgE-FLAG tag fussion protein to assay for expression and export of FlgE (Smith *et al.*, 2009). A two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR are required for activation of σ^{54} (Beier & Frank, 2000; Spohn & Scarlato, 1999). FlgS is a soluble, cytoplasmic protein that autophosphorylates and then transfers the phosphate to FlgR, which is then capable of activating transcription (Beier & Frank, 2000). FlgR-phosphate activates transcription by stimulating isomerization of a closed complex between σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) and the promoter to an open complex (Brahmachary *et al.*, 2004). FlgR differs from most other σ^{54}-dependent activators in that it lacks a DNA-binding domain and does not bind an upstream sequence to activate transcription. Rather FlgR engages σ^{54}-holoenzyme directly from solution (Brahmachary *et al.*, 2004). The environmental or cellular cue that begins FlgS/FlgR signal transduction is unknown. Some σ^{54}-dependent genes are responsive to exposure to low pH or heat shock response, but these stresses appear to affect expression of the RpoN regulon indirectly (Merrell *et al.*, 2003; Roncarati *et al.*, 2007). The flagellar protein export apparatus is required for expression of the RpoN regulon. Inactivation of genes encoding components of the *H. pylori* export apparatus results in decreased levels of FlgE (Allan *et al.*, 2000; Schmitz *et al.*, 1997). DNA microarray analysis showed that inactivation of

flhA results in reduced transcript levels of σ^{54}-dependent genes (Niehus *et al.*, 2004). In addition, we have shown that *flhB* is required for expression of two σ^{54}-dependent reporter genes (Smith *et al.*, 2009). The reason disruption of the export apparatus interferes with expression of the RpoN regulon in *H. pylori* is not known.

Once the hook reaches a specific length of approximately 60 nm for *H. pylori* the hook-length control protein FliK interacts with the hook cap at the tip of the nascent flagellum and $FlhB_C$ inside the cell to indicate mature hook length (Ryan *et al.*, 2005b; Williams *et al.*, 1996). Interactions between FliK and $FlhB_C$ stimulate a conformational change in the export apparatus that results in a switch in substrate specificity to filament-type substrates (Minamino *et al.*, 1999a). The conformational change in the export apparatus is irreversible and is accompanied by a site-specific, autocatalytic cleavage at a conserved NPTH motif within $FlhB_C$ (Fraser *et al.*, 2003; Minamino & Macnab, 2000a). Substitutions at the cleavage site inhibit processing of FlhB and export of filament-type, but not rod-/hook-type, substrates (Smith *et al.*, 2009; Wand *et al.*, 2006). Disruption of *fliK*, which is also a σ^{54}-dependent gene, results in loss of motility and formation of abnormally long hook structures, referred to as poly-hooks, rather than normal flagella (Pereira, 2005; Ryan *et al.*, 2005b). The poly-hook structures are characteristic of *fliK* mutants in *S. typhimurium* and are thought to result from a delay in the switch in substrate specificity of the export apparatus (Williams *et al.*, 1996). Inactivation of *fliK* in *H. pylori* stimulates expression of RpoN-dependent flagellar genes (Kamal *et al.*, 2007; Ryan *et al.*, 2005b; Smith *et al.*, 2009).

The next structure to assemble is the hook-filament junction which includes FlgK. The *flgK* gene is σ^{54}-dependent and co-transcribed with the upstream gene *hp1120* which does not have a known function (Spohn & Scarlato, 1999). We have successfully captured this σ^{54}-

dependent promoter to fuse to different reporter genes (Pereira & Hoover, 2005). The hook-filament junction is followed by the minor flagellin protein FlaB. FlaB is a homolog (58% identical) of the major flagellin FlaA (Suerbaum *et al.*, 1993). FlaB localizes close to the hook and makes up about 10% of the total filament (Kostrzynska *et al.*, 1991). The *flaB* gene is σ^{54}-dependent (Spohn & Scarlato, 1999; Suerbaum *et al.*, 1993), and we have also captured this promoter to fuse to different reporter genes (Brahmachary *et al.*, 2004). In addition we have a polyclonal antiserum that cross-reacts with both FlaB and FlaA to assay for their expression and export (Pereira & Hoover, 2005).

FlaA is the last protein assembled in the flagellum structure to complete the filament. The *flaA* gene is σ^{28}-dependent (Leying *et al.*, 1992), and we have captured this promoter to fuse to different reporter genes (Brahmachary *et al.*, 2004). Transcription of σ^{28}-dependent genes is negatively regulated by the anti-sigma factor FlgM (Colland *et al.*, 2001; Josenhans *et al.*, 2002). *S. typhimurium* FlgM is a filament-type substrate for the flagellar protein export apparatus and is exported from the cytoplasm once the hook-basal body complex is completed (Hughes *et al.*, 1993), thus coupling expression of the late flagellar genes with assembly of the flagellum. In *H. pylori*, disrupting genes encoding components of the flagellar protein export apparatus inhibits expression of FliA-dependent genes (Schmitz *et al.*, 1997), indicating that *H. pylori* FlgM may be secreted by the export apparatus as it is in *S. typhimurium*. However, there is recent evidence suggesting that *H. pylori* FlgM localizes in the soluble cytoplasmic fraction of the cell and is not secreted (Rust *et al.*, 2009).

Research Summary

To investigate the effects of FlhB mutations on expression of the flagellar reporter genes I replaced the entire coding region *flhB* in *H. pylori* 43504 with a *cat* cassette (confers chloramphenicol resistance) by allelic exchange. As expected from previous reports (Allan *et al.*, 2000; Wand *et al.*, 2006), the resulting Δ*flhB:cat* mutant was non-motile. I tried unsuccessfully to complement the Δ*flhB* mutant by introducing a copy of *flhB* in the *hp0405* locus. The *hp0405* gene, which encodes a NifU-like protein, is not required for motility or flagellar gene expression, and we had introduced genes into this locus previously for complementation experiments (Pereira & Hoover, 2005). The reason complementation in the *hp0405* site failed is unclear. Possibly, a *cis*-acting regulatory element needed for expression of *flhB* was missing or contextual factors may have interfered with expression of *flhB* in the *hp0405* locus (Ye *et al.*, 2007). Therefore, we reintroduced the wild-type *flhB* allele into its native locus from a suicide vector that carried *ermB* (confers erythromycin resistance) immediately downstream of the cloned *flhB*. Reintroduction of wild-type *flhB* into the native locus restored motility, thus verifying that no genes downstream of *flhB* are required for motility and that no other mutations that affect motility were introduced during construction of the strain with the original Δ*flhB:cat* mutation.

Expression of the two RpoN-dependent reporter genes was reduced approximately 10-fold in the Δ*flhB:cat* mutant compared to wild type. Disruption of either *rpoN* or *flgR* resulted in a similar reduction in expression of these reporter genes indicating that the low level of expression in the Δ*flhB:cat* mutant was essentially background. Expression of the *flaA'-'xylE* reporter gene was also diminished in the Δ*flhB:cat* mutant. Our findings on expression of the RpoN- and FliA-dependent reporter genes are consistent with the previous report that disruption

of *flhB* results in reduced levels of hook protein and the two flagellins in *H. pylori* (Allan *et al.*, 2000). In contrast to the RpoN- and FliA-dependent reporter genes, expression of *flgI'-'xylE* in the Δ*flhB:cat* mutant did not differ significantly from wild type. As expected from motility assays, reintroduction of the wild-type *flhB* allele into its native locus restored expression of the RpoN- and FliA-dependent reporter genes to wild-type levels.

In light of these results, I wished to determine how processing-deficient FlhB variants affected expression of the *H. pylori* RpoN and FliA regulons. Amino acid residues Asn-265 and Pro-266 are located at the predicted cleavage site of *H. pylori* FlhB. Variants of FlhB in which Asn-265 was changed to an alanine or Pro-266 was altered to a glycine were constructed by site-directed mutagenesis and expressed in *H. pylori*. FlhB autocleavage was examined in *H. pylori* using a polyclonal antibody generated against $FlhB_C$. In contrast to wild-type FlhB, the $FlhB^{N265A}$ and $FlhB^{P266G}$ variants were not cleaved *in vivo*. These substitutions also prevented export of filament-type substrates but not rod-/hook-type substrates. Expression of RpoN-dependent and FliA-dependent reporter genes in strains bearing the FlhB variants was near wild-type levels. This result indicates that the export apparatus containing the FlhB variants adopts a conformation that allows activation of the RpoN regulon. Alternatively, these data indicate that if there is an inhibitor of the RpoN regulon that is transported out of the cell by the flagellar protein export apparatus, it is a rod-/hook-type substrate.

Interestingly, the FlhB variants responded differently in the absence of FliK. Disrupting *fliK* in the Δ*flhB:cat* mutant failed to restore expression of RpoN-dependent reporter genes indicating that the inhibitory effect of the Δ*flhB:cat* mutation is not due to the inability to export FliK. Disrupting *fliK* in the strain that produce $FlhB^{N265A}$ significantly inhibited expression of RpoN- and FliA-dependent reporter genes but had little effect on expression of these reporter

genes in the strain producing FlhB^{P266G}. These findings suggest that FliK interacts with FlhB^{N265A} to stabilize the export apparatus in a conformation that allows the export apparatus to carry out one or more functions necessary for expression of the RpoN and FliA regulons (Smith *et al.*, 2009).

In a second study we investigated the effects of different *flhA* alleles on expression of the RpoN regulon. The *flhA* gene was disrupted by replacing the 5'-end of the gene and part of the upstream intergenic region the the *cat* cassette, and the resulting mutant transformed with the reporter genes. Our results agree with the finding that an *flhA* mutant has decreased expression of the RpoN regulon (Niehus *et al.*, 2004). The Δ*flhA* mutant showed a similar pattern to the Δ*flhB* mutant: the two RpoN-dependent reporter genes and the FliA-dependent reporter gene showed decreased expression while the RpoD-dependent reporter gene showed no change.

In contrast to previous results, a disruption of *flhA* after codon 77 (*flhA77*) enhanced expression of RpoN-dependent reporter genes. In this experiment the *cat* cassette was inserted 231 bp downstream of the translational start (corresponding to codon 77) to disrupt the gene, and the reporter genes were introduced. In the *flhA*77 mutant expression of the RpoD-dependent gene was not changed and the FliA-dependent reporter gene was decreased similar to the Δ*flhA* mutant. However, expression of the two RpoN-dependent reporters was stimulated about 8-fold in this mutant. Enhanced transcription of the RpoN regulon in this strain could be due to a disruption in signaling from the export apparatus. The effect of this mutation on the RpoN regulon is not due to changes in expression of HP1042, the gene immediately downstream of *flhA*. The *flhA77* mutation prevented export of both rod-/hook-type and filament-type substrates. This suggests that a negative regulator of the RpoN regulon is not exported by the export apparatus as part of the normal transcriptional control of the RpoN regulon. Instead, the *flhA77*

mutation may produce a truncated protein that localizes to the export apparatus and stabilizes a conformation of the export apparatus that stimulates expression of RpoN-dependent genes.

To examine the extracellular proteome of *H. pylori* a new method was developed in our laboratory that involves growing cultures in defined minimal media supplemented with β-cyclodextran instead of serum (Bruggraber *et al.*, 2004). Extracellular proteins were then directly analyzed by liquid chromatography-mass spectrometry. Previous studies using 2-D gel electrophoresis identified about 20 secreted proteins (Bumann *et al.*, 2002). Our method detected all but one of these proteins as well as identified many more putative secreted proteins (Smith *et al.*, 2007). The extracellular proteome of wild-type *H. pylori* (ATCC 43504) conatianed 10 different flagellar proteins; none of which were basal body or export apparatus proteins. While useful for identification of extracellular proteins, this method unfortunately proved costly and unreliable for analysis of flagellar proteins.

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CHAPTER 2

DECIPHERING BACTERIAL FLAGELLAR GENE REGULATORY NETWORKS IN THE GENOMIC ERA[1]

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Abstract

Synthesis of the bacterial flagellum is a complex process involving dozens of structural and regulatory genes. Assembly of the flagellum is a highly-ordered process, and in most flagellated bacteria the structural genes are expressed in a transcriptional hierarchy that results in the products of these genes being made as they are needed for assembly. Temporal regulation of the flagellar genes is achieved through sophisticated regulatory networks that utilize checkpoints in the flagellar assembly pathway to coordinate expression of flagellar genes. Traditionally flagellar transcriptional hierarchies are divided into various classes. Class I genes, which are the first genes expressed, encode a master regulator that initiates the transcriptional hierarchy. The master regulator activates transcription a set of structural and regulatory genes referred to as class II genes, which in turn affect expression of subsequent classes of flagellar genes. We review here the literature on the expression and activity of several known master regulators, including FlhDC, CtrA, VisNR, FleQ, FlrA, FlaK, LafK, SwrA, and MogR. We also examine the Department of Energy Joint Genomes Institute database to make predictions about the distribution of these regulators. Many bacteria employ the alternative sigma factors σ^{54} and/or σ^{28} to regulate transcription of later classes of flagellar genes. Transcription by σ^{54}-RNA polymerase holoenzyme requires an activator, and we review the literature on the σ^{54}-dependent activators that control flagellar gene expression in several bacterial systems, as well as make predictions about other systems that may utilize σ^{54} for flagellar gene regulation. Finally, we review the prominent systems that utilize σ^{28} and its antagonist, the anti-σ^{28} factor FlgM, along with some systems that utilize alternative mechanisms for regulating flagellar gene expression.

Introduction

The flagellum is the locomotion organelle for many species of bacteria from diverse phyla. Motility is required for several important microbial processes, including tactic responses (e.g., chemotaxis, aerotaxis, magnetotaxis), colonization of plant and animal hosts, biofilm formation, bacterial development, and dispersion of bacteria. Bacterial species differ with respect to the number of flagella they possess and their distribution across the cell surface. Despite these differences, all bacterial flagella consist of the following basic structures: i) the basal body, which anchors the flagellum in the bacterial cell envelope and contains the motor that powers flagellar rotation; ii) the hook, which is a flexible, curved rod that acts as a universal joint to transfer torque to the filament when it is not aligned with the axis of the rod; and iii) the filament, which acts as a propeller to push against the surrounding medium to propel the bacterium forward (Macnab, 1996). The basal body is the most complex of these structures, consisting of three ring structures in Gram-negative bacteria, a rod, a rotary motor, and a flagellar protein export apparatus. The export apparatus is a type III secretion system that is required for translocation of most of the flagellar proteins that localize outside the cell membrane (Minamino & Macnab, 1999). More details on the structure and function of the bacterial flagellum can be found in a recent review (Chevance & Hughes, 2008).

Bacterial flagellar biosynthesis is a complex and ordered process requiring the coordinated and temporal regulation of dozens of genes via a transcriptional hierarchy. The organization of flagellar genes varies greatly among bacteria. In some bacteria the flagellar genes are arranged within a few operons that are clustered together within the chromosome (e.g., *Sinorhizobium meliloti*). In contrast, the flagellar genes in *Helicobacter pylori* are arranged in over 20 operons that are scattered around the chromosome.

Temporal regulation of flagellar genes ensures that the structural proteins of the flagellum are produced as they are needed for assembly of the nascent flagellum. In the assembly pathway, the basal body is generated first, followed by the hook and then the filament (Fig. 2.1). Sequential expression of flagellar genes is achieved through the integration of regulatory networks that control the expression of different sets of flagellar genes. These regulatory networks are responsive to specific checkpoints in flagellar biosynthesis which helps coordinate flagellar gene regulation with assembly. Temporal regulation of flagellar genes is also subject to developmental control in many bacterial species that exhibit a dimorphic lifestyle (Section II.B). These bacteria include *Caulobacter crescentus*, which is the paradigm for developmental control of flagellar gene expression, as well as the budding, prosthecate bacteria (which include *Hyphomonas* spp., *Hyphomicrobium* spp., *Pedomicrobium* spp., and *Rhodomicrobium vannielli*), *Kineococcus radiotolerans*, and several members of the phylum Planctomycetes. In addition, some bacteria elaborate different arrangements of flagella depending on environmental conditions. For example, *Vibrio parahaemolyticus* expresses a polar flagellum for swimming motility and a separate set of lateral flagella for swarming motility across surfaces or in viscous medium (Section II.D.3). Examples of other bacteria that possess functional dual flagella systems include some *Aeromonas* spp., *Azospirillum brasilense* and *Rhodospirillum centenum* (see review (Merino *et al.*, 2006)).

As might be expected, considerable variation exists in the architecture of the regulatory networks that control flagellar biosynthesis in different bacterial species (Fig. 2.2). Here we review some of the prominent paradigms for flagellar gene regulation. In addition, we examine databases of bacterial genomic sequences to predict how flagellar regulatory networks might operate in select bacterial species where flagellar gene regulation has not been studied.

To identify genomes of flagellated bacteria for our analysis, homology searches were done for four key flagellar biosynthetic proteins: FliG, the flagellar rotor protein; FliM, the rotor-switch protein; FlgE, the hook protein; and FlhA, an essential component of the flagellar protein export apparatus. Bacterial genomes in the United States Department of Energy Joint Genome Institute Integrated Microbial Genomes database (DOE JGI, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) were searched for the genes encoding these proteins. Bacterial species whose genomes contained orthologs (reciprocal best BLASTP hits) of at least three of these proteins were predicted to be flagellated for at least part of their life cycle. After examining 865 sequenced bacterial genomes, 442 genomes representing 303 unique species were predicted to be flagellated using this criterion. This estimation has certain pitfalls since some pathogenic organisms such as *Shigella, Bordetella pertussis*, and *Yersinia pestis* (which are not included in the above estimate) possess all four of these key flagellar genes yet do not produce flagella apparently due to the highly antigenic nature of the structure (Akerley & Miller, 1993; Parkhill *et al.*, 2001; Tominaga *et al.*, 1994).

Master Regulators

In traditional flagellar gene transcriptional hierarchies the first genes to be transcribed encode regulatory proteins that initiate transcription of the early structural genes. This regulator is referred to as the master regulator, and it recognizes elements in the promoter regulatory regions of genes whose products are required at the earliest steps of flagellar assembly. Traditionally, genes encoding the master regulator are termed class I genes. A variety of master regulators have been identified in different bacterial species (Table 2.1), but for many systems a

master regulator is yet to be identified. The following sections focus on various master regulators that have been described to date.

FlhDC

FlhDC is the most extensively studied master regulator and is found in members of the phylum Proteobacteria including *Salmonella typhimurium* (Kutsukake *et al.*, 1990), *Escherichia coli* (Liu & Matsumura, 1994), *Serratia liquefaciens* (Givskov *et al.*, 1995), *Proteus mirabilis* (Furness *et al.*, 1997), *Yersinia enterocolitica* (Young *et al.*, 1999), *Xenorhabdus nematophilus* (Givaudan & Lanois, 2000), *Ralstonia solanacearum* (Tans-Kersten *et al.*, 2004), *Burkholderia glumae* (Kim *et al.*, 2007), *Erwinia carotovora* (Cui *et al.*, 2008) and *Azotobacter vinelandii* (Leon & Espin, 2008). Expression of *flhDC* is controlled by several regulators (Fig. 2.3) including but not limited to the heat shock proteins DnaK, DnaJ and GrpE, which respond to changes in temperature (Li *et al.*, 1993; Shi *et al.*, 1993); H-NS, which responds to changes in pH (Soutourina *et al.*, 1999; Soutourina *et al.*, 2002); OmpR, which responds to osmolarity (Shin & Park, 1995); and cAMP-CAP, which responds to the availability of carbon sources (Kutsukake, 1997; Soutourina *et al.*, 1999). Additional signals that affect *flhDC* expression include quorum sensing in *Y. pseudotuberculosis* and *E. coli* (Atkinson *et al.*, 2008; Sperandio *et al.*, 2002)as well as transition to a solid surface in *P. mirabilis* (Hatt & Rather, 2008). In addition to environmental inputs , expression of flagellar master regulators is also subject to cell cycle control. This is most apparent in *C. crescentus*, which has a dimorphic life cycle in which only one of the cell types is flagellated (see Section II.B). In *E. coli* cultures in which cell division was synchronized, transcription of *flhDC* was observed to increase immediately following cell division until it reached a steady-state level in the middle of the cell division cycle (Pruss & Matsumura, 1997). As described in the following sections, *flhDC* is not unique with

regard to the multiple environmental signals that mediate its expression through global regulators. Thus, transcription of other master regulator genes is likely also regulated in a cell cycle-dependent fashion similar to *E.coli flhDC*.

FlhDC activity and levels in the cell are further regulated (Fig. 2.3). FlhDC is regulated at the level of message stability by the global RNA-binding protein CsrA (Wei *et al.*, 2001) and protein stability by the protease ClpXP (Claret & Hughes, 2000a; Tomoyasu *et al.*, 2003). *S. typhimurium* FliZ, a DNA-binding regulatory protein, enhances FlhDC stability probably by down regulating expression of ClpXP or inducing transcription of another factor that directly stabilizes FlhDC (Kutsukake *et al.*, 1999; Lanois *et al.*, 2008; Saini *et al.*, 2008). *X. nematophilus* FliZ has been shown to have a similar function, but instead of acting indirectly, it binds to the *flhDC* promoter region to stimulate transcription (Lanois *et al.*, 2008). A final example of a factor that influences FlhDC activity is FliT, a chaperone that facilitates export of the filament cap. FliT negatively regulates FlhDC activity by sequestering the protein and preventing it from activating transcription (Kutsukake *et al.*, 1999; Yamamoto & Kutsukake, 2006). Since *fliT* is expressed late in the transcriptional hierarchy, the interactions between FliT and FlhDC would function as a negative feedback mechanism to turn off expression of the earlier flagellar genes.

The crystal structure of the FlhDC complex was recently solved (Wang *et al.*, 2006). In contrast to earlier work reporting a $FlhD_2C_2$ structure (Claret & Hughes, 2000b; Claret & Hughes, 2002; Liu & Matsumura, 1996), the crystal structure revealed a $FlhD_4C_2$ hexameric complex (Wang *et al.*, 2006). FlhD and FlhC consist of predominantly alpha-helical structures, and FlhC contains a unique zinc-binding domain (Campos *et al.*, 2001; Wang *et al.*, 2006). FlhDC recognizes sites located approximately 28 to 88 bp upstream of the transcriptional start

site of its target genes, which overlap the -35 promoter elements of these genes (Liu & Matsumura, 1994). The FlhDC binding site contains 17-18 bp imperfect inverted repeats separated by a 10-11 bp spacer (Claret & Hughes, 2002). Upon binding its sites, FlhDC interacts with the C-terminal domain of RNA polymerase α subunit to activate transcription by recruiting σ^{70}-RNA polymerase holoenzyme (σ^{70}-holoenzyme) to the promoter (Liu *et al.*, 1995; Wang *et al.*, 2006).

FlhDC activates transcription of genes whose products include components of the flagellar protein export apparatus, basal body, hook and the regulatory proteins FliA and FlgM (Claret & Hughes, 2002; Frye *et al.*, 2006; Gillen & Hughes, 1993; Liu & Matsumura, 1994; Pruss *et al.*, 2001). These genes are referred to as class II or middle genes. FlhDC acts as a global transcriptional activator of some genes unrelated to flagella biosynthesis including ones involved in anaerobic respiration using dimethyl sulfoxide, nitrate or nitrite as a terminal electron acceptor (Pruss *et al.*, 2001; Pruss *et al.*, 2003).

One of the flagellar genes under control of FlhDC is *fliA* which encodes the alternative sigma factor σ^{28}. Expression of class III or late genes, including the flagellin FliC, is σ^{28}-dependent (Kutsukake *et al.*, 1990; Ohnishi *et al.*, 1990). FlgM is an anti-sigma factor that interacts with σ^{28} to prevent transcription of the class III genes (Section IV). Inhibition of class III genes is alleviated upon completion of the basal body-hook structure when the flagellar protein export apparatus switches substrate specificity and transports FlgM out of the cell (Hughes *et al.*, 1993).

From our search of the DOE JGI database, FlhDC appears to be restricted to the β- and γ-Proteobacteria (Table 2.1). FlhDC orthologs were identified in 58 species, all of which are members of the β- or γ-Proteobacteria. Species in these two subgroups make up about 42% of

the 303 sequenced species that we predicted to be flagellated. Species that contain FlhDC orthologs constitute 44% of the β- and γ-Proteobacteria that we predicted to be flagellated, suggesting that there is diversity with respect to master regulators even within members of these groups.

CtrA

In the α-Proteobacterium *C. crescentus* the complexity of the flagellar gene hierarchy is superseded by the elegant transcriptional regulation of the cell cycle. During the *C. crescentus* cell cycle a flagellated swarmer cell differentiates into a sessile, stalked cell which undergoes asymmetric cell division to give rise to a new swarmer daughter cell. An excellent description of the regulatory network controlling the *C. crescentus* cell cycle can be found in a recent paper by Collier and co-workers and the figures therein (Collier *et al.*, 2007). CtrA is a response regulator that is involved in controlling the cell cycle in *C. crescentus*, as well as serving as the master regulator of the flagellar gene hierarchy (Quon *et al.*, 1996). Flagellar assembly is so intimately connected to the cell cycle that mutations in some flagellar genes can arrest the cell cycle (Shapiro & Maizel, 1973; Yu & Shapiro, 1992)

Expression of *ctrA* is driven from two promoters (Domian *et al.*, 1999). Transcription from the distal promoter is induced by the upstream cell cycle regulator GcrA and is repressed due to methylation of a GANTC motif within the promoter by CcrM, the downstream cell cycle regulator (Holtzendorff *et al.*, 2004; Reisenauer & Shapiro, 2002). Both *ctrA* promoters are subject to autoregulation as CtrA phosphate (CtrA~P) represses transcription from the distal promoter but induces transcription from the proximal promoter (Domian *et al.*, 1999). CtrA~P not only acts as a transcriptional regulator but also binds to sites near the origin of replication to inhibit chromosome replication and cell division (Quon *et al.*, 1998). Because of its role in

chromosome replication and cell division CtrA~P must be turned over within the cell to allow continuation of the cell cycle (Domian *et al.*, 1997). Precise modulation of CtrA levels is achieved by localization of CtrA~P to the stalk-cell pole by RcdA at the proper time in the cell cycle and co-localization of the protease ClpXP by CpdR (Iniesta *et al.*, 2006; Jenal & Fuchs, 1998; McGrath *et al.*, 2006). CpdR activity is inhibited by phosphorylation (Iniesta *et al.*, 2006). A single phosphorelay involving CckA receives signals from the cell cycle to control both CtrA and CpdR (Biondi *et al.*, 2006; Iniesta *et al.*, 2006; Jacobs *et al.*, 1999).

Flagellar genes make up a major portion of the genes controlled by CtrA (Laub *et al.*, 2002). At class II flagellar gene promoters, CtrA~P binds TTAA direct repeats (or slight deviations of this motif) that are separated by a critical 7 bp spacer (Ouimet & Marczynski, 2000; Quon *et al.*, 1996) and overlap the -35 promoter element (Reisenauer *et al.*, 1999). An additional CtrA-binding half-site (TTAACCAT) has also been identified (Laub *et al.*, 2000; Laub *et al.*, 2002). Binding of CtrA to sites in the promoter regulator regions of class II genes activates transcription with σ^{73}-RNA polymerase holoenzyme, which is the primary form of RNA polymerase holoenzyme in the bacterium (Reisenauer & Shapiro, 2002; Wu *et al.*, 1998). Class II genes encode components of the basal body and flagellar protein export apparatus, as well as the regulatory proteins RpoN (σ^{54}) and FlbD (Laub *et al.*, 2002; Quon *et al.*, 1996). CtrA binds another set of flagellar gene promoters and is required for transcription of these genes. Unlike traditional class II genes, disruptions in these genes (which encode components of the basal body and chemotaxis proteins - *flgBC*, *fliE*, *flmABCDEFGH*), do not inhibit expression of the subsequent flagellar genes (Boyd & Gober, 2001; Leclerc *et al.*, 1998). The alternative sigma factor σ^{54} is responsible for transcription of the class III and IV flagellar genes (see

Section III.A) which encode the remaining basal body, hook and flagellin proteins (Anderson *et al.*, 1995; Brun & Shapiro, 1992).

Searching the DOE JGI database revealed CtrA orthologs in all of the α-Proteobacteria that are predicted to be flagellated, one *Campylobacter* species, four *Clostridium* species, and one member of the phylum Thermotoga (*Petrotoga mobilis*). In *Hyphomonas neptunium*, which has a dimorphic life cycle similar to that of *C. crescentus*, *ctrA* is in an apparent operon with the flagellar genes *flhF* and *motR*, suggesting a role for CtrA in flagellar gene regulation in this bacterium (Badger *et al.*, 2006) Predicted CtrA-binding sites were identified *in silico* in the promoter regulatory regions of flagellin genes in α-Proteobacteria *Brucella melitensis*, *Mesorhizobium loti*, *S. meliloti* and *Agrobacterium tumefaciens*, suggesting a role for CtrA in the regulation of flagellar biogenesis in these bacteria (Hallez *et al.*, 2004). If CtrA functions as a regulator of these genes, it probably is not the master regulator since flagellins are required late in flagellar assembly. Moreover, in *S. meliloti* another master regulator as been identified (Section II.C), and CtrA from *Silicibacter* sp. TM1040 does not serve as the master regulator of flagellar genes (Miller & Belas, 2006).

VisNR

S. meliloti is a symbiotic, nitrogen-fixing, member of the α-Proteobacteria. The *S. meliloti* flagellar gene hierarchy is controlled by the master regulator VisNR and a second regulator Rem. The *visNR* and *rem* genes are part of a 56-kb island that contains all of the genes required for flagellar assembly, function and chemotaxis (Rotter *et al.*, 2006; Sourjik *et al.*, 1998; Sourjik *et al.*, 2000). This region of the chromosome, called the flagellar regulon, contains approximately 50 genes (Rotter *et al.*, 2006; Sourjik *et al.*, 1998). VisNR controls expression of

rem, and Rem subsequently controls expression of the class II flagellar genes. In this regulatory cascade *visNR* are referred to as class IA genes and *rem* as a class IB gene (Rotter *et al.*, 2006).

Expression of *visNR* is controlled by at least three regulatory networks, Sin-ExpR, ExoS-ChvI, and CbrA. SinR and SinI produce the quorum sensing molecules N-acyl homoserine lactones which are detected by ExpR (Hoang *et al.*, 2004; Marketon *et al.*, 2002). ExpR either directly or indirectly down regulates expression of *visNR* as population density and concentration of N-acyl homoserine lactones increase (Hoang *et al.*, 2008). ExoS is a membrane-bound sensor kinase which, together with its cognate response regulator ChvI, regulates extracellular polysaccharide (EPS) production (Cheng & Walker, 1998). ExoR is a periplasmic regulator that also regulates EPS production through ExoS-ChvI (Wells *et al.*, 2007). Transposon insertions in *exoR* or *exoS* result in excess EPS production (Doherty *et al.*, 1988) and a non-motile, aflagallated phenotype (Yao *et al.*, 2004). Lack of flagella in these mutants is due to a down regulation of *visNR* and is independent of the Sin-ExpR quorum sensing system (Hoang *et al.*, 2008). CbrA, another regulator of EPS production as well as other genes important for symbiosis in the host legume, also affects expression of *visN* (Gibson *et al.*, 2007).

VisN and VisR have a similar predicted secondary structure and are proposed to form an active heterodimer. Based on the predicted secondary structure, VisN and VisR are LuxR-type transcriptional activators (Sourjik *et al.*, 2000). Members of the LuxR protein family consist of a DNA-binding helix-turn-helix motif and a ligand-binding receptor domain (Fuqua & Winans, 1994). VisNR effector molecules are yet to be identified. Variability in the ligand-binding domains of VisN and VisR suggest these proteins bind distinct effector molecules (Sourjik *et al.*, 2000). Although the VisNR binding site in the *rem* promoter regulatory region has not been identified, VisNR likely activates transcription with σ^{70}-holoenzyme from two different

promoters upstream of *rem* (Rotter *et al.*, 2006). Rem is an OmpR-type transcriptional activator that directly controls expression of class II genes by activating transcription with σ^{70}-holoenzyme (Hoang *et al.*, 2008; Rotter *et al.*, 2006). Rem recognizes a 17-bp imperfect tandem repeat that overlaps the -35 element by 3 bp in its target promoters (Rotter *et al.*, 2006). The close proximity of the Rem-binding site to the promoter is consistent with the transcriptional activation model for OmpR-type regulators (Busby & Ebright, 1994; Rotter *et al.*, 2006).

σ^{54}-dependent master regulators

Some master regulators function with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). This form of RNA polymerase holoenzyme binds its target promoter to form a closed complex but cannot proceed further in transcription initiation in the absence of an activator (Reitzer *et al.*, 1987; Sasse-Dwight & Gralla, 1988). The activator stimulates the conversion of the closed complex to an open promoter complex in a reaction that requires ATP hydrolysis by the activator (Popham *et al.*, 1989). Hundreds of σ^{54}-dependent activators have been described to date. These activators function with σ^{54}-holoenzyme to transcribe genes involved in a variety of processes, including nitrogen assimilation and fixation, H_2 metabolism, transport of sugars and dicarboxylic acids, degradation of aromatic compounds, response to phage shock, and pilin production, as well as flagellar biosynthesis (Studholme & Dixon, 2003). Most σ^{54}-dependent activators have a three-domain architecture consisting of a N-terminal regulatory domain, a central activation domain, and a C-terminal DNA-binding domain (Schumacher *et al.*, 2006). The activation domain, the most conserved of these domains, is an ATPase that belongs to the AAA+ superfamily, members of which are involved in a variety of cellular functions (Neuwald *et al.*, 1999; Ogura & Wilkinson, 2001). Activators of σ^{54}-holoenzyme generally bind to upstream activation sequences (UAS; sometimes referred to as

bacterial enhancers) that are often located 70 bp or more upstream of the target promoter (Reitzer & Magasanik, 1986). After binding the UAS the activator engages σ^{54}-holoenzyme bound at the promoter through DNA looping (Su *et al.*, 1990). DNA looping is often facilitated by an auxiliary DNA-binding protein, such as integration host factor (IHF), which binds to a site between the promoter and the UAS (Hoover *et al.*, 1990).

FleQ

The master regulator FleQ from *Pseudomonas aeruginosa* is a σ^{54}-dependent activator (Arora *et al.*, 1997). Several factors have been identified that modulate expression of *P. aeruginosa fleQ*, including the alternative sigma factor AlgT (σ^{E}) which regulates EPS production (Tart *et al.*, 2005). AlgT is required for expression of AmrZ, a regulator that binds to and represses the *fleQ* promoter (Tart *et al.*, 2006). Thus, similar to the situation in *S. meliloti*, when EPS production is turned on flagellar assembly is turned off in *P. aeruginosa*. Interestingly, FleQ appears to bind and repress a set of promoters for EPS production genes. This repression is relieved by high concentrations of cyclic diguanylate (c-di-GMP), which FleQ appears to bind despite the fact that it does not possess any known c-di-GMP-binding motifs (Hickman & Harwood, 2008). Vfr, a homolog of *E. coli* CAP, also binds the *fleQ* promoter and prevents σ^{70}–holoenzyme from binding at the -10 promoter element (Dasgupta *et al.*, 2002).

FleQ activates transcription of class II genes encoding components of the basal body, motor, and export apparatus as well as the regulator proteins FleN and FleSR. FleN negatively regulates the activity of FleQ through direct interactions to restrict flagellar assembly to a single flagellum (Dasgupta *et al.*, 2000; Dasgupta & Ramphal, 2001). A FleQ-binding site with dyad symmetry was identified 67 bp upstream of the *fleSR* transcriptional start site, and a potential IHF binding site was identified between this site and the *fleSR* promoter (Jyot *et al.*, 2002). A

maltose-binding protein-FleQ (MBP-FleQ) fusion protein was also reported to recognize sites immediately downstream of the promoters of three other flagellar genes (*flhA*, *fliE* and *fliL*) (Jyot *et al.*, 2002). The authors of this study demonstrated by site-directed mutagenesis the importance of the site downstream of the *flhA* promoter for expression. These downstream sites, however, lack homology with the FleQ-binding site at *fleSR* as well as with each other, and appear to be positioned too close to the promoter (located from 5 to 18 bp from the transcriptional start sites) to allow FleQ to contact σ^{54}-holoenzyme via DNA looping. Thus, it is unclear how FleQ might activate transcription from these downstream sites. It is possible that FleQ contacts σ^{54}-holoenzyme by a different mechanism than DNA looping (e.g., by tracking along the DNA after binding to the downstream sites). Alternatively, a contaminating protein may have been responsible for the reported binding activity since relatively high levels MBP-FleQ (1.0 – 7.5 μM) were used in the DNA binding assays (Jyot *et al.*, 2002). If this is the case, then the contaminating protein may interact with FleQ and have an important regulatory role.

One of the class II operons activated by FleQ encodes the FleSR two-component system (discussed in section III.B) which is required for expression of class III genes (Correa *et al.*, 2000). FleR is a σ^{54}-dependent activator that stimulates transcription of the class III genes, which encode the remaining basal body components, hook and hook associated proteins (Dasgupta *et al.*, 2003). Class IV genes are σ^{28}-dependent and encode the flagellin, chemotaxis proteins and the regulatory protein FlgM (Dasgupta *et al.*, 2003; Frisk *et al.*, 2002; Starnbach & Lory, 1992).

FlrA

Vibrio cholerae FlrA, an ortholog of FleQ, is another example of a σ^{54}-dependent activator that serves as a flagellar master regulator (Klose & Mekalanos, 1998). Similar to the link between motility and EPS production in *P. aeruginosa* and *S. meliloti*, expression of

virulence genes and flagellar genes in *V. cholerae* is mutually exclusive (Gardel & Mekalanos, 1996). ToxR and H-NS have been shown to play a role in this process. ToxR, which is an activator of virulence genes (DiRita, 1992), represses motility while H-NS directly or indirectly represses virulence genes and induces expression of *flrA* (Ghosh *et al.*, 2006).

The regulatory network controlling flagellar gene expression in *V. cholerae* is similar to that of *P. aeruginosa*. Expression of class II genes is initiated by the σ^{54}-dependent activator FlrA. As in *P. aeruginosa*, two of the class II genes encode the regulatory proteins FlrBC which form a two-component system required for expression of the σ^{54}-dependent class III genes. Similarly, the class IV genes are σ^{28}-dependent in *V. cholerae* (Prouty *et al.*, 2001). One significant difference between the two systems is *P. aeruginosa fliA* (σ^{28}) is not regulated by FleQ and σ^{54} (Dasgupta *et al.*, 2003), whereas *fliA* is a class II gene in *V. cholerae* (Prouty *et al.*, 2001). In addition, *V. cholerae* produces multiple flagellin proteins, the majority of which are encoded by class IV genes, but the gene encoding the core flagellin FlaA is a class III gene (Prouty *et al.*, 2001).

FlaK and LafK

As mentioned previously, some bacteria possess a polar flagellar system that is utilized for swimming motility and a lateral flagellar (*laf*) system that is expressed for swarming motility. We will only briefly discuss the regulation of such dual flagellar systems and refer the reader to two recent reviews for more information on the subject (McCarter, 2004; Merino *et al.*, 2006). The best characterized dual flagellar systems are in *V. parahaemolyticus* and *Aeromonas hydrophila*. Within each of these bacterial species, the two flagellar systems apparently do not to share structural or regulatory genes (McCarter, 2004). In addition, the genes for each system are unlinked. For example, in *V. parahaemolyticus* the genes required for the polar system are

distributed among five clusters on chromosome I, while the genes encoding components of the lateral flagella are arranged in two clusters on chromosome II (Kim & McCarter, 2000; Stewart & McCarter, 2003).

The regulatory networks that control the polar and lateral flagellar systems are very similar to those of *V. cholerae* and *P. aeruginosa*. In *V. parahaemolyticus*, the polar flagellum master regulator, FlaK, is dispensable for polar flagellum biosynthesis as the lateral flagella master regulator, LafK, compensates for its loss. FlaK cannot replace LafK, however, in expression of the *laf* system (Kim & McCarter, 2004). Expression of the *V. parahaemolyticus laf* system is induced by iron-limitation and stalling of polar flagellum rotation (McCarter *et al.*, 1988; McCarter & Silverman, 1989). Stalling of polar flagellum rotation occurs when the bacterium encounters a solid surface or viscous environment. Thus, the polar flagellum functions as a tactile sensor for the cell.

As in *V. cholerae* and *P. aeruginosa*, the class III and class IV genes in both flagellar systems of *V. parahaemolyticus* are dependent on σ^{54} and σ^{28}, respectively, for their expression (McCarter, 2004; Merino *et al.*, 2006). Distinct σ^{54}-dependent activators are used for expression of the class III genes in the two flagellar systems; and likewise distinct σ^{28} and anti-σ^{28} proteins are employed in regulating expression of the class IV genes in the two flagellar systems. Thus, transcriptional control of the polar and lateral flagellar systems in *V. parahaemolyticus* is mediated through parallel regulatory networks.

Predicting σ^{54}-dependent master regulators

One of the difficulties in predicting FleQ/FlrA-type master regulators from genome sequences is that σ^{54} is involved in many diverse processes and bacteria can possess several different σ^{54}-dependent activators, each targeting a different set of genes. All σ^{54}-dependent

activators share a high degree of homology, particularly within their AAA+ domains, and there does not appear to be any outstanding motifs within FleQ/FlrA-type master regulators that distinguish them from many other σ^{54}-dependent activators. While the presence of a gene encoding a σ^{54}-dependent activator within a cluster of flagellar genes would suggest a role for this activator in flagellar biosynthesis, additional information would be required to predict if the activator served as a master regulator or was involved in later stages of flagellar assembly.

An alternative strategy for predicting whether a given bacterium utilizes a FleQ/FlrA-type master regulator is to search for potential σ^{54}-dependent promoters upstream of class II genes. Promoters recognized by σ^{54}-holoenzyme contain conserved elements located approximate 12 and 24 bp upstream of the transcriptional start site of the gene. Barrios and co-workers identified consensus sequences 5'-TTGCW-3' and 5'-TGGCACGR-3' (where W is A or T and R is G or A) for the -12 and -24 elements, respectively, based on a comparison of 186 known or potential promoter sequences (Barrios *et al.*, 1999). The GC and GG dinucleotides (underlined) are the most conserved elements in the consensus. Spacing between these dinucleotides is absolutely critical since insertion or deletion of a single base pair results in loss of promoter function (Buck, 1986). These features facilitate identification of potential σ^{54}-type promoters from genome sequences.

We used the Pattern Locator program (Mrazek & Xie, 2006) (http://www.cmbl.uga.edu/software/patloc.html) to search the intergenic regions of the genomes of several diverse bacterial species for the motif 5'-TGGYAYNNNNN{TT}(1)GCW-3' (parentheses indicate that only one of the two residues preceding the GC doublet must be a T). This motif was chosen to take into account the variability in σ^{54}-type promoter sequences yet maintain enough stringency to minimize the number of sequences gleaned from the search.

Potential σ^{54}-type promoters in the correct orientation were identified upstream of putative class II flagellar genes in several bacterial genomes (Table 2.2). All of these bacteria belonged to the β-, γ-, or δ-Proteobacteria groups, suggesting that FleQ/FlrA-type master regulators are restricted to these groups. A caveat of this approach is that sequences of σ^{54}-type promoters in some bacteria may vary from the consensus. Indeed, the -24 element of σ^{54}-type promoters of flagellar genes in *H. pylori* and other ε-Proteobacteria has the consensus 5'-WGGAAC-3' (Pereira *et al.*, 2006), which would not have been detected in our motif search.

Other master regulators

In the Firmicutes *Bacillus subtilis* and *Listeria monocytogenes* two additional early flagellar gene regulators have been identified (Table 2.1). SwrA (also referred to as SwrAA) in *B. subtilis* is required for swimming motility in liquid and swarming motility on solid surfaces (Calvio *et al.*, 2005; Kearns *et al.*, 2004). Binding of SwrA to flagellar promoters has not been demonstrated, but changes in expression of *swrA* affect expression of the 31-gene *fla*/*che* operon (all genes in this operon are involved in flagellar biosynthesis and chemotaxis) and the overall flagellation of the cell (Kearns & Losick, 2005; Marquez-Magana & Chamberlin, 1994). The promoter of the *fla*/*che* operon deviates slightly from the σ^{A} consensus, which accounts for its dependence on SwrA (Kearns & Losick, 2005). The *swrAB* gene, which is immediately downstream of *swrA* and is co-transcribed with it, is involved in the increase in flagellation associated with swarming motility. SwrAB is a membrane-bound regulatory protein and appears to be required for proteolysis of SwrA (Calvio *et al.*, 2005). Expression of the *fla*/*che* operon is also directly regulated by the two-component system DegS-DegU which globally controls changes in gene expression during stationary phase. DegU phosphate (DegU~P) binds the σ^{A} promoter of the *fla*/*che* operon and enhances SwrA-dependent transcription (Calvio *et al.*, 2008;

Kobayashi, 2007; Tsukahara & Ogura, 2008). In addition, DegU~P appears to enhance expression of *swrA* from an upstream σ^A promoter while a second promoter that is σ^D-dependent (Section IV.A) also drives *swrA* expression (Calvio *et al.*, 2008).

SwrA orthologs are found only in three other members of the genus: *B. amyloliquefaciens, B. licheniformis* and *B. pumilus*. Orthologs of SwrAB are more widespread than SwrA in the phylum Firmicutes. Several Firmicutes that we do not predict to be flagellated contain SwrAB orthologs, while some of the predicted flagellated Firmicutes lack SwrAB orthologs. Thus, SwrAB appears to have other roles outside flagellar biogenesis.

L. monocytogenes MogR is technically not a master regulator since it functions as a repressor of early flagellar genes rather than a transcriptional activator. MogR represses expression of flagellar genes in a non-hierarchal fashion during intracellular growth or growth at 37° C (Grundling *et al.*, 2004; Shen & Higgins, 2006) by binding TTTT-N_5-AAAA motifs that overlap the -35 promoter element of flagellar gene operons (Shen & Higgins, 2006). MogR activity is controlled by an antagonist, GmaR, the expression of which is subject to temperature regulation through DegU. At lower temperatures GmaR is expressed, sequesters MogR through protein-protein interactions, and allows expression of flagellar genes. Interestingly, GmaR is a functional glycosyltransferase for the flagellin FlaA and is believed to be the first prokaryotic protein identified to control expression of its enzymatic substrate (Shen *et al.*, 2006).

MogR orthologs occur in all of the *Listeria* spp. we predicted to be flagellated and a subset of the predicted flagellated *Bacillus* spp. (see Section IV.A for further discussion about this subset). MogR orthologs were not found in species predicted to be non-flagellated suggesting a conserved function for MogR and its orthologs in regulation of flagellar genes. As expected from its bifunctional nature, GmaR orthologs are found in many diverse species, some

of which appear to be flagellated and others which are not. All of the *Listeria* spp. (but not all of the Firmicutes) that contained MogR orthologs also contained GmaR orthologs.

The response regulator FtcR in the α-Proteobacterium *Brucella melitensis* is a final example of a flagellar master regulator. A cognate sensor kinase to regulate FtcR activity has yet to be identified. Expression of *ftcR* is at least partially controlled by a quorum sensing system (Leonard *et al.*, 2007). FtcR binds to sites located upstream of the open reading frame of *fliF*, which encodes the MS-ring of the basal body, activating transcription of *fliF* and subsequent production of hook and flagellin (Leonard *et al.*, 2007). FtcR orthologs are found in many of the predicted flagellated α-Proteobacteria overlapping with orthologs of both CtrA and VisNR in many of these species including *S. meliloti* and *A. tumefaciens*, but not *C. crescentus*. How or if these multiple regulatory systems are integrated to control flagellar biosynthesis in these bacteria is unknown. Clearly, more work is needed to dissect the complex regulatory networks that initiate the flagellar gene transcriptional hierarchy in these non-paradigm systems.

RpoN (σ^{54}) regulators

As alluded to previously, many organisms utilize σ^{54}-holoenzyme for transcription of class III and IV flagellar genes. Utilization of different σ^{54}-dependent activators for regulation of late flagellar genes provides an additional level of temporal control to coordinate gene expression with assembly of gene products (Fig. 2.2). Examples of activators of σ^{54}-holoenzyme that control expression of class III and class IV flagellar genes are discussed in the following sections.

To predict class III genes that are dependent on σ^{54} for their expression in previously uncharacterized systems, we followed the same strategy outlined above (Section II.D.4) using

the Pattern Locator program (Mrazek & Xie, 2006). Potential σ^{54}-type promoters were found upstream of class III genes in at least one representative from the α-, β-, γ-, and δ- Proteobacteria but not any other groups of bacteria (Table 2.3). Based the data from Tables 2 and 3, σ^{54}-type promoters appear to be more prevalent upstream of class III genes indicating that σ^{54}-dependent expression of middle and late flagellar genes may be more common than σ^{54}-dependent master regulators.

FlbD

As previously mentioned (Section II.B), *C. crescentus* utilizes σ^{54} and the σ^{54}-dependent activator FlbD to control expression of class III and IV genes (Fig. 2.2, (Brun & Shapiro, 1992; Ramakrishnan & Newton, 1990)). Expression of both *rpoN* and *flbD* is CtrA-dependent (Laub *et al.*, 2002). FlbD is a σ^{54}-dependent activator with three functional domains: a response regulator, AAA+ ATPase and DNA-binding domains (Ramakrishnan & Newton, 1990). Activity of FlbD is both positively and negatively regulated through interactions with FliX, a membrane-bound protein that senses completion of the MS-ring, rotor switch, and export apparatus intermediate (Mohr *et al.*, 1998; Muir & Gober, 2001; Muir & Gober, 2002; Muir & Gober, 2004). The *fliX* gene is a class II gene under control of CtrA (Mohr *et al.*, 1998).

FlbD and FliX orthologs are found in several α- Proteobacteria suggesting a conserved function in flagellar gene regulation (Muir & Gober, 2004). Searching the DOE JGI database, we found that all the species that contained FliX orthologs also contained FlbD orthologs. Bacteria that contained FliX orthologs included *Acidiphilium cryptum*, *Bradyrhizobium* spp., *Magnetospirillum magneticum*, *Maricaulis maris*, *Methylobacterium* spp., *Nitrobacter* spp., *Parvibaculum lavamentivorans*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*. We predict potential σ^{54}-type promoters upstream of class III genes in all of these organisms (Table

2.3). In *C. crescentus* and all of the organisms listed above *fliX* is divergently transcribed from *flgI* indicating a possible functional or evolutionary connection between FliX and FlgI.

Basal body assembly, FliX, and FlbD are all required for cell division in *C. crescentus*, (Mohr *et al.*, 1998; Muir & Gober, 2004; Yu & Shapiro, 1992). However, introduction of a variant FlbD or FliX that retain activity independent of basal body assembly restores normal cell division (Muir & Gober, 2001; Muir & Gober, 2004). Thus, FlbD activity ultimately appears to influence progression of the cell-cylce and is itself subject to cell-cycle control through a FliX-independent mechanism (Muir *et al.*, 2005; Wingrove *et al.*, 1993). FlbE was originally believed to be the cell-cycle dependent regulator of FlbD activity (Wingrove & Gober, 1996) but was later shown to be a structural component of the flagellum probably causing an indirect effect via FliX (Muir & Gober, 2001).

FlbD binds to flagellar transcription regulatory (*ftr*) sites located about 100 bp upstream of class III and IV genes to activate transcription (Benson *et al.*, 1994; Mullin *et al.*, 1994; Ramakrishnan & Newton, 1990; Wingrove *et al.*, 1993). An *ftr* site is also located upstream of the class II gene *fliF* and overlaps the CtrA-dependent promoter (Van Way *et al.*, 1993). FlbD binds this site and acts as a repressor of *fliF* (Benson *et al.*, 1994; Mullin *et al.*, 1994).

FleR/FlrC

The two-component systems FleS/FleR in *P. aeruginosa* and FlrB/FlrC in *V. cholorae* are required for transcriptional activation of σ^{54}–dependent class III genes(Dasgupta *et al.*, 2003; Klose & Mekalanos, 1998; Prouty *et al.*, 2001; Ritchings *et al.*, 1995). FleS and FlrB are the sensor kinases in these two-component systems, while FleR and FlrC are the response regulators and activators of σ^{54}-holoenzyme (Klose & Mekalanos, 1998; Ritchings *et al.*, 1995). Phosphorylation of FlrC by FlrB-phosphate is required for flagellar biosynthesis (Correa *et al.*,

2000), but the cellular signals that influence FlrB (or FleS) autophosphorylation have not been identified. Unlike other sensor kinases, FlrB and FleS are predicted to be soluble proteins rather than membrane-bound. In contrast to most activators of σ^{54}-holoenzyme, FlrC binds to sites downstream of its target promoters to activate transcription (Correa & Klose, 2005). FlrC recognizes sites located between 24 to 95 bp and 11 to 114 bp downstream of the transcriptional start but still in the non-coding portion of *flaA* (encodes a flagellin) and *flgK* (encodes hook-associated protein 1), respectively. When relocated to a position 295 bp upstream of either the *flaA* or *flgK* promoter, the FlrC-binding site from the *flaA* promoter-regulatory region allowed FlrC to activate transcription indicating that it functions as a bacterial enhancer (Correa & Klose, 2005).

FlgR

In the ε-Proteobacteria *C. jejuni* and *H. pylori* σ^{54} and σ^{28} are used for transcription of flagellar genes similar to the *V. cholerae* and *P. aeruginosa* systems (Fig. 2.2). A master regulator in these ε-Proteobacteria has yet to be identified. In *H. pylori* at least three regulons have been defined based on the sigma factor required for their transcription (Niehus *et al.*, 2004). Genes encoding the components of the basal body require the primary sigma factor σ^{80} (Beier *et al.*, 1997; Porwollik *et al.*, 1999; Schmitz *et al.*, 1997); genes encoding the hook, hook-associated proteins and a minor flagellin require σ^{54} (Spohn & Scarlato, 1999; Suerbaum *et al.*, 1993); and genes encoding the filament cap and the major flagellin require σ^{28} (Colland *et al.*, 2001; Kim *et al.*, 1999; Leying *et al.*, 1992). A two-component system composed of the sensor kinase FlgS plus the response regulator and σ^{54}-dependent activator FlgR regulates expression of the RpoN regulon (Beier & Frank, 2000; Spohn & Scarlato, 1999).

H. pylori FlgR lacks the C-terminal DNA-binding domain that is characteristic of most σ^{54}-dependent activators. Instead of binding an UAS, FlgR contacts σ^{54}–holoenzyme directly from solution (Brahmachary *et al.*, 2004). *C. jejuni* FlgR contains a DNA-binding domain, but this domain is dispensable indicating that this FlgR activates transcription by a similar mechanism (Joslin & Hendrixson, 2008). Expression of FlgR in *C. jejuni* is also phase variable with addition or contraction of a nucleotide within any of several homopolymeric tracts resulting in a truncated, non-functional protein (Hendrixson, 2006). Like FleS and FlrB, FlgS is predicted to be soluble rather than membrane-bound, and the cellular signals that affect FlgS autophosphorylation are unknown. Disruptions in genes encoding components of the flagellar protein export apparatus in *H. pylori* or *C. jejuni* , however, inhibit expression of the RpoN regulon (Allan *et al.*, 2000; Hendrixson & DiRita, 2003; Niehus *et al.*, 2004; Schmitz *et al.*, 1997). In addition, loss of the hook-length control protein FliK in both of these bacteria stimulates expression of the RpoN regulon (Kamal *et al.*, 2007; Ryan *et al.*, 2005b). These results suggest that similar to *C. crescentus* FliX, the activity of FlgS is controlled by interactions with the flagellar protein export apparatus or basal body structure. Alternatively, the export apparatus may secrete an inhibitor of the RpoN regulon, similar to its role in the export of FlgM to alleviate inhibition of the FliA regulon. In either model, the flagellar protein export apparatus could serve a role in communicating the progression of flagellar assembly to the RpoN regulon.

Flagellar biogenesis in *H. pylori* may also be influenced by regulated proteolysis of σ^{54}. *H. pylori* HP0958, a previously uncharacterized protein, was found to protect σ^{54} from proteolysis and allow expression of the middle and late flagellar genes (Pereira & Hoover, 2005; Ryan *et al.*, 2005a). HP0958 was originally identified as a protein that interacted with σ^{54} in a high throughput genetic screen that employed the yeast two-hybrid system (Rain *et al.*, 2001).

The mechanism by which HP0958 protects σ^{54} from proteolysis is not known. One possibility is that HP0958 acts as a chaperone to assist σ^{54} in binding core RNA polymerase. HP0958 also interacts with the flagellar export apparatus component FliH in the yeast two-hybrid system (Rain *et al.*, 2001), although the significance of these interactions is not known. HP0958 possesses two CxxC motifs near its C-terminus that are predicted to form a zinc ribbon that is possibly involved in binding nucleic acids. Thus, HP0958 may have multiple functions in *H. pylori*. Orthologs of HP0958 are found in a wide variety of bacteria, not all of which are flagellated.

FleT

In the α-Proteobacteria *Rhodobacter sphaeroides* a four-tiered flagellar gene hierarchy is proposed similar to that in *V. cholerae* and *P. aeruginosa*. *R. sphaeroides* possesses a *fleQ* ortholog that encodes a master regulator, which controls expression of the *fleT* operon. The *fleT* operon encodes class II structural genes as well as FleT, which is a σ^{54}-dependent activator required for expression of class III genes (Poggio *et al.*, 2005). We address FleT separately here because of its unusual architectural features. FleT consists of only the AAA+ ATPase domain (Poggio *et al.*, 2005). *R. sphaeroides* FleQ is also unusual in that it lacks the N-terminal regulatory domain. FleT and FleQ appear to be constitutively active, suggesting that the activities of these proteins are regulated at the level of expression. Both FleQ and FleT are required for expression of class III genes, which encode components of the basal body, export apparatus and the hook (Poggio *et al.*, 2005). FleQ binds to a site upstream of *fleT* to activate transcription. FleT inhibits FleQ activity at its own promoter while apparently enhancing FleQ activity at class III gene promoters. Sequences upstream of the *fliO* promoter are not required for transcriptional activation of this class III gene (Poggio *et al.*, 2005), suggesting that

FleQ/FleT-mediated transcriptional activation does not require an UAS at target genes. Additional information on unconventional transcriptional activators such as FlgR and FleT can be found in a recent review (Beck *et al.*, 2007).

FliA (σ^{28}) and FlgM

In many of the systems discussed thus far, expression of the late flagellar genes requires the alternative sigma factor σ^{28}. The flagellar protein export apparatus plays an essential role in regulating σ^{28} activity. In *S. typhimurium* the flagellar protein export apparatus is made up of the membrane spanning proteins FlhA, FlB, FliO, FliP, FliQ and FliR that form a specialized pore at the base of the basal body. In addition, the cytoplasmic proteins FliJ (a chaperone), FliI (an ATPase), and FliH (a regulator of FliI) function as part of the export apparatus (Minamino & Macnab, 1999; Minamino *et al.*, 2000a; Minamino & MacNab, 2000b). Two of the membrane-bound components, FlhA and FlhB, have large cytoplasmic domains (Kutsukake *et al.*, 1994b; Minamino *et al.*, 1994). The cytoplasmic domain of FlhB is processed by an autocleavage mechanism (Ferris *et al.*, 2005; Minamino & Macnab, 2000a). Processing of FlhB coincides with completion of the mature hook which is signaled to FlhB by interactions with FliK, the hook length control protein (Hirano *et al.*, 1994; Minamino & Macnab, 2000a; Williams *et al.*, 1996). FlhB processing correlates with a switch in substrate specificity of the export apparatus (Fraser *et al.*, 2003; Minamino & Macnab, 2000a). Prior to the switch in substrate specificity the export apparatus secretes rod- and hook-type substrates. Following the switch, the export apparatus displays an increased affinity for filament-type substrates, which includes the filament cap protein and flagellins (Minamino *et al.*, 1999b). The regulatory protein FlgM is an anti-σ^{28} factor (Ohnishi *et al.*, 1992) and is secreted from the cytoplasm via the export apparatus as a

filament-type substrate (Hughes *et al.*, 1993; Kutsukake, 1994; Minamino *et al.*, 1999a). Both *fliA* and *flgM* are early genes (Hughes *et al.*, 1993; Kutsukake *et al.*, 1990), but FlgM inhibits σ^{28} activity until the hook-basal body complex is complete and the late gene products are required (Karlinsey *et al.*, 2000). FliA binds to promoters upstream of genes encoding hook associated proteins, filament cap, flagellin, and motor proteins that have a TAAA-N_{15}-GCCGATAA consensus that corresponds to the -35 and -10 elements of σ^{70}-type promoters (Kutsukake *et al.*, 1990). Interestingly, FliA acts as a chaperone to facilitate the secretion of FlgM by the export apparatus (Aldridge *et al.*, 2006). FlgM secretion by the flagellar protein export apparatus has also been demonstrated in *V. cholerae* which produces a polar, sheathed flagellum (Correa *et al.*, 2004).

In the cytoplasm FlgM associates with both free σ^{28}, which prevents it from interacting with core RNA polymerase and also with σ^{28}-RNA polymerase holoenzyme (σ^{28}- holoenzyme), which destabilizes the holoenzyme complex (Chadsey *et al.*, 1998; Chadsey & Hughes, 2001; Ohnishi *et al.*, 1992). FlgM has limited secondary and tertiary structure. The N-terminus of the protein does not form a stable structure and the C-terminus forms non-rigid α-helices (Daughdrill *et al.*, 1998). The C-terminal region, however, forms a rigid helical structure upon binding of FlgM to σ^{28} (Daughdrill *et al.*, 1997). FlgM sequesters σ^{28} through interactions with conserved sigma regions 2.1, 3.1, 4.1 and 4.2(Chadsey & Hughes, 2001; Kutsukake *et al.*, 1994a), but only interacts with region 4 to destabilize σ^{28}- holoenzyme (Chadsey & Hughes, 2001). The crystal structure of *Aquifex aeolicus* σ^{28}/FlgM complex confirmed these observations and showed that FlgM binding causes extensive rearrangement of the conserved sigma domains so that they are tightly packed with FlgM wrapped around the outside (Sorenson *et al.*, 2004).

The association of FlgM with σ^{28} in the cytoplasm stabilizes σ^{28} by protecting it from Lon protease (Barembruch & Hengge, 2007).

SigD and other systems

The flagellar transcription factor σ^{28} was originally characterized in *B. subtilis* where it is referred to as σ^{D} or SigD (Helmann *et al.*, 1988). SigD controls both late flagellar gene expression and genes required for separation of daughter cells after cell division (Kearns & Losick, 2005; Margot *et al.*, 1999). SigD promoters are upstream of several flagellar gene operons that are distant from the *fla*/*che* operon and encode the hook associated proteins, filament cap and flagellin as well as FlgM (Serizawa *et al.*, 2004). FlgM functions with SigD in a similar manner as FliA in *S. typhimurium* (Caramori *et al.*, 1996; Fredrick & Helmann, 1996). A weak SigD-dependent promoter is located upstream of the *fla*/*che* operon (Estacio *et al.*, 1998), but transcription of this operon is mainly driven from the SwrA-dependent σ^{A} promoter as described previously (Section II.E). The *sigD* gene is the next to last gene in the *fla*/*che* operon, and the gene immediately downstream designated *swrB* encodes a SigD activator that is required for swarming motility (Estacio *et al.*, 1998; Kearns *et al.*, 2004; Werhane *et al.*, 2004).

Functional homologs of FlgM can be difficult to predict from genome sequences due to the lack of well conserved primary structure. Many FlgM orthologs can be found by searching for orthologous group COG2747 or PFAM group PF04316. Outside of *S. typhimurium*, functional FlgM homologs have been identified in *V. cholerae* (Correa *et al.*, 2004), *P. aeruginosa* (Frisk *et al.*, 2002), and *H. pylori* (Colland *et al.*, 2001). *C. jejuni* has a FlgM homolog, but this protein appears to play only a minor role in regulation of flagellin genes (Hendrixson & DiRita, 2003). Recently, three FlgM clades were defined by comparing a group of 77 FlgM sequences from diverse bacteria (Pons *et al.*, 2006).

When the DEO JGI database was searched FliA/SigD orthologs were found in 552 genomes representing 300 species of which 359 genomes representing 230 species were predicted to be flagellated. These species make up 76% of the species we predicted to be flagellated. Not all FliA/SigD orthologs are involved in flagellar biogenesis. For example, the alternative sigma factor WhiG from *Streptomyces* spp. is a FliA/SigD ortholog, but this protein is involved in sporulation and glycogen biosynthesis rather than flagllar biogenesis. One pattern that emerged from the search for FliA/SigD orthologs was that the majority (83%) of species that lack FliA/SigD orthologs belong to the α-Proteobacteria group. Indeed, the only members of the α-Proteobacteria that contained FliA orthologs are *Erythrobacter* spp., *Loktanella vestfoldensis*, *Novosphingobium aromaticivorans*, *R. sphaeroides*, *Roseovarius* spp., *Sphingomonas* spp., *Sphingopyxis alaskensis*, and *Zymomonas mobilis*. With the exception of *N. aromaticivorans*, all of these bacteria also possess FlgM orthologs. All other predicted flagellated α-Proteobacteria presumably utilize σ^{54} for transcription of late flagellar genes as in *C. crescentus*. Temporal regulation of the late flagellar genes in *C. crescentus* is achieved through post-transcriptional regulation by FlbT which binds to the 5' non-coding portion of the flagellin transcript and prevents its translation until completion of the hook-basal body complex (Anderson & Gober, 2000). Orthologs of FlbT are found in many α-Proteobacteria, including *R. sphaeroides* and *Rosovarius* spp. suggesting that temporal control of late flagellar genes in *R. sphaeroides* and *Roseovarius* spp. could be mediated by both FlgM and FlbT.

A second pattern that emerged is that a subset of *Bacillus* spp. lack SigD orthologs. These species include *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* and are the same species that were found to have orthologs of the flagellar gene repressor MogR. In addition, disruption of the export apparatus component FlhA in *B. thuringiensis* did not inhibit

expression of the flagellin gene as would be predicted if FlgM-SigD regulation was operating in this bacterium (Ghelardi *et al.*, 2002). These findings suggest that during the course of evolution these *Bacillus* species acquired a flagellar gene regulatory network that differs from that found in many other members of the genus.

Another interesting observation from the search for FliA/SigD orthologs is seen in the Spirochetes. *Borrelia* spp. lack FliA/SigD orthologs as well as σ^{28}-type promoter consensus sequences upstream of their flagellar genes. Rather, the flagellar genes of *Borrelia* spp. are predicted to have σ^{70}-type promoters (Ge *et al.*, 1997; Sal *et al.*, 2008). Two recent studies have shown that in *B. burgdorferi* regulates expression of flagellins at a post-transcriptional level, and this regulation is dependent on formation of the hook-basal body complex (Motaleb *et al.*, 2004; Sal *et al.*, 2008). While other Spirochetes including *Leptospira* spp. and *Treponema* spp. have FliA/SigD orthologs, at least for *T. denticola* a similar post-transcriptional regulation appears to control expression of flagellins (Limberger *et al.*, 1999).

A final interesting twist on the search for FliA/SigD orthologs is found in *Buchnera aphidicola* which is the only predicted flagellated γ-Proteobacteria that lacks a FliA ortholog. This endosymbiotic bacterium is non-motile due to the absence of the genes encoding the flagellin, but produces hundreds of basal body-hook structures that cover its cell surface (Maezawa *et al.*, 2006). These structures are predicted to function in *B. aphidicola* symbiosis, and given the metabolic expense required to produce so many of these structures this prediction seems likely.

Conclusions

Tremendous diversity exists within the transcriptional regulatory networks that bacteria employ to coordinate flagellar gene expression with assembly of the flagellum. These regulatory systems are highly sophisticated and are often responsive to a variety of environmental signals and cellular cues. From a few paradigms, we have learned much about how bacteria are able to assemble complex cellular structures like the flagellum with precision and efficiency. Still, we have much to learn about the molecular mechanisms that control flagellar gene expression in these paradigms. Moreover, we are only beginning to understand the multiformity of regulatory systems that control flagellar biogenesis in other bacteria. The ready availability of proteomic and genomic experimental tools coupled with the wealth of accessible genomic sequence data will undoubtedly lead to new findings that will expand even more our appreciation of the variety of mechanisms used by bacteria to regulate transcription of flagellar gene hierarchies. As we begin to elucidate these mechanisms in more detail, it is likely that we will discover common threads that unite these diverse regulatory networks.

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Table 2.1 Master regulators of flagellar genes

Name	Group (Subgroup)	Representative Bacteria	Gene Locus[a]
FlhDC	Proteobacteria (β/γ)	*Salmonella typhimurium* LT2	STM1925 STM1924.S
CtrA	Proteobacteria (α)	*Caulobacter crescentus* CB15	CC3035
VisNR Rem[b]	Proteobacteria (α)	*Sinorhizobium meliloti* 1021	SMc03015 SMc03016 SMc03046
FtcR	Proteobacteria (α)	*Brucella melitensis* 16M	BMEII0158
FleQ FlrA[c]	Proteobacteria (β/γ/δ)	*Pseudomonas aeruginosa* PAO1 *Vibrio cholerae* O395	PA1097 VC0395_A1721
SwrA	Firmicutes	*Bacillus subtilis* 168	BSU35230
MogR	Firmicutes	*Listeria monocytogenes* EGD	Lmo0674

[a]Locus tag for genes in the representative bacteria are in the same order as the names.
[b]VisNR and Rem are two separate regulators that are both required for expression of downstream flagellar genes.
[c]FlrA and FleQ are orthologous σ^{54}-dependent activators.

Table 2.2 Potential σ^{54}-dependent promoters upstream of putative class II genes

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential Promoter[c]	Distance[d]
β-Proteobacteria	*Azoarcus* sp. BH72	*fliE*	Azo2713	GTGGCACGTGGTTTGCT	72
		fliF (+12)	Azo2716	CTGGCACTTTAGCTGCA	120
γ-Proteobacteria	*Aeromonas hydrophila* hydrophila ATCC 7966	*fliE* (+25)	Aha_1364	TTGGCACCCTAATTGCT	41
	Pseudoalteromonas haloplanktis TAC125[e]	*fliE* (+27[f])	PSHAa0791	GTGGCACGTTTTGTGCT	84
	Thiomicrospira crunogena XCL-2[e]	*fliE* (+12)	Tcr_1443	TTGGCATATGAATTGCT	80
	Xanthomonas campestris pv. campestris ATCC 33913[e]	*flhF* (+5)	Xcc1908	TTGGCACACCGCATGCA	160
		fliL (+4)	Xcc1920	TTGGCACGCTGATTGCA	33
δ-Proteobacteria	*Geobacter sulfurreducens* PCA[e]	*fliL* (+2)	Gsu0420	CTGGCATTTCGGTTGCA	59
		flhA (+6)	Gsu3056	CTGGCACAACGGTTGCA	51

[a]The numbers in parentheses indicate the number of flagellar or chemotaxis genes that could form an operon with the given gene.
[b]Locus tag for genes with potential σ^{54}-dependent promoters.
[c]Sequence of the potential σ^{54}-dependent promoter with the conserved dinucleotides underlined.
[d]Distance in base pairs between the potential promoter and the predicted translational start of the gene.
[e]Bacterium is also predicted to have potential σ^{54}-dependent promoters upstream of class III genes (Table 2.3).
[f]Divergently transcribed from *flaM*, which encodes a σ^{54}-dependent activator.

Table 2.3 Potential σ^{54}-dependent promoters upstream of putative class III genes

Group	Bacterium	Gene (Operon)[a]	Locus[b]	Potential Promoter[c]	Distance[d]
α-Proteobacteria	*Acidiphilium cryptum* JF-5	*flgE*	Acry_0110	TGGCACGCTTGCTGCA	94
	Bradyrhizobium japonicum USDA 110	*flgF* (+3)	Blr5838	TTGGCACGGCTTTCGCT	113
		flgI (+3)[e]	Blr5838	GTGGCACAGCACTCGCA	25
	Maricaulis maris MCS10	*flgF* (+3)	Mmar10_1946	TGGCACGCCGCGTGCA	65
		flgI (+3)[e]	Mmar10_1952	TGGCACGCCACTGGCA	35
		fliC (+2)	Mmar10_1961	TGGCACCGCGCTTGCT	73
		motA	Mmar10_2419	TGGCACCGCGCTTGCT	72
	Magnetospirillum magneticum AMB-1	*flgE*	Amb1389	TTGGCATCGACCTTGCA	43
		flgI (+4)[e]	Amb3824	TTGGCATGGGTTTCGCA	50
	Methylobacterium extorquens PA1	*flgI* (+3)[e]	Mext_3041	TGGCACGGCCCTGGCA	46
	Nitrobacter winogradskyi Nb-255	*flgI* (+3)[e]	Nwi_1111	TGGCACGGGTCTCGCT	69
		flgF (+3)	Nwi_1122	TGGCACGCATTTCGCT	109
	Parvibaculum lavamentivorans DS-1	*flgI* (+3)[e]	Plav_2556	TGGCACGGCTTTCGCT	40
		flgF (+3)	Plav_2562	TGGCACAGCCCCTGCA	18
	Rhodopseudomonas palustris BisA53	*flgI* (+3)[e]	Rpe_1531	TGGCACGGCGCTGGCA	65
		flgF (+3)	Rpe_1538	TGGCACGGCTTTCGCT	110
	Rhodospirillum rubrum ATCC 11170	*flgB* (+4)	Rru_A2842	TGGCACGGGTCATGCA	59
		flgI (+4)[e]	Rru_A2849	TGGCACGACATTAGCA	92
β-Proteobacteria	*Nitrosomonas europaea* ATCC 19718	*flgB* (+11)	Ne0302	CTGGCACGATTCCTGCT	34
		fliK ([f])	Ne2088	GTGGCATGAGAATTGCT	43
δ-Proteobacteria	*Desulfovibrio vulgaris* vulgaris Hildenborough	*flgE*	Neu0307	CTGGCACGGCTCGTGCT	42
		MCP[g]	Neu0750	ATGGCACTGCTCTTGCT	184
		fliD (+1)	Neu0863	GTGGCATCTGGATTGCA	54
		fliC ([h])	Neu1441	TTGGGATCGTGTTTGCT	37
		MCP (+2)[g]	Neu1962	TCGGTATCATCTCCGCA	23

	Geobacter sulfurreducens PCA[i]	*flgB* (+12)	Gsu0407	CTGGTACGGCTTTTGCT	35
		flgJ (+4)	Gsu3046	TTGGCACATAACATGCT	60
	Syntrophus aciditrophicus SB	*flgB* (+8)[j]	Syn_01467	GTGGTATGTGCTTTGCT	25
γ-Proteobacteria	*Pseudoalteromonas haloplanktis* TAC125[i]	*fliC* (+3)	PSHAa0781	TTGGCACAAAACCTGCT	77
		fliS (+1)	PSHAa0786	TTGGCATTATAATTGCT	46
		motY	PSHAa2115	TAGGCATGTAATTTGCT	98
	Shewanella baltica OS155	*cheW* (+18)	Sba1_2949	TTGGCACATGAATTGCT	34
	Thiomicrospira crunogena XCL-2[i]	MCP[g]	Tcr_0570	CTGGCATTGGGTTTGCT	41
		cheA (+1)	Tcr_0750	GTGGCACGGTCATTGCT	90
		MCP[g]	Tcr_0759	TTGGCACGGTCATTGCT	39
		fliD (+18)[k]	Tcr_1449	ATGGCATTTTGTATGCT	65
		flgI (+3)	Tcr_1467	ATGGCATACCTAATGCT	51
		flgB (+3)	Tcr_1474	TTGGCATTCGGATTGCT	88
		fliS	Tcr_2133	ATGGCATGCTGTCTGCT	108
	Xanthomonas campestris pv. campestris ATCC 33913[i]	*flgB* (+4)	Xcc1952	CTGGCACAAACATTGCT	33

[a]The numbers in parentheses indicate the number of flagellar or chemotaxis genes that could form an operon with the given gene.
[b]Locus tag for genes with potential σ^{54}-dependent promoters.
[c]Sequence of the potential σ^{54}-dependent promoter with the conserved dinucleotides underlined.
[d]Distance in base pairs between the potential promoter and the predicted translational start of the gene.
[e]Divergently transcribed from *fliX* gene encoding a FlbD regulator (Section III.A).
[f]Divergently transcribed from *fleSR* genes encoding a two-component σ^{54}-dependent regulatory system (Section III.B).
[g]Gene encodes a methyl-accepting chemotaxis protein.
[h]Divergently transcribed from *flaG* gene encoding a flagellin.
[i]Organism is also predicted to have potential σ^{54}-dependent promoters upstream of class II genes (Table 2).
[j]Upstream genes encode a potential two-component σ^{54}-dependent regulatory system.
[k]Putative two-component σ^{54}-dependent regulatory system downstream in operon.

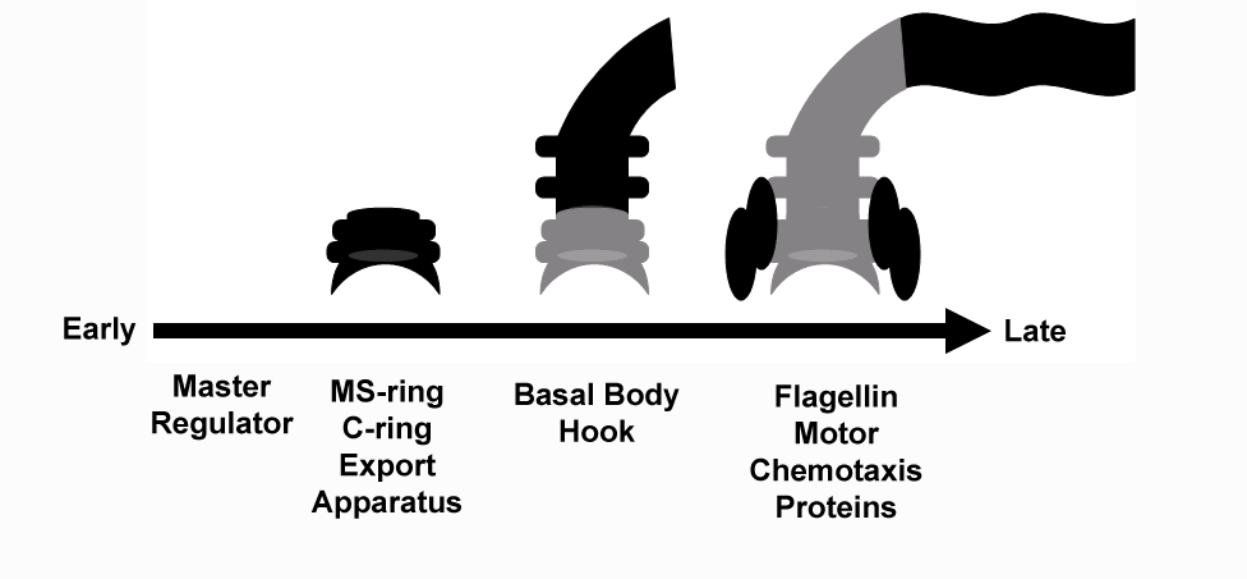

Figure 2.1 Assembly of the Gram-negative flagellum. Temporal control of flagellar genes ensures that flagellar genes are expressed as their products are required for assembly. Completion of the basal body-hook structure is a key checkpoint for coordinating assembly with gene expression in many bacteria.

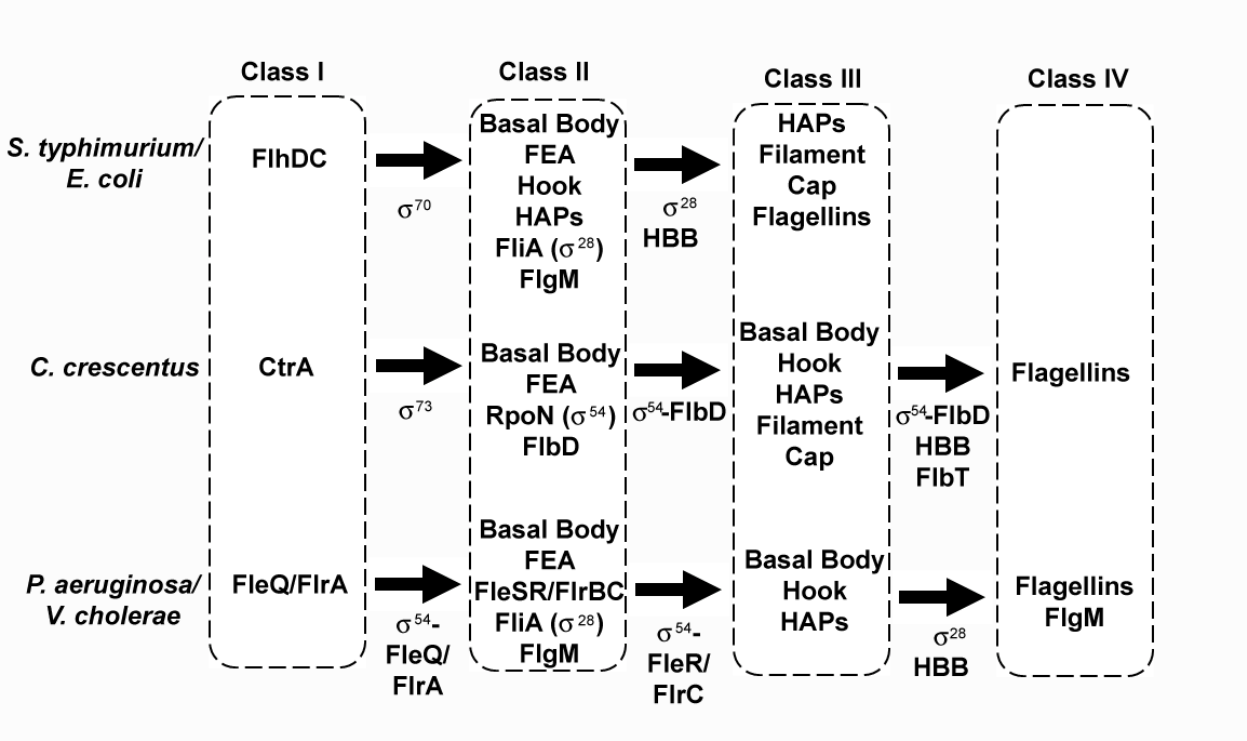

Figure 2.2 Paradigms for flagellar gene hierarchies. Flagellar gene regulatory networks are compared for three different systems. Class I genes encode the master regulators. Regulatory proteins encoded by Class II genes include the σ^{28} (FliA), the anti-σ^{28} factor (FlgM), and σ^{54}-dependent regulators FlbD in *C. crescentus*, FleRS in *P. aeruginosa* and FlrBC in *V. cholerae*. Abbreviations for class II genes are as follows: flagellar protein export apparatus (FEA) and hook associated proteins (HAPs). Regulation of Class III/IV genes is governed by completion of the hook-basal body complex (HBB).

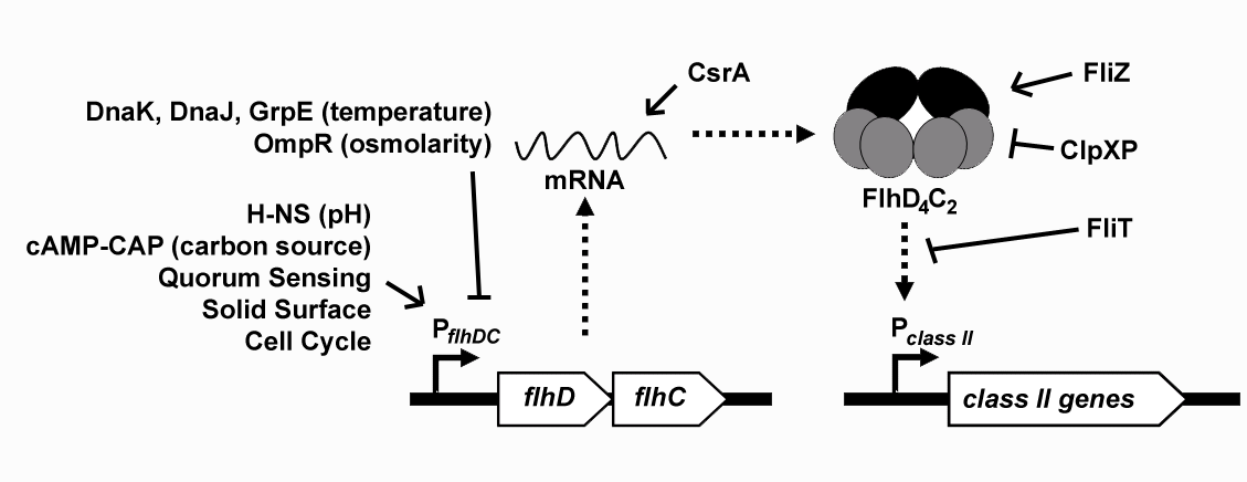

Figure 2.3 Regulation of the flagellar master regulator FlhDC. Multiple regulatory proteins influence expression of *flhDC* in response to various environmental factors, and still other regulators influence mRNA stability, protein stability, or activity. Arrowheads indicate a positive effect while blunt-ends indicate a negative effect.

CHAPTER 3

HELICOBACTER PYLORI FlhB PROCESSING-DEFICIENT VARIANTS AFFECT FLAGELLAR ASSEMBLY BUT NOT FLAGELLAR GENE EXPRESSION[1]

[1] Smith, T. G., L. Pereira and T. R. Hoover. 2009. *Microbiology*. 155: 1170 - 1180

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Summary

Regulation of the *Helicobacter pylori* flagellar gene cascade involves the transcription factors σ^{54} (RpoN), employed for expression of genes required midway through flagellar assembly, and σ^{28} (FliA) required for expression of late genes. Previous studies revealed that mutations in genes encoding components of the flagellar protein export apparatus block expression of the *H. pylori* RpoN and FliA regulons. FlhB is a membrane-bound component of the export apparatus that possesses a large cytoplasmic domain ($FlhB_C$). The hook length control protein, FliK, interacts with $FlhB_C$ to modulate the substrate specificity of the export apparatus. $FlhB_C$ undergoes autocleavage as part of the switch in substrate specificity. Consistent with previous reports, deletion of *flhB* in *H. pylori* interfered with expression of RpoN-dependent reporter genes, while deletion of *fliK* stimulated expression of these reporter genes. In the Δ*flhB* mutant, disrupting *fliK* did not restore expression of RpoN-dependent reporter genes, suggesting that the inhibitory effect of the Δ*flhB* mutation is not due to the inability to export FliK. Amino acid substitutions (N265A and P266G) at the putative autocleavage site of *H. pylori* FlhB prevented processing of FlhB and export of filament-type substrates. The FlhB variants supported wild-type expression of RpoN- and FliA-dependent reporter genes. In the strain producing $FlhB^{N265A}$ expression of RpoN- and FliA-dependent reporter genes was inhibited when *fliK* was disrupted. In contrast, expression of these reporter genes was unaffected or slightly stimulated when *fliK* was disrupted in the strain producing $FlhB^{P266G}$. *H. pylori* HP1575 (FlhX) shares homology with the C-terminal portion of $FlhB_C$ ($FlhB_{CC}$) and can substitute for $FlhB_{CC}$ in flagellar assembly. Disrupting *flhX* inhibited expression of a *flaB* reporter gene in the wild type but not in the Δ*fliK* mutant or strains producing FlhB variants suggesting a role for FlhX or $FlhB_{CC}$ in normal expression of the RpoN regulon. Taken together, these data indicate

that the mechanism by which the flagellar protein export apparatus exerts control over the *H. pylori* RpoN regulon is complex and involves more than simply switching substrate specificity of the flagellar protein export apparatus.

Introduction

Bacterial flagellar biosynthesis involves transcriptional hierarchies that coordinate flagellar gene expression with assembly. Flagellar gene hierarchies are well characterized in a few bacteria including *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Caulobacter crescentus* (Chilcott & Hughes, 2000; Wu & Newton, 1997). Flagellar gene regulation in ε-Proteobacteria, such as *Helicobacter pylori* and *Campylobacter jejuni*, shares some similarities with these established paradigms but also differs significantly. As in *S. typhimurium*, *H. pylori* flagellar genes required late in assembly, such as the major flagellin (FlaA) and filament cap protein, are transcribed by σ^{28} (FliA)-RNA polymerase holoenzyme (Kim *et al.*, 1999; Leying *et al.*, 1992); and as in *C. crescentus*, many *H. pylori* flagellar genes require σ^{54} (RpoN) for their transcription (Spohn & Scarlato, 1999; Suerbaum *et al.*, 1993). Transcription of genes needed early in *H. pylori* flagellar assembly are dependent on σ^{80} (RpoD), the primary *H. pylori* sigma factor (Beier *et al.*, 1997; Porwollik *et al.*, 1999; Schmitz *et al.*, 1997). Expression of early flagellar genes generally requires a transcriptional activator referred to as the master regulator. While a master regulator has yet to been identified in *H. pylori*, some studies have shown that the quorum-sensing autoinducer synthase, LuxS, affects motility by altering expression of early and late flagellar genes (Loh *et al.*, 2004; Osaki *et al.*, 2006; Rader *et al.*, 2007). The effect of LuxS on flagellar gene expression, however, is strain specific (Joyce *et*

al., 2000; Lee *et al.*, 2006), and may be due to global changes in gene expression caused by decreased fitness in the *luxS* mutant rather than loss of quorum sensing (Lee *et al.*, 2006).

The *H. pylori* RpoN regulon contains genes that encode proteins needed midway through flagellar assembly including the hook protein FlgE and the minor flagellin FlaB (Niehus *et al.*, 2004; Spohn & Scarlato, 1999). Transcription of the RpoN regulon is controlled by a two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR (Beier & Frank, 2000; Spohn & Scarlato, 1999), which activates transcription with σ^{54}-RNA polymerase holoenzyme without binding upstream activation sequences in the DNA (Brahmachary *et al.*, 2004). The cellular cues that initiate FlgS/FlgR signal transduction are unknown. Some RpoN-dependent genes are responsive to exposure to low pH or heat shock response, but these stresses appear to affect expression of the RpoN regulon indirectly (Merrell *et al.*, 2003; Roncarati *et al.*, 2007).

Transcription of the *H. pylori* FliA regulon is negatively regulated by the anti-sigma factor FlgM (Colland *et al.*, 2001; Josenhans *et al.*, 2002). *S. typhimurium* FlgM is secreted by the flagellar protein export apparatus upon completion of the hook-basal body complex resulting in expression of the FliA-dependent flagellar genes (Hughes *et al.*, 1993). In *H. pylori* disrupting the flagellar protein export apparatus inhibits expression of FliA-dependent genes (Schmitz *et al.*, 1997), indicating that the *H. pylori* export apparatus may secrete FlgM. Inactivation of the *H. pylori* export apparatus also results in decreased levels of some RpoN-dependent gene products (Allan *et al.*, 2000; Schmitz *et al.*, 1997), and reduced transcript levels of RpoN-dependent genes (Niehus *et al.*, 2004). Similarly, disruption of the *C. jejuni* flagellar protein export apparatus inhibits expression of RpoN-dependent reporter genes (Hendrixson & DiRita,

2003). The mechanism that couples export apparatus function with expression of RpoN-dependent genes in *H. pylori* or *C. jejuni* is unknown.

The flagellar protein export apparatus is a type III secretion system (T3SS) that transports flagellar substrates across the cell membrane (Minamino & Macnab, 1999). In *S. typhimurium* the export apparatus consists of six membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) and three cytoplasmic proteins (FliH, FliI and FliJ) (Macnab, 2003). FlhB possesses a large C-terminal cytoplasmic domain referred to as $FlhB_C$ (Kutsukake *et al.*, 1994b; Minamino *et al.*, 1994). The export apparatus transports rod-/hook-type substrates until completion of the hook-basal body complex, at which point it switches substrate specificity to filament-type substrates (Minamino *et al.*, 1999a). The switch in substrate specificity involves FlhB and the hook length control protein, FliK. Completion of the mature hook is communicated by FliK to $FlhB_C$ which is believed to cause conformational changes that are required for the switch between export states (Williams *et al.*, 1996). $FlhB_C$ undergoes autocleavage at a conserved NPTH motif which is required for the switch in substrate specificity as substitutions at the cleavage site inhibit processing of FlhB and export of filament-type substrates (Fraser *et al.*, 2003; Minamino & Macnab, 2000a). The C-terminal subdomain of $FlhB_C$ ($FlhB_{CC}$) remains tightly associated with the rest of FlhB following processing (Minamino & Macnab, 2000). FlhB homologs in the virulence factor T3SS used by pathogenic bacteria also undergo autocleavage, and like $FlhB_{CC}$ the C-terminal subdomains of these proteins remain associated with the rest of the protein after autocleavage (Deane *et al.*, 2008; Minamino & Macnab, 2000a; Zarivach *et al.*, 2008). FlhB processing is proposed to serve as a molecular clock to regulate temporal gene expression with the spatial assembly of the flagellum (Ferris *et al.*, 2005).

H. pylori has an open reading frame (HP1575) that shares homology with $FlhB_{CC}$ (Tomb *et al.*, 1997; Wand *et al.*, 2006). Orthologs of HP1575 occur in several other bacteria and are referred to as FlhX proteins (Pallen *et al.*, 2005; Wand *et al.*, 2006). Wand and coworkers reported that FlhX can functionally substitute for $FlhB_{CC}$ in *H. pylori* (Wand *et al.*, 2006) suggesting that in the absence of $FlhB_{CC}$, FlhX can associate with the N-terminal subdomain of $FlhB_C$ ($FlhB_{CN}$).

As observed in *S. typhimurium*, disruption of *H. pylori fliK* (*hp0906*) results in reduced motility and formation of poly-hook structures due to a delay in the switch between export states (Ryan *et al.*, 2005b; Williams *et al.*, 1996). Inactivation of *fliK* in *H. pylori* and *C. jejuni* stimulates expression of RpoN-dependent flagellar genes (Kamal *et al.*, 2007; Ryan *et al.*, 2005b). Based on the role of FliK and FlhB in substrate switching, we wished to determine how processing-deficient FlhB variants would affect expression of the *H. pylori* RpoN and FliA regulons. FlhB variants were constructed in which the two residues at the cleavage site, Asn-265 and Pro-266, were replaced with alanine and glycine, respectively, and expressed in *H. pylori*. In contrast to wild-type FlhB, $FlhB^{N265A}$ and $FlhB^{P266G}$ variants did not undergo detectable autocleavage. FlhB processing was required for wild-type motility and export of filament-type substrates. FlhB processing did not influence flagellar gene expression as FlhB processing-deficient strains expressed RpoN- and FliA-dependent reporter genes close to wild-type levels. Disrupting *fliK* in the strain that produced $FlhB^{N265A}$ inhibited expression of RpoN- and FliA-dependent reporter genes but not in the strain producing $FlhB^{P266G}$. We also observed that *flhX* was required for optimal expression of the RpoN-dependent reporter gene *flaB'-'xylE* in an otherwise wild-type background but not in strains that produced $FlhB^{N265A}$ or $FlhB^{P266G}$. These

findings provide a valuable framework for dissecting the mechanism that couples export apparatus function with flagellar gene expression in *H. pylori*.

Methods

Bacterial strains and growth conditions

H. pylori ATCC 43504 was grown on tryptic soy agar supplemented with 5% horse serum at 37 °C in a 2% O_2/5% CO_2/93% N_2 atmosphere. When necessary media was supplemented with 30 μg chloramphenicol ml^{-1}, 30 μg kanamycin ml^{-1}, and 10 μg erythromycin ml^{-1}. *Escherichia coli* DH5α and BL21 pLysS were grown at 37 °C with shaking in Luria Bertani broth or brain-heart infusion broth (BHI) supplemented with 100 μg ampicillin ml^{-1}, 30 μg chloramphenicol ml^{-1} or 150 μg erythromycin ml^{-1}.

Motility Assay

H. pylori motility was assessed using semisolid medium containing Mueller-Hinton broth supplemented with 0.4% (w/v) noble agar, 10% (v/v) horse serum and 10 μM $FeSO_4$. A sterile toothpick was used to stab the cells into the agar, and plates were incubated at 37 °C in a microaerobic atmosphere for up to 7 days.

Construction of mutants

H. pylori flhB (HP0770), *fliK* (HP0906) and *flhX* (HP1575) including 500 bp of flanking DNA sequence (See Table 3.1 for primer sequences), were amplified from *H. pylori* strain 26695 genomic DNA using Pfu Turbo Hotstart DNA polymerase (Stratagene). Amplicons were incubated with Taq DNA polymerase (Promega) and then cloned into pGEM-T (Promega). The resulting plasmids, pTS45 (*flhB*), pTS69 (*fliK*) and pTS68 (*flhX*), were used as templates for inverse PCR with primers that introduced EcoRI sites and annealed to sequences immediately

upstream of the translational start or downstream of the translational stop. Amplicons were circularized using T4 DNA ligase, digested with EcoRI, and a 1.1 kb EcoRI fragment containing a chloramphenicol transacetylase gene (*cat*) was cloned into the vector (Wang & Taylor, 1990). Alternatively, after cutting with EcoRI the overhanging ends were filled in using T4 DNA polymerase and a 1.1 kb EcoRV fragment containing a *Streptococcus pneumoniae* erythromycin resistance gene (*ermB*) was cloned into the vector (Stabb & Ruby, 2002). The antibiotic resistant cassette completely replaced the target gene in the final constructs, which were used as suicide vectors in *H. pylori*. Plasmids were introduced into *H. pylori*, and transformants selected as described previously (Brahmachary *et al.*, 2004). Replacement of the target gene on the chromosome with *cat* or *ermB* was confirmed by PCR.

Complementation of Δ*flhB* mutant

Plasmid pTS45 was used as a template for site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene). Primers that annealed 11 bp downstream of the translational stop of *flhB* were used to introduce a unique EcoRV site (Table 3.1) into which *ermB* was cloned. The resulting plasmid (pTS50) was introduced into a *H. pylori* Δ*flhB:cat* mutant to restore the wild-type *flhB* allele. Transformants were selected on solid medium containing erythromycin and screened for chloramphenicol sensitivity. This same strategy was used to introduce *flhB*(N265A) and *flhB*(P266G) alleles. Site-directed mutagenesis was used to introduce the desired substitutions in *flhB* in pTS50 using the primers indicated in Table 3.1, and the resulting plasmids were introduced in *H. pylori* Δ*flhB:cat* as described above. The *flhB* alleles in selected transformants were PCR amplified, and amplicons were sequenced at the University of Georgia Integrated Biotechnology Laboratory to confirm that the expected mutations were present and no other mutations had been introduced.

FLAG-tagged-FlgE construction

The *flgE* gene (HP0870) was amplified from *H. pylori* strain 26695 genomic DNA using Taq DNA polymerase and primers indicated in Table 3.1. The forward primer, containing a SalI site, annealed 100 bp upstream of the translational start, and the reverse primer, containing a KpnI site, replaced the translational stop with sequence encoding the FLAG epitope (DYKDDDDK). Amplicons were cloned into pGEM-T, confirmed by sequencing and then subcloned into the shuttle vector pHel3 (Heuermann & Haas, 1998) to obtain pTS14 which was introduced into *H. pylori* strains by natural transformation (Brahmachary *et al.*, 2004).

Protein export assays

H. pylori stains were grown in BHI (pH 6.5) supplemented with 0.4% (w/v) β-cyclodextran and 30 μg kanamycin ml^{-1} for strains harboring pTS14. Cultures were incubated at 37 °C with shaking in a 10% O_2/5% CO_2/10% H_2 atmosphere for 24 h. Before harvesting cells were vortexed for 10 sec to increase shearing of flagella. Cells were separated from the medium by centrifugation for 20 min at ~4,000 ***g***. The resulting supernatant was further clarified by centrifugation for 15 min at ~19,000 ***g***, mixed 3:1 with ice-cold 25% (w/v) trichloroacetic acid (TCA) and incubated at 0°C for 15 min. Precipitated proteins were collected by centrifugation for 10 min at ~11,000 ***g***. The protein pellet was washed three times with acetone, air dried, and resuspended in 25 mM Tris-buffered saline (pH 7.4). The cell pellet from the initial centrifugation step was resuspended in 3 ml PBS (pH 7.4) and lysed by two passages through a French press at ~13,800 kPa. Cellular debris was removed by centrifugation for 10 min at ~4,000 ***g*** and membranes were removed by centrifugation for 60 min at ~100,000 ***g***. Proteins in the resulting supernatant were collected by TCA precipitation as described above. Protein concentrations of the samples were measured using the bicinchoninic acid protein assay (Pierce)

following the manufacturer's instructions. Equivalent amounts of protein were analyzed for FlgE-FLAG, FlaB and FlaA by western blotting.

His-tagged-FlhB$_C$ purification

A 457 bp fragment encoding *H. pylori* FlhB$_c$ was PCR amplified from *H. pylori* 26695 genomic DNA using Taq DNA polymerase and primers indicated in Table 3.1. The resulting product was cloned into pGEM-T, sequenced and subcloned into pET-28 (Novagen) to generate a hexahistidine-tagged-fusion. Recombinant protein was over-produced in *E. coli* BL21 pLysS by inducing expression for 3.5 h with 0.5 mM IPTG in a 1 L culture grown to mid-log phase. His-tagged-FlhB$_C$ protein was purified using nickel-NTA (Qiagen) affinity chromatography following the supplier's instructions. Purified His-tagged-FlhB$_C$ was sent to Cocalico Biologicals to immunize a New Zealand white rabbit.

Western blots

Antiserum directed against *H. pylori* FlaB was described previously (Pereira & Hoover, 2005). Antiserum directed against the FLAG epitope was purchased from Sigma. Antiserum directed against FlhB$_c$ was affinity purified by adapting a previously described method (Pereira & Hoover, 2005). Primary antibodies were detected by enhanced chemiluminescence using a peroxidase-conjugated goat anti-rabbit antibody (MP Biomedicals).

XylE assays

XylE reporter gene fusions were described previously (Brahmachary *et al.*, 2004; Pereira & Hoover, 2005). Whole-cell XylE assays were carried as described (Brahmachary *et al.*, 2004). XylE activity was reported as micromoles of product formed per minute per 10^8 *H. pylori* cells. XylE activity for each strain was determined from at least 10 statistical replicates from two or

more biological replicates. Student's *t* test was used to determine the standard deviation with 95% confidence intervals ($P = 0.05$).

Results

Deletion of *fliK* or *flhB* has different consequences for *H. pylori* flagellar gene expression.

Disruption of *H. pylori fliK* results in reduced motility and elevated transcript levels of RpoN-dependent genes (Ryan *et al.*, 2005b). Building upon this observation, we examined how deletion of *fliK* influenced expression of flagellar reporter genes in which promoter regulatory regions of selected flagellar genes were fused to a promoter-less *Pseudomonas putida xylE* (encoding catechol dioxygenase). The different constructs included *flgI'-'xylE*, which is dependent on RpoD (*flgI* encodes the P-ring protein); *flaB'-'xylE* and *hp1120'-'xylE*, which are dependent on RpoN (*flaB* encodes the minor flagellin; *hp1120* encodes a protein of unknown function and is part of an operon with *flgK* which encodes hook-associated protein 1); and *flaA'-'xylE*, which is dependent on FliA (*flaA* encodes the major flagellin)(Brahmachary *et al.*, 2004; Pereira & Hoover, 2005). Reporter genes were introduced into *H. pylori* strains on the shuttle vector pHel3 (Heuermann & Haas, 1998), and XylE activities were measured using a whole cell colorimetric assay. Consistent with the previous report (Ryan *et al.*, 2005b) we found the Δ*fliK:cat* mutation stimulated expression of the RpoN-dependent reporter genes 3.5- to 6.5-fold compared to wild-type (Fig. 3.1).

Expression of the *flaA'-'xylE* reporter gene in the Δ*fliK:cat* mutant was similar to wild type (Fig. 3.1). In contrast, Ryan *et al*. (2005) found that disrupting *fliK* resulted in decreased *flaA* transcript levels. One possibility for this discrepancy is that the *flaA'-'xylE* reporter gene may lack elements that affect *flaA* transcript stability. This seems plausible as elements that

destabilize flagellin transcripts have been described in other bacteria (Anderson & Gober, 2000). Consistent with the report by Ryan *et al*. (2005) expression of the RpoD-dependent *flgI'-'xylE* reporter gene in the Δ*fliK:cat* mutant was similar to that in the wild-type strain (Fig. 3.1).

To investigate the role of FlhB on expression of the flagellar reporter genes we deleted *flhB* in *H. pylori* ATCC 43504. As expected from previous reports (Allan *et al.*, 2000; Wand *et al.*, 2006), the resulting Δ*flhB:cat* mutant was non-motile (Fig. 3.2). We tried unsuccessfully to complement the Δ*flhB:cat* mutant by introducing *flhB* into the HP0405 locus (data not shown). HP0405 encodes a NifU-like protein that is not required for motility or flagellar gene expression (Pereira & Hoover, 2005). Complementation in the HP0405 locus may have failed because a *cis*-acting regulatory element needed for expression was missing or contextual factors (Ye *et al.*, 2007) may have interfered with expression of *flhB* in the HP0405 locus. We replaced the Δ*flhB:cat* allele with the wild-type *flhB* allele in its native locus, and motility was restored (Fig. 3.2) confirming that no other mutations had been introduced inadvertently in the original Δ*flhB:cat* strain that affected motility.

Expression of the two RpoN-dependent reporter genes was reduced approximately 10-fold in the Δ*flhB:cat* mutant compared to wild type (Fig. 3.1). Disruption of *rpoN* or *flgR* resulted in a similar reduction in expression of these reporter genes (data not shown). Expression of the *flaA'-'xylE* reporter gene was diminished ~2.5-fold relative to wild type in the Δ*flhB* mutant (Fig. 3.1). These findings were consistent with the previous report that disruption of *H. pylori flhB* results in reduced levels of hook protein and both flagellins (Allan *et al.*, 2000). In contrast to the RpoN- and FliA-dependent reporter genes, expression of *flgI'-'xylE* in the Δ*flhB:cat* mutant was not inhibited (Fig. 3.1). Reintroduction of the wild-type *flhB* allele restored expression of the RpoN- and FliA-dependent reporter genes to levels that were

comparable to wild-type (<1.5-fold difference), except for expression of the *flaA'-'xylE* reporter gene which was slightly enhanced (~1.7-fold) in the *flhB* restored strain for unknown reasons (Fig. 3.1).

FlhB processing-deficient variants support wild-type expression of RpoN-dependent reporter genes.

We postulated that enhanced expression of the RpoN-dependent reporter genes in the Δ*fliK:cat* mutant resulted from a delay in the switch between export states. Therefore, we reasoned that in *H. pylori* strains producing FlhB processing-deficient variants the export apparatus might be locked in the rod-/hook-type conformation and over-express the RpoN regulon. The predicted cleavage site in *H. pylori* FlhB is between Asn-265 and Pro-266. *H. pylori* FlhB variants were generated in which Asn-265 was replaced with alanine, or Pro-266 was replaced with glycine. These amino acid changes were chosen since equivalent substitutions in *S. typhimurium* FlhB interfere with cleavage (Fraser *et al.*, 2003). *H. pylori* strains expressing FlhB^{N265A} or FlhB^{P266G} were severely reduced in their motility (Fig. 3.2). These findings were consistent with the decrease in motility observed in *S. typhimurium* expressing the equivalent FlhB variants (Fraser *et al.*, 2003) and were also consistent with the finding that substitutions in the conserved NPTH motif in the *Yersinia* T3SS homolog YscU abolishes export of translocators (Sorg *et al.*, 2007). This result, however, contradicts the results of Wand *et al.* (2006) who reported that the *H. pylori* FlhB ^{P266G} variant does not affect motility. The reason for this discrepancy is not due to the *H. pylori* strains used. Expressing the FlhB ^{P266G} variant in *H. pylori* J99 (the same strain used by Wand *et al.*) resulted in similarly reduced motility (data not shown). Wand and co-workers introduced a FLAG tag at the C-terminal of the FlhB^{P266G} variant which may have influenced the activity, conformation and/or processing of this protein in *H.*

pylori allowing normal motility. Consistent with this scenario, Wand and co-workers observed that the FLAG-tagged FlhB^{P266G} variant was processed at a secondary site when expressed in *E. coli*. The researchers were unable to detect FlhB and FlhB variants in *H. pylori* to see if this processing also occurred in *H. pylori* (Wand *et al.*, 2006).

Affinity purified antibodies that recognize *H. pylori* FlhB$_C$ were used in western blot assays to examine FlhB proteins. In membrane preparations from wild-type *H. pylori* a band with an estimated 34-kDa molecular mass was observed (Fig. 3.3), which is close to the predicted mass of a protein consisting of the FlhB transmembrane domain and FlhB$_C$ N-terminal subdomain (30-kDa). In some blots a faint band that migrated close to the predicted mass of full-length FlhB (41-kDa) was also observed. Neither of these bands were detectable in the Δ*flhB:cat* mutant, but both were present in the strain in which wild-type *flhB* had been restored (Fig. 3.3). These results confirm that *H. pylori* FlhB is processed *in vivo* similarly to *S. typhimurium* FlhB (Minamino & Macnab, 2000a) and verify previous work that demonstrated processing of *H. pylori* FlhB recombinant proteins in *E. coli* (Wand *et al.*, 2006). In contrast to wild-type FlhB, FlhB^{N265A} and FlhB^{P266G} were not detectably processed (Fig. 3.3), indicating that the amino acid substitutions in these FlhB variants inhibit cleavage of the protein. These results are consistent with the previous report that the FlhB^{P266G} variant expressed in *E. coli* is deficient in normal processing (Wand *et al.*, 2006).

The FlhB$_{CC}$ subdomain remains associated with the rest of FlhB following autocleavage (Deane *et al.*, 2008; Minamino & Macnab, 2000a; Zarivach *et al.*, 2008). Structural and biochemical studies indicate that the C-terminal subdomain of FlhB T3SS homologs remains intimately associated with the N-terminal subdomain following autocleavage (Deane *et al.*, 2008; Riordan & Schneewind, 2008; Zarivach *et al.*, 2008). However, we were unable to detect a

cross-reacting protein corresponding to the *H. pylori* $FlhB_{CC}$ subdomain (10 kDa) in the membrane preparations. In a similar study with *Yersinia* YscU, the C-terminal subdomain of this FlhB homolog was not detected in cellular extracts of wild-type *Y. enterocolitica* (Riordan & Schneewind, 2008). The fate of *H. pylori* $FlhB_{CC}$ in our assay is unknown. $FlhB_{CC}$ may have dissociated from the rest of FlhB *in vivo* or during the membrane isolation procedure. The affinity-purified antibodies did recognize purified $FlhB_{CC}$ (data not shown), indicating that the inability to detect $FlhB_{CC}$ in the membrane fractions was not due to poor cross-reactivity.

In the strain expressing the $FlhB^{N265A}$ variant all four reporter genes were expressed at levels slightly higher than wild-type levels (~1.5-fold difference), similar to the levels observed when *flhB* was restored in the native locus (Fig. 3.1). In the strain expressing the $FlhB^{P266G}$ variant, only the *flaB'-'xylE* reporter gene was expressed at levels that were significantly different from wild-type levels (~1.9-fold difference, Fig. 3.1). Taken together, these observations indicate that processing of FlhB is not required for normal expression of these representative genes from all three *H. pylori* flagellar regulons. In addition, FlhB processing and FliK apparently mediate different effects on the structure and/or function of the export apparatus since the FlhB processing-deficient variants did not stimulate expression of the RpoN-dependent reporter genes to the same extent as the Δ*fliK:cat* mutant (Fig. 3.1).

Disruption of *fliK* in FlhB processing-deficient strains has distinct consequences on flagellar gene expression.

Since FliK is a rod-/hook-type substrate (Minamino *et al.*, 1999b), we reasoned that inhibition of the RpoN-dependent reporter genes in the Δ*flhB:cat* mutant might result from the failure to export FliK. This does not appear to be the case, though, since disrupting *fliK* in the Δ*flhB:cat* mutant did not significantly alter expression of the RpoN-dependent reporter genes

(Fig. 3.4). We also disrupted *fliK* in strains that expressed the FlhB variants. Inactivating *fliK* in the strain that produced FlhB^{N265A} severely inhibited expression of the RpoN-dependent *flaB'-'xylE* reporter gene and inhibited expression of the FliA-dependent *flaA'-'xylE* reporter gene ~2-fold compared to FlhB^{N265A} parent strain (Fig. 3.4). Expression of the RpoN-dependent *hp1120'-'xylE* reporter gene was also inhibited in this strain, while expression of the RpoD-dependent *flgI'-'xylE* reporter gene was not significantly changed (data not shown). In contrast, disrupting *fliK* in the strain that produced FlhB^{P266G} had little effect on expression of the flagellar reporter genes with the exception of *hp1120'-'xylE* which was slightly stimulated (Fig. 3.4, data not shown). Thus, despite the fact that FlhB^{N265A} and FlhB^{P266G} are both deficient in autocleavage, these proteins elicit very different effects on the RpoN and FliA regulons in the absence of FliK.

FlhB^{N265A} or FlhB^{P266G} variant strains are defective in exporting filament-type substrates.

H. pylori strains producing FlhB^{N265A} or FlhB^{P266G} were tested for the ability to secrete rod-/hook-type and filament-type substrates. For these assays extracellular fractions from strains grown in liquid medium were analyzed by western blot for the presence of the hook protein FlgE fused to the FLAG epitope (FlgE-FLAG, a rod-/hook-type substrate) or FlaB and FlaA (filament-type substrates). Flagellar proteins present in the extracellular fraction result from flagella shearing off, and/or failure of flagellar subunits to polymerize with the nascent flagellum. Strains producing FlhB^{N265A} or FlhB^{P266G} expressed and exported FlgE-FLAG (Fig. 3.5a) but failed to export FlaA or FlaB even though both flagellins were present in the soluble cytoplasmic fractions from these strains (Fig. 3.5b). In this regard the strains producing the FlhB variants appear similar to the Δ*fliK:cat* mutant in this assay. In the strain producing the FlhB^{N265A} in the absence of FliK, FlgE-FLAG, FlaB and FlaA levels are drastically reduced in the soluble

cytoplasmic fraction, confirming the results of the reporter gene assays. The phenotype of the strain producing FlhB^{P266G} in the absence of FliK appeared similar to that of the other FlhB processing-deficient variants in that FlaA and FlaB are present in the soluble cytoplasmic fraction but are not exported (Fig. 3.5).

FlhX is required for optimal expression of *flaB* in a wild-type background but not in strains producing FlhB processing-deficient variants.

To determine if FlhX (HP1575) influences flagellar gene expression, we constructed a Δ*flhX*:*cat* mutant and monitored expression of the RpoN-dependent *flaB*'-'*xylE* reporter gene in the resulting strain. Expression of the reporter gene was inhibited in the Δ*flhX*:*cat* mutant ~2-8 fold relative to wild-type (Fig. 3.6). This result was unexpected since, consistent with the observations of Wand *et al.* (2006) disruption of *flhX* had no observable effect on motility (data not shown). The Δ*flhX*:*cat* allele was introduced into the strains that expressed FlhB^{N265A} and FlhB^{P266G} variants. In contrast to the strain with a wild-type *flhB* allele, disrupting *flhX* in these strains had little effect on expression of the *flaB*'-'*xylE* reporter gene (Fig. 3.6). Disrupting *flhX* in the Δ*fliK*:*cat* mutant inhibited expression of the *flaB*'-'*xylE* reporter gene slightly but not to the degree seen with the wild-type background (Fig. 3.6). Taken together these data suggest a role for FlhX in *H. pylori* flagellar biosynthesis.

Discussion

Consistent with previous studies that showed expression of the *H. pylori* RpoN and FliA regulons is inhibited by disrupting any one of several genes encoding components of the flagellar protein export apparatus (Allan *et al.*, 2000; Jenks *et al.*, 1997; Niehus *et al.*, 2004; Porwollik *et al.*, 1999; Schmitz *et al.*, 1997), we show here that deletion of *flhB* inhibits expression of both

RpoN- and FliA-dependent reporter genes (Fig. 3.1). Inhibition of the FliA regulon may result from failure of the Δ*flhB*:*cat* mutant to export FlgM via the export apparatus. A sheath contiguous with the outer membrane surrounds the *H. pylori* flagellum (Geis *et al.*, 1993), but this does not necessarily preclude FlgM secretion since *Vibrio cholerae*, which also possesses a sheathed flagellum, secretes FlgM from the cytoplasm via the flagellar protein export apparatus (Correa *et al.*, 2004). The FliA-dependent *flaA'-'xylE* reporter gene was expressed at levels near wild type in strains that produced the $FlhB^{N265A}$ or $FlhB^{P266G}$ variants, but these strains appear to be deficient in export of filament-type substrates (Fig. 3.5). Therefore, if the *H. pylori* export apparatus secretes FlgM, we predict it does so prior to the switch in substrate specificity to filament-type substrates. This is also consistent with the observation that the FliA-dependent reporter gene was expressed at wild-type levels in the Δ*fliK*:*cat* mutant.

The molecular basis for the link between the export apparatus and the RpoN regulon is less obvious. The export apparatus possibly secretes a factor that inhibits expression of the RpoN regulon. Alternatively, conformational changes in the export apparatus may be communicated through protein-protein interactions to the RpoN regulon. FlgS is a reasonable candidate for such interactions since the activity of this sensor kinase could be modulated by interactions with the export apparatus.

Our study confirmed a previous report that disruption of *H. pylori fliK* stimulates expression of RpoN-dependent flagellar genes (Ryan *et al.*, 2005b). Since *fliK* is part of the *H. pylori* RpoN regulon (Niehus *et al.*, 2004), FliK could function as part of a negative feedback loop to down regulate the RpoN regulon. We predict FliK mediates its effect on gene expression by influencing the conformation and/or activity of the export apparatus. Increased expression of the RpoN regulon in the Δ*fliK:cat* mutant may result from a delay in the switch in substrate

specificity of the export apparatus. This delay could influence the RpoN regulon if, as discussed above, the export apparatus secretes an inhibitor of the RpoN regulon or modulates the activity of FlgS.

To investigate if the phenotype of the Δ*fliK:cat* mutant resulted from a delay in the substrate specificity switch, we generated *H. pylori* FlhB variants that were defective in autocleavage (Fig. 3.3) and appear unable to export filament-type substrates (Fig. 3.5). FlhB is part of a family that includes homologs from virulence factor T3SS, and autocleavage at the conserved NPTH motif appears to occur universally in FlhB family members (Deane *et al.*, 2008; Riordan & Schneewind, 2008; Zarivach *et al.*, 2008). Based on studies of similar amino acid substitutions in *S. typhimurium* FlhB and FlhB T3SS homologs, we predicted the export apparatus in these strains to be locked in the rod-/hook-type conformation. If the enhanced expression of the RpoN-dependent reporter genes in the Δ*fliK:cat* mutant was due to a delay in the switch in substrate specificity of the export apparatus, we would have expected to observe enhanced expression of these reporter genes in the strains that produced the FlhB processing-deficient variants. Contrary to this prediction, strains producing the $FlhB^{N265A}$ or $FlhB^{P266G}$ variant expressed the flagellar reporter genes close to wild-type levels (Fig. 3.1). This expectation for enhanced expression of the RpoN-dependent reporter genes, however, is based on the assumption that the export apparatus only exists in either the rod-/hook-type or filament-type conformations. The export apparatus might assume additional conformations during flagellar assembly. With this in mind, a significant distinction between the Δ*fliK:cat* mutant and the FlhB processing-deficient variants is that the export apparatus in the Δ*fliK:cat* can switch substrate specificity. Poly-hooks produced by *fliK* mutants often have filaments attached (Ryan *et al.*, 2005b), while the export apparatus containing $FlhB^{N265A}$ or $FlhB^{P266G}$ appears incapable of

switching substrate specificity. Thus, the export apparatus in the Δ*fliK*:*cat* mutant may persist in a conformation that stimulates expression of the RpoN regulon while the export apparatus in strains producing the processing-deficient FlhB variants is unable to assume this conformation.

Two recent studies reported the crystal structures of the cytoplasmic domains of FlhB T3SS homologs *E. coli* EscU, *S. typhimurium* SpaS and *Shigella flexneri* Spa40 (Deane *et al.*, 2008; Zarivach *et al.*, 2008). These studies reveal the structural changes that take place in FlhB T3SS homologs following autocleavage and also further elucidate the mechanism of autocleavage. These FlhB homologs contain a central β-sheet surrounded by four α-helices. The NPTH motif is located between β1 and β2 strands forming a type II β-turn that undergoes autocleavage via a mechanism involving cyclization of the asparagine residue (Ferris *et al.*, 2005; Zarivach *et al.*, 2008). Autocleavage does not affect the protein fold but rather generates localized electrostatic and conformational changes that create a unique surface which is believed to influence interactions with other components of the export apparatus (Deane *et al.*, 2008). Based on the structural analysis of these FlhB T3SS homologs, the reasons that *H. pylori* $FlhB^{N265A}$ and $FlhB^{P266G}$ were deficient in processing are because replacing Asn-265 with Ala removes the reactive asparagine residue and replacing Pro-266 with Gly disrupts an essential type II β-turn in the NPTH loop (Zarivach *et al.*, 2008).

Disrupting *fliK* in the strain that produced $FlhB^{N265A}$ resulted in a dramatic decrease in the expression of the two RpoN-dependent reporter genes, as well as a ~2-fold decrease in expression of the *flaA'-'xylE* reporter gene. In contrast, disrupting *fliK* in the strain that produced $FlhB^{P266G}$ resulted in no change or slightly increased expression of the RpoN- and FliA-dependent reporter genes (Fig. 3.4). In the absence of structural data for the FliK-FlhB interaction, the molecular basis of the synergistic, negative effect of the $FlhB^{N265A}$ variant and

Δ*fliK:cat* mutation is difficult to predict. Possibly, FliK interacts with $FlhB^{N265A}$ to overcome a barrier within the export apparatus that prevents expression of the RpoN and FliA regulons. Similar interactions between FliK and wild-type FlhB may occur but may no longer be needed for expression of the RpoN and FliA regulons upon autocleavage of FlhB. Macnab and co-workers isolated mutations within *flhB* that restored motility in a *S. typhimurium fliK* mutant (Williams *et al.*, 1996). These mutations occurred at two highly conserved positions (Gly-293 and Ala-298) within $FlhB_{CC}$, and slowed the rate at which FlhB was cleaved (Minamino & Macnab, 2000a). Structural studies with *S. flexneri* $Spa40_C$ suggest that these substitutions in FlhB disrupt packing of the β2 strand and α2 helix around the region of the NPTH loop thus influencing the ability of the NPTH loop to adopt the conformation needed for switching substrate specificity of the export apparatus independent of FliK (Deane *et al.*, 2008).

We observed that disruption of *flhX* in *H. pylori* resulted in decreased expression of the RpoN-dependent *flaB'-'xylE* reporter gene (Fig. 3.6). This inhibition, however, was not seen in strains that produced the $FlhB^{N265A}$ or $FlhB^{P266G}$ variant. We infer from these results that $FlhB_{CC}$ can dissociate from $FlhB_{CN}$ following autocleavage and be replaced with FlhX and that association of $FlhB_{CC}$ or FlhX is required for optimal expression of the RpoN regulon. In the processing-deficient FlhB variants, $FlhB_{CC}$ remains linked to $FlhB_{CN}$, so FlhX is not required for wild-type expression of the RpoN regulon in strains that produce these FlhB variants. FliK may facilitate the dissociation of $FlhB_{CC}$ from $FlhB_{CN}$, which could explain why disruption of *flhX* in the Δ*fliK*:*cat* mutant had less of an impact on expression of the *flaB'-'xylE* reporter gene than in the wild-type.

Clearly, the molecular mechanisms that govern the function of the flagellar protein export apparatus and the conformational changes within the export apparatus that influence substrate

specificity are complex. Further defining the role FlhB plays in these processes will be important for understanding how the export apparatus influences expression of the *H. pylori* RpoN and FliA regulons.

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Table 3.1 Oligonucleotide primers used for this study	
flhB forward	CAT GCC TTT AAA ATC GTA TTC TAG
flhB reverse	CTT TAA TGG TGC TTT ATC TTT TCT
flhB inverse forward	TTC CCG TGG CGA TAG TCT TCG CT
flhB inverse reverse	TTC GGT TTT TTC TTC TTC AGC CA
fliK forward	CGT TTC AAA TAG TAA CTC TTC TTG
fliK reverse	CCG TTA CTT CCC TTA AAG CG
fliK inverse forward	AGA ATT CTT TTA AGC AAG TTT GCT CTA TAA T
fliK inverse reverse	AGA ATT CGG TTA TCC TTTT TTA AAA CAA CTC
flhX forward	GAC CGC CAT AGA AAG TTC AAA
flhX reverse	CAG GGC AAT TCG CTA AAC TCC
flhX inverse forward	AGA ATT CTC GGA ATG TAA AGT TAA AAC GAT
flhX inverse reverse	AGA ATT CCT AAT ACC CTA AAG TCA AAG CGT
flhB EcoRV forward	CCC GCT TTT AAG CCC TAA AAA AAC ACG ATA TCA AAA GGC TTT AGC TAT TCC
flhB EcoRV reverse	GGA ATA GCT AAA GCC TTT TGA TAT CGT GTT TTT TTA GGG CTT AAA AGC GGG
flhB(N265A) forward	GTC GTG GTT ACT GCC CCC ACC CAT TAC GCC
flhB(N265A) reverse	GGC GTA ATG GGT GGG GGC AGT AAC CAC GAC
flhB(P266G) forward	GTC GTG GTT ACT AAC GGC ACC CAT TAC GCC GTC
flhB(P266G) reverse	GAC GGC GTA ATG GGT GCC GTT AGT AAC CAC GAC
flgE FLAG forward	GTC GAC CCC CTA TTT TCT TAT GAA TTT GG
flgE FLAG reverse	GGT ACC TTA TTT ATC ATC ATC ATC TTT ATA ATC TTG CTT AAG ATT CAA TAG GGT G
$FlhB_C$ forward	CAT ATG GAT TAT AAA GAT GAT GAT GAT AAA ATC AAA CGC CGC CAA TAC ACC AAC
$FlhB_C$ reverse	AAG CTT TTA AAG AGG TTT AAT GAT CTT TTG TTT TTG G

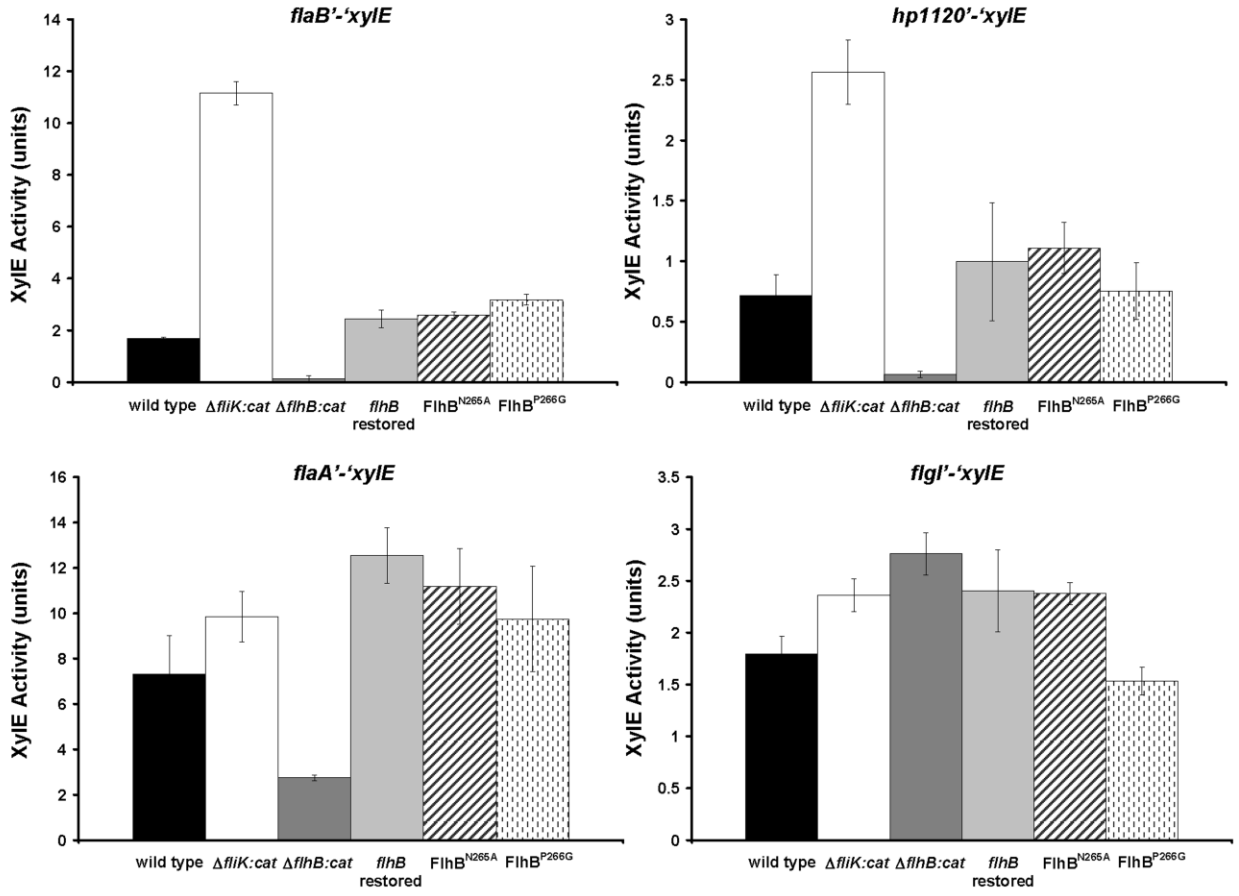

Figure 3.1 Expression of flagellar reporter genes in various *H. pylori* strains. XylE activities were measured in whole cell assays for the reporter genes indicated in *H. pylori* ATCC 43504 (wild type, black), Δ*fliK:cat* (white), Δ*flhB:cat* (dark gray), a strain in which the wild-type *flhB* allele was restored (*flhB* restored, light gray), *flhB*(N265A) ($FlhB^{N265A}$, stripped), or *flhB*(P266G) ($FlhB^{P266G}$, stippled). One unit of XylE activity corresponds to one μmol of product formed min^{-1} $(10^8$ cells$)^{-1}$. Means are reported for five statistical replicates with error bars indicating the range of values with 95% confidence (P=0.05) as determined by Student's t test.

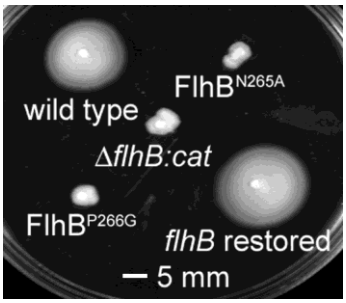

Figure 3.2 Motility of *H. pylori* strains in a semisolid medium. *H. pylori* strains shown are wild type, Δ*flhB:cat*, a strain in which the wild-type *flhB* allele was restored (*flhB* restored) and strains expressing $FlhB^{N265A}$ or $FlhB^{P266G}$ variants. Bar, 5 mm.

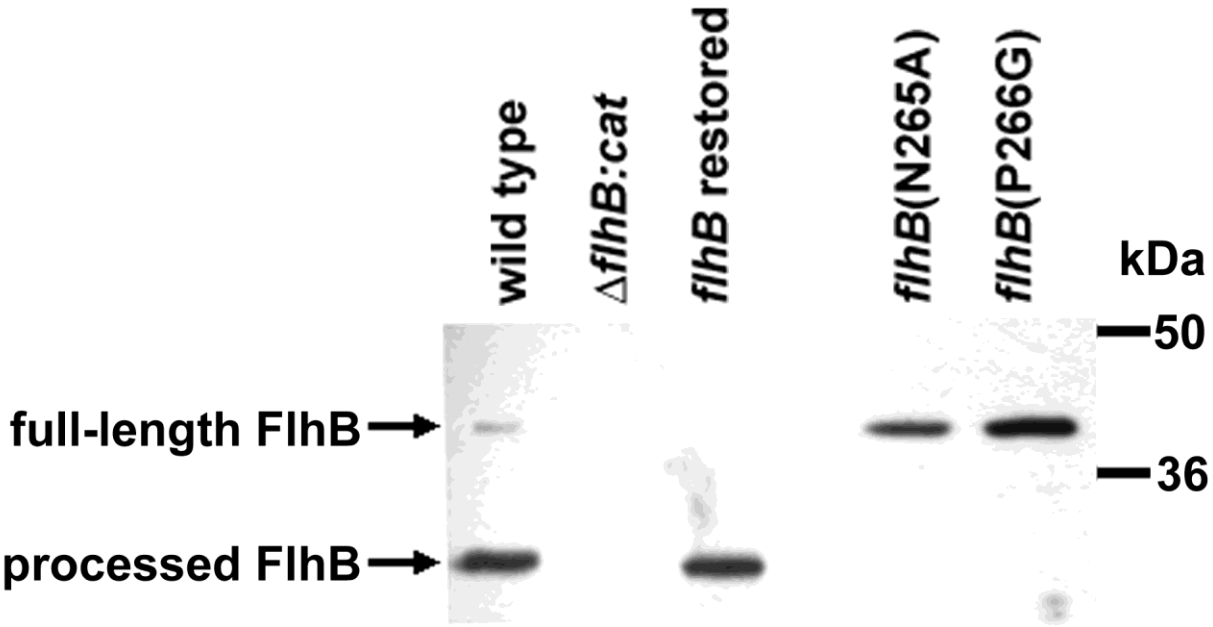

Figure 3.3 Western blot analysis of FlhB in *H. pylori* strains. Membrane fractions from strains bearing the following *flhB* alleles were analyzed: wild type, Δ*flhB:cat*, *flhB* restored, *flhB*(N265A) and *flhB*(P266G). Purified membrane fractions containing approximately 20 μg of total protein were loaded in each lane and proteins were separated by SDS-PAGE. FlhB proteins were detected in western blots using affinity-purified antibodies directed against $FlhB_C$. Arrows indicate the position of full-length and processed FlhB. Locations of protein standards and their molecular masses (kDa) are indicated.

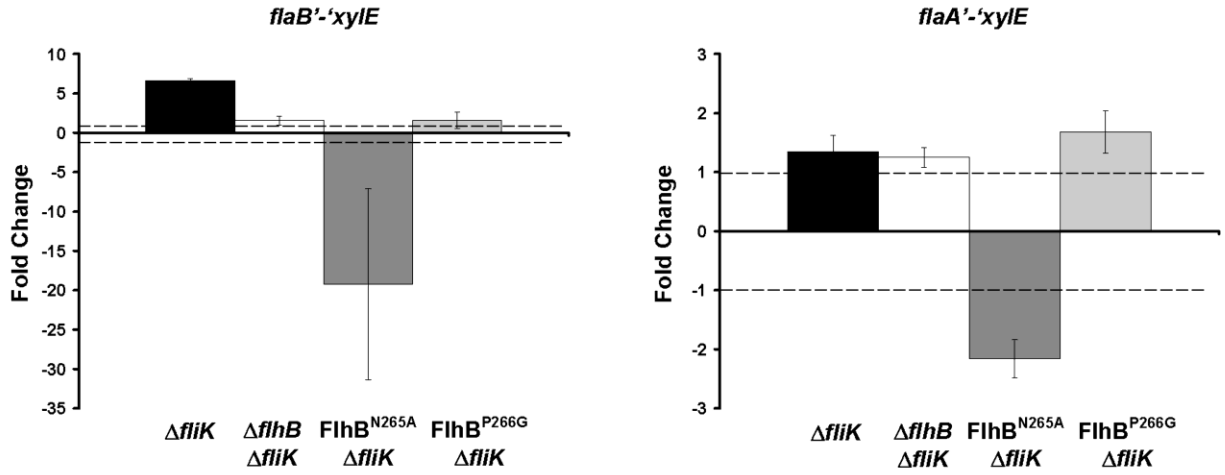

Figure 3.4 Changes in expression of flagellar reporter genes due to *fliK* disruption in *H. pylori* strains with various *flhB* alleles. XylE activities indicated were measured in strains with the following genotypes: Δ*fliK:cat* (wild type Δ*fliK*, black), Δ*flhB:cat* Δ*fliK:ermB* (Δ*flhB* Δ*fliK*, white), Δ*fliK:cat flhB*(N265A) (FlhB^{N265A} Δ*fliK*, dark gray) and Δ*fliK:cat flhB*(P266G) (FlhB^{P266G} Δ*fliK*, light gray). Activities of these strains were compared with those of the corresponding parental strain: wild type, Δ*flhB:cat*, *flhB*(N265A) and *flhB*(P266G). The fold change is reported as negative if expression decreased when *fliK* was disrupted and positive if expression increased. The dotted lines at 1 and -1 indicate no change in expression between the parent and Δ*fliK* strain. Error bars indicating the range of values with 95% confidence (P=0.05) as determined by Student's *t* test.

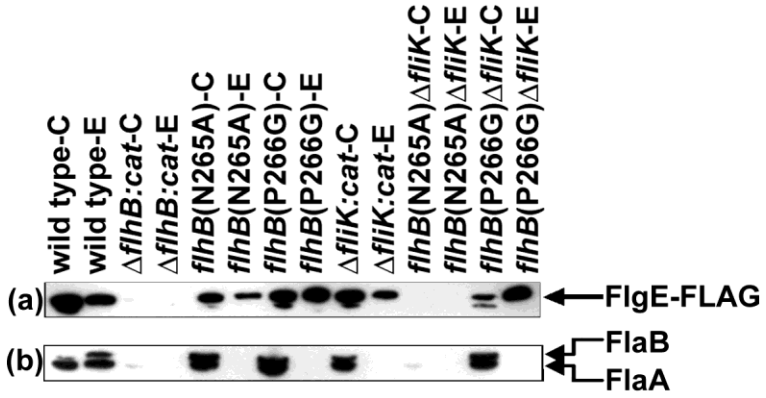

Figure 3.5 Assay for export of hook and flagellin proteins in various *H. pylori* strains. (a) Western blot analysis of hook protein FlgE with a C-terminal FLAG-tag fussion (FlgE-FLAG) in soluble cytoplasmic (C) and extracellular (E) fractions. The following strains bearing the FlgE-FLAG expression vector, pTS14, were analyzed: wild type, Δ*flhB:cat, flhB*(N265A), *flhB*(P266G), Δ*fliK:cat*, *flhB*(N265A) Δ*fliK:cat* and *flhB*(P266G) Δ*fliK:cat*. Approximately 15 μg of total protein were loaded in each C-lane and 20 μg of total protein in each E-lane. FlgE-FLAG was detected in western blots with antibodies directed against the FLAG epitope. (b) Western blot analysis of flagellins in soluble cytoplasmic (C) and extracellular (E) fractions. The same protein samples and similar amounts of protein were loaded as in (a). Flagellins were detected in western blots with anti-serum directed against FlaB. The anti-serum also cross-reacts with FlaA, which is slightly smaller than FlaB.

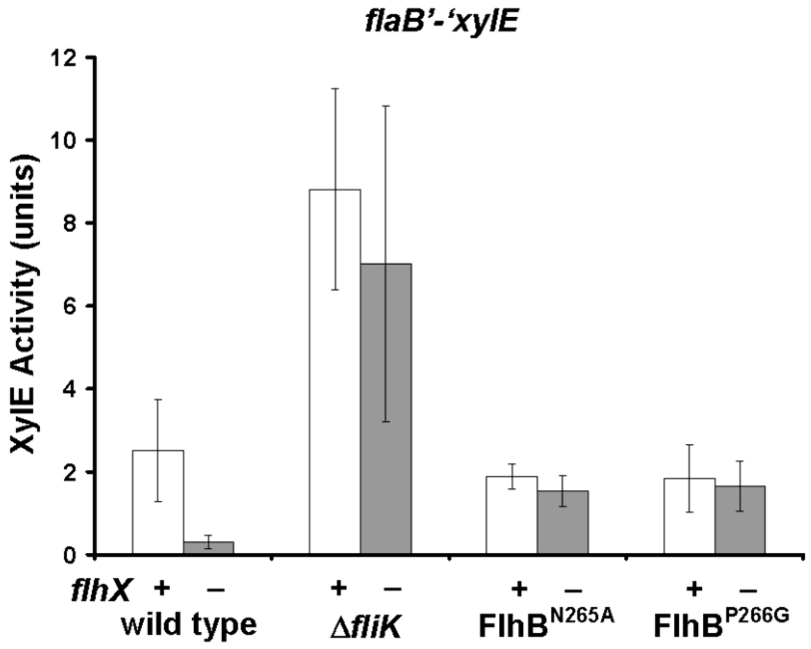

Figure 3.6 Effect of *flhX* deletion on expression of the *flaB'-'xylE* reporter gene in *H. pylori* strains with different *flhB* or *fliK* alleles. Reporter gene activities were measured in various strains: wild type, Δ*fliK:cat*, *flhB*(N265A), and *flhB*(P266G) that either possessed or lacked *flhX*. One unit of XylE activity corresponds to one μmol of product formed min^{-1} $(10^8$ cells$)^{-1}$. Means are reported for five statistical replicates with error bars indicating the range of values with 95% confidence (P=0.05) as determined by Student's *t* test.

CHAPTER 4

FLAGELLAR PROTEIN EXPORT APPARATUS FUNCTION IS NOT REQUIRED FOR EXPRESSION OF σ^{54}-DEPENDENT FLAGELLAR GENES IN *HELICOBACTER PYLORI*

Abstract

The Gram-negative, ε-Proteobacteria *Helicobacter pylori* is a significant human pathogen infecting a large percentage of the worldwide population and causing acute gastritis leading to peptic ulcers and gastric carcinoma in some infected individuals. Many essential colonization factors have been identified in *H. pylori* including flagellar motility. Flagellum assembly requires the coordinated expression over 40 proteins utilizing all three sigma factors (RpoD, RpoN and FliA) found in the cell. Transcription of the *H. pylori* RpoN regulon is controlled by the FlgS/FlgR two-component regulatory system. Previous studies in *H. pylori* showed that expression of the RpoN- and FliA-dependent flagellar genes requires a functional flagellar protein export apparatus. Specifically FlhA, a component of the flagellar protein export apparatus, was identified as having a potential regulatory role in transcription of these regulons. In the present study we found that some mutations in *flhA* resulted in decreased expression of RpoN-dependent reporter genes, while other mutations resulted in enhanced expression of these reporter genes. These differences do not appear to be due to polar effects on the gene immediately downstream of *flhA* which is *hp1042*. Homologs of *hp1042* are downstream of *flhA* in many of the ε-Proteobacteria whose genomes have been sequenced, suggesting a possible role for HP1042 in flagellar biosynthesis or function. Disruption of *hp1042* in *H. pylori* ATCC

43504 did not affect motility or expression of RpoN- or FliA-dependent reporter genes. However, HP1042 appears to have post-translational effects on the hook protein FlgE and the flagellins FlaA and FlaB, possibly affecting glycosylation of these proteins. Based on these results the differences in gene expression may reflect distinct consequences of *flhA* disruption due to the predicted size of a resulting truncated FlhA protein. Regardless of the cause of the differences between the *flhA* alleles, all of the *flhA* mutants were severely inhibited in export of both rod-/hook-type substrates and filament-type substrates even though these substrates were present in the cytoplasm of the *flhA* mutant strains. This strongly suggests that a negative regulator of the RpoN regulon is not exported from the cell as a substrate of the flagellar protein export apparatus as part of a regulatory mechanism that governs expression of the RpoN regulon.

Introduction

In *Helicobacter pylori* two to six polar, sheathed flagella drive motility which is required for colonization of the human gastric mucosa (Eaton *et al.*, 1992; Goodwin *et al.*, 1985; Schreiber *et al.*, 2005). The bacterial flagellum is made up of three parts: the basal body, hook and filament. Located at the base of the basal body the flagellar protein export apparatus is a type-three secretion system required for translocation of most of the extracellular components of the flagellum (Hirano *et al.*, 2003; Minamino & Macnab, 1999). In several bacterial species transcription of genes encoding components of the flagellum have been shown to be responsive to assembly of those components. The export apparatus plays a crucial role in controlling flagellar assembly as well as gene expression in these bacteria. In *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) once formation of the hook is completed the export apparatus undergoes a switch in substrate specificity and begins to export filament-type substrates

(Minamino *et al.*, 1999). FlgM is an anti-σ^{28} factor that inhibits transcription of σ^{28}-dependent flagellar genes (FliA regulon), which includes genes encoding flagellin as well as other flagellar proteins that are required late in the assembly of the flagellum (Ohnishi *et al.*, 1992). Upon completion of the hook-basal body complex, FlgM is exported from the cell as a filament-type sybstrate by the export apparatus, which alleviates the inhibition of FlgM on expression of the FliA-dependent flagellar genes (Hughes *et al.*, 1993).

In *Caulobacter crescentus* formation of the export apparatus, MS-ring and rotor switch is required for transcription initiation by σ^{54} (RpoN) which is employed for expression of middle and late flagellar genes (Wu & Newton, 1997). Prior to assembly of the export apparatus-basal body intermediate, the regulatory protein FliX interacts with the σ^{54}-dependent activator FlbD to disrupt its DNA-binding activity and prevent transcription of middle and late genes (Mohr *et al.*, 1998; Muir & Gober, 2001; Ramakrishnan & Newton, 1990). Upon completion of export apparatus-basal body intermediate FliX responds to an unidentified signal and switches its activity to stimulate FlbD-mediated transcriptional activation of the RpoN-dependent genes (Muir & Gober, 2004).

Flagellar gene regulation in *H. pylori* is similar to that in *C. crescentus* in that RpoN is required for expression of middle flagellar genes, but is also similar to flagellar gene regulation in *S. typhimurium* in that FliA and FlgM control expression of late flagellar genes (Colland *et al.*, 2001; Josenhans *et al.*, 2002; Spohn & Scarlato, 1999). A two-component regulatory system composed of the sensor kinase FlgS and the response regulator FlgR is required to activate transcription of the *H. pylori* RpoN regulon. FlgS and FlgR differ from most two-component regulatory system proteins in that FlgS is not membrane bound and FlgR does not bind DNA (Beier & Frank, 2000; Brahmachary *et al.*, 2004). The genes encoding FlgR and FlgS are

unlinked – *flgS* (locus number HP0244) appears to be in an operon with *flgI* (encodes flagellar P-ring protein) while *flgR* (locus number HP0703) appears to be in an operon with several other genes that are not involved in motility.

It is not known what, if any, signal is sensed by *H. pylori* FlgS to regulate expression of the RpoN regulon. Several lines of evidence, however, indicate that expression of the RpoN regulon is linked to the activity of the flagellar protein export apparatus. Inactivation of the genes encoding various components of the export apparatus, including FlhA, FlhB, FliQ or FliI, result in decreased expression of the RpoN-dependent genes *flgE* and *flaB,* which encode the hook protein and a minor flagellin, respectively (Allan *et al.*, 2000; Foynes *et al.*, 1999; Jenks *et al.*, 1997). In addition, results from a genome-wide DNA microarray analysis of *H. pylori* revealed that disruption of *flhA* results in decreased transcript levels of RpoN-dependent genes (Niehus *et al.*, 2004). In the closely related ε-Proteobacterium *Campylobacter jejuni*, disruption of FlhA, FlhB, FliP or FliR results in decreased expression of RpoN-dependent reporter genes (Hendrixson & DiRita, 2003). Although the hook length control protein FliK is not part of the export apparatus, it plays a key role in the switch in specificity from rod-/hook-type to filament-type substrates (Williams *et al.*, 1996). Inactivation of *fliK* results in enhanced expression of RpoN-dependent genes in *H. pylori* as well as *C. jejuni* (Kamal *et al.*, 2007; Ryan *et al.*, 2005; Smith *et al.*, 2009). FliK is secreted by the export apparatus as a rod-/hook-type substrate, but failure to secrete FliK does not appear to be responsible for the inhibition of the RpoN regulon in export apparatus mutants as inactivation of *fliK* in a *flhB* mutant background fails to restore expression of RpoN-dependent reporter genes (Smith *et al.*, 2009).

It is not known how the export apparatus controls expression of the RpoN regulon, but it believed to modulate the activity of FlgS. In support of this hypothesis, expression of a

constitutively active form of FlgR in *C. jejuni flhA*, *flhB* or *fliP* mutants results in a partially restoration of expression of RpoN-dependent reporter genes (Joslin & Hendrixson, 2009). Based on the current observations there are two plausible models for how the export apparatus might affect FlgS activity. In the first model, FlgS may be responsive to the conformation of the export apparatus, while in the second model FlgS activity may be regulated by a substrate of the export apparatus. Arguing against the second model, Joslin and Hendrixson (2009) screened ~65,000 transposon insertion mutants but were unable to identify insertions that restored expression of a RpoN-dependent reporter gene in *C. jejuni flhA*, *flhB* or *fliP* deletion mutants. While this study argues against the existence of a negative regulator of the RpoN regulon secreted by the export apparatus, it is possible that such a factor is essential for cell viability or is encoded by a gene upstream of an essential gene. We report here that some insertions within *H. pylori flhA* result in enhanced expression of RpoN-dependent genes. Since these insertions interfere with the ability of the export apparatus to secrete both rod-/hook-type and filament-type substrates, these results suggest that the conformation rather than the secretory function of the export apparatus is responsible for modulating FlgS activity.

Results

Insertions in *H. pylori flhA* allow expression of RpoN-dependent reporter genes

FlhA is an integral membrane protein with eight predicted membrane spanning domains (McMurry *et al.*, 2004) and a large cytoplasmic domain ($FlhA_C$). In *S. typhimurium* FlhA along with Fluke (*flk*) appear to sequester the cytoplasmic domain of FlhB ($FlhB_C$) to prevent premature substrate switching (Hirano *et al.*, 2009). A Fluke ortholog has not been identified in *H. pylori*, but FlhA probably functions in a similar manner in *H. pylori* and *S. typhimurium*. In

addition, FlhA or $FlhA_C$ have been shown to interact with all other export apparatus components except FliR indicating an important role for FlhA in assembly of the export apparatus (McMurry *et al.*, 2004).

To investigate the role of FlhA in flagellar gene regulation we initially disrupted *flhA* with a chloramphenicol resistance (*cat*) cassette immediately following codon 77 (referred to as *flhA77* allele), taking advantage of an Eco47III site at this location. Previous studies by Niehus *et al*. (2004) on the function of *H. pylori flhA* in flagellar gene expression were done using an *flhA* allele in which a kanamycin resistance cassette had been inserted into a HindIII site overlapping codons 456 and 457. We wished to create a similar *flhA* mutation, but the *flhA* from *H. pylori* 26695 lacked this HindIII site. Therefore, we introduced a unique EcoRI site by site-directed mutagenesis that overlapped codons 454 and 455 of *flhA* and then inserted the *cat* cassette into this site, creating the *flhA454* allele. A third *flhA* allele was constructed in which a region corresponding to 90 nucleotides upstream of the predicted start codon of *flhA* through codon 77 of the gene was replaced with the *cat* cassette (referred to as Δ*flhA*). All three *flhA* alleles were introduced individually into *H. pylori* ATCC 43504. Based on the predicted structure of FlhA, the first 77 amino acids of the protein include the first two membrane-spanning helices, while the first 454 amino acids include the entire transmembrane domain of FlhA along with part of $FlhA_C$ (McMurry *et al.*, 2004). Motility was similarly reduced in all three of the *flhA* mutants (Fig. 4.1, data not shown).

Disruption of *flhA* in *H. pylori* has been reported to interfere with expression of the middle (RpoN-dependent) and late (FliA-dependent) flagellar genes, but not expression of early (RpoD-dependent) flagellar genes (Niehus *et al.*, 2004). Consistent with this previous observation, expression of an RpoD-dependent *flgI'-'xylE* reporter gene was unaffected in strains

containing *flhA77* and *flhA454* mutant alleles (Table 4.1). For unknown reasons expression of the *flgI'-'xylE* reporter gene was significantly higher in the Δ*flhA* mutant when compared to wild type. Also consistent with this previous report, expression of two RpoN-dependent reporter genes, *flaB'-'xylE* and *hp1120'-'xylE* was significantly reduced in the Δ*flhA* mutant compared to wild type (Table 4.2). The expression levels of these RpoN-dependent reporter genes in the Δ*flhA* mutant were similar to those observed in an *rpoN* mutant (data not shown). Unexpectedly, however, expression of the RpoN-dependent reporter genes was significantly higher in *H. pylori* strains bearing the *flhA77* or *flhA454* alleles compared to the wild-type strain (Table 4.2). The only exception was *hp1120'-'xylE* in the *flhA454* mutant which was expressed at wild-type levels. To verify these results the *flhA* mutations were reconstructed in a naive strain and reporter gene activities were measured in these strains. The enhanced expression of the RpoN-dependent reporter genes was observed for the reconstructed *flhA454* mutant strain, but not the *flhA77* mutant strain. This led us to investigate additional factors that could be responsible for the observed phenotype.

All of the *flhA* mutants displayed reduced expression of a FliA-dependent reporter gene, *flaA'-'xylE* (Table 4.3). Interestingly, in the *flh77* mutant strain in which the RpoN regulon was up-regulated, expression of the *flaA'-'xylE* reporter gene was down-regulated to a greater extent than in the Δ*flhA* mutant. We infer from this result that a member of the RpoN regulon may negatively regulate the FliA regulon.

HP1042 does not affect motility or flagellar gene expression

We first considered that downstream or polar effects might be responsible for the different *flhA* phenotypes. The gene immediately downstream of *flhA* is HP1042, an uncharacterized gene that possesses DHH and DHHA1 domains. The DHH family of proteins

are predicted to have phosphoesterase activity and include the single-stranded DNA exonuclease RecJ (Aravind & Koonin, 1998). The DHHA1 domain (DHH associated domain) is diagnostic of DHH subfamily 1 members, which include RecJ and alanyl tRNA synthetase, an observation that has led to the suggestion that this domain may have a nucleic acid binding function (Aravind & Koonin, 1998). The *flhA* and *hp1042* open reading frames are separated by only 24 nucleotides suggesting that these genes are co-transcribed. This gene organization is conserved in other ε-Proteobacteria, including *Campylobacter* ssp., *Wolinella succinogenes* and *Thiomicrospira denitrificans,* but not *Arcobacter butzleri*. The association of *hp1042* with *flhA* in all of these organisms suggested HP1042 might be involved in flagellar biogenesis, and perhaps account for the differences observed in the *flhA* mutants.

To determine if altered (either increased or decreased) expression of HP1042 was responsible for the up regulation of the RpoN regulon in the *flhA77* mutant, *hp1042* was disrupted in both the wild-type background and the strain bearing the *flhA77* allele. For these experiments a Δ*hp1042:cat* mutation was introduced into wild-type *H. pylori* 43504 and a Δ*hp1042:erm* mutation was introduced into the strain bearing the *flhA77* allele. In addition, *hp1042* was placed under control of the *hp1563* promoter and inserted into the HP0405 locus on the chromosome to try to express HP1042 at a constitutively low level. The HP1563 promoter was used previously to complement a flagellar regulatory gene mutation (Douillard *et al.*, 2009), and the HP0405 locus was used previously to express *H. pylori* genes from a non-native locus (Pereira & Hoover, 2005).

Disruption of *hp1042* in the wild-type background did not affect motility (Fig. 4.1), nor did it affect expression of the flagellar reporter genes (Table 4.1, 4.2 and 4.3). When *hp1042* was disrupted in the *flhA77* mutant, expression of the RpoN-dependent reporter genes was still

up regulated, although to a slightly lesser extent than that observed in the *flhA77* mutant alone. Similarly, when HP1042 was introduced with a non-native promoter in the HP0405 locus in the *flhA77* mutant expression of the RpoN-dependent reporter genes was still up regulated (Table 4.2). Although we do not know if HP1042 was successfully expressed from the HP0405 locus, these results suggest that the altered expression of HP1042 is not responsible for the up regulation of the RpoN regulon in the *flhA77* and *flhA454* mutants.

flhA mutants are defective in export of both rod-/hook-type and filament-type substrates

We wished to verify that the *flhA* mutants were deficient in exporting flagellar proteins. To examine export of rod-/hook-type substrates we constructed a reporter gene that encoded a fusion of the FLAG epitope to the carboxy-terminus of the hook protein FlgE. The FlgE-FLAG protein was expressed from the *flgE* promoter regulatory region and was carried on the shuttle vector pHel3 (Smith *et al.*, 2009). Commercially available monoclonal antibodies directed against the FLAG tag were used to monitor synthesis and export of the FlgE-FLAG protein. To test for export of filament-type substrates we used antiserum directed against *H. pylori* minor flagellin FlaB. Since *H. pylori* FlaB and the major flagellin FlaA share 58% amino acid identity the antiserum directed against FlaB cross-reacted with FlaA. *H. pylori* strains were grown in serum-free liquid medium and the cells were collected by centrifugation. Extracellular proteins in the resulting supernatant liquid were concentrated by TCA precipitation and analyzed by western blotting. Cell pellets were resuspended in buffer and lysed. The crude cell extracts were subjected to low speed centrifugation followed by high speed ultracentrifugation. Proteins in the resulting supernatant liquids were concentrated by TCA precipitation and analyzed by western blotting. Consistent with the results from the XylE reporter gene assays, the RpoN-dependent FlgE-FLAG protein was observed in the soluble cytoplasmic fractions of the *flhA77* and *flhA454*

mutant strains, but not the Δ*flhA* mutant (Fig. 4.2A). The FlgE-FLAG protein was detected in the extracellular fraction of the wild-type strain, but not those of the *flhA* mutant strains except for a small amount in the extracellular fraction of the *flhA77* mutant which could be due to cell lysis (Fig. 4.2A). Similarly, both *flhA77* and *flhA454* mutants but not the Δ*flhA* mutant produced but did not export the RpoN-dependent gene product FlaB (Fig. 4.2B). In agreement with the XylE reporter gene assay, all three of the *flhA* mutants failed to produce FliA-dependent FlaA. Taken together, these results verified that strains with the *flhA77* or *flhA454* alleles were able to express RpoN-dependent flagellar genes, but were unable to export the products of these genes.

The Δ*hp1042:cat* mutant had an interesting phenotype with regard to the physical properties of the FlgE-FLAG protein and the flagellins. The FlgE-FLAG protein was degraded into several stable products in the extracellular fraction of the Δ*hp1042:cat* mutant (Fig. 4.2A). In addition, the full-length form of the FLAG-FlgE protein was slightly larger than that in the parental wild-type strain. The extracellular fraction of the Δ*hp1042:cat* mutant had very little protein that cross-reacted with the FlaB antiserum where FlaA and FlaB were expected to migrate, but did have two new cross-reactive proteins that migrated significantly higher with an apparent molecular weight of 86 and 91 kDa (Fig. 4.2C).

We postulate that the changes in FlgE-FLAG and flagellins in the Δ*hp1042:cat* mutant are due to changes in the glycosylation of these proteins. *H. pylori* FlaA and FlaB are known to be glycosylated by pseudaminic acid (Schirm *et al.*, 2003), but post-translational modification of FlgE in *H. pylori* has not been demonstrated. We postulate that in the Δ*hp1042:cat* mutant the hook and flagellin proteins are hyperglycosylated, resulting in proteins with increased molecular weights. In addition, hyperglycosylation of FlgE-FLAG could affect the structure of the protein and target it for degradation by *H. pylori* proteases that are present extracellularly (Smith *et al.*,

2007). Moreover, hyperglycosylation of the flagellins could block epitopes on the proteins that are recognized by the FlaB antiserum, which may account for the weak cross-reactivity of these proteins in the western blot. Interestingly, if the hook protein and flagellins are hyperglycosylated in the Δ*hp1042:cat* mutant, this does not have a detectable effect on motility. How HP1042 might influence glycosylation of the hook protein and the flagellins is not obvious, but it may affect the activity of enzymes involved in glycosylation pathway.

Discussion

Assembly of the bacterial flagellum is a highly ordered process that involves the coordinated regulation of over 40 structural and regulatory genes. Transcriptional hierarchies for flagellar gene expression result in temporal regulation of these genes. This transcriptional hierarchy ensures that the products of the flagellar genes are made as they are required in the assembly process. In *H. pylori* flagellar genes can be divided into three regulons that are controlled by the three sigma factors within this bacterium. Considerable evidence has accumulated implicating the flagellar protein export apparatus in regulating expression of the *H. pylori* RpoN flagellar regulon. We envision two competing models for how the export apparatus influences expression of the *H. pylori* RpoN regulon.

In one model, the activity of FlgS is influenced by the conformation of the export apparatus. For example, when the export apparatus is initially assembled it would be in a conformation that favors export of rod-/hook-type substrates. FlgS could sense this conformation, either through direct interactions with the export apparatus or indirectly, stimulating the autokinase activity of FlgS and allowing phosphorylation of FlgR. FlgR-phosphate would then activate transcription of the RpoN-dependent flagellar genes. Following a

conformational change in the export apparatus that favors export of filament-type substrates, FlgS would no longer be active and transcription of the RpoN regulon would cease.

In a second model, the export apparatus affects expression of the RpoN regulon by secreting a factor that inhibits transcription of the RpoN-dependent genes. In this model the role of the export apparatus in regulating expression of the RpoN regulon would be analogous to the way in which it modulates expression of the FliA regulon in *S. typhimurium* by secreting that anti-σ^{28} factor, FlgM. However, unlike *S. typhimurium* FlgM which is a filament-type substrate, any inhibitor of the *H. pylori* RpoN regulon would be anticipated to be a rod-/hook-type substrate. Using domain searches no candidates for a secreted inhibitor have been identified.

We attempted to distinguish between these two models by analyzing the effect of *flhA* mutations on expression of the RpoN regulon. We found that two RpoN-dependent reporter genes were not expressed in a Δ*flhA* mutant, consistent with previous results which showed reduced transcript levels for all the RpoN-dependent genes in an *flhA* mutant (Niehus *et al.*, 2004). In contrast, we observed here that when *flhA* was disrupted with insertion of a *cat* cassette at codon 77 (*flhA77*) or codon 454 (*flhA454*) that expression of the RpoN-dependent reporter genes was stimulated. Interestingly, the *flhA* mutant used by Niehus *et al*. (2004) for these previous experiments was an insertion mutation that was near the site (codon 456) where *flhA454* is interrupted. We do not know the reason for the differences between these two studies. It is possible that the discrepancy is due to strain differences. We used *H. pylori* ATCC 43504, while Niehus used strains G27 and a motile revertant of strain 26695 (*H. pylori* 26695 is non-motile due to a frameshift in *fliP*, which encodes a component of the flagellar protein export apparatus).

Alternatively, the differences may be attributed to additional amino acids that were inadvertently introduced to the ends of the truncated FlhA proteins in our study. A fragment containing the start of *flhA* and the end of the end of the *cat* cassette was amplified from the *flhA77* mutant and sequenced. The insertion of the *cat* cassette into *flhA* at codon 77 resulted in the addition of 96 amino acid residues to the carboxy-terminus of the truncated FlhA protein. A high percentage of these extra amino acid residues were charged (10 Asp or Glu residues and 11 Arg or Lys residues). If the truncated FlhA protein is localized to the export apparatus, these charged regions could interact with other components of the export apparatus and influence their conformation. Based on the predicted topological arrangement of FlhA we expect that this extra region would be at the end of the second transmembrane spanning helix and localized on the cytoplasmic side of the membrane. A study by Wand *et al.* (2006) lends some evidence to this hypothesis that the juxtaposition of a highly charged peptide may affect the conformation of the export apparatus. They reported that an FlhB variant with an amino acid substitution at the site of autocleavage (Pro266 changed to Gly) was able to support wild-type motility in *H. pylori* (Wand *et al.*, 2006), while we observed that the same substitution inhibited motility and export of filament-type substrates (Smith *et al.*, 2009). To detect FlhB, Wand *et al.* (2006) introduced the FLAG-tag at the carboxy-terminus of the FlhB variant, which could have compensated for the substitution at the autocleavage site and restored at least partial function to the protein. Like the additional amino acids on the truncated FlhA, the FLAG tag is highly charged (5 of 8 amino acids in the FLAG-tag are Asp and 2 are Lys). The carboxy-terminal sequence of the FlhA protein expressed from the *flhA454* allele has not yet been determined, but we are currently working to sequence this region to determine if a highly charged sequence was similarly introduced at the end of this protein.

Another possible explanation for the differences between our study and that of Niehus *et al.* (2004) is the way in which gene expression was monitored. Niehus *et al.* (2004) used DNA microarrays to examine transcript levels, while we used reporter genes in which the promoter regions of select flagellar genes were fused to a promoter-less *xylE* gene. The reporter gene assays could be insensitive to potential post-transcriptional regulatory events (e.g., transcript stability) mediated by the export apparatus. The western blot analysis (Fig 4.2) argues against this explanation showing that the *flhA77* and *flhA454* mutants express the FlgE-FLAG protein and FlaB while the Δ*flhA* mutant does not. Thus, it seems unlikely that the discrepancy in the two studies can be attributed to the different methods used to monitor gene expression.

Finally, the differences between our results and those of Niehus *et al*. (2004) could be due to extragenic mutations that occur at a high frequency when insertions are made in *flhA*. Phase variation of flagellar gene expression has been shown to occur at a reasonably high frequency in *C. jejuni* and involves the addition or deletion of nucleotides within homopolymeric adenine or thymine tracts within the transcriptional regulatory gene *flgR* (Hendrixson, 2006). As mentioned previously, *H. pylori* strain 26695 is non-motile due to a frameshift within *fliP* and motile variants can be obtained from this strain. Thus, it is possible that we picked up an extragenic mutation within a gene encoding a negative regulator of the RpoN regulon during the construction of the *flhA* insertion mutants which was responsible for the increased expression of RpoN-dependent reporter genes in these strains. To test this hypothesis we attempted to express a wild-type copy of *flhA* from its native promoter within the HP0405 locus on the chromosome or from an inducible promoter on the pHEL-3 plasmid to see if the complemented strains showed the enhanced expression of RpoN-dependent genes. We were unable to complement the loss of motility in any of the *flhA* mutants, suggesting that *flhA* was not expressed in the complemented

strains. One reason for our failure to complement the *flhA* mutants may be that we did not reintroduce a functional *flhA*. The source of *flhA* for these experiments was *H. pylori* 22695 genomic DNA, which as indicated previously is non-motile. Thus, it is possible that the *H. pylori* 22695 strain that we used has acquired an *flhA* mutation. We plan to address this issue by cloning *flhA* from a motile *H. pylori* strain whose genome has been sequenced, such as J99 or G27, for the complementation assays.

Rust *et al*. (2009) recently suggested a role for FlhA in the FlgM-mediated down regulation of the *H. pylori* FliA regulon. These researchers reported that the *H. pylori* anti-σ^{28} factor, FlgM may not be exported by the export apparatus as it is in *S. typhimurium*. *H. pylori* FlgM appears to localize predominately to the cytoplasm where it may interact with $FlhA_C$. Rust *et al*. propose a model in which FlhA sequesters FlgM or modulates its activity. Interestingly, this same group showed that disrupting *flgM* partially restored expression of the RpoN regulon in an *flhA* mutant (Niehus *et al.*, 2004). We do not believe that we inadvertently picked up *flgM* mutations during the construction of the *flhA77* and *flhA454* strains since expression of the *flaA-xylE* reporter gene was down regulated in both of these strains, suggesting these strains have a functional FlgM. In fact, the *flaA-xylE* reporter gene was down regulated to a greater extent in the *flhA77* and *flhA454* mutatns than in the Δ*flhA* mutant. We infer from this observation that the up regulation of the RpoN regulon in the *flhA77* and *flhA454* mutants may be responsible for the enhanced down regulation of the *flaA-xylE* reporter gene. We postulate that one or more gene products of the RpoN regulon inhibit expression of the FliA regulon. In support of this hypothesis the *flaA-xylE* reporter gene is over expressed in an *rpoN* mutant compared to wild type (data not shown) . Alternatively, the truncated FlhA proteins expressed in the *flhA77* and *flhA454* mutants may enhance the inhibitory effect of FlgM on the FliA regulon.

To understand the molecular mechanism by which the export apparatus affects expression of the RpoN regulon it is imperative to determine if the mutant strains possessing the *flhA77* and *flhA454* alleles produce truncated FlhA proteins that are competent for assembly of the export apparatus. McMurry *et al*. (2004) systematically analyzed the cytoplasmic portions of FlhA and found that the soluble N-terminal sequence which preceded the first transmembrane helix was required for FlhA function. Thus, it is possible that this small portion of FlhA is sufficient to stimulate expression of the RpoN regulon. If the truncated FlhA proteins are not expressed stably and associated with the export apparatus it is difficult to envision how the *flhA77* and *flhA454* alleles could affect expression of the RpoN regulon differently than the Δ*flhA* allele. We have antiserum directed against a peptide corresponding to the first 20 amino acid residues of the amino-terminus of FlhA, but unfortunately we have not been able to reproducibly detect FlhA in wild-type *H. pylori* using this antiserum. This is likely due to the extremely low amounts of FlhA in the cell (only 2-12 copies of FlhA per flagellated cell).

Understanding the basis for the disparity in results with the two *flhA77* strains that we constructed is another issue that must be resolved. The difference between the two strains cannot be attributed to the manner in which the suicide vector recombined with the target gene. PCR analysis of genomic DNA from the two *flhA77* strains indicated that in both cases the chromosomal copy of *flhA* had been replaced with the copy from the suicide plasmid that carried the *cat* cassette insertion (i.e., there had been a double crossover event rather than integration of the suicide vector into the chromosome). We have sequenced most of the coding regions of the two *flhA77* alleles but have not found any differences in the sequences. We plan to sequence the remaining coding regions as well as the upstream regions to determine if there are any nucleotide sequence differences that might account for the different phenotypes of these strains.

In conclusion, we show here that a functional export apparatus is not required for expression of the *H. pylori* RpoN flagellar regulon. Unfortunately, because of the caveats mentioned above this observation does not allow us to distinguish between potential models for how the export apparatus controls expression of the RpoN regulon. Nevertheless, as we analyze the *flhA* mutants more thoroughly we anticipate being able to differentiate between these competing models. We also showed here that the effects of the *flhA* mutations on the RpoN regulon are not due to polar effects on *hp1042*. Interestingly, HP1042 appears to have a significant post-translational effects on the hook protein and flagellins in *H. pylori*. We speculate that HP1042 prevents the flagellins, and perhaps also the hook protein, from becoming hyperglycosylated, and plan further experiments to test this hypothesis.

Materials and Methods

Bacterial strains and culture conditions

H. pylori ATCC 43504 was cultured at 37 °C in a microaerobic atmosphere as described previously (Smith *et al.*, 2009) on tryptic soy agar supplemented with 5% horse serum supplemented with 30 μg/ml chloramphenicol, 30 μg/ml kanamycin, or 10 μg/ml erythromycin as required. *Escherichia coli* DH5α was cultured at 37 °C with shaking in Luria Bertani broth supplemented with 100 μg/ml ampicillin or 30 μg/ml chloramphenicol or brain-heart infusion broth (BHI) with 150 μg/ml erythromycin. *H. pylori* motility was assessed using semisolid Mueller-Hinton medium supplemented with 10% horse serum as described previously (Smith *et al.*, 2009).

Construction of *H. pylori* mutants

H. pylori flhA (HP1041) fragments of ~700 bp, ~975 bp, and 1 kb (for the Δ*flhA:cat*, *flhA77*and *flhA454* alleles respectively) were amplified from *H. pylori* strain 26695 genomic DNA using *Taq* DNA polymerase (Promega) and the primers indicated in Table 4.4. HP1042 including 500 bp of flanking DNA sequence was amplified from *H. pylori* strain 26695 genomic DNA using Pfu Turbo Hotstart DNA polymerase (Stratagene) and the primers indicated in Table 4.4. The *hp1042* amplicon was incubated with Taq DNA polymerase (Promega) at 72 °C for 10 min, and all the amplicons were cloned into pGEM-T (Promega) creating pLP120 (pGEM- *flhA*), pLP78 (pGEM- *flhA77*), pLP113 (pGEM- *flhA454*) and pTS74 (pGEM-*hp1042*). To constuct the Δ*flhA:cat* allele the pLP120 plasmid was digested with HindIII and Eco47III, and the overhanging ends were filled in using T4 DNA polymerase (Promega). This was ligated with a 1.3 kb SmaI fragment containing a chloramphenicol transacetylase (*cat*) gene from pSKAT4 (Wang & Taylor, 1990). To construct the *flhA77*allele the plasmid pLP78 was digested with Eco47III and ligated with a 1.3 kb SmaI fragment containing *cat*. To construct the *flhA454* allele the plasmid pLP113 was used as a template for site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene). Primers that annealed 1362 bp downstream of the translational start of *flhA* (corresponding to codon 454) were used to introduce a unique EcoRI site (Table 4.4) into which a 1.3 kb EcoRI fragment containg *cat* was cloned. To construct the Δ*hp1042:cat* allele the plasmid pTS74 was used as a template for inverse PCR using primers that introduced an EcoRV site and annealed to sequences immediately upstream of the translational start or downstream of the translational stop. Amplicons were circularized using T4 DNA ligase, digested with EcoRV, and a 1.3 kb EcoRI fragment containing *cat* with the ends filled-in was cloned into the vector. Alternatively, a 1.1 kb EcoRV fragment containing a *Streptococcus*

pneumoniae erythromycin resistance gene (*ermB*) was cloned into the vector (Stabb & Ruby, 2002). Plasmids were introduced into *H. pylori* by natural transformation, and transformants selected as described previously (Brahmachary *et al.*, 2004). Introduction of *cat* or *ermB* in the target gene on the chromosome was confirmed by PCR.

To construct the *hp1563'*-'*hp1042* fusion, a ~1.5 kb fragment containing *hp1042* and a ~250 bp fragment containing the promoter regulatory region upstream of HP1563 were amplified from *H. pylori* strain 26695 genomic DNA using Pfu Turbo Hotstart DNA polymerase (Stratagene) and the primers indicated in Table 4.4. The forward primer annealing at the start of *hp1042* and the reverse primer annealing upstream of *hp1563* introduced 14 bp of overlapping sequence and a unique EcoRI site. The resulting amplicons were mixed together in a 1:1 ratio and cycled with Pfu Turbo Hotstart DNA polymerase without primers. The resulting product was used as template in a second PCR using Pfu Turbo Hotstart DNA polymerase, the forward primer annealing upstream of *hp1563* and the reverse primer annealing downstream of *hp1042*. The resulting amplicon was incubated with Taq DNA polymerase and cloned into pGEM-T. The resulting plasmid (pTS106) and the HP0405 vector were digested with SpeI and SacII and ligated together using T4 DNA ligase. Construction of the HP0405 vector to introduce genes into the HP0405 locus was described previously (Pereira & Hoover, 2005). For this work the *cat* was replaced with *ermB* in this vector. The construct was introduced into *H. pylori flhA*77 by natural transformation and transformants selected using 10 μg/ml erythromycin.

Modified XylE assay

Construction of XylE reporter gene fusions were described previously (Brahmachary *et al.*, 2004; Pereira & Hoover, 2005). *H. pylori* stains containing the XylE reporter gene fusions were cultured for 36 hrs at 37 °C in a microaerobic atmosphere on tryptic soy agar supplemented

with 5% horse serum supplemented with 30 μg/ml kanamycin. Cells were resuspended in 50 mM K_2HPO_4 (pH 7.0). Absorbance of the cell suspensions was measured at 600 nm using a Shimadzu UV-160U spectrophotometer. Cell suspensions and 0.9 ml aliquots of 3 mM catechol (prepared with the resuspension buffer) were incubated for 5 min at 37 °C, 0.1 ml of the cell suspension was added to the catechol and reactions were incubated for 2 min at 37 °C. To stop the reactions 0.5 ml of 1 M sodium carbonate was added, and the cells were removed by centrifugation at ~16,000 g for 2 min. The absorbance of each reaction was measured at 375 nm using a Shimadzu UV-160U spectrophotometer. The final absorbance of the reaction was multiplied by 22.7 μmol of product formed/min and divided by the absorbance at 600 nm to give units of μmol/min/10^8 *H. pylori* cells. XylE activity for each strain was determined from at least 6 statistical replicates from two or more biological replicates. Student's t-test was used to determine the standard deviation with 95% confidence intervals (P = 0.05).

Western Blots

Construction of pTS14 which contains a FlgE-FLAG-tag fussion was described previously (Smith *et al.,* 2009). *H. pylori* strains containing pTS14 were cultured in BHI (pH 6.5) supplemented with 0.4% β-cyclodextran and 30 μg/ml kanamycin in a sealed microaerobic bottle at 37 °C with slow shaking for 24 hrs. Cells were separated from the medium and protein was concentrated using trichloroacetic acid as described previously (Smith *et al.,* 2009). Protein concentrations were measured using the bicinchoninic acid protein assay (Pierce) following the manufacturer's instructions, and equivalent amounts of total protein were analyzed by western blotting. A monoclonal peroxidase conjugated antibody directed against the FLAG epitope (Sigma) and enhanced chemiluminescence were used to detect FlgE-FLAG. Antiserum directed against *H. pylori* FlaB (Pereira & Hoover, 2005), a peroxidase-conjugated goat anti-rabbit

antibody (MP Biomedicals) and enhanced chemiluminescence were used to detect FlaB and FlaA.

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Table 4.1 *flgI'-'xylE* activity from selected *H. pylori* strains

Strain	XylE activity[a]	Fold change[b]
Wild type	0.92 ± 0.16[c]	-
Δ*flhA*	1.7 ± 0.21	1.8
flhA77	0.95 ± 0.22	1.00
flhA454	1.2 ± 0.87	1.3
Δhp1042:cat	1.2 ± 0.16	1.3
flhA77 Δhp1042:ermB	0.97 ± 0.041	1.00
flhA77 hp0405:hp1563'-'hp1042	0.57 ± 0.23	0.62

[a] Average XylE actvity given in units of μmol/min/10^8 *H. pylori* cells for six statistical replicates from two biological replicates.
[b] Fold change of average XylE activity from the mutant strain compared to the wild type. Light gray shading indicates a ≤ 1.5 average fold change and dark gray ≤ 2.0 average fold change.
[c] Standard deviations indicates 95% confidence intervals (P=0.05) calculated using Student's *t* test.

Table 4.2 XylE activity of RpoN-dependent reporter genes from selected *H. pylori* strains

Strain	*flaB'-'xylE* XylE activity[a]	*flaB'-'xylE* fold change[b]	*hp1120'-'xylE* XylE activity	*hp1120'-'xylE* fold change
Wild type	0.65 ± 0.26[c]	-	0.44 ± 0.100	-
Δ*flhA*	0.30 ± 0.068	0.46	0.25 ± 0.012	0.57
flhA77	5.4 ± 3.00	8.3	1.2 ± 0.15	2.7
flhA454	1.5 ± 0.025	2.3	0.43 ± 0.15	0.98
Δhp1042:cat	0.72 ± 0.300	1.1	0.44 ± 0.036	1.00
flhA77 Δhp1042:ermB	2.5 ± 0.71	3.8	0.62 ± 0.042	1.4
flhA77 hp0405: hp1563'-'hp1042	1.6 ± 0.67	2.5	1.00 ± 0.067	2.3

[a] Average XylE actvity given in units of µmol/min/10^8 *H. pylori* cells for six statistical replicates from two biological replicates.
[b] Fold change of average XylE activity from the mutant strain compared to the wild type. Light gray shading indicates a ≤ 1.5 average fold change and dark gray ≤ 2.0 average fold change.
[c] Standard deviations indicates 95% confidence intervals (P=0.05) calculated using Student's *t* test.

Table 4.3 *flaA'-'xylE* activity from selected *H. pylori* strains

Strain	XylE activity[a]	Fold change[b]
Wild type	2.2 ± 1.5[c]	-
Δ*flhA*	1.3 ± 0.700	0.59
flhA77	0.600 ± 0.16	0.27
flhA454	0.52 ± 0.17	0.24
Δhp1042:cat	2.4 ± 0.12	1.1
flhA77 Δhp1042:ermB	0.45 ± 0.0800	0.200
flhA77 hp0405:hp1563'-'hp1042	0.56 ± 0.28	0.25

[a] Average XylE actvity given in units of μmol/min/10^8 *H. pylori* cells for six statistical replicates from two biological replicates.
[b] Fold change of average XylE activity from the mutant strain compared to the wild type. Light gray shading indicates a ≤ 1.5 average fold change and dark gray ≤ 2.0 average fold change.
[c] Standard deviations indicates 95% confidence intervals (P=0.05) calculated using Student's *t* test.

Table 4.4 Oligonucleotide primers used for this study	
ΔflhA forward	5’ CAC TTG CAC CTC ACA AGA AC 3’
ΔflhA reverse	5’ CTT CAG TAA CCC TAG TAG AGC 3’
flhA77 forward	5’ ATG GCA AAC GAA CGC TCC AAA TTA G 3’
flhA77 reverse	5’ GCC ATG CGA TGA ATA AAA AGA GAC 3’
flhA454 forward	5’ GAC TCT CTT TTT ATT CAT CGC ATG 3’
flhA454 reverse	5’ CAA GAC TTT CAT GGC CTC TTC 3’
flhA454 EcoRI forward	5’ CCC CCA ACG CAT TAT GAA TTC AAA CTT AAA GGC ATT GTG 3’
flhA454 EcoRI reverse	5’ CAC AAT GCC TTT AAG TTT GAA TTC ATA ATG CGT TGG GGG 3’
hp1042 forward	5’ GTT ACA TGA AAA AAT CCC CAT TAA 3’
hp1042 reverse	5’ GTG TTT GGT TGG GTG GTA TGA 3’
hp1042 inverse forward	5’ GGA TAT CAA CTA AGC TGT TTA GAA AAA ACT AA 3’
hp1042 inverse reverse	5’ GGA TAT CTC TCC TTT TTT ATC AAT TAT TTA TCC 3’
hp1042 overlap forward	5’ GGG GGA ATT CCC CCG GAT AAA TAA TTG ATA AAA AAG 3’
hp1563 forward	5’ TAA CGA TCC TTT TTG TAT GAT 3’
hp1563 overlap reverse	5’ GGG GGA ATT CCC CCT ATC GTA ACT CCT TAA GTG T 3’

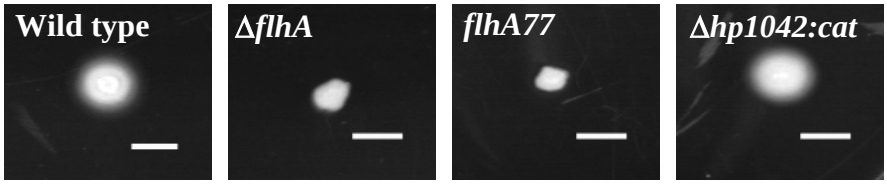

Figure 4.1 Motility of selected *H. pylori flhA* and *hp1042* strains. *H. pylori* strains with the indicated genotypes were stab inoculated in semisolid medium and incubated for up to 7 days in a microaerobic atmosphere at 37 °C. Scale bar is 5 mm.

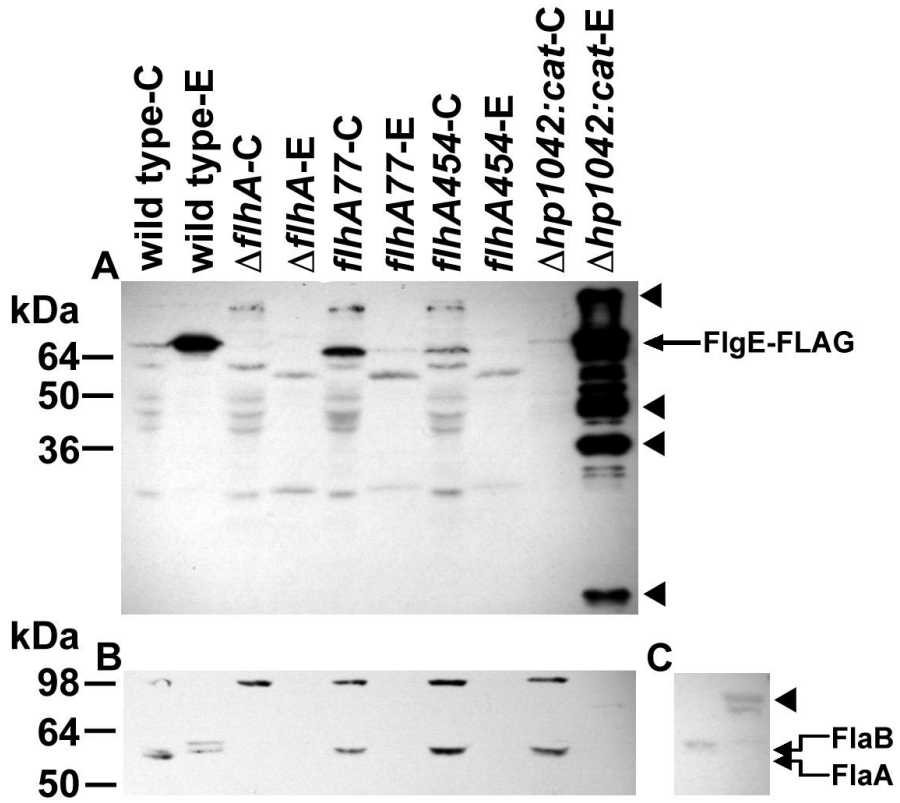

Figure 4.2 Western blot analyses of hook and flagellin proteins exported from selected *H. pylori* strains. (A) Western blot analysis of hook FlgE-FLAG fusion protein in soluble cytoplasmic (C) and extracellular (E) protein fractions. The strains indicated along the top containing the FlgE-FLAG expression vector pTS14 were analyzed using 15 µg total protein for each cytoplasmic sample and 20 µg for each extracellular sample. (B) Western blot analysis of flagellins FlaB and FlaA in soluble cytoplasmic and extracellular protein fractions. The same protein samples and similar amounts of protein were anlayzed as in part A. (C) Western blot analysis of flagellins showing only the Δ*hp1042:cat* C and E samples. The same protein samples and similar amounts of protein were anlayzed as in part B. Arrowheads indicate unique proteins in the Δ*hp1042:cat*-E sample.

CHAPTER 5

DIRECT ANALYSIS OF THE EXTRACELLULAR PROTEOME FROM TWO STRAINS OF *HELICOBACTER PYLORI*[1]

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[1] Smith, T. G., J. Lim, M. V. Weinberg, L. Wells, and T. R. Hoover. 2007. *Proteomics*. 7: 2240–2245

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Abstract

Helicobacter pylori extracellular proteins are of interest because of possible roles in pathogenesis, host recognition and vaccine development. We utilized a unique approach by growing two strains (including one non-sequenced strain) in a defined serum-free medium and directly analyzing the proteins present in the culture supernatants by LC-MS/MS. Over 125 proteins were identified in the extracellular proteomes of two *H. pylori* strains. Forty-five of these proteins were enriched in the extracellular fraction when compared to soluble cell-associated protein samples. Our analysis confirmed and expanded on the previously reported *H. pylori* extracellular proteome. Extracellular proteins of interest identified here included *cag* pathogenicity island protein Cag24 (CagD); proteases HP0657 and HP1012; a polysaccharide deacetylase, HP0310, possibly involved in hydrolysis of acetyl groups from host *N*-acetylglucosamine residues or from residues on the cell surface; and HP0953, an uncharacterized protein that appears to be restricted to *Helicobacter* species that colonize the gastric mucosa. In addition, our analysis found eight previously unidentified outer membrane proteins and two lipoproteins that could be important cell surface proteins.

Dataset Brief

Colonization of the human gastric mucosa by *Helicobacter pylori* can result in acute gastritis that may proceed to peptic ulcer disease, gastric carcinoma, or B-cell mucosa-associated lymphoid tissue lymphoma (Graham *et al.*, 1992; Marshall *et al.*, 1985; Megraud *et al.*, 1989). *H. pylori* infects a large percentage of the worldwide population, but most infected individuals remain asymptomatic (Dooley *et al.*, 1989). The purpose of this study was to identify extracellular proteins from *H. pylori* since extracellular proteins, such as vacuolating cytotoxin

(VacA), have known roles in pathogenesis (Figueiredo *et al.*, 2005). Previous studies of *H. pylori* extracellular proteins relied on the resolution of protein spots using 2-dimensional gel electrophoresis (Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Kim *et al.*, 2002). In the current study, we utilized a defined serum-free growth medium and a direct LC-MS/MS analysis of the culture supernatant to identify extracellular proteins from a sequenced and a non-sequenced strain of *H. pylori*, 26695 and ATCC 43504, respectively (Tomb *et al.*, 1997). The use of a non-sequenced strain demonstrated that direct LC-MS/MS analysis could be applied to other *H. pylori* strains, including clinical isolates, in the future.

The two strains were grown on plates supplemented with 5% horse serum (Invitrogen, Grand Island, NY) in a microaerobic environment for 36- 48h. Cells were harvested from the plate, resuspended in Hank's balanced salts solution, and used to inoculate 25 ml of a defined serum-free medium prepared as described by Bruggraber *et al.* (2004) in 160 ml sealed glass serum bottles. The headspace was flushed with 5%CO2/10%H2 and O2 was added to a final concentration of 5%. Cultures were inoculated to OD600 of ~0.05 and grown 24 h at 37°C with shaking to OD600 0.2 to 0.4 AU. Cells were separated from the medium by centrifugation for 10 min at 5,000 x g. The resulting supernatant was filtered through a 0.22 sterile filter to remove residual cells, and extracellular proteins were concentrated by precipitation with 25% trichloroacetic acid (TCA) for 15 min followed by centrifugation for 10 min at 10,000 x g. After washing the protein pellet with acetone twice, the sample was resuspended in 25 mM tris-buffered saline (pH 7.4). The soluble cell-associated (cytoplasmic and periplasmic) proteins were isolated from the cell pellet by resuspending in 2 ml of phosphate buffered saline and lysing the cells by two passages through a French press at 1,000 bar followed by centrifugation for 20 min at 5,000 x g and then 45 min at 100, 000 x g (Backert *et al.*, 2005). Following the high

speed centrifugation step the supernatant was removed, and the protein was concentrated by TCA precipitation as described for the extracellular proteins.

Protein samples were denatured with 1 M urea, reduced with 10 mM dithiothreitol for 1 h at 52 °C, carboxyamidomethylated with 55 mM iodoacetamide in the dark for 45 min, and then digested by 3 µg trypsin (Promega, Madison, WI) in 40 mM NH4HCO3 overnight at 37 °C. After digestion, the peptides were acidified with 200 µl of 1 % trifluoroacetic acid (TFA), desalted using reverse phase (C18 spin column, The Nest Group, Southborough, MA), then lyophilized in a Speed Vac. Peptides were resuspended with 39 µL of mobile phase A (0.1 % formic acid, FA, in water) and 1 µL of mobile phase B (80 % acetonitrile, ACN, and 0.1 % formic acid in water) and filtered with 0.2 µm filters (Nanosep, PALL, East Hills, NY). The samples were loaded off-line onto a nanospray tapered capillary column (75 uM by 8.5 cm, PicoFrit, New Objective, Woburn, MA) self-packed with C18 reverse-phase (RP) resin (5 uM, Waters, Milford MA) in a Nitrogen pressure bomb and then separated via 160-min linear gradient of increasing mobile phase B at a flow rate of approximately 200 nL/min after split. LC-MS/MS analysis was performed on a Finnigan LTQ mass spectrometer (Thermo Electron Co., San Jose, CA) equipped with a nanoelectrospray ion source. A full MS spectrum (350-2000 m/z) was collected followed by 8 CID MS/MS spectra (normalized collision energy of 34% with a 2.2 m/z isolation width and dynamic exclusion set at 2).

The resulting MS/MS spectra were searched against a non-redundant *H. pylori* (released on July 31, 2006) database obtained from the National Center for Biotechnology Information (NCBI) using the TurboSequest algorithm (BioWorks 3.2, Thermo Finnigan), as well as a reverse *H. pylori* database to estimate the false positive rate (FPR) and the false discovery rate (FDR) of peptide identification. The SEQUEST parameters were set to allow 2.0 Da of precursor

ion mass tolerance with average mass and 0.3 Da of fragment ion tolerance with monoisotopic mass. Three missed internal cleavage sites by trypsin were allowed. Mass increases of 15.99 and 57.02 Da were allowed for oxidized methionine and alkylated cysteines, respectively. The results of the SEQUEST search were filtered to establish the FPR and the FDR using the Sequest scores (ScoreFinal, Sf) and probability score (P). The SEQUEST criteria were determined at Sf ε0.45 or P ε30 with FDR <0.4% at the protein level with the *H. pylori* database for assignment of a protein by 2 or more unique peptides and at Sf ε0.75 or P ε80 with FDR <0.5% at the protein level for proteins assigned by only 1 peptide. All reported proteins were identified by 2 or more unique peptides. See Table 5.2 (supporting information) for additional information supporting the identity of each protein reported in Table 5.1 including sequence coverage, number of peptides, Turbo-Sequest scores, charge state, Sf, P and peptide sequences. See Table 5.3 (supporting information) for additional information on the criteria used for identification of all proteins including the Sf, P and FDR. All spectra used to assign proteins are available upon request.

A problem associated with analysis of the *H. pylori* extracellular proteome is distinguishing between proteins that are extracellular due to secretion and those present due to cell lysis (Bumann *et al.*, 2002; Schraw *et al.*, 1999; Vanet & Labigne, 1998). To address this issue, proteins enriched in the culture supernatant were identified by comparing the average number of unique peptides assigned from three extracellular samples with the average number of unique peptides assigned from the three corresponding cell-associated samples following stringent filtering and assignment. In addition, only proteins that were identified in at least two of the three extracellular samples were included in the analysis. A protein was considered

enriched in the extracellular fraction if the ratio of the average number of unique peptides assigned from the extracellular samples to that of the cell-associated samples was >1.5.

A total of 172 different proteins from the two strains were identified in the cell-associated samples and 130 proteins from the extracellular samples were identified in at least two biological replicates (Tables 5.4 and 5.5, Supporting information). After applying the criterion described above, 45 of the extracellular proteins were considered enriched in the extracellular fraction (Table 5.1). Eleven of the 45 extracellular proteins were identified previously as part of the *H. pylori* extracellular proteome (Bumann *et al.*, 2002; Kim *et al.*, 2002).

Many of the proteins reported previously to be in the *H. pylori* extracellular proteome, but not appearing in Table 5.1, were identified in the extracellular samples but did not meet the criterion for enrichment in the extracellular fraction (Tables 5.4 and 5.5). Most notably urease (UreB, HP0072) is one of the most abundant extracellular proteins in cultures of *H. pylori* (Bumann *et al.*, 2002) and was one of the most abundant proteins that we identified in the extracellular fraction (on average UreB represented 8% of the total signal). UreB was also one of the most abundant proteins in the cell-associated fraction such that the ratio of the average number of unique peptides assigned from the extracellular samples to that of the cell-associated samples was below 1.5 (Table 5.5). Other extracellular proteins reported previously, that were identified in extracellular samples but failed to meet our criterion for enrichment, included flavodoxin (HP1161), HP0231 and thioredoxin (HP0824) which were reported by Bumann *et al.* (2002); HP0129, HP0305, HP0720, HP0835, HP0902, HP0913, HP0973 and HP1564, which were reported by Kim *et al.* (2002).

None of the previously identified flagellar proteins were identified using this approach, including flagellar hook protein FlgE (HP0870), hook cap protein FlgD (HP0907), hook-length

control protein FliK (HP0906), and hook-basal body complex protein FliE (HP1557) (Bumann *et al.*, 2002). This was not an unexpected result for strain 26695 since this strain has a frameshift mutation in *fliP*, which encodes a component of the flagellar protein export apparatus (Josenhans *et al.*, 2000). For *H. pylori* 43504 the major flagellin FlaA (HP0601) and filament cap protein FliD (HP0752) were detected in extracellular samples but were not enriched in the extracellular fraction (Tables 5.4 and 5.5). Other proteins previously reported to be in the extracellular proteome but not reproducibly detected in extracellular samples included HP0367, thiol-disulfide interchange protein (HP0377) (Bumann *et al.*, 2002), HP0721, ssDNA-binding protein (HP0827), ribosomal protein L11 (HP1202) and ribosomal protein L1 (HP1201) (Kim *et al.*, 2002).

Over half of the proteins in Table 5.1 (28 of 45) have putative signal peptides for type II secretion in Gram-negative prokaryotes as predicted by artificial neural networks and hidden Markov model using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). One extracellular protein, γ-glutamyltranspeptidase (Ggt; HP1118), has a signal sequence at its N-terminus (MRRSFLK) that matches the twin arginine motif [(S/T)RRXFLK] found in proteins exported via the Tat pathway (Berks, 1996). *H. pylori* appears to have a functional Tat protein secretion system since *H. pylori tatA* complements an *Escherichia coli tatA* mutant (Sargent *et al.*, 1999). Some of the proteins appearing in Table 5.1 are clearly cytoplasmic proteins including, glutamate dehydrogenase (HP0380), citrate synthase (HP0026) and enolase (HP0154). Applying a more stringent criterion for enrichment in the extracellular fraction by increasing the ratio to >2, eliminates most of the predicted cytoplasmic proteins but also eliminates several predicted secreted proteins.

Several proteins in Table 5.1 are either outer membrane or periplasmic proteins. Dipeptide-binding protein (HP0298), copper ion binding protein (HP1073), carbonic anhydrase (HP1186), propyl cis-trans isomerase (HP0175), catalase (HP0875) and HP1286 have all been shown to localize to the periplasm or are believed to be periplasmic proteins (Harris & Hazell, 2003; Marcus *et al.*, 2005). These proteins may be released from *H. pylori* upon cell lysis or outer membrane blebbing and be more stable in the growth medium than in the periplasm, which could account for their apparent enrichment in the extracellular fraction. Consistent with this hypothesis, Bumann and co-workers reported that *H. pylori* cytoplasmic proteins were stable when added to growing *H. pylori* cultures (Bumann *et al.*, 2002). The nine outer membrane proteins and two lipoproteins (HP1456 and HP1457) identified here are significant in that they are probably abundant proteins and localize to the cell surface.

Several proteins in Table 5.1 have not been reported previously in the extracellular proteome, including a *cag* pathogenicity island (*cag*PAI) protein Cag24 (HP0545), two predicted Zn-dependent proteases (HP0657 and HP1012), a predicted polysaccharide deacetylase (HP0310) and a protein of unknown function (HP0953). The *cag24* locus, also designated as *cagD*, is found in a majority of clinical isolates (Sadakane *et al.*, 1999), but little is known about its role. Disruption of *cagD* was reported to interfere with induction of interleukin (IL)-8 in host cells, but the authors speculated that this result was due to polar effects on downstream genes (Tummuru *et al.*, 1995). The mode by which CagD is enriched in the extracellular fraction under the growth conditions used in our assay is unclear. CagD enrichment does not appear to be dependent on *cag*PAI mediated type IV secretion since the type IV effector CagA was present in the cell-associated samples but was not enriched in the extracellular fraction (Table 5.5). Thus, although the *cag*PAI type IV secretion system is functional in the strains examined here it does

not appear to be induced under the assayed growth conditions. This result was not unexpected since induction of the type IV secretion apparatus requires bacterium to host cell contact (Matthysse, 1987). CagD has a predicted signal sequence for type II secretion (Table 5.1), which may account for its enrichment in the extracellular proteome.

Protease HtrA (HP1019) was reported previously as an extracellular protein (Bumann *et al.*, 2002). We also observed that HtrA was enriched in the extracellular fraction, as well as several other proteases, indicating that extracellular proteases may play an important role in the growth and survival of *H. pylori*.

HP0310 is predicted to belong to a family of polysaccharide deacetylases (COG0726), members of which catalyze the hydrolysis of either *N*-linked acetyl groups from *N*-acetylglucosamine residues or *O*-linked acetyl groups from *O*-acetylxylose residues (Psylinakis *et al.*, 2005). HP0310 may deacetylate *N*-acetylglucosamine residues in mucin or other host glycoproteins. Alternatively, HP0310 may function as a peptidoglycan *N*-acetylglucosamine deacetylase to modulate properties of the murein layer of *H. pylori*. If HP0310 is involved in peptidoglycan modification it could play an important role in pathogenesis as recent studies showed that components of *H. pylori* peptidoglycan induce proinflammatory responses in epithelial cells that result in nuclear factor (NF)-κB activation and IL-8 induction (Viala *et al.*, 2004). This proinflammatory response is dependent on the host protein Nod1, which is an intracellular pathogen-recognition receptor with specificity for Gram-negative peptidoglycan, and the *cag*PAI type IV secretion apparatus, which is required for the delivery of peptidoglycan into host cells (Viala *et al.*, 2004).

Another uncharacterized protein, HP0953, lacks conserved domains but is present in all three *H. pylori* strains whose genomes have been sequenced as well as *Helicobacter acinonychis*,

which colonizes the gastric mucosa of large felines. Other closely related ε-proteobacteria, including *Helicobacter hepaticus* and *Campylobacter jejuni*, lack HP0953 homologs suggesting that HP0953 is unique to gastric *Helicobacter* species. While growth conditions in this experiment do not attempt to mimic the environment of the gastric mucosa, other experimental methods could be used to investigate whether HP0953 is involved with survival of these gastric *Helicobacter* species. In Table 5.1, HP0953 is indicated only for strain 26695 because the ratio of enrichment in the extracellular fraction was slightly less than 1.5 (~1.4) for strain 43504 (Table 5.5).

In summary, the results of this study confirm the previous identification of several proteins in the *H. pylori* extracellular proteome and provide evidence for the existence of additional extracellular proteins that may have important roles in the pathogenesis of *H. pylori*. Further characterization of extracellular proteins identified here will elucidate their roles in *H. pylori* physiology and may lead to new strategies for the control and/or surveillance of *H. pylori* infection.

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Table 5.1. *H. pylori* proteins enriched in the extracellular fraction

Gene locus[a]	Name	Description	Ratio[b]	Signal peptide[c]	Strain	Matching peptides[d]	Reference
HP0127	*omp4, horB*	*Helicobacter* outer membrane protein family	ND[e]	Yes	43504	AAD07197, ABF84193	
HP0275	*addB*	jhp0260 homolog, ATP-dependent nuclease	ND, ND[e]	Yes	Both	AAD07339, ABF84344	
HP0294	*aimE*	aliphatic amidase	ND[e]	No	43504	NP_223877, NP_208030	(Jungblut *et al.*, 2000)
HP0298	*dppA*	periplasmic dipeptide-binding protein	ND[e]	Yes	43504	AAD07367, NP_223004, ABF84367	
HP0330	*ilvC*	ketol-acid reductoisomerase	ND[e]	No	43504	NP_223032, ABF84401	
HP0472	*omp11, horE*	*Helicobacter* outer membrane protein family	ND, ND[e]	Yes	Both	AAD07540, ABF84516	
HP0657	*ymxG*	processing protease	ND, ND[e]	Yes	Both	NP_223320, NP_207451, AAD06183	
HP0672	*aspB*	member of the PLP dependent aminotransferase superfamily clan	ND, ND[e]	No	Both	NP_223333, AAD07733	
HP0706	*omp15, hopE*	*Helicobacter* outer membrane protein family	ND[e]	Yes	43504	AAD07756, ABF84758	
HP0710		*Helicobacter* outer membrane protein family	ND[e]	Yes	43504	NP_223588, AAD06437	
HP0912	*omp20*	*Helicobacter* outer membrane protein family	ND[e]	Yes	43504	AAD07960, NP_207704, NP_223566	(Kim *et al.*, 2002)
HP1073	*copP*	copper ion binding protein	ND[e]	No	43504	AAD08120	
HP1173		HPAG1_1112 homolog	ND[e]	Yes	26695	AAD08225, ABF85179	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000)
HP1186		carbonic anhydrase	ND, ND[e]	Yes	Both	NP_223829, ABF85193, BAE66646	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP1395	*omp30*	*Helicobacter* outer membrane protein	ND[e]	Yes	26695	AAD08440, NP_208186, ABF85542	
HP1457		HPAG1_1458 homolog, lipoprotein	ND, ND[e]	Yes	Both	AAD08499, ABF85525, NP_224068	

HP1501	*omp32*	*Helicobacter* outer membrane protein family	ND[e]	No	43504	NP_224112, NP_208292	
HP0887	*vacA*	vacuolating cytotoxin	ND[e], 4.4[f]	Yes	Both	AAD04290, P55981, AAC77450	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Kim *et al.*, 2002)
HP0175		propyl cis-trans isomerase	8	Yes	43504	NP_206974, NP_222882	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Kim *et al.*, 2002; Sabarth *et al.*, 2002)
HP1019	*htrA*	serine protease	7.8, 2.5[f]	Yes	Both	NP_223124, ABF84495	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP1454		jhp1347 and HPAG1_1461 homolog	5, 1.8[f]	Yes	Both	AAD08497, ABF85528	(Bumann *et al.*, 2002; Jungblut *et al.*, 2000)
NH[g]	*hopZ*	Similar to omp19 (HP0896), omp2 (HP0025) and omp27 (HP1177)	3.1	Yes	43504	CAB42974	
HP0154	*eno*	enolase, phosphopyruvate hydratase	3	No	43504	NP_222863, ABF84219, P48285	(Jungblut *et al.*, 2000)
HP0953		HPAG1_0937 homolog	3	Yes	26695	AAD08007, ABF85004	
HP1098		jhp1024 homolog, cysteine-rich protein C, conserved secreted protein	3	Yes	43504	AAD08141, NP_223741, ABF85103	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP0243	*napA*	neutrophil activating protein	2.9	No	26695	AAD07308, AAG28155, AAF37841	(Backert *et al.*, 2005; Jungblut *et al.*, 2000)
HP1456	*lpp20*	membrane-associated lipoprotein Lpp20	2.9, 4.2[f]	Yes	Both	AAK56938, ABF85526	
HP0875	*katA*	Catalase	2.3	No	43504	AAD07923, NP_207669	(Backert *et al.*, 2005; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP1012	*pqqE*	putative zinc protease	2.3	Yes	43504	NP_223130, AAD05993, NP_207802	(Jungblut *et al.*, 2000)
HP1285		jhp1205 homolog, acid phosphatase	2.3	Yes	43504	ABF85295, CAD21745	(Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP1118	*ggt*	gamma-glutamyltranspeptidase	2.1	Yes	43504	AAD08162, NP_223763, ABF85123	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP0229	*omp6*	*Helicobacter* outer membrane protein family	2	Yes	43504	AAD07298, AAD05801,	

						NP_207027, NP_222935	
HP0011	*groES, hspA*	co-chaperone protein	1.8, 2[f]	No	Both	AAL86899, ABF84078	(Backert *et al.*, 2005; Jungblut *et al.*, 2000)
HP0310		jhp0295 and HPAG1_0312 homolog, polysaccharide deacetylase	1.8	No	43504	NP_223015, AAD05872, ABF84379, NP_207108	
HP0380	*gdhA*	glutamate dehydrogenase	1.8	No	43504	AAD07448, NP_207178	(Jungblut *et al.*, 2000)
HP0570	*pepA*	aminopeptidase a/i	1.8	No	43504	AAD07638, AAD06098	(Jungblut *et al.*, 2000)
HP1458	*trxC*	Thioredoxin	1.8	No	43504	NP_224069, NP_208249	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Kim *et al.*, 2002)
HP0026	*gltA*	citrate synthase	1.7	No	43504	AAD07097, ABF84091	(Jungblut *et al.*, 2000)
HP1350	*prc*	carboxyl-terminal protease	1.7	Yes	43504	AAD08394, ABF85364	(Backert *et al.*, 2005; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP0166	*ompR*	response regulator	1.6	No	43504	NP_222873, ABF84229	
HP0410	*hpaA*	putative neuraminyllactose-binding hemagglutinin	1.6	Yes	43504	AAD07478, NP_207208, ABF85049	(Backert *et al.*, 2005; Jungblut *et al.*, 2000; Sabarth *et al.*, 2002)
HP0545	*cagD*	cag pathogenicity island protein Cag24	1.6	Yes	43504	AAD07611, AAN74227, NP_207341, ABF84588	
HP1266	*nuoG*	NADH-ubiquinone oxidoreductase, NQO3 subunit	1.6	No	43504	NP_223905, ABF85277	
HP1286	*yceI*	jhp1206 homolog, conserved secreted protein	1.6	Yes	26695	AAD08331, ABF85294	(Backert *et al.*, 2005; Bumann *et al.*, 2002; Jungblut *et al.*, 2000; Kim *et al.*, 2002)
HP1375	*lpxA*	UDP-N-acetylglucosamine acyltransferase	1.6	No	43504	NP_224007, AAD06863, AAD08418	

a) Location of the gene corresponding to the matching peptides in *H. pylori* 26695 genome.
b) Average number of unique peptides assigned from three extracellular samples divided by average number of unique peptides assigned from three cell-associated samples.

c) Prediction of signal peptide based on artificial neural networks and hidden Markov model using SignalP 3.0 Server.
d) Accession numbers of sequences in the database that matched assigned peptides.
e) Not determinable because zero peptides where assigned from three cell-associated samples.
f) Ratio for strain 43504 given first.
g) No homolog in *H. pylori* 26695 genome. See description.

Table 5.2 Supporting data for the identification of reported proteins[a]

Description	Matching peptides	Entry	Coverage (%)	Seq[b]	Score	CS[c]	Sf[d]	P[e]	Peptides(M*:+15.99, C#:+57.02)
co-chaperone protein	AAL86899, ABF84078	26695-1-sec	10.2	3	1.91	2	0.73	98	(K)EGDVIAFGK(Y)
						2	0.68	66	(K)C#VKEGDVIAFGK(Y)
						1	0.5	56	(K)EGDVIAFGK(Y)

a) Example is given for one entry. Complete supporting information is available on the world-wide web under http://www.wiley-vch.de/contents/jc_2120/2007/pro200600875_s.pdf

b) Sequences

c) Charge state

d) Final score

e) Probablility

Table 5.3 Critiria for identification of proteins

Entry	Total peptide	Criteria (protein)[a]	F-proteins[b]	R-proteins[c]	FDR (protein)[d]	Criteria (peptide)[a]	F-peptides[b]	R-peptides[c]	FDR (peptide)[d]
26695-1	7148	Sf ³0.45 or P ³30	126	0	0.00%	Sf ³0.75 or P ³80	516	3	0.58%
26695-2	7463	Sf ³0.45 or P ³30	125	1	0.80%	Sf ³0.75 or P ³80	510	0	0.00%
26695-3	6818	Sf ³0.45 or P ³30	232	2	0.86%	Sf ³0.75 or P ³80	750	5	0.67%
26695-4	7264	Sf ³0.45 or P ³30	260	2	0.77%	Sf ³0.75 or P ³80	949	7	0.74%
43504-1	5861	Sf ³0.45 or P ³30	99	0	0.00%	Sf ³0.75 or P ³80	439	0	0.00%
43504-2	7862	Sf ³0.45 or P ³30	165	1	0.61%	Sf ³0.75 or P ³80	720	2	0.28%
43504-3	9818	Sf ³0.45 or P ³30	228	0	0.00%	Sf ³0.75 or P ³80	886	4	0.45%
43504-4	9654	Sf ³0.45 or P ³30	231	0	0.00%	Sf ³0.75 or P ³80	982	7	0.71%
26695-cyto	10274	Sf ³0.45 or P ³30	314	1	0.32%	Sf ³0.75 or P ³80	1297	10	0.77%
26695-sec	9986	Sf ³0.45 or P ³30	213	0	0.00%	Sf ³0.75 or P ³80	964	5	0.52%
43504-cyto	10344	Sf ³0.45 or P ³30	294	2	0.68%	Sf ³0.75 or P ³80	1294	10	0.77%
43504-sec	10128	Sf ³0.45 or P ³30	160	0	0.00%	Sf ³0.75 or P ³80	827	3	0.36%
				Average	0.34%				0.49%
			Standard Deviation		0.004				0.003

a) Sf = final score, P = probablility score
b) *H. pylori* database forward
c) *H. pylori* database reverse
d) False discovry rate (%)

Table 5.4 Total Extracellular Proteins[a]

Gene locus[b]	Name	Description	Peptide coverage strain 26695[c]	Peptide coverage strain 43504[c]
HP0010	*groEL*	heat shock protein	46.7	56.3
HP0011	*groES, hspA*	co-chaperone	4.7	7.3
HP0026	*gltA*	citrate synthase	8.3	20.3
HP0027	*icd*	isocitrate dehydrogenase	7.0	10.7
HP0068	*ureG*	urease accessory protein	6.7	3.0
HP0072	*ureB*	urease beta subunit	27.3	31.3
HP0073	*ureA*	urease alpha subunit	14.3	18.3
HP0105		jhp0097 homolog, S-ribosylhomocysteine lyase	0.0	2.3
HP0106	*metB*	cystathionine gamma-synthase	2.7	4.0
HP0109	*dnaK*	heat shock protein 70	9.0	20.3
HP0127	*omp4, HorB*	*Helicobacter* outer membrane protein family	0.0	1.3
HP0129		jhp0118 homolog	3.3	3.3
HP0154	*eno*	enolase, phosphopyruvate hydratase	3.7	2.0
HP0166	*ompR*	response regulator	3.0	1.7
HP0170		HPAG1_0166 and jhp0156 homolog	2.7	2.0
HP0175		propyl cis-trans isomerase	5.3	8.0
HP0176	*tsr, fba*	fructose-bisphosphate aldolase	8.0	10.3
HP0177	*efp*	translation elongation factor EF-P	3.0	4.3
HP0183	*glyA*	serine hydroxymethyltransferase	2.3	0.0
HP0192	*frdA*	fumarate reductase, flavoprotein subunit	0.0	1.3
HP0198	*ndk*	nucleoside diphosphate kinase	0.0	1.3
HP0218		HPAG1_0198 and jhp0204 homolog, phospholipid-binding protein	2.7	0.0
HP0224	*msrA*	peptide methionine sulfoxide reductase	11.0	10.7
HP0229	*omp6*	*Helicobacter* outer membrane protein family	0.0	1.3
HP0231		jhp0216 homolog, disulphide isomerase	7.7	10.3
HP0232		secreted protein involved in flagellar motility	2.7	1.7
HP0243	*napA*	neutrophil activating protein	9.7	7.7
HP0264	*clpB*	ATP-dependent protease binding subunit	0.0	3.3
HP0275	*addB*	jhp0260 homolog, ATP-dependent nuclease	2.3	2.7
HP0294	*aimE*	aliphatic amidase	4.0	3.7

HP0298	*dppA*	periplasmic dipeptide-binding protein	1.7	4.7
HP0305		jhp0290 homolog	8.0	12.0
HP0310		HPAG1_0312 and jhp0295 homolog, polysaccharide deacetylase	2.3	5.7
HP0318		HPAG1_0321 and jhp0301 homolog, Pyridoxamine 5'-phosphate oxidase	5.3	7.0
HP0330	*ilvC*	ketol-acid reductoisomerase	0.0	1.3
HP0380	*gdhA*	glutamate dehydrogenase	3.0	5.7
HP0389	*sodB*	superoxide dismutase	10.7	14.3
HP0390	*tagD*	adhesin-thiol peroxidase	8.0	13.3
HP0410	*hpaA*	putative neuraminyllactose-binding hemagglutinin	2.3	4.3
HP0468		HPAG1_0444 homolog	2.3	3.0
HP0472	*omp11, HorE*	*Helicobacter* outer membrane protein family	2.7	6.7
HP0480	*yihK*	jhp0432 homolog, fusA-homolog	2.3	9.0
HP0485		jhp0437 homolog, catalase-like protein	5.0	2.3
HP0500	*dnaN*	DNA polymerase III beta-subunit	2.7	3.7
HP0512	*glnA*	glutamine synthetase	4.7	6.3
HP0514	*rplI*	ribosomal protein L9	3.0	3.7
HP0545	*cag24*	cag pathogenicity island protein (cag24)	0.0	1.7
HP0547	*cagA*	cag pathogenicity island protein	0.0	5.3
HP0558	*fabF*	beta ketoacyl-acyl carrier protein synthase II	2.7	4.3
HP0559	*acpP*	acyl carrier protein	1.7	1.7
HP0570	*pepA*	aminopeptidase a/i	14.0	12.7
HP0589	*oorA*	ferredoxin oxidoreductase, alpha subunit	0.0	2.7
HP0590	*oorB*	ferredoxin oxidoreductase, beta subunit	0.0	2.0
HP0599	*hylB*	hemolysin secretion protein precursor	4.7	6.0
HP0601	*flaA*	flagellin A	0.0	4.0
HP0617	*aspS*	aspartyl-tRNA synthetase	2.3	0.0
HP0620	*ppa*	inorganic pyrophosphatase	0.0	2.0
HP0649	*aspA*	aspartate ammonia-lyase	5.3	2.3
HP0653	*pfr*	nonheme iron-containing ferritin	0.0	2.3
HP0657	*ymxG*	processing protease	4.7	2.3
HP0672	*aspB*	member of the PLP dependent aminotransferase superfamily clan	3.0	2.3
HP0690	*thl, fadA*	acetyl coenzyme A acetyltransferase, thiolase	5.0	8.3
HP0691		3-oxoadipate coA-transferase subunit A	0.0	6.3
HP0706	*omp15, HopE*	*Helicobacter* outer membrane protein family	0.0	2.7
HP0710		*Helicobacter* outer membrane protein family	0.0	1.3

HP0719		HPAG1_0704 and jhp0657 homolog	2.3	0.0
HP0720			0.0	2.0
HP0752	*fliD*	flagellar hook-associated protein 2	0.0	3.3
HP0779	*acnB*	aconitase B	9.7	9.3
HP0795	*tig*	trigger factor	2.7	2.7
HP0824	*trxA*	thioredoxin	5.0	7.0
HP0825	*trxB*	thioredoxin reductase	3.3	10.0
HP0829	*guaB*	inosine-5'-monophosphate dehydrogenase	2.7	1.7
HP0835	*hup*	histone-like DNA-binding protein HU	1.7	3.0
HP0875		catalase	10.0	36.7
HP0887	*vacA*	vacuolating cytotoxin	25.0	26.7
HP0891		jhp0824 homolog, acyl coenzyme A thioesterase	3.0	0.0
HP0900	*hypB*	hydrogenase expression/formation protein	1.7	2.7
HP0902		jhp0839 homolog	3.0	2.0
HP0912	*omp20*	*Helicobacter* outer membrane protein family	0.0	2.3
HP0913	*omp21, HopB*	*Helicobacter* outer membrane protein family	0.0	1.7
HP0953		HPAG1_0937 homolog	3.0	3.7
HP0958		jhp0892 homolog, sigma54-stabilizing protein	3.0	2.7
HP0973		HPAG1_0954 homolog	0.0	3.3
HP1012	*pqqE*	putative zinc protease	2.3	2.3
HP1019	*htrA*	serine protease	10.0	15.7
HP1037	*pepQ*	putative proline peptidase	1.7	3.7
HP1038	*aroQ*	3-dehydroquinase type II DHQase	2.0	0.0
HP1043		response regulator	0.0	1.3
HP1067	*cheY*	chemotaxis protein	1.7	1.7
HP1073	*copP*	copper ion binding protein	0.0	1.3
HP1098		jhp1024 homolog, cysteine-rich protein C, conserved secreted protein	0.0	8.0
HP1099	*eda*	2-keto-3-deoxy-6-phosphogluconate aldolase	2.0	4.7
HP1100	*edd*	6-phosphogluconate dehydratase	2.7	3.0
HP1104	*cad*	cinnamyl-alcohol dehydrogenase	0.0	3.0
HP1118	*ggt*	gamma-glutamyltranspeptidase	5.0	7.7
HP1134	*atpA*	ATP synthase F1, subunit alpha	0.0	3.0
HP1161	*fldA*	flavodoxin	15.7	22.0
HP1173		HPAG1_1112 homolog	2.3	0.0
HP1177	*omp27, hopQ*	*Helicobacter* outer membrane protein family	4.3	5.0
HP1178	*deoD*	purine-nucleoside phosphorylase	1.3	0.0

HP1186		carbonic anhydrase	2.3	2.0
HP1195	*fusA*	translation elongation factor EF-G	10.0	5.7
HP1199	*rplL*	ribosomal protein L7/L12	4.0	0.0
HP1205	*tuf*	translation elongation factor EF-Tu	9.3	9.3
HP1246	*rps6*	ribosomal protein S6	0.0	1.3
HP1256	*frr, rrf*	ribosome releasing factor	0.0	1.7
HP1266	*nuoG*	NADH-ubiquinone oxidoreductase, NQO3 subunit	1.3	2.7
HP1275	*algC*	phosphomannomutase	0.0	3.0
HP1285		jhp1205 homolog, acid phosphatase	3.0	2.3
HP1286	*yceI*	jhp1206 homolog, conserved secreted protein	8.3	2.0
HP1293	*rpoA*	DNA-directed RNA polymerase, alpha subunit	2.3	1.7
HP1325	*fumC*	fumarase	5.0	0.0
HP1350	*prc*	carboxyl-terminal protease	1.3	2.3
HP1375	*lpxA*	UDP-N-acetylglucosamine acyltransferase	2.0	1.7
HP1395	*omp30, HorL*	*Helicobacter* outer membrane protein family	2.0	0.0
HP1398	*ald*	alanine dehydrogenase	0.0	5.3
HP1422	*ileS*	isoleucyl-tRNA synthetase	2.3	2.0
HP1441	*ppi*	peptidyl-prolyl cis-trans isomerase B	0.0	1.3
HP1454		jhp1347 and HPAG1_1461 homolog	8.0	5.0
HP1456	*Lpp20*	membrane-associated lipoprotein Lpp20	5.7	10.7
HP1457		HPAG1_1458 homolog, lipoprotein	3.0	2.0
HP1458		thioredoxin	0.0	3.0
HP1501	*omp32*	*Helicobacter* outer membrane protein family	0.0	2.0
HP1512		iron-regulated outer membrane protein	0.0	2.0
HP1561	*ceuE*	jhp1469 homolog, periplasmic iron-binding protein,	0.0	2.0
HP1562		jhp1470 homolog, periplasmic iron-binding protein	5.7	2.3
HP1563	*tsaA*	alkyl hydroperoxide reductase	25.7	31.7
HP1564		periplasmic binding protein	13.3	12.7
	hopZ	closest match omp19 (HP0896), omp2 (HP0025) and omp27 (HP1177)	3.7	11.3

a) All proteins identified in the extracellular samples from at least two biological replicates.
b) Location of the gene corresponding to the matching peptides in *H. pylori* 26695 genome.
c) Average number of unique peptides assigned from three extracellular samples.

Table 5.5 Total Cell-associated Proteins[a]

Gene locus[b]	Name	Description	Peptide coverage strain 26695[c]	Ratio strain 26695[d]	Peptide coverage strain 43504[c]	Ratio strain 43504[d]
HP0003	*kdsA*	3-deoxy-d-manno-octulosonic acid 8-phosphate synthetase	2.3	0.3	2.3	0.3
HP0010	*groEL*	heat shock protein	54.3	0.9	74.3	0.8
HP0020	*nspC*	carboxynorspermidine decarboxylase	4.0	NR[e]	2.3	0.4
HP0025	*omp2*	outer membrane protein	3.0	0.8	0.0	NR[f]
HP0026	*gltA*	citrate synthase	11.7	0.7	11.3	>1.5[g]
HP0027	*icd*	isocitrate dehydrogenase	13.0	0.5	21.7	0.5
HP0068	*ureG*	urease accessory protein	6.0	1.1	6.0	0.5
HP0072	*ureB*	urease beta subunit	24.3	1.1	31.0	1.0
HP0073	*ureA*	urease alpha subunit	16.3	0.9	24.7	0.7
HP0105		jhp0097 homolog, S-ribosylhomocysteine lyase	0.0	NR[f]	4.3	0.5
HP0106	*metB*	cystathionine gamma-synthase	3.0	0.9	4.0	1.0
HP0109	*dnaK*	heat shock protein 70	21.3	0.4	32.7	0.6
HP0110	*grpE*	co-chaperone	2.3	0.9	5.0	NR[e]
HP0115	*flaB*	flagellin B	0.0	NR[f]	3.3	NR[e]
HP0129		jhp0118 homolog	4.3	0.8	7.0	0.5
HP0154	*eno*	enolase, phosphopyruvate hydratase	2.7	1.4	0.7	>1.5[g]
HP0166	*ompR*	response regulator	5.3	0.6	1.0	>1.5[g]
HP0170		HPAG1_0166 and jhp0156 homolog	3.7	0.7	6.3	0.3
HP0175		propyl cis-trans isomerase	7.0	0.8	1.0	>1.5[g]
HP0176	*tsr, fba*	fructose-bisphosphate aldolase	7.7	1.0	17.0	0.6
HP0177	*efp*	translation elongation factor EF-P	4.7	0.6	8.3	0.5
HP0182	*lysS*	lysyl-tRNA synthetase	0.0	NR[f]	2.3	NR[e]
HP0183	*glyA*	serine hydroxymethyltransferase	4.7	NR[e]	5.7	0.3
HP0192	*frdA*	fumarate reductase, flavoprotein subunit	5.7	0.2	8.3	0.2
HP0194	*tpi*	triosephosphate isomerase	2.0	NR[e]	0.0	NR[f]
HP0195	*fabI*	enoyl-acyl carrier protein reductase (NADH)	3.3	NR[e]	2.3	NR[e]
HP0198	*ndk*	nucleoside diphosphate kinase	0.0	NR[f]	6.3	0.2
HP0210	*htpG*	chaperone, heat shock protein	10.0	NR[e]	14.3	NR[e]
HP0221		nifU-like protein	0.0	NR[f]	5.3	NR[e]
HP0224	*msrA*	peptide methionine sulfoxide reductase	14.7	0.8	10.0	1.1
HP0229	*omp6*	*Helicobacter* outer membrane protein family	1.3	NR[e]	0.0	>1.5[g]

HP0231		jhp0216 homolog, disulphide isomerase	7.0	1.1	9.0	1.1
HP0232		secreted protein involved in flagellar motility	3.0	0.9	2.7	0.6
HP0233		jhp0218 homolog, Glutathionylspermidine synthase	0.0	NR[f]	5.7	NR[e]
HP0243	*napA*	neutrophil activating protein	3.3	>1.5[g]	5.7	1.4
HP0264	*clpB*	ATP-dependent protease binding subunit	9.0	NR[e]	10.7	0.3
HP0267		jhp0252 homolog, chlorohydrolase	2.0	NR[e]	0.0	NR[f]
HP0294	*aimE*	aliphatic amidase	6.7	0.6	0.0	>1.5[g]
HP0296	*rplU*	ribosomal protein L21	1.3	NR[e]	0.0	NR[f]
HP0298	*dppA*	periplasmic dipeptide-binding protein	1.7	1.0	0.0	>1.5[g]
HP0305		jhp0290 homolog	6.7	1.2	9.7	1.2
HP0306	*hemL*	glutamate-1-semialdehyde 2,1-aminomutase	3.0	NR[e]	5.0	NR[e]
HP0310		HPAG1_0312 and jhp0295homolog, polysaccharide deacetylase	4.3	0.5	3.0	>1.5[g]
HP0318		HPAG1_0321 and jhp0301 homolog, Pyridoxamine 5'-phosphate oxidase	5.7	0.9	8.3	0.8
HP0322		jhp0305 homolog, poly E-rich protein	4.3	NR[e]	4.3	NR[e]
HP0349	*pyrG*	CTP synthetase	0.0	NR[f]	2.0	NR[e]
HP0371	*fabE*	biotin carboxyl carrier protein	2.0	NR[e]	2.7	NR[e]
HP0380	*gdhA*	glutamate dehydrogenase	7.0	0.4	3.0	>1.5[g]
HP0389	*sodB*	superoxide dismutase	9.3	1.1	18.3	0.8
HP0390	*tagD*	adhesin-thiol peroxidase	12.3	0.6	15.3	0.9
HP0399	*rpsA*	ribosomal protein S1	16.7	NR[e]	12.7	NR[e]
HP0402	*pheT*	phenylalanyl-tRNA synthetase, beta subunit	0.0	NR[f]	2.7	NR[e]
HP0404		protein kinase C inhibitor hit family protein	0.0	NR[f]	2.3	NR[e]
HP0410	*hpaA*	putative neuraminyllactose-binding hemagglutinin	4.0	0.6	2.7	>1.5[g]
HP0417	*metG*	methionyl-tRNA synthetase	6.7	NR[e]	5.0	NR[e]
HP0468		HPAG1_0444 homolog	7.0	0.3	4.0	0.8
HP0470	*pepF*	oligoendopeptidase F	7.0	NR[e]	8.3	NR[e]
HP0480	*yihK*	jhp0432 homolog, fusA-homolog	4.3	0.5	6.0	1.5
HP0485		jhp0437 homolog, catalase-like protein	4.0	1.3	1.7	1.4
HP0486		*Helicobacter* outer membrane protein family	2.7	NR[e]	0.0	NR[f]
HP0500	*dnaN*	DNA polymerase III beta-subunit	10.7	NR[e]	10.3	0.4
HP0512	*glnA*	glutamine synthetase	6.3	0.7	5.3	1.2
HP0514	*rplI*	ribosomal protein L9	6.3	0.5	3.3	1.1
HP0516		ATP-dependent hsl protease	0.0	NR[f]	1.7	NR[e]
HP0543	*cagF*	putative outer membrane protein cag 22	1.3	NR[e]	1.3	NR[e]
HP0545	*cagD*	cag pathogenicity island protein cag 24	1.3	NR[e]	1.0	>1.5[g]
HP0547	*cagA*	cag pathogenicity island protein	0.0	NR[f]	49.3	0.1

HP0558	*fabF*	beta ketoacyl-acyl carrier protein synthase II	6.0	0.4	3.7	1.2
HP0559	*acpP*	acyl carrier protein	3.0	0.6	3.0	0.6
HP0561	*fabG*	3-ketoacyl-acyl carrier protein reductase	4.0	NR[e]	3.7	NR[e]
HP0564		jhp0511and HPAG1_0543 homolog	2.0	0.7	3.3	NR[e]
HP0570	*pepA*	aminopeptidase a/i	7.0	1.3	11.0	>1.5[g]
HP0589	*oorA*	ferredoxin oxidoreductase, alpha subunit	0.0	NR[f]	9.7	0.3
HP0590	*oorB*	ferredoxin oxidoreductase, beta subunit	6.0	0.1	4.0	0.5
HP0591	*oorC*	ferredoxin oxidoreductase, gamma subunit	3.0	NR[e]	2.0	NR[e]
HP0599	*hylB*	hemolysin secretion protein precursor	12.7	0.4	10.3	0.6
HP0601	*flaA*	flagellin A	0.0	NR[f]	20.3	0.2
HP0604	*hemE*	uroporphyrinogen decarboxylase	4.7	NR[e]	4.3	NR[e]
HP0617	*aspS*	aspartyl-tRNA synthetase	7.7	0.3	6.0	NR[e]
HP0618	*adk*	adenylate kinase	3.0	NR[e]	2.7	NR[e]
HP0620	*ppa*	inorganic pyrophosphatase	0.0	NR[f]	6.0	0.3
HP0630	*mda66*	modulator of drug activity	7.0	NR[e]	2.3	NR[e]
HP0643	*gltX*	glutamyl-tRNA synthetase	4.3	NR[e]	2.3	NR[e]
HP0649	*aspA*	aspartate ammonia-lyase	6.7	0.8	7.7	0.3
HP0654			0.0	NR[f]	2.0	NR[e]
HP0658	*gatB*	Glu-tRNA(Gln) amidotransferase, subunit B	5.0	NR[e]	3.3	NR[e]
HP0690	*thl, fadA*	acetyl coenzyme A acetyltransferase, thiolase	9.3	0.5	8.0	1.0
HP0691		3-oxoadipate coA-transferase subunit A	2.0	NR[e]	5.3	1.2
HP0695	*hyuA*	hydantoin utilization protein A	7.3	NR[e]	0.0	NR[f]
HP0696		N-methylhydantoinase	11.0	NR[e]	0.0	NR[f]
HP0719		HPAG1_0704 and jhp0657 homolog	2.7	0.9	0.0	NR[f]
HP0720			2.7	NR[e]	2.7	0.8
HP0752	*fliD*	flagellar hook-associated protein 2	0.0	NR[f]	3.0	1.1
HP0773		HPAG1_0758 homolog, denitrification protein	3.7	NR[e]	0.0	NR[f]
HP0779	*acnB*	aconitase B	21.7	0.4	22.3	0.4
HP0783		HPAG1_0769 and jhp0720 homolog	0.0	NR[f]	1.7	NR[e]
HP0786	*secA*	preprotein translocase subunit	4.7	NR[e]	2.3	NR[e]
HP0795	*tig*	trigger factor	6.7	0.4	5.0	0.5
HP0824	*trxA*	thioredoxin	8.7	0.6	15.3	0.5
HP0825	*trxB*	thioredoxin reductase	10.7	0.3	23.0	0.4
HP0829	*guaB*	inosine-5'-monophosphate dehydrogenase	9.3	0.3	7.0	NR[e]
HP0830	*gatA*	Glu-tRNA(Gln) amidotransferase, subunit A	3.0	NR[e]	1.3	NR[e]
HP0835	*hup*	histone-like DNA-binding protein HU	5.0	0.3	7.0	0.4

HP0865	*dut*	deoxyuridine 5'-triphosphate nucleotidohydrolase	1.3	NR[e]	0.0	NR[f]
HP0875		catalase	6.7	1.5	15.3	>1.5[g]
HP0891		jhp0824 homolog, acyl coenzyme A thioesterase	4.3	0.7	0.0	NR[f]
HP0900	*hypB*	hydrogenase expression/formation protein	5.3	0.3	9.0	0.3
HP0902		jhp0839 homolog	2.7	1.1	4.0	0.5
HP0912	*omp20*	*Helicobacter* outer membrane protein family	1.7	NR[e]	0.0	>1.5[g]
HP0913	*omp21, HopB*	*Helicobacter* outer membrane protein family	0.0	NR[f]	1.3	1.3
HP0930	*surE*	stationary phase survival protein	1.3	NR[e]	0.0	NR[f]
HP0953		HPAG1_0937 homolog	0.0	>1.5[g]	2.7	1.4
HP0958		jhp0892 homolog, sigma54-stabilizing protein	6.3	0.5	4.7	0.6
HP0973		HPAG1_0954 homolog	4.0	NR[e]	5.0	0.7
HP0974	*pmg*	phosphoglycerate mutase	6.0	NR[e]	0.0	NR[f]
HP1012	*pqqE*	putative zinc protease	2.3	1.0	0.0	>1.5[g]
HP1014	*hdhA*	7-alpha-hydroxysteroid dehydrogenase	2.0	NR[e]	2.0	NR[e]
HP1027			0.0	NR[f]	1.7	NR[e]
HP1037	*pepQ*	putative proline peptidase	7.3	0.2	13.0	0.3
HP1038	*aroQ*	3-dehydroquinase type II	2.3	0.9	2.7	NR[e]
HP1042		phosphoesterase protein family	2.0	NR[e]	0.0	NR[f]
HP1043		response regulator	2.3	NR[e]	3.7	0.4
HP1067	*cheY*	chemotaxis protein	1.3	1.3	1.7	1.0
HP1099	*eda*	2-keto-3-deoxy-6-phosphogluconate aldolase	3.0	0.7	3.3	1.4
HP1100	*edd*	6-phosphogluconate dehydratase	4.7	0.6	3.7	0.8
HP1103	*glk*	glucokinase	3.3	NR[e]	7.0	NR[e]
HP1104	*cad*	cinnamyl-alcohol dehydrogenase	7.7	0.3	5.7	NR[e]
HP1110	*porA*	pyruvate ferredoxin oxidoreductase, alpha subunit	8.0	NR[e]	6.0	NR[e]
HP1111	*porB*	pyruvate ferredoxin oxidoreductase, beta subunit	5.3	NR[e]	10.0	NR[e]
HP1118	*ggt*	gamma-glutamyltranspeptidase	5.0	1.0	3.7	>1.5[g]
HP1123	*slyD*	peptidyl-prolyl cis-trans isomerase	3.0	NR[e]	9.3	NR[e]
HP1132	*atpD*	ATP synthase F1, subunit beta	13.0	NR[e]	9.7	NR[e]
HP1134	*atpA*	ATP synthase F1, subunit alpha	15.0	NR[e]	16.0	0.2
HP1161	*fldA*	flavodoxin	16.0	1.0	17.3	1.3
HP1177	*omp27*	*Helicobacter* outer membrane protein family	10.3	0.4	4.0	1.3
HP1178	*deoD*	purine-nucleoside phosphorylase	2.0	0.7	3.0	NR[e]
HP1179	*deoB*	phosphopentomutase	2.3	NR[e]	3.0	NR[e]
HP1190	*hisS*	histidyl-tRNA synthetase	0.0	NR[f]	2.3	NR[e]
HP1195	*fusA*	translation elongation factor EF-G	16.7	0.6	22.7	0.3

HP1196	*rps7*	ribosomal protein S7	2.0	NR[e]	0.0	NR[f]
HP1199	*rplL*	ribosomal protein L7/L12	2.7	1.5	6.3	NR[e]
HP1200	*rplJ*	ribosomal protein L10	1.3	NR[e]	0.0	NR[f]
HP1201	*rplA*	ribosomal protein L1	3.7	NR[e]	2.3	NR[e]
HP1202	*rplK*	ribosomal protein L11	3.0	NR[e]	3.0	NR[e]
HP1203	*nusG*	transcription termination factor	0.0	NR[f]	3.0	NR[e]
HP1205	*tuf*	translation elongation factor EF-Tu	24.7	0.4	39.3	0.2
HP1218	*purD*	glycinamide ribonucleotide synthetase	2.0	NR[e]	4.7	NR[e]
HP1256	*frr, rrf*	ribosome releasing factor	3.0	NR[e]	6.7	0.3
HP1266	*nuoG*	NADH-ubiquinone oxidoreductase, NQO3 subunit	3.7	0.4	1.7	>1.5[g]
HP1275	*algC*	phosphomannomutase	2.7	NR[e]	5.0	0.6
HP1285	*jhp1205*	acid phosphatase	2.3	1.3	1.0	>1.5[g]
HP1293	*rpoA*	DNA-directed RNA polymerase, alpha subunit	10.3	0.2	19.3	NR[e]
HP1304	*rpl6*	ribosomal protein L6	5.0	NR[e]	0.0	NR[f]
HP1307	*rpl5*	ribosomal protein L5	2.7	NR[e]	2.7	NR[e]
HP1313	*rpsC*	ribosomal protein S3	1.3	NR[e]	0.0	NR[e]
HP1320	*rpsJ*	ribosomal protein S10	0.0	NR[f]	2.0	NR[e]
HP1325	*fumC*	fumarase	7.3	0.7	11.7	NR[e]
HP1350	*prc*	carboxyl-terminal protease	2.0	0.7	1.3	>1.5[g]
HP1375	*lpxA*	UDP-N-acetylglucosamine acyltransferase	3.3	0.6	1.0	>1.5[g]
HP1398	*ald*	alanine dehydrogenase	4.0	NR[e]	5.0	1.1
HP1422	*ileS*	isoleucyl-tRNA synthetase	8.0	0.3	8.7	0.2
HP1458		thioredoxin	3.3	NR[e]	0.0	>1.5[g]
HP1495	*tal*	transaldolase	2.3	NR[e]	0.0	NR[f]
HP1512		iron-regulated outer membrane protein	3.0	NR[e]	0.0	NR[f]
HP1527			2.7	NR[e]	0.0	NR[f]
HP1555	*tsf*	translation elongation factor EF-Ts	7.7	NR[e]	12.0	NR[e]
HP1561	*ceuE*	jhp1469 homolog, periplasmic iron-binding protein	0.0	NR[f]	3.7	0.5
HP1562		jhp1470 homolog, periplasmic iron-binding protein	8.7	0.7	6.3	NR[e]
HP1563	*tsaA*	alkyl hydroperoxide reductase	28.7	0.9	49.3	0.6
HP1564		periplasmic binding protein clan	11.0	1.2	11.0	1.2
HP1588		jhp1494 and HPAG1_1536 homolog	4.3	NR[e]	0.0	NR[f]

a) All proteins identified in the soluble cell-associated samples from at least two biological replicates in one strain. Note proteins from Table 5.1 not appearing in this table were not reprodicibly identified in the other strain.
b) Location of the gene corresponding to the matching peptides in *H. pylori* 26695 genome.

c) Average number of unique peptides assigned from three extracellular samples divided by average number of unique peptides assigned from three cell-associated samples.
d) Average number of unique peptides assigned from three cell-associated samples.
e) Not reproducibly identified in extracellular samples from this strain.
f) Not reproducibly identified in extracellular or cell-associated samples from this strain.
g) See Table 5.1 for actual ratio.

CHAPTER 6

CONCLUSION

Impact of Studies

The goal of these studies was to elucidate further the molecular mechanisms that control expression of flagellar genes in the bacterium *Helicobacter pylori*. The studies described here have direct relevance to human health and welfare since understanding how *H. pylori* regulates production of its flagella could lead to new methods for detection, treatment or prevention of *H. pylori* infection. Because *H. pylori* is closely related to other human pathogens, such as *Campylobacter* and *Arcobacter*, my studies on flagellar biosynthesis will also serve as an important model for other medically important organisms.

Chapter 2, "Deciphering bacterial flagellar gene regulatory networks in the genomic era", provided a much needed comprehensive review of flagellar gene regulation in diverse bacterial species. In addition, several predictions about the distribution of different regulatory schemes were included. Hopefully, these predictions will help guide future work on flagellar gene regulatory networks in more diverse bacteria.

In Chapter 3, studies investigating the roles of FlhB, FliK and FlhX in expression of flagellar genes in *H. pylori* were described which advanced our understanding of how the flagellar protein export apparatus influences flagellar gene expression in *H. pylori*. These studies revealed that as in *S. typhimurium*, FlhB undergoes autocleavage at a specific site in *H. pylori*. At the time these studies were undertaken the apparently universal nature of FlhB

autocleavage was not yet recognized. In addition, amino acid substitutions at the autocleavage site interfered with processing of *H. pylori* FlhB and prevented the export of filament-type substrates.

The FlhB processing-deficient variants did not affect expression of RpoN-dependent or FliA-dependent flagellar reporter genes. This finding was particularly interesting for at least two reasons. First, we had expected that the FlhB processing-deficient variants would stimulate expression of the RpoN regulon similar to loss of the hook length control protein, FliK (Ryan *et al.*, 2005). We thought that loss of FliK stimulated expression of the RpoN regulon by causing a delay in the substrate specificity switch of the export apparatus. We expected that the FlhB variants would lock the export apparatus in the rod-/hook-type conformation and would therefore result in enhanced expression of the RpoN-dependent reporter genes. Thus we were surprised to find that the RpoN-dependent reporter genes were expressed at wild-type levels in the strains that produced the FlhB processing-deficient variants. While this finding has impacted our thinking of how the export apparatus might influence expression of the RpoN regulon, it does not rule out the possibility that the conformation of the export apparatus is the signal that is communicated to the RpoN regulon. Novel conformational changes may take place within the export apparatus independent of FlhB autocleavage.

A second reason the observations on FlhB processing-deficient variants were interesting is that we expected these proteins to interfere with expression of the *flaA-xylE* reporter gene. This expectation was based on the assumption that FlgM is a filament-type substrate in *H. pylori* as it is in *S. typhimurium*. Since strains producing the *H. pylori* FlhB processing-deficient variants were unable to export filament-type substrates yet were able to support wild-type expression levels of the *flaA-xylE* reporter gene suggesting that *H. pylori* FlgM is not secreted as

a filament-type substrate. Thus, if FlgM is secreted by the flagellar protein export apparatus in *H. pylori* it may be secreted prior to the switch to filament-type substrate conformation. Alternatively, in light of the recently proposed model (Rust *et al.*, 2009) it is possible that FlgM is not secreted by the flagellar protein export apparatus, which would also be consistent with our observations.

The studies presented in Chapter 3 also characterized the role of FliK in flagellar gene regulation more fully by demonstrating that disruption of *fliK* in a *flhB* deletion mutant does not restore expression of the RpoN regulon. This observation suggests that the failure to secrete FliK by mutants that lack a functional flagellar protein export apparatus, such as the *flhB* mutant, is not responsible for inhibition of the RpoN regulon in these mutants. Finally, the work presented in Chapter 3 demonstrated a role for FlhX, a protein that shares homology with the $FlhB_{CC}$ subdomain, in regulation of the RpoN-dependent genes in *H. pylori*. Interestingly, FlhX is required for optimal expression of RpoN-dependent reporter genes in the wild-type *H. pylori* strain, but not in strains that possess FlhB processing-deficient variants. We infer from these data that FlhX may substitute for $FlhB_{CC}$ to achieve optimal expression of the RpoN regulon. This model predicts that $FlhB_{CC}$ can be displaced from the export apparatus following autocleavage of FlhB. $FlhB_{CC}$ could be released into the cytoplasm of the cell, or it could be secreted from the cell by the export apparatus. If $FlhB_{CC}$ is secreted via the export apparatus, it may be secreted along with FliK if interactions between $FlhB_{CC}$ and FliK are sufficiently strong. I attempted to test this hypothesis but was unable to detect $FlhB_{CC}$ unequivocally by western blot analysis of cellular or extracellular fractions of *H. pylori* (data not shown).

The studies on FlhA described in Chapter 4 have the potential for the highest impact with regard to understanding how the export apparatus influences expression of the RpoN regulon, but

require further characterization of the mutations and strains that were generated. The results of my studies showed that expression of the RpoN regulon occurs in strains in which the flagellar protein export apparatus is completely deficient in protein secretion. Unfortunately, I was unable to show conclusively that truncated FlhA protein is expressed and localized to the export apparatus, which is critical for verifying that this mutant protein is affecting expression of the RpoN regulon. Attempts to express a truncated FlhA protein that consisted of only the first 77 amino acid residues did not result in the enhanced expression of the RpoN-dependent reporter genes. However, it is unclear if a truncated FlhA was actually expressed since attempts to express a wild-type copy of *flhA* in this background failed to restore motility, and I was unable to identify any FlhA proteins in *H. pylori* membrane fractions by western blotting. These experiments were also undertaken before sequencing the *flhA77* allele revealed the product of this allele contained a significant amount of additional primary sequence that could be responsible for the observed phenotype. Future work should attempt to address this issue by cloning the *flhA77* allele with the additional primary sequence and expressing it in the Δ*flhA* mutant background.

Another potentially interesting result from the studies presented in Chapter 4 was the unexpected effect of loss of HP1042 had on the FlgE-FLAG protein and flagellins. Inactivation of *hp1042* resulted in an increase in the apparent size of some of the FlgE-FLAG protein and its degradation in the extracellular fraction. Similarly, the sizes of the flagellins in the extracellular fraction of the *hp1042* mutant were significantly larger. We postulate that in the absence of HP1042 the FlgE-FLAG protein and flagellins are hyperglycosylated which accounts for the apparent increase in size of these proteins. This could also account for the degradation of the

FlgE-FLAG protein, perhaps by affecting the ability of the protein to fold properly and making it more susceptible to proteolysis.

Finally, in Chapter 5, "Direct analysis of the extracellular proteome from two strains of *Helicobacter pylori*", a novel method was developed for analysis of the *H. pylori* proteome. This collaborative work was initiated to develop a method for assessing the ability of *H. pylori* strains to export flagellar proteins. The method did not turn out to be a viable approach for analyzing flagellar protein export since only a few of the known flagellar proteins that are external to the cell membrane were enriched in the extracellular fraction. The study was successful, however, in identifying new proteins in the extracellular fraction. Specifically, this work has been cited in other studies of CagD (Cendron *et al.*, 2009) and extracellular proteases (Lower *et al.*, 2008).

Future Directions

Regulation of flagellar gene expression in *H. pylori* and other closely related bacteria is a fertile area for new and exciting discoveries. For example, the master regulator that initiates the flagellar gene transcriptional hierarchy in *H. pylori* and related bacteria has yet to be discovered. The molecular mechanisms that govern the various flagellar regulons in *H. pylori* require further characterization, and investigators have only begun to characterize the role of flagellar protein glycosylation and the regulation of this process in *H. pylori*. In addition, several potential flagellar genes of unknown function have been identified in *H. pylori* through DNA microarray analysis and bioinformatic approaches.

With regard to future directions of projects directly related to the work presented here, studies are already underway to investigate the role of $FlhB_{CC}$ and FlhX in expression of RpoN-dependent flagellar genes. A strain expressing a truncated FlhB variant that lacks $FlhB_{CC}$ has

been constructed, and the Δ*flhX* mutation has also been introduced in this background. These strains will be used to test the hypothesis that FlhX can susbstitute for $FlhB_{CC}$ to allow expression of the RpoN and FliA regulons. We believe that this hypothesis will test valid since FlhX can substitute for $FlhB_{CC}$ in facilitating assembly of a functional flagellum (Wand *et al.*, 2006). Expression of the RpoN-dependent reporter genes in the strain that lacks the $FlhB_{CC}$ subdomain, however, could be lower than in wild-type *H. pylori* since FlhX may be only transiently associated with the export apparatus.

As indicated previously, disruption of *flgM* was shown by Niehus *et al.* (2004) to partially restore expression of RpoN-dependent genes in an *flhA* insertion mutant. While we cannot duplicate this exact experiment since our *flhA* insertion mutants are not deficient in expression of the RpoN-dependent reporter genes, we can determine if disruption of *flgM* restores expression of the RpoN-dependent reporter genes in our Δ*flhA* strain. We could also obtain the *flhA* insertion mutant that Niehus *et al.* used for their studies to see if we can duplicate their results. If we find that disruption of *flgM* in the *flhA* deletion mutant restores expression of the RpoN-dependent reporter genes we can determine if the same is true for our Δ*flhB* mutant. I have already constructed a Δ*flgM* mutant, and expression of the *flaA-xylE* reporter gene in this strain was up regulated consistent with the expected phenotype of a *flgM* mutant.. The results of these experiments will tell us if the effect of FlgM on the RpoN regulon requires a truncated FlhA protein or not. Depending on the outcome of these experiments, we can disrupt *flgM* in strains expressing the FlhB processing-deficient variants and the FlhB variant lacking the $FlhB_{CC}$ subdomain to determine the influence of $FlhB_C$ on FlgM activity.

I have also disrupted several genes of unknown function in *H. pylori* that are predicted to be part of the RpoN regulon and examined the phenotypes of the resulting mutant strains. These

genes included HP0367, HP1076, and HP1154. Expression of these genes was reported to be dependent on RpoN and/or FlgS based on a DNA microarray analysis (Niehus *et al.*, 2004). In addition, we identified potential RpoN-dependent promoters upstream of these genes using the motif identification program Pattern Locator (Mrazek & Xie, 2006). HP1076 is upstream and in the opposite orientation of the gene encoding the nickel transporter NixA. HP1076 has no predictable function but is highly conserved among the sequenced ε-Proteobacteria. HP1154 is a paralog of HP1377, and both are FliW orthologs. FliW is reported to be a chaperon for flagellin proteins similar to FliS (Titz *et al.*, 2006). HP1154 and HP1377 interact with FlaA while FliS interacts with FlaB in *H. pylori* in a yeast two-hybrid system (Rain *et al.*, 2001).

When *hp0367*, *hp1076* and *hp1154* isogenic mutants were constructed, only *hp0367* was required for motility (Fig 6.1, work is currently underway to construct an *hp1377* isogenic and *hp1154*/*hp1377* double mutant to more accurately determine their effect on motility since the products of these genes may have the same function in *H. pylori*). HP0367 has no predictable function but together with HP0366 appears to be exclusively found in *H. pylori*. *hp0367* is the first gene of a predicted operon with *hp0366* whose product is involved in biosynthesis of pseudaminic acid, which is the sugar used to modify the *H. pylori* flagellins (Schirm *et al.*, 2003). The Δ*hp0367:cat* mutation is likely to have polar effects on *hp0366*, which could account for the loss of motility. The Δ*hp0367:cat* mutant expresses both FlaA and FlaB, but appears to be able to export only FlaA (Fig. 6.1). These data suggest that either HP0367 plays a role in export of FlaB or glycosylation of FlaB is required for its export. Further characterization of the *hp0367-hp0366* operon and its products will allow us to test these hypotheses. In addition, strains will be constructed to determine if the genes of unknown function in the RpoN regulon

that do not have an apparent role in motility are required for colonization or other cellular functions.

Finally, continued work on the *flhA* alleles and their effects on flagellar gene expression are needed. Specifically, complementation analysis is needed to eliminate the possibility of an extragenic mutation. In addition, the two *flhA77* alleles that elicit different phenotypes and the regions upstream of these genes need to be sequenced to see if there are any differences in their sequence that could account for the phenotypic differences. Similarly, the *flhA454* allele needs to be sequenced to determine what additional primary structure is fused to the carboxy-terminus of the predicted FlhA truncated protein. We also need to verify that the *flhA* insertion mutants are expressing truncated FlhA proteins that are required for the enhanced expression of the RpoN-dependent reporter genes. The most convincing way of showing this would be to express the *flhA77* and *flh454* alleles in the Δ*flhA* strain, which we are presently attempting. Alternatively introducing the Δ*flhA* allele in the *flhA77* or *flhA454* mutants should result in loss of expression of the RpoN-dependent reporter genes. Such a result would argue strongly against the idea that an extragenic mutation was responsible for the phenotypes and support the prediction that the *flhA77* and *flhA454* mutants are expressing truncated FlhA proteins. Finally, the *flhA77* and *flhA454* alleles should be introduced in other strains, such as strain J99, to ensure that strain 43504 does not contain unique genes responsible for the observed phenotype.

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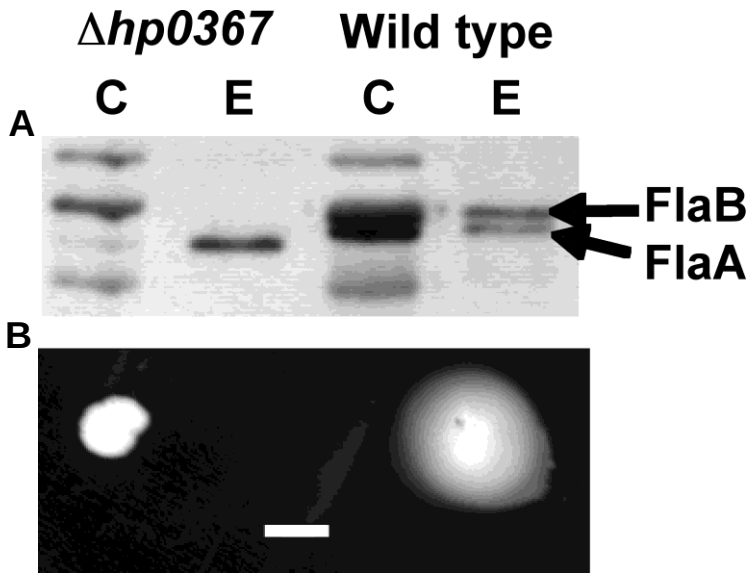

Figure 6.1 Phenotypes of a Δ*hp0367:cat* mutant compared to wild type. (A) Western blot analysis of flagellins FlaB and FlaA in soluble cytoplasmic (C) and extracellular (E) protein fractions of indicated *H. pylori* strains. Assay for export of flagellins was preformed as described previously (Smith *et al.*, 2009). (B) Motility assay showing *H. pylori* strains Δ*hp0367:cat* (left) and wild type (right). *H. pylori* strains were stab inoculated in semisolid (0.4% agar) Mueller-Hinton medium supplemented with 10% horse serum and incubated for up to 7 days in a microaerobic atmosphere at 37 °C. Scale bar is 5 mm.