CONTROL OF THE RpoN FLAGELLAR REGULON IN *HELICOBACTER PYLORI*

by

LARA ELIZABETH PEREIRA

(Under the Direction of Timothy R. Hoover)

ABSTRACT

In *Helicobacter pylori* essential flagellar components including rod and hook proteins, and the minor flagellin FlaB are dependent on σ^{54} for their expression. The novel *H. pylori* protein HP0958 was initially observed to interact with σ^{54} in a yeast two-hybrid assay. Inactivation of *hp0958* resulted in non-motile, non-flagellate phenotype. Expression of σ^{54}-dependent *flaB'-'xylE* and *orf1120'-'xylE* reporter gene fusions was reduced in the mutant; this decrease was due to lower σ^{54} levels. HP0958 was shown to influence σ^{54} at a post-translational level by reducing the turnover of σ^{54} in *H. pylori*.

Mutational analysis of *hp0906* revealed that it is functionally equivalent to the hook-length control protein FliK. *XylE*-reporter gene fusions indicated a stimulation of the RpoN regulon and a slight increase in the FliA (σ^{28}) regulon. Inactivation of the flagellar export apparatus component *flhA* resulted in downregulation of the FliA regulon, whereas the RpoN regulon was either stimulated or downregulated depending on the site of *flhA* inactivation. These data suggest that both the RpoN and FliA regulons are coupled to the function of the flagellar export apparatus. A model to explain this mode of regulation is proposed.

The sequence of σ^{54}-dependent promoters is highly conserved among bacteria. However, in *H. pylori*, there is a substitution of A for C at the –23 position. In other bacteria, this base change at –23 results in a reduction in promoter affinity. To determine if *H. pylori* has a preference for the base at this position, the promoter affinity of σ^{54} was examined. *Escherichia coli* maltose-binding protein fused to the amino-terminus of *H. pylori* σ^{54} enabled purification of a MBP-σ^{54} chimeric protein which bound preferentially to promoter sequences with an A instead of C at –23 when examined by electromobility shift assay. *In vivo* expression of *flaB'-'xylE* reporter gene with various base substitutions at –23 further demonstrated the different promoter specificity of *H. pylori* σ^{54}. Additionally, *H. pylori* σ^{54} displayed different affinities for its various promoters. Since FlgR, the transcriptional activator of σ^{54}-holoenzyme does not bind DNA to activate transcription, this suggests a means by which *H. pylori* differentially regulates expression of σ^{54}-dependent flagellar genes.

INDEX WORDS: *Helicobacter pylori*, flagellar synthesis, RpoN, σ^{54}, transcriptional activation, flagellar export apparatus, promoter specificity

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DEDICATION

This is dedicated to my parents, whose unconditional love, support and sacrifices made it possible for me to reach for the stars. You have my love and deepest respect.

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And finally for those of you who know me, this wouldn't be complete without a certain something…nothing but *disco* from here on out!!

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Chapter 1

Introduction and Literature Review

Overview of Microbiological Characteristics

Barry Marshall and Robyn Warren first isolated *Helicobacter pylori* in 1982 from gastric biopsy specimens of patients with peptic ulceration (109, 184). Initially classified as *Campylobacter pyloridis*, its name was later changed to *Campylobacter pylori* (108). Although *H. pylori* shares many similarities with *Campylobacter*, it differs in aspects such as fatty acid content and 16S rRNA sequence (59, 175). The bacterium was therefore transferred to a new genus, *Helicobacter*, and finally renamed *H. pylori* in 1989 (58). Thus far, the only known human reservoir of *H. pylori* is the human gastric environment. Related species, such as *H. mustelae* and *H. canis*, which infect ferrets and dogs respectively, have also been isolated (51, 170). By 1984, it became evident that *H. pylori* is responsible for peptic ulceration and chronic gastritis, and its infection can lead to serious health conditions such as gastric cancer and non-Hodgkin's lymphoma (20, 47, 113, 147). The study of this microorganism and its pathogenesis is therefore crucial to develop effective treatments for an infection. The following is a review of the basic microbiology of *H. pylori* which highlights the differences between this microorganism and many Gram-negative bacteria particularly in the area of flagellar biogenesis.

Morphology

H. pylori belongs to the epsilon subdivision of Proteobacteria, whose other members include the rumen bacterium *Wolinella succinogenes*, and *Campylobacter* species such as *C.*

jejuni and *C. coli*. It is a Gram-type negative, spiral-shaped bacterium having a length of 0.5 μm to 5 μm and is 0.5-1 μm wide (Fig. 1). The shape can vary, being predominantly rod-like when cultured on solid media and coccoid when grown on solid or in liquid medium for a prolonged period of time (22, 30). Although metabolically active, these coccoid forms cannot be cultured *in vitro* (22, 60). The coccoid form is thought to play a role in survival when the bacterium is subjected to nutrient starvation or antibiotic treatment.

Each cell has four to six unipolar sheathed flagella that are essential for motility. Each flagellum is about 30 μm long and 2.5 nm thick and possess a terminal bulb which is an extension of the flagellar sheath (56). This sheath is contiguous with the outer membrane and is thought to function in protecting the filament proteins from dissociation in the low pH environment of the stomach (56). The flagellar sheath is a bi-layered membrane composed of phospholipids, lipopolysaccharides, and proteins such as the flagellar sheath protein HpaA (56, 80). A glycocalyx or capsule-like layer has been observed, in addition to electron-dense granular, polyphosphate aggregates that may serve as an energy source (40).

Cell wall and lipopolysaccharide layer (LPS)

The cell surface of an *H. pylori* cell is relatively hydrophilic and negatively-charge (165). The physical organization of the cell envelope is similar to that in other Gram-type negative bacteria. However, the cell wall and outer membrane are unique in some aspects. As in other bacteria, *H. pylori* peptidogylcan consists of the amino sugars N-acetyl glucosamine and N-acetyl-muramic acid linked by a β-1,4 glycosidic bond. Adjacent glycan strands are cross-linked via di-aminopimelic acid (DAP)-D-alanine peptide cross-bridges (35, 37). However, unlike many Gram-type negative bacteria, DAP-DAP cross-links in the murein of *H. pylori* are absent

and its peptidoglycan also lacks muropeptide trimers and tetramers (35, 37, 48, 66). Compared to *E. coli* and some other Gram-type negative bacteria, *H. pylori* has a high proportion of muropeptides and short glycan chain lengths (35, 48, 66). This short glycan chain length and low peptide cross-linking of *H. pylori* may result in peptidoglycan that is weakly held together, a feature that may enable *H. pylori* to change its morphology from spiral to coccoid.

H. pylori has the ability to synthesize all precursors required for peptidogylcan assembly (39, 40, 48, 107, 145). It also has penicillin-binding proteins (PBPs) that are involved in the synthesis and modification of peptidoglycan (65). Initially, three high molecular weight PBPs, PBP1A, PBP2, and PBP3, were identified in *H. pylori*, although a fourth low molecular weight PBP, PBP4, in addition to five other potential PBPs have been described (38, 65, 95). High molecular weight PBPs have transglycolase and/or transpeptidase activities, while low molecular weight PBPs have carboxypeptidase and/or endopeptidase activites (65, 95). Some bacteria, such as *Escherichia coli*, are able to recycle peptidoglycan components (57). However, based on analysis of the *H. pylori* genome, it is uncertain if murein turnover occurs in *H. pylori*.

Lipopolysaccharide (LPS) is a key component of the outer membrane of Gram negative bacteria. Depending on the growth media used or the age of the culture, *H. pylori* produces two types of LPS . One of these is high-molecular weight smooth form LPS (S-LPS), which has an O side chain, core oligosaccharide and lipid A. S-LPS is produced by fresh isolates of *H. pylori*, while strains that have been repeatedly subcultured on solid media produce a low molecular weight, rough form R-LPS, that lacks the O side chain (129, 131). The core oligosaccharide contains a number of saccharides, including fucose, D-mannose, D-glucose and D-galactose. Structures for the lipid A components of both S-LPS and R-LPS have been elucidated (130). R-LPS has a larger proportion of lipid A than S-LPS, and both forms have lipid A that is

underphosphorylated and underacylated when compared with that from enterobacterial lipid A . This is thought to be the reason for the low endotoxic property of *H. pylori* LPS. The O antigen has extended chains with fucosylated and non-fucosylated N-acetyllactosamine units (12). Repeating units of O side chains have been shown to mimic Type 2 Lewis blood group antigens (Le^x and Le^y) in structure (12). Since these blood group antigens are expressed in the gastric mucosa of normal individuals, this mimicry may camouflage the bacterium and hence aid colonization.

H. pylori Genome

Two strains of *H. pylori*, 26695 and J99, have been sequenced using a whole genome random sequencing approach (8, 179). *H. pylori* strain 26695 was isolated from a gastric patient in the United Kingdom before 1987, and strain J99 was isolated in the USA in 1994 from a patient with duodenal ulcers (8). The genome sizes of *H. pylori* 26695 and J99 are about 1.67 Mb and 1.64 Mb, respectively (8). These genome sizes are similar to that of *Hemophilus influenzae* and are about a third of the genome size of *E. coli*. The G + C composition of both genomes averages 39% (9, 107, 179).

The *H. pylori* genomes from these two strains are highly conserved with respect to gene content, gene order and function. Sequence variation between these two strains usually occurs in the third position of a codon, therefore the primary sequence of the encoded protein is highly conserved. Approximately 1495 and 1552 open reading frames (ORFs) have been identified in strains J99 and 26695, respectively, and between 6-7% of these genes are specific to each strain (9). Thus there is some level of variation between these and other *H. pylori* strains. For instance, compared to strain NCTC11638, strains J99 and 26695 have deletions in certain genes of the cag pathogenicity island which encodes proteins involved in the production and delivery of the cagA

toxin to host gastric epithileal cells (8). Also, each *H. pylori* strain studied thus far contains its own restriction-modification system (8). Based upon sequence similarity to proteins of known function, about 58% of the gene products of both strains have been assigned a putative function. About 18% of the gene products are conserved but have no known function, and about 23% are specific to the *H. pylori* strains (9).

Close to 40 % of *H. pylori* isolates contain plasmids ranging in size from 1.5 kb to 23.3 kb, but thus far, none have been found to contain recognized virulence factors (9, 39). A few of these plasmids have been used to construct stable shuttle vector systems for gene transfer in *H. pylori* (69). A number of *H.pylori* strains are naturally competent for DNA uptake, with actively growing *H. pylori* cells being more competent than slow-growing or resting cells (46). This competency, combined with recombination events, provides a mechanism for the diversity observed in these species.

Replication, Transcription and Translation

H. pylori contains homologues to the subunits of DNA polymerase III, the enzyme required for DNA replication, including DnaE, DnaN, DnaX, DnaQ and HolB (8, 39, 40). *H. pylori* also contains the genes encoding homologues to all enzymes involved in initiation and DNA chain elongation with the exception of DnaC (9, 40). This protein has also been shown to be absent in other bacteria including *Mycobacterium tuberculosis* and the spirochete *Treponema pallidum* (8, 39, 40).

The transcription and translation apparatus is similar to those found in other Gram-type negative bacteria. The enzyme RNA polymerase is involved in synthesis of mRNA from a DNA template. This enzyme first interacts with a sigma factor to form a holoenzyme and is then directed to a particular promoter sequence to initiate transcription. There is a notable difference

in the RNA polymerase genes when compared to other known prokaryotes. The *rpoB* and *rpoC* genes, which encode the β and β` subunits of RNA polymerase in other bacteria, are fused in *H. pylori* (9, 19, 40). This gene fusion is also present in other helicobacters and in *W. succinogenes* but does not occur in *Campylobacter* species (194). In *H. pylori*, fusion of these β and β` subunits is not important for viability or virulence since a *H. pylori* strain in which *rpoB* and *rpoC* are separated by insertion mutagenesis is viable and able to colonize and multiply in C57BL/6 mice if (194).

Analysis of the J99 and 26695 genomes revealed three sigma factors – RpoD (σ^{80}; the housekeeping sigma factor in *H. pylori*), RpoN (σ^{54} or σ^{N}) and FliA (σ^{28}) (8, 19, 39, 40). *H. pylori* lacks other common alternative sigma factors such as the stationary-phase sigma factor (RpoS) or the heat shock sigma factor (RpoH) (19).

H. pylori has homologues to the three termination factors, NusA, NusB, and Rho (8, 179). Computational analysis of the complete genome using algorithm programs such as GeSTer (genome scanner for terminators) and RNAfold, have shown that inverted repeats followed by a stretch of T-residues are extremely rare in *H. pylori* (181, 185). Thus, transcription termination in this organism is likely to be primarily rho-dependent.

Translation components identified in *H. pylori* include 21 ribosomal proteins that constitute the 30S subunit of the ribosome and 31 orthologues of the 50S subunit proteins have been identified (8, 107). This microorganism also contains translation initiation factors, four peptide chain elongation factors (EF-G, EF-P, EF-Ts, and EF-Tu), and the three release factors, RF-1, RF-2 and RRF (8, 107).

H. pylori has the ability to produce all of the aminoacyl-rRNA synthetases with the exception of asparaginyl- and glutaminyl-tRNA synthetases (8, 39, 40, 107). Usually, both

$tRNA^{Glu}$ and $tRNA^{Gln}$ are amiacylated with glutamate by glutamyl-tRNA synthetase. Glutaminyl-tRNA can be produced by amidation of glutamate to glutamine (107). This process may occur in *H. pylori,* but it is possible that one of the two copies of glutamyl-tRNA synthetase genes may function as a glutaminyl-tRNA synthetase. A similar transamination process has been suggested for the formation of asparaginyl-tRNA synthetase from aspartate-charged tRNAs. However, more studies are required to clearly explain the mechanism of amidation of aspartate-charged tRNA and the formation of glutamine-tRNA.

Respiration and Metabolism

Respiration results in the formation of ATP from generation of a transmembrane proton-motive force. This conversion of the proton electrochemical gradient across the bacterial cytoplasmic membrane into ATP is done by ATP synthase. The genes for the membrane-embedded F_o proton-channeling complex (a, b and c subunits) and the catalytic F_1 (α, β, γ, δ and ε) are present in *H. pylori* (88). *H. pylori* maintains a constant proton motive force at external pH values between 3 and 7. The proton motive force drives not only ATP synthesis, but also flagellar rotation and solute uptake (88, 169). The biochemical and genome analysis of *H. pylori* suggest a simple respiratory apparatus involving dehydrogenases that include some NADPH-linked enzymes. The reducing equivalents are passed to menaquinone, then through cytochrome bc1 complex and soluble cytochrome c to a single oxidase (39, 88, 107, 169).

Metabolism in *H. pylori* appears to be to that of other aerobic respiring bacteria with the potential for significant ATP yields via oxidative phosphorylation. Analysis of the genome sequences show that *H. pylori* possesses homologs of genes for the Entner-Douderoff pathway, suggesting that glucose is used as an energy source, which is consistent with published data (8,

39, 107, 179). Pyruvate is produced primarily from lactate, L-alanine, L- serine and D-amino acids, rather than glucose or malate (8, 39, 107, 179). The tri-carboxylic acid cycle is similar to branched anaerobic tricarboxylic acid cycle in *E. coli*, with two exceptions: succinyl CoA is generated from 2-oxo-glutarate rather than succinate, and fumarate is generated from aspartate rather than malate (39, 107). Pyruvate can be fermented to acetate or ethanol owing to the presence of alcohol dehydrogenase activity (39, 107).

The gastric environment, which consists primarily of mucin, is unlikely to be of nutritional value to *H. pylori*. This bacterium is rather limited in its use of oxidizable carbon substrates, so it is possible that other energy sources are utilized by *H. pylori*. Indeed, it has been shown that *H. pylori* possesses a membrane-bound hydrogenase that releases electrons from hydrogen for further oxidation-reduction and energy-generating steps (143). The source of hydrogen is bacteria present in the large intestine. Fermentation reactions result in the production of compounds such as acetate and butyrate, with hydrogen gas as a by-product. The hydrogen gas produced has been shown to be present in the gastro-intestinal tract of rodents and humans, and since hydrogen is not an energy substrate for the host, it may serve as a significant energy source for *H. pylori*. This may play a role in the pathogen's ability to persist in the human host. Experimental data have shown that mutants unable to oxidize hydrogen are unsuccessful in the colonization of mice (143). It has also been demonstrated that *H. pylori* grows well in glucose-free medium supplemented with the amino acids arginine, aspartate, asparagine, glutamate, glutamine and serine as sole substrates, suggesting the bacterium has the ability to utilize amino acids as carbon and energy sources (115).

Another potential energy source for *H. pylori* is acetone, which is produced in the body by spontaneous decarboxylation of acetoacetate. Both *H. pylori* strains, 26695 and J99, have

been found to possess genes encoding a potential acetone carboxylase, which initiates acetone metabolism by converting it to acetoacetate (164). Acetoacetate can be further metabolized to acetyl-CoA and the genes for the conversion of acetone to acetyl-CoA are clustered. There are a number of differences in the central metabolism of strains 26695 and J99, such as the ability of J99 to ferment pyruvate to acetate, while 26695 cannot (39). Since both strains possess genes that encode a putative acetone carboxylase, it strongly suggests that acetone metabolism is important and may contribute significantly to the bacterium's ability to colonize and persist in its host.

H. pylori uses the dissociated fatty acid synthesis pathway and based on genome sequence analysis, its mode of fatty acid synthesis is similar to that of *E. coli* (87). *H. pylori* also has the genes required for C_2 or short-chained fatty-acid catabolism. However, no holomogs to genes needed for the oxidation of long chain fatty acids have been found in either J99 or 26695 (39, 87, 107).

Nutritional Requirements

H. pylori requires the amino acids arginine, histidine, leucine, isoleucine, valine, methionine and phenylalanine, which is consistent with genome sequence information (8, 39, 107, 157). These requirements, however, vary with the strain, with some strains also requiring alanine or serine for growth (157). *H. pylori* has the genes required to synthesize cofactors and vitamins, such as biotin, folate, heme, molybdopterin, pantothenate, riboflavin, thioredoxin and nicotinamide adenine dinucleotide (39, 107, 157). It also has the ability synthesize and hydrolyze polyphosphate for the storage and latter use of the essential element phosphorus (39). The sequenced strains of *H. pylori* have all the genes required for biosynthesis of cofactors and vitamins, except vitamin B_{12} and thiamine, which need to be supplemented for growth (39).

H. pylori lacks the enzymes for *de novo* synthesis of purines suggesting that it cannot make them from serine, glycine or formate (8, 39, 40, 107). However, it does have homologs to genes required for purine salvage as well as interconversion enzymes. It also has homologs to genes required for the *de novo* synthesis of UTP and CTP, and this has been demonstrated experimentally (8, 39, 40, 107).

H. pylori can incorporate nitrogen from urea into amino acids via the production of ammonia (107). It also has iron uptake systems such as the ferric citrate transport system and the TonB-dependent iron-uptake system (39, 76, 191). *H. pylori* does not appear to have the ability to produce siderophores . The heavy-metal transporter NixA is also produced by *H. pylori* and this is required for nickel ion uptake, which is essential for activity of the enzyme urease (15). *H. pylori* also has uptake systems for copper and molybdate, which are required in trace amounts (39).

In summary, *H. pylori* is capable of synthesizing the cofactors necessary for growth and can acquire important inorganic elements, although it is limited in its ability to use sulfur. It is auxotrophic for at least seven amino acids and for purines. It has no complex sugar transport system and lacks homologues to enzymes required for degradation of complex sugars, suggesting that complex sugars are not major energy sources. *H. pylori* requires complex basal medium with blood or serum supplementation for growth (64). A serum-free medium, which includes brain-heart infusion and 0.1-1% β-cyclodextrin also permits growth rates comparable to those in complex media (4, 26). In addition, a defined medium consisting of glucose, amino acids, vitamins and cofactors has been developed for the growth of *H. pylori* (157). It is grown under microaerobic conditions at 30-37^{o}C, with about 5 % atmospheric carbon dioxide. The reason that atmospheric carbon dioxide enhances growth is unclear, although it is speculated that

the presence of carbon dioxide may reflect the conditions present in the gastric environment, since carbon dioxide would be produced from bicarbonate excreted from surface epithelial cells and produced by the *H. pylori* urease enzyme. Also, two homologs of carbonic anhydrase are present in the genome sequences, suggesting that these putative enzymes may play a role in conversion of carbon dioxide to bicarbonate (89).

Pathogenesis

The costs associated with the diseases caused by an *H. pylori* infection in the United States are estimated at almost $6 billion annually (167). The deaths associated with peptic ulcer disease average approximately 6500 per year (167). Understanding the pathogenesis of this microorganism is crucial for the development of new and effective treatments.

Epidemiology

H. pylori infection occurs worldwide. The prevalence of infection differs in various parts of the world, with a higher number of cases in the developing countries. Both males and females are infected, and some studies have shown that both sexes are infected at about the same rates, but one study suggested that males have a slightly higher risk for infection (156, 176).

An *H. pylori* infection is usually naturally acquired in childhood and once established within the gastric mucosa it can persist for life if left untreated (40, 176). *H. pylori* causes gastric inflammation, peptic ulcer disease, and has been linked to gastric cancer and non-Hodgkin's lymphomas of the stomach (20, 21, 47, 113, 146, 147). The nature and severity of the disease depends on host characteristics, bacterial strain and environmental factors.

Possible sources of *H. pylori* infection are thought to be animals and water supplies (40, 126). Although the mode of transmission is not clearly understood, several routes have been

described. The first, and perhaps least common, involves the introduction of endoscopes or tubes from an infected person to another individual. Endoscopists or gastroenterologists are thought to be more at risk for *H. pylori* infection (2, 40, 126). *H. pylori* has been isolated from dental plaque and saliva, so oral-oral transmission may be a possibility (31, 93). Fecal-oral transmission has also been suggested, although conclusive evidence remains to be found (126, 178). *H. pylori* also converts to a coccoid form which has been reported to survive for several years in river water, and it has been suggested that water contaminated with these coccoid forms is a possible source of *H. pylori* infection (75, 126).

Colonization and Persistence

Several factors are involved in the colonization, adhesion and virulence of *H. pylori*. Animal models have been used to demonstrate *H. pylori* virulence factors such as urease and flagella, which are discussed in more detail below. Other *Helicobacter* species, which include *H. felis* and *H. mustelae*, infecting cat and ferrets, respectively, have been used extensively as animal models (50, 51, 101). Gnotobiotic piglets have been used as an animal model for *H. pylori*, as have Mongolian gerbils (43, 44, 186). These animals display similar patterns to a human infection, exhibiting gastritis, ulceration, and carcinoma (101, 186). Therefore the use of these animal models has shed considerable light on the colonization and/or virulence factors of *H. pylori.* Some of these factors are discussed below.

(1) Motility

Motility due to flagella enable *H. pylori* to move through the viscous environment of the mucin layer that lines the stomach. *H. pylori* mutants that either lack or have non-functional flagella are severely impaired in their ability to establish a successful infection in gnotobiotic

piglets (44, 106, 169). Motility is pH dependent and does not occur at a pH below 4 (125). Enhanced motility has been observed at pH 5 when compared to motility at a neutral pH (116). The structure and biogenesis of *H. pylori* flagella will be discussed in more detail in the latter sections.

(2) Urease

The 550-kDa enzyme urease produced by *H. pylori* enables the bacterium to maintain a pH-neutral microenvironment (42). It catalyzes the hydrolysis of urea to produce ammonia and carbamate, the latter compound decomposing to another molecule of ammonia and carbonic acid; the net effect of these reactions is an increase in pH. The genes encoding *H. pylori* urease and urease accessory proteins include *ureABIEFGH* (33, 99). With the exception of *ureI*, all the genes share homology with urease genes from bacteria such as *Klebsiella aerogenes* and *Ureaplasma urealyticum* (128). The genes *ureA* and *ureB* encode the two structural units of the enzyme, and nickel ions are present in the active site. The accessory genes *ureI*, *ureE*, *ureF*, *ureG* and *ureH*, are essential for synthesis of a catalytically active urease (33, 99). These genes encode proteins that interact with the enzyme and aid in the delivery of nickel ions to the active site (128). In addition, two accessory genes *hypA* and *hypB* have been shown to be required for urease activity (144). Mutations in these genes affect the activity of the membrane-bound nickel-containing hydrogen uptake hydrogenase enzyme, which results in a deficiency in nickel sequestration and thus urease activity. Urease activity is lost at a pH below 5, which raises questions about its localization (128, 159). Studies have demonstrated that a significant portion of urease is present in the cytoplasm and that a fraction of it is associated with the outer membrane (41, 149). Urease is released as a result of autolysis of a fraction of *H. pylori* cells and

becomes adsorbed to the surface of the remaining intact bacteria. *H. pylori* containing only cytoplasmic urease is susceptible to acid (94).

(3) Oxidative Stress Enzymes

To combat oxidative stress that it encounters in the stomach environment, *H. pylori* possesses a number of enzymes, which include superoxide dismutase (SOD), catalase, alkylhydroperoxide reductase and thioredoxin-linked thiol peroxidase (40, 139, 142, 148). The enzyme SOD breaks down superoxide produced in polymorphonuclear leukocytes and macrophages. Catalase protects the bacterium against the damaging effects of hydrogen peroxide which is released from phagocytes. Alkylhydroperoxide reductase catalyzes the reduction of alkylhydroperoxide to the corresponding alcohol. Thiol peroxidase removes hydrogen peroxide in a metal-catalyzed oxidation reaction to protect enzymes sensitive to oxidative stress. An additional enzyme, methionine sulfoxide reductase (Msr) is also involved in oxidative stress resistance in *H. pylori*. Msr belongs to the group of oxidoreductases that reduce oxidized sulfur residues in methionines (133). An *msr* mutant of *H. pylori* is sensitive to oxidative stress agents such as paraquat and it is compromised in its ability to colonize the gastric mucosa of mice (3). The MdaB protein has also been shown to play a role in oxidative stress resistance and *mdaB* mutants are deficient in the colonization of mouse stomachs (183).

(4) **Adhesins**

Outer membrane proteins, phospholipids, glycolipids and blood group-antigen-binding adhesins have all been shown to be involved in adherence to either mucin or gastric mucosa epithelial cells (145). *H. pylori* adhesins bind to several carbohydrate ligands on epithileal cells. These include BabA, which binds to fucosylated glycoproteins, and a protein that binds to laminin, a basement membrane sialylated glycoprotein. Studies have also shown that *H. pylori*

LPS is involved in adherence to gastric epithileal cells (182). In addition to epithileal cells, *H. pylori* neutrophil-activating protein can bind to mucin that is sulfated and/or sialylated (134).

(5) Cytotoxin-associated gene antigen

Another factor involved in the pathogensis of *H. pylori* is the cytotoxin-associated gene antigen which is encoded by *cagA*. Although CagA has been extensively studied, its exact mode of action is still unclear. Studies have shown that CagA is secreted from *H. pylori* into the cytoplasm of host epithelial cells to which the bacteria are attached. A type IV secretion system encoded by the cag pathogenicity island is involved in this process.

Once inside the host cell, CagA is phosphorylated at a specific tyrosine residue, although the host cell kinase involved remains to be identified. Upon phosphorylation, the CagA toxin is believed to affect cytoskeletal plasticity and signal transduction within the host cell. It is thought that cag pathogenicity island is linked to the bacterium's ability to cause gastric cancer.

(6) Mucinase

Mucins are high molecular weight, oligomeric proteins that are sulfated and glycosylated. These glycoproteins are a major component of the mucous layer present near the gastric epithelial cells (177). Mucins usually protect epithelial cells by preventing colonization of microbes. Gastric mucin prevents *H. pylori* from adhering to gastric epithelial cells by competing with the cell surface for *H. pylori* antigens (180). Although *H. pylori* mucinase activity has not been shown to be important for colonization or virulence, its ability to weaken the mucous barrier may contribute to *H. pylori* colonization. The exact mode of action against mucin is unclear, but a study by Slomiany and co-workers suggested that *H. pylori* produces a protease that has endopeptidase activity which cleaves regions of the mucin glycoprotein molecule that lack carbohydrates, resulting in the formation of glycopeptides (163). However, another study

demonstrated that degradation of mucin may be the result of the destabilizing effects of the pH change that occurs at the mucosal surface when *H. pylori* urease hydrolyses urea (161). Further research is required to elucidate if *H. pylori* does possess mucinase activity.

(7) Other factors involved in colonization, persistence, and virulence

H. pylori causes tissue damage by induction of gastric inflammation and disruption of the gastric mucosal barrier (20, 21). Phospholipase A digests phospholipids of cell membranes (100). Other toxins include the vacuolating cytotoxin A, VacA, which produces vacuoles in gastric epithelial cells (36). This vacuole formation compromises the mucosal barrier and when the epithilial barrier has been disrupted, *H. pylori* causes a significant increase in epithileal cell apoptosis. LPS also mimics Lewis blood group antigens and may be involved in regulation of the immune response (145).

There are other factors that affect the gastric epithelial layer. One such factor is interleukin 8 (IL-8). IL-8 is a small peptide secreted by the cell and is a potent inflammatory mediator that recruits and activates neutrophils resulting in inflammation that is responsible for superficial gastritis (40). *H. pylori* strains are able to induce IL-8 secretion from gastric cells from both gastric biopsies and cell lines (73). The host platelet activating factor (PAF) is also involved in pathogenesis. PAF is a phospholipid mediator and stimulates gastric acid secretion. *H. pylori* can metabolize the non-ulcerogenic precursor lyso-PAF that is produced normally in healthy persons, to PAF, which is a potent ulcerogenic agent (166).

The production of nitric oxide by inducible nitric oxide synthase induction in macrophages and gastric epithelial cells is also associated with immune activation and tissue injury (40, 107). An *H. pylori* infection stimulates the production of the hormone gastrin which in turn stimulates

parietal cells in the stomach to hypersecrete acid (111). Table 1 summarizes the various mechanisms by which *H. pylori* damages the gastric mucosa.

Treatment

Therapy for an *H. pylori* infection involves ten days to two weeks of one or two effective antibiotics, such as amoxicillin, tetracylcine, metronidazole, or clarithromycin (112). Other treatments include rantidine bismuth citrate, bismuth subsalycilate, or a proton pump inhibitor (112). Acid suppression by proton pump inhibitor in conjunction with the antibiotics helps alleviate ulcer-related symptoms (i.e., abdominal pain, nausea), helps heal gastric mucosal inflammation, and may enhance efficacy of the antibiotics against *H. pylori* at the gastric mucosal surface.

Currently, eight *H. pylori* treatment regimens are approved by the United States Food and Drug Administration (FDA). However, several other combinations have been used successfully here in the USA. Antibiotic resistance and patient noncompliance are the two major reasons for treatment failure. Eradication rates of the eight FDA-approved regimens range from 61% to 94% depending on the regimen used. Overall, triple therapy regimens have shown better eradication rates than dual therapy. In addition, FDA studies have shown that a longer length of treatment (14 days versus 10 days) results in better eradication rates (http://www.cdc.gov/ulcer/md.htm#treatment).

Flagellar Structure and Assembly

A single *H. pylori* cell usually has four to six unipolar sheathed flagella, each of which is about 30 μm long (56). The sheath is an extension of the outer membrane and is believed to

prevent the depolymerization of flagellin proteins in the surrounding acidic environment and is also thought to mask flagellar epitoopes (56). A 29 kDa protein HpaA is specifically localized to the flagellar sheath. Although initially thought to be a hemagglutinin, it was later proven that the protein is not an adhesin for erythrocytes or gastric epithelial cells (79).

As occurs in most Enterobactericiae, *H. pylori* flagella consist of three main parts: the basal body, which contains proteins required for movement and is embedded in the cell envelope; the hook, which is a flexible structure that links the basal body to the third component, the external curved flagellar filament (169). Rotation of the rod within the basal body is transferred to the filament via the hook, resulting in the propagation of waves that move along the length of the filament and push against the surrounding medium to propel bacteria in the opposite direction (105, 169). The structure of a single flagellum is illustrated in Figure 2.

Flagellar assembly in bacteria is a highly ordered process and has been studied extensively in organisms such as *E. coli* and *Caulobacter crescentus.* The first component of the flagellar apparatus to be assembled is the MS ring, which is formed in the cytoplasmic membrane and is encoded by *fliF* (81). The MS ring acts as a base for the proteins FlgB, FlgC and FlgG, which make up the flagellar rod, and it also acts as a mounting plate for the flagellar export apparatus (72). In addition, it serves as the anchor for the motor rotation proteins and motor switch proteins. The P and L rings are formed in the periplasmic space and outer membrane, respectively, and are encoded by *flgI* and and *flgH* (81). The proteins of the P and L rings are secreted via the Sec pathway. The hook protein is encoded by *flgE* and the hook-associated protein FliD, is localized at the tip of the filament and promotes elongation of the filament (90, 138). In some bacteria such as *E. coli* and *Salmonella enterica* serovar Typhimurium, the filament is composed of a single flagellin FliC, whereas *Vibrio cholerae* has

five flagellins, of which only one is essential for motility (105, 110). In *H. pylori*, the filament is composed of a major flagellin protein FlaA and minor protein FlaB. Both flagellins are required for full motility and colonization (85, 103, 172).

The proteins of the filament, hook and rod are secreted by a pathway specific for flagellar proteins, the type III secretion system. Proteins exported by this pathway lack signal peptides and do not undergo peptide cleavage (120).The flagellar export apparatus has been well studied in bacteria like *S. enterica* serovar Typhimurium and *E. coli*, and consists of six membrane proteins, FlhA, FlhB, FliQ, FliP, FliO and FliR, and three cytoplasmic proteins, FliH, FliI and FliJ (120) . The membrane components are thought to be located in a central pore within the basal-body MS-ring. FliI is an ATPase which provides the energy required for the translocation of flagellar proteins (45). FliH is a regulatory protein that prevents FliI from hydrolyzing ATP until the flagellar export apparatus is competent to transport flagellar proteins (122). FliJ is a general chaperone protein that prevents premature aggregation of export substrates in the cytoplasm (120).

In enteric bacteria, proteins that are known to be exported through the flagellar export apparatus include the basal body proteins (FlgB, FlgC, FlgF and FlgG), hook and hook-associated proteins (FlgE, FlgD, FlgK and FlgL), filament proteins and the regulatory protein FlgM (anti-σ^{28} factor) (70, 71, 97). Recently, two additional export substrates have been identified – FlgJ, a muramidase that is thought to puncture the peptidoglycan layer allowing growth of the flagellum, and FliK, a protein involved in hook-length control (118, 119, 121, 135). Once transported through the export apparatus, the flagellar proteins assemble at the distal end of the nascent structure (105).

Initially, rod/hook-type substrates are exported. The completion of the hook is sensed by the C-terminal domain of FliK and this information is communicated to the C-terminal cytoplasmic domain of FlhB, causing a conformational change in FlhB and a switch in specificity from rod/hook-type substrates to filament-type substrates (70, 71, 97, 118, 119, 121, 123). It is not clear how FliK senses a completion of the hook structure, but it is proposed that the N-terminal of FliK senses the appropriate hook length and transmits this information to its C-terminal, possible via a structural change (123). This results in a productive interaction between the C-termini of FliK and FlhB causing a switch in substrate specificity. Mutants lacking FliK are non-motile and produce abnormally long hook structures referred to as polyhooks (123).

In *H. pylori*, an understanding of the flagellar assembly is still relatively limited. At least forty proteins are predicted to be involved in flagellar biosynthesis, some of which are listed in Table 2. Some flagellar genes of unknown function have been proposed to be paralogs of well characterized proteins. For instance, *orf0908* (*flgE`*) is thought to be a *flgE* paralog, while *orf1126* (*flaB`*) is suggested to be a paralog of the minor flagellin *flaB*. Inactivation of *orf0908* resulted in a non-motile phenotype in *H. pylori*, suggesting an essential role in motility (Brahmachary, P., unpublished data).

Studies have established the function of several flagellar genes in *H. pylori* including *flgE*, *fliD*, *flaA* and *flaB* (90, 103, 136, 138, 168, 172). Experiments have also identified and characterized the function of all but two components (*fliR* and *fliO*) of the export apparatus in *H. pylori*, which include *fliH, fliI, fliP, fliQ, flhA* and *flhB* (6, 8, 78, 83, 136, 151, 160, 179). A *fliJ* homolog has not been identified in *H. pylori* yet. As with other flagellar genes in *H. pylori*, most of the genes encoding components of the flagellar export apparatus are scattered through out the

genome, unlike *S. typhimurium* and *E. coli*, where many flagellar genes are clustered in well-defined genomic regions.

Chemotaxis and Mechanism of Motion

H. pylori is thought to move actively toward the gastric epithelial layer to prevent being washed away by mucous flow and avoid cellular damage caused by stomach acid (127, 190). Experimental data have shown that *H. pylori* exhibits chemotactic activity towards compounds such as urea, sodium bicarbonate and a few amino acids such as glutamine and histidine (127, 169). Mucin is also a chemoattractant , but since this is a compound having a fairly high molecular weight, it is likely that breakdown products of mucin are responsible for the chemotactic activity shown by *H. pylori* (161).

The biological system involved in chemotaxis has been well studied in *E. coli* and *S. typhimurium* (1, 10). Response of the bacterial cell to attractants or repellents is mediated by the methyl-accepting chemotaxis proteins (MCPs). The MCP consists of a periplasmic ligand interaction domain and a cytoplasmic signaling and adaptation domain (1, 10). Other proteins involved in this system include CheA, CheY, CheW and CheZ. CheA and CheY are part of a two-component system, with CheA being the sensor histidine kinase and CheY the response regulator. CheW is the receptor-coupling factor and CheZ enhances the dephosphorylation of CheY-phosphate (1, 10).

When a MCP binds a ligand, a conformational change occurs in the cytoplasmic domain of the protein and this change is recognized by a CheA-CheW complex, which is bound to the MCP by CheW (1, 10). If a repellent binds to a MCP, autophosphorylation of CheA occurs and phosphate is transferred to CheY. CheY-phosphate then binds directly to FliM, which is present

in the flagellar motor switch complex, and clockwise rotation of the flagellum occurs resulting in tumbling of the cell (1, 10). If an attractant binds the MCP, phosphorylation of CheA is suppressed, leading to less CheY-phosphate. CheZ also increases the conversion of CheY-phosphate to CheY (1, 10). The result of decreased CheY-phosphate levels is that the cell tumbles less and the runs towards the attractant increase.

Approximately nine chemotaxis genes in *H. pylori* have been identified. These include genes encoding a bifunctional CheAY protein, CheY, CheW, three MCPs (*tlpA, tlpB* and *tlpC*) and three CheV proteins (18, 52, 179). CheV, which has been previously identified in *Bacillus subtilis*, contains an amino-terminal CheW homologous domain linked to a response regulator domain of the CheY family. The three MCPs of *H. pylori* are predicted to be membrane-bound and associate with the cytoplasmic proteins CheA and the linker protein CheW (18). In *E. coli,* chemoreceptors and their associated cytoplasmic proteins cluster predominantly at the cell poles (104, 162). No correlation has been observed between the localization of clusters and the direction of swimming. The role of this clustering in unknown, but it may enhance communication between chemoreceptors or mediate signal amplification. It would be of interest to determine if MCPs in *H. pylori* are clustered in a similar manner and if they are localized to the flagellated pole.

Homologs of the methyltransferase CheR and the methylesterase CheB, which are involved in the chemotactic adaptation response, are absent in *H. pylori* (52). The absence of CheR and CheB in *H. pylori* suggest that the adaptive response of this bacteria to chemotactic signals is different from the well established chemotaxis pathway in *E. coli* and *S. typhimurium*. An ortholog of CheZ, which stimulates the dephosphorylation of CheY-phosphate is also absent from *H. pylori*(52). The functions of *H. pylori* chemotaxis proteins are summarized in Table 3.

Overview of Flagellar Gene Regulation in *H. pylori*.

All three sigma factors found in *H. pylori*, σ^{80}, σ^{28}, and σ^{54}, are involved in the transcription of different sets of flagellar genes (16, 168, 179). The housekeeping sigma factor, σ^{80}, regulates transcription of genes encoding some of the components of the basal body, proteins required for the export of flagellar proteins, and those involved in chemotaxis (18, 151, 168). The alternative sigma factor σ^{28} controls transcription of the major flagellin gene (*flaA)*, the filament cap protein (*fliD)*, *flaG* (function unknown) and the flagellin chaperone protein (*fliS)* (90, 103, 179). The other alternative sigma factor, σ^{54}, is involved in the regulation of genes encoding the structural proteins of the rod, the hook, and the hook-associated proteins, as well as the minor flagellin FlaB (138, 168). Transcription of σ^{54}-dependent genes requires the activator FlgR, which is a homologue to the NtrC family of transcriptional activators (168). The activator FlgR is part of a two-component signal transduction system and is phosphorylated by the sensor kinase FlgS. Unlike most other σ^{54}-dependent activators, FlgR lacks the DNA-binding domain and does not bind an enhancer or upstream activation sequence to stimulate transcription of its target genes (23). FlgR is encoded within an operon that has been suggested to be expressed constitutively (168). The FlgS gene appears to be part of a later class of flagellar operons which may contribute to the temporal regulation of σ^{54}-dependent flagellar genes.

Studies have recently evaluated the role of the flagellar protein export apparatus in transcription of flagellar genes. There is evidence to suggest a link between the export apparatus and the expression of flagellar genes. In *H. pylori*, mutations in *flhA* and *flhF* affected both the RpoN and FliA regulons (136). FlhF belongs to the signal recognition particle family, which has a role in polar flagellar placement and flagella numbers (86). Inactivation of *flhA* and *flhF* in *H. pylori* resulted in a reduction in of transcripts of the RpoN and FliA regulons (136). A similar

trend was seen in the close relative *C. jejuni*. The use of a reporter gene demonstrated that a mutation in *flhA, flhB* or *fliP*, each resulted in a 50- to 1000-fold reduction in σ^{54}-dependent gene expression (68). However, σ^{28}-dependent *flaA* expression was not affected. It is unclear if these mutations directly affect flagellar gene expression or if unknown components, that would otherwise be exported through the apparatus, accumulate and negatively affect flagellar synthesis. Alternatively, the export apparatus could communicate with the FlgS/FlgR two component system. Since FlgS is a cytoplasmic protein, it is possible that it interacts with components of the flagellar protein export apparatus, resulting in autophosphorylation of FlgS, phosphate donation to FlgR and the subsequent stimulation of the RpoN regulon.

Additional levels of flagellar gene regulation are also present in *H. pylori*. An anti-σ^{28} factor FlgM was identified. Its interaction with σ^{28} inhibits transcription of σ^{28}-dependent genes (34). The study which demonstrated the reduction in RpoN-dependent transcripts in a *flhA* mutant background also showed that a *flhA/flgM* double mutant resulted in levels of RpoN-dependent transcripts higher than wild-type levels, suggesting that FlgM influences the RpoN regulon possibly by accumulating at the export apparatus of the *flhA* mutant and blocking access to FlgS (136). This observation further highlights the complexity of flagellar gene regulation in *H. pylori.* One of the objectives of this study is to investigate the role of the export apparatus in flagellar gene expression in *H. pylori*.

In addition to the role of regulatory proteins like σ^{54}, FlgR and FlgM in flagellar biogenesis, environmental factors may also be involved in regulation of these genes. It has been shown that conditions such as growth medium, certain salt concentrations, and even temperature, affect transcription of *flaB* in *Campylobacter coli*, a close relative of *H. pylori* (7). In *H. pylori*, growth medium supplements did not affect the transcription of flagellin genes (116). However,

microarray analysis suggests that a lower pH results in an increase in *flaB* transcription, while results based on a *flaB*-reporter gene fusion showed no change in expression at a low pH (5, 137). Although there is no direct evidence for the effect of environmental factors of *H. pylori* flagellar gene expression, it is likely that this organism varies its flagellin content to alter the physical properties of the flagellum so that it can adapt to changes in pH and viscosity in the gastric environment.

A more detailed discussion of the three sigma factors mentioned above and their role in flagellar gene regulation will be done in a later section. Genes involved in flagellar gene regulation are listed in Table 4.

Flagellar Gene Transciptional Hierarchies

Where it has been examined extensively, the expression of flagellar genes is controlled by a transcription hierarchy. In *S. typhimurium*, expression of class I genes in response to an unknown cellular cue initiates flagellar biogenesis. Class I genes encode two master regulatory proteins, FlhC and FlhD, which are required for the expression of class II genes (14, 91). Transcription of class II genes is mediated by σ^{70}-RNA polymerase holoenzyme (91). Class II genes encode basal body proteins and hook proteins, as well as σ^{28} and an anti-σ^{28} factor, FlgM (105, 140). Upon completion of the basal body-hook structure, FlgM is exported out of the cell via this flagellar structure (74). The resulting decrease in FlgM levels allow σ^{28}-RNA polymerase to initiate transcription of the class III flagellar genes which includes genes for chemoreception and the flagellin gene (74, 141).

In *C. crescentus*, an early signal in the cell cycle, perhaps DNA replication, initiates activation of the master trancriptional regulator CtrA (153, 192). CtrA activates expression of

class II genes in conjunction with σ^{70}-RNA polymerase holoenzyme, the primary RNA polymerase holoenzyme in *C. crescentus* (192). This results in the production and assembly of the MS-ring and motor switch complex, as well as the expression of σ^{54} and the σ^{54}-dependent activator FlbD. FlbD and σ^{54} are required for the expression of the class III and class IV genes, which include the basal body-hook complex and flagellar filament proteins, respectively. FlbD, which is phosphorylated by the histidine kinase FlbE, is thought to concentrate at the mid-cell sight and at the pole in predivisional cells. Later in the cell cycle FlbE, which is in the swarmer compartment, autophosphorylates and transfers phosphate to FlbD, resulting in swarmer compartment-specific expression of late flagella promoters (153). Two check points are present in the assembly of flagellar components – completion of the MS-ring complex is required for expression of class III genes and completion of basal body-hook complex is required for transcription of class IV genes (192).

The expression of flagellar genes in *H. pylori* shares some similarities with the hierarchies described for *S. typhimurium* and *C. crescentus*. A model for flagellar biogenesis in *H. pylori* is illustrated in Figure 4. Like *C. crescentus*, *H. pylori* σ^{54} and its activator FlgR is required for expression of the basal body-hook complex (168). However, like *S. typhimurium*, another alternative sigma factor, σ^{28}, is required for expression of *flaA* and filament-associated proteins (90, 103). Flagellar biosynthesis in *H. pylori* is most similar, but not identical, to that of the human pathogen *Vibrio cholerae*. Like *H. pylori*, flagellar biogenesis in *V. cholerae* involves both σ^{54} and σ^{28} (152). Unlike *H. pylori, V. cholerae* has two σ^{54}-dependent activators, FlrA and FlrC (152). FlrA, which is the product of a class I gene, activates σ^{54}-holoenzyme allowing expression of class II genes which encode structural components such as the MS ring, switch, export apparatus, FliA and FlrC. FlrC, along with σ^{54}-holoenzyme then activates expression of

class III genes which include the basal body, hook and filament genes (152). Class IV genes, which include the motor genes and additional filament genes, are σ^{28}-dependent (152).

It is not clear if a check point(s) in flagellar assembly exists in *H. pylori*, although there is likely to be one. Evidence in support of this is that mutations in genes encoding components of the secretory apparatus and the MS-ring result in a significantly lower level of expression of *flaA, flaB* and *flgE*, suggesting that completion of this apparatus is a check point for expression of σ^{54}- and σ^{28}-dependent genes (151). Further research is required to elucidate if other possible check points exist.

Sigma Factors

Sigma factors are proteins that bind reversibly to core RNA polymerase, which is the catalytic form of RNA polymerase (67). Association of core RNA polymerase with a sigma subunit forms a holoenzyme that can recognize specific promoter sequences (62, 67). Bacteria usually contain multiple sigma factors, each confering a different sequence specificity to the holoenzyme. One of these sigma factors is the "housekeeping" sigma, which is responsible for transcription of most of the genes within the bacterium, while the other sigma factors are alternative sigma factors that are involved in expression of specific sets of genes.

Three sigma factors have been identified in *H. pylori*: σ^{80} (RpoD), σ^{54} (RpoN) and σ^{28} (FliA) (8, 168, 179). *H. pylori* lacks homologs of the stationary phase sigma factor RpoS (σ^{S}), as well as the heat-shock protein-specific RpoH (σ^{H}) (8, 19, 179). The primary sigma factor in *H. pylori* is σ^{80} and is essential for the bacteria, while σ^{54} and σ^{28} appear to be involved only in flagellar synthesis (19, 82, 168). The three sigma factors and their role in gene regulation in *H. pylori* are discussed below.

Sigma 70 Family

The 78 kDa sigma factor is the primary sigma factor in *H. pylori* and is referred to as σ^{80} (19). As such, σ^{80} is essential for viability in *H. pylori* (17). Based on studies of *E. coli* σ^{70}, the primary sigma factor in this bacterium consists of four regions (Figure 5) (17). Regions I and II are involved in DNA melting (62). Regions II and IV recognize and bind to the –10 and –35 regions, respectively, of the promoter sequence (62)(Fig. 5). In *E. coli*, the consensus sequence of the –10 region is TATAAT, while the –35 consensus is TTGACA (62). In *H. pylori* however, the sequences of these two regions differ considerably from the *E. coli* consensus sequence (19). For instance, the sequence of the –10 region of *vacA* is TAAAAA, sharing only four out of the six base positions of the *E. coli* consensus sequence. The –35 sequence of *vacA* gene lacks consensus altogether. Furthermore, when the –10 sequence of *vacA* in *H. pylori* is changed to that of the consensus sequence, the resulting mutant shows a 15-fold lower transcription level relative to the wild-type –10 sequence (49). This suggests that *H. pylori* σ^{70} has a different promoter specificity. Morever, recognition of the *tac* promoter is very poor with *H. pylori* σ^{80}-holoenzyme when compared with the recognition shown by *E. coli* σ^{70}-holoenzyme (17). This difference in promoter recognition may be related to the spacer region between regions I and II of *H. pylori* σ^{80} (17). The spacer region (amino acids 209-454), which connects subregions 1.2 and 2.1, has an important role in promoter recognition. It was originally called "spacer" because it was believed to have no other role than that of connecting functional domains. When the spacer region segment in *E. coli* σ^{70} is substituted with that from *H. pylori* σ^{80}, the resulting protein is unable to drive transcription from *E. coli* promoters (17). However, when the spacer segment of σ^{80} is replaced with that from *E. coli* σ^{70}, the recognition of *E. coli* promoters is substantially improved (17). This suggests that the spacer segment of *H. pylori* is

distinct in that it is incompatible with the transcriptional machinery of *E. coli* but is adapted to regulate the efficiency of transcription initiation in its own ecological niche.

H. pylori σ^{80} is required for the transcription of some flagellar genes (Table 4). Studies have shown that the *fliI-fliQ* operon (encodes flagellar export proteins) and the stress-responsive operon containing *cheY* are regulated by σ^{80} (18, 151). In addition, sequence analysis of the *H. pylori* genome suggests that σ^{80} also regulates expression of chemotaxis genes and genes encoding components of the basal body (8, 179). Table 5 lists some of the σ^{80}-dependent promoters in *H. pylori*. The differences in the –10 and –35 sequences of these promoters versus the consensus sequences are highlighted.

Sigma 28 Family

H. pylori σ^{28} (FliA) recognizes the –10 and –35 region of promoter sequences upstream of specific flagellar genes. Unlike the primary sigma factor σ^{80}, region 1, which is the activation response domain, is absent in σ^{28} (61, 67). However, the highly conserved region 2, as well as regions 3 and 4 are present (61, 67). σ^{28} is involved in the transcription of class III flagellar genes, which include the major flagellin gene *flaA*, as well as the *fliD* operon, which includes the genes *flaG, fliD* and *fliS*. FlaG is a polar flagellin, while FliD is the filament capping protein, and FliS is essential for flagellar elongation (90, 103, 179).

H. pylori contains an anti- σ^{28} factor HP1122, which is similar to *S. typhimurium* FlgM in its mode of action (34). Analysis of the *H. pylori* genome sequence initially failed to identify a FlgM homolog. The degree of sequence identity between FlgM from *Bacillus subtilis* and *S. typhimurium*, however, is rather low, so it is not surprising that a FlgM homologue in *H. pylori* could not be identified based on sequence similarity. A high throughput yeast two-hybrid screen

identified a specific 8.6-kDa protein, HP1122, that interacts with σ^{28} (34). Closer inspection of the C-terminal portion of HP1122 revealed limited homology with FlgM, and this portion of HP1122 was shown to interact with region IV of σ^{28} (34). Inactivation of *H. pylori orf1122* resulted in a greater than two-fold increase of *flaA* transcription (34). In addition, overexpression of HP1122 in *H. pylori* decreased the level of *flaA* transcription to half of that observed in the wild-type strain (34, 82). These observations suggest strongly that HP1122 is an anti-σ^{28} factor; hence this protein is referred to as FlgM.

An anti-sigma factor can inhibit transcription either by blocking interaction between the sigma factor and the core RNA polymerase or by preventing promoter recognition by the sigma factor. Using a three-hybrid system consisting of FlgM, σ^{28} and RNA polymerase, it was found that HP1122 prevents the interaction between σ^{28} and the β-subunit of RNA polymerase (34). This is similar to FlgM action in *S. typhimurium*, where FlgM binds to σ^{28} and interferes with σ^{28}-holoenzyme formation (34, 82, 98, 104).

H. pylori FlgM lacks the N-terminal region found in *S. typhimurium* FlgM, with the protein from *H. pylori* strain 26695 missing the first 20 amino acid residues and the protein from *H. pylori* strain J99 lacking the first 29 amino acid residues found in *S. typhimurium* FlgM (34, 82). FlgM is exported out of the cell through the flagellar type III secretion apparatus in enteric bacteria and the N-terminal region of FlgM is required for recognition by the flagellar protein secretion system (74, 96). Thus, the absence of a significant portion of this region in *H. pylori* FlgM suggests that another mode of action is involved in relieving σ^{28} from the inhibitory action of FlgM. Alternatively, export signaling sequences could reside elsewhere within *H. pylori* FlgM. It has also been suggested that transcriptional control of *orf1122* is σ^{54}-dependent (82). This scenario is unlikely for two reasons. First, spacing between the -12 and -24 elements of the

proposed σ^{54}-dependent promoter upstream of *orf1122* is incorrect. Second, this model would predict that levels of FlgM would be low when σ^{28} is initially expressed (which would result in the premature expression of the late flagellar genes) and high during later stages of flagellar biogenesis (which would inhibit expression of the late σ^{28}-dependent flagellar genes).

Sigma 54

The σ^{54} factor associates with core RNA polymerase to form a holoenzyme that recognizes specific promoters at elements that are located 12 and 24 nucleotides upstream from the transcription initiation site (19, 25, 53). The consensus sequences of the –12 and –24 regions are TTGCW (where W is T or A) and TGGYAY (where Y is C or T), respectively (13). A large number of bacterial species possess σ^{54}, and this highly conserved sigma factor performs a variety of functions. It is involved in nitrogen fixation in bacteria such as *Bradyrhizobium japonicum* and *Rhodobacter sphaeroides* (32, 114). It plays a role in C_4-dicarboxylate transport in *Agrobacterium tumefaciens* (193). The σ^{54}-holoenzyme is also involved in regulation of genes related to flagellar biosynthesis in several bacterial species such as *Caulobacter crescentus, Vibrio cholera*, and *H. pylori* (124, 152, 168).

The σ^{54}-holoenzyme binds to the promoter to form a stable closed complex, but this complex is unable to undergo isomerization to an open complex that is competent to initiate transcription (24, 25). Isomerization of the closed complex to an open complex requires a transcriptional activator, which generally binds to sites located 70-100 base pairs upstream of the promoter (132, 150, 173). These activator binding sites, or upstream activation sequences (UAS) can often function when moved several kilobases away from the promoter (155). This property is reminiscent of enhancers in eukaryotes and these activator binding sites are sometimes referred

to as bacterial enhancers (132). After binding to the UAS, the activator contacts the closed complex through DNA looping (158, 171) . Productive interactions between the activator and σ^{54}-holoenzyme lead to open complex formation in a reaction that requires ATP hydrolysis by the activator (102, 187).

The σ^{54} factor generally consists of three functional regions (Figure 6) (174, 189). Region I, a glutamine-rich region, is involved in the response to the activator to form an open complex (54, 55). Region II, which is acidic, appears to affect the rate of open-complex formation (28, 188). Region III, which consists of a helix-turn-helix (HTH) and an RpoN box, is responsible for core-binding, DNA-binding and and may also be involved in signal transduction from the activator via Region I (27, 29, 63, 117).

H. pylori σ^{54} is different in many aspects from σ^{54} proteins found in other bacteria (Figure 7). Although *H. pylori* σ^{54} contains a conserved leucine-rich region in region I, it lacks the glutamine-rich portion present in this region . *H. pylori* σ^{54} also lacks the acidic region II. The *H. pylori* σ^{54}, however, does have the HTH and the RpoN box in region III found in other σ^{54} proteins. The sequence of the *H. pylori* RpoN box, RRTITKYR, is slightly different from the consensus sequence RRTVAKYR, suggesting that this difference may influence DNA binding.

These differences may be related to the activator of *H. pylori* σ^{54}, which is FlgR. This protein is an NtrC homologue and is part of a two-component signal transduction system along with its cognate sensor FlgS (16, 168). Activators of σ^{54}-holoenzyme usually have three functional domains: a N-terminal domain that is involved in regulation, a central domain required for ATP hydrolysis and activation, and a C-terminal domain that is responsible for DNA-binding (150). FlgR lacks the DNA-binding domain, and it has been demonstrated that it does not activate transcription from an UAS or enhancer (23). The σ^{54}-dependent activators from

Chlamydia trachomatis, and possibly other *Chlamydia* species, similarly lack DNA-binding domains, indicating that FlgR is not unique in this regard (92).

Comparison of the promoters of σ^{54}-dependent flagellar genes in *H. pylori* reveals that the consensus sequence of the –24 region differs from those recognized by σ^{54} in most other bacteria. The consensus sequence for the –24 region of *H. pylori* σ^{54}-dependent promoters is TGGAAC, while the consensus sequence from other bacteria is TGGCAC (13). When C is substituted with A in enteric bacteria, the affinity of σ^{54}-holoenzyme for the promoter is considerably reduced (11). This suggests that *H. pylori* σ^{54} has a different specificity for the bases in the –24 region it recognizes, although it is not unique in this regard since putative σ^{54}-dependent in other ε–proteobacteria such as *Campylobacter coli*, also possess A instead of C at -23.

Flagellar genes regulated by σ^{54} in *H. pylori* are indicated in Table 7. Genes recently identified by microarray analysis include *hp1076, hp1233, hp1154, hp1155* and *hp0869* (136). Inactivation of *hp0176* and *hp1233* resulted in defective flagella with impaired sheaths (136). The gene *hp0869* is the only one that has no apparent association with flagellar biogenesis.

Recently it was found that *H. pylori* σ^{54} may be involved in transcription of the two genes *neuA*, a CMP-sugar synthetase, and *flmD*, a glycosyl transferase-like gene (84). These two genes overlap by seven nucleotides and were found to have a σ^{54}-like promoter about 25 bp upstream from the ATG start codon of *neuA*. However, whole transcriptome microarray analysis suggest that transcription of *neuA/flmD* is not σ^{54}-dependent (136).

Experimental evidence suggests that FlmD is responsible for the glycosylation of flagellin proteins, which may be required for proper secretion of late flagellar proteins through the flagellar type III secretion system (84). The glycosylation may also help stabilize the flagellin

proteins prior to, or during the secretion process. Evidence in support of this is that both FlaA and FlaB flagellins have abundant glycosylation motifs, and have been found to be glycosylated in wild-type *H. pylori* (84). Moreover, a *flmD* mutant produces flagellins in very low amounts that do not appear to be glycosylated. This suggests that either a lower level of transcription or translation of flagellin genes occurs in this mutant, or non-glycosylated flagellin proteins are less stable that the glycosylated proteins.

The factors that affect expression of the RpoN regulon genes are not clearly understood. As mentioned earlier, there are several potential levels of regulation, including environmental factors and the flagellar export apparatus. Additional levels of σ^{54} –dependent flagellar gene regulation may exist in *H. pylori.* A protein-protein interaction map of *H. pylori* suggests that a novel protein, HP0958, interacts with σ^{54} (154). Homologs of HP0958 occur in a variety of bacteria, most of which have σ^{54} suggesting that HP0958 plays a role in regulating σ^{54} activity or levels in *H. pylori*. This study investigated the role of HP0958 in *H. pylori* and it was demonstrated to be required for the accumulation of σ^{54}.

Homologs of HP0958 share 20-34% amino acid identity with HP0958 and are found in *Aquifex aeolicus, Borrelia burgdorferi, Treponema pallidum, Chlamydia pneumoniae, C. trachomatis* and *C. jejuni*. The occurrence of HP0958 homologs in the chlamydial species is interesting given that these bacteria also have FlgR-like σ^{54}-dependent activators. It is possible that additional levels of σ^{54} regulation are needed for appropriate control of gene expression in bacteria that rely on activators that function without an enhancer or UAS.

Summary of Research

As described in the review above, *H. pylori* differs from several Gram negative bacteria in a number of ways. In particular, flagellar biogenesis in this microorganism appears to be distinct from models that have been well established in bacteria like *E. coli* and *C. crescentus*. Since the use of animal models, such as gnotobiotic piglets, has demonstrated that motility is essential for *H. pylori* to establish an infection in the gastric mucosa (44), the study of flagellar biogenesis is therefore considerably important and may lead to the identification or development of new methods of control or detection. Elucidating flagellar synthesis in *H. pylori* would also provide insight into alternative methods of flagellar production and assembly and reveal novel aspects of gene regulation in general.

Although the role of the sigma factor σ^{54} in *H. pylori* flagellar synthesis has been demonstrated before, little is known about factors that affect its synthesis and/or activity. The purpose of my study was to identify and examine such factors. My focus was on a subset of flagellar genes that are under the control of the regulatory protein σ^{54}. Here I examined the role of the novel protein HP0958 on σ^{54} function, the role of the flagellar export apparatus on the RpoN regulon, as well as the distinct promoter specificity of *H. pylori* σ^{54}. Results reveal novel aspects of control of the RpoN regulon and show that its regulation occurs on multiple levels. Data will show that there is post-translational regulation of σ^{54} and the promoter affinity of σ^{54} also plays a role in flagellar gene regulation. Additionally, coordination between the flagellar export apparatus and σ^{54}-dependent gene expression is demonstrated. These data shed light on flagellar biogenesis in this important human pathogen and highlights additional levels of gene regulation that bacteria are capable of employing.

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Table 1: Putative mechanisms by which *H. pylori* alters gastric physiology.

Effect on Gastric Physiology	**Mechanism**
Induction of gastric inflammation	IL-8 secretion Neutrophil adherence to endothelial cells Platelet-activating factor Lipopolysaccharide
Disruption of gastric mucosal barrier	Phospholipases Mucinase Vacuolating cytotoxin Reactive oxygen metabolites Induction of inducible nitric oxide synthase Induction of apoptosis
Altered gastrin-gastric homeostasis	Decreased somatostatin release Hypergastrinemia Diminished response of parietal cells to gastrin

Table 2: Known and potential flagellar and flagellar-associated genes in *H. pylori* strain 26695.

Genes encoding structural proteins	**Putative function**	**Genes required for flagellar assembly**	**Putative function**
fliF	MS ring protein	*flhA (flbA)*	Export apparatus
flgI	P ring protein	*flhF*	Unknown
flgH	L ring protein	*fliP*	Export apparatus
fliE	MS ring/rod adaptor	*fliQ*	Export apparatus
flgC	Proximal rod protein	*fliI*	Export apparatus
flgB	Proximal rod protein	*fliR*	Unknown
flgG	Distal rod protein	*flaG*	Modification of flagellin
flgG`	FlgG homolog	*fliH*	Export apparatus
flgE	Hook protein	*fliS*	Facilitates flagellin export
flgD	Hook capping protein	*fliY*	Unknown
flgE`	FlgE homolog	*flmH (flaG)*	Modification of flagellin
flgK	Hook-associated protein I	*flmA (flaA1)*	Modification of flagellin
fliD	Filament cap	*flhB*	Export Apparatus
flaA	Flagellin A		
flaB	Flagellin B		
flaB`	Flagellin B homolog		
fliG	Rotor compnent		
fliM	Motor/switch		
fliN	Motor/switch		
motA	Motility enabling protein		
motB	Motility enabling protein		

Table 3: Chemotaxis genes and their associated function in *H. pylori*.

Gene	Putative function of encoded protein
cheA	Sensor histidine kinase
cheY	Response regulator
cheV	CheW-CheY homolog
cheW	Receptor-coupling factor
tlpA	Methyl-accepting chemotaxis protein
tlpB	Methyl-accepting chemotaxis protein
tlpC	Methyl-accepting chemotaxid transducer

Table 4: Genes encoding regulatory proteins involved in flagellar synthesis.

Gene	**Function**
rpoD	σ^{80}
fliA	σ^{28}
orf1122	Anti-σ^{28} factor
rpoN	σ^{54}
flgR	Activator of σ^{54}-holoenzyme
flgS	Sensor kinase for FlgR
orf0958	Regulator of σ^{54}?

Table 5: σ^{80}-dependent flagellar genes in *H. pylori*.

Flagellar Genes/operons
flgG
flhB
fliE
fliI-fliQ
flgG
flhF
motA-motB
fliL
fliP-fliP`
fliN
fliR
flgI
flaG
flgH
fliF-fliG-fliH

Table 6. Sequence comparisons of some *H. pylori* σ^{80} promoters.

Gene	Nucleotide sequence and positions
σ^{80} dependent promoters	-35 -10 +1 aaa-t-tg-TAtaaT
vacA	ATCTTGTTGAGTTTTATGTTTATTTACCTTAATTTGATAAAAGTTTAATA
katA	AATTGAATCTTGTTAGTCTTTGTATAACAAATTATGTAATAATCACCACA
cagB P2	CGATTCACATTTTTACCCACAATTAACAAAAATTTGCTAAAATGAAAGAA
cheY	ATTATTGTCGGTCAAGGTAATTTTAATCAAAACATGCTATTATTTGGAAT
ureA	GAACGCTTCTGTTAATCTTAGTAAATCAAAACATTGCTACAATTACATCC
cagA	GATTAATGAAAAAAAAAAAAAATGCTTGATATTGTTGTATAATGAGAATG

Table 7: σ^{54}-dependent flagellar genes in *H. pylori*.

Gene	Function
flaB	Minor flagellin protein
flgE	Hook protein
flgB	Proximal rod protein
flgC	Proximal rod protein
flgK	Hook-associated protein
flgL	Hook-associated protein
fliK	Hook length control protein
hp1076	Unknown function, interacts with flagellar chaperone FliS and rod protein FlgB
hp1233	FlgJ homolog
hp1154	Unknown function, possible role in peptidoglycan synthesis
hp1155	Putative glycosyltransferase, role in peptidoglycan synthesis
hp0869	HypA protein

Figure 1. Morphology of *Helicobacter pylori*. Transmission electron micrograph of *H. pylori* with four sheathed polar flagella. The terminal bulb is indicated by the arrow. Bar = 1.0 μm.

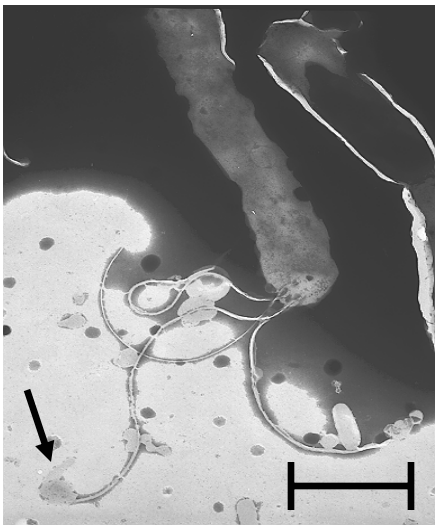


Figure 2. Flagellar components. The main parts include the basal body, rod, hook, and filament. The external flagellar proteins are secreted by a specific export apparatus. Flagellar rotation is driven by a transmembrane proton gradient. The direction of rotation is determined by the interaction of the motor/switch complex with the chemotaxis regulator CheY.

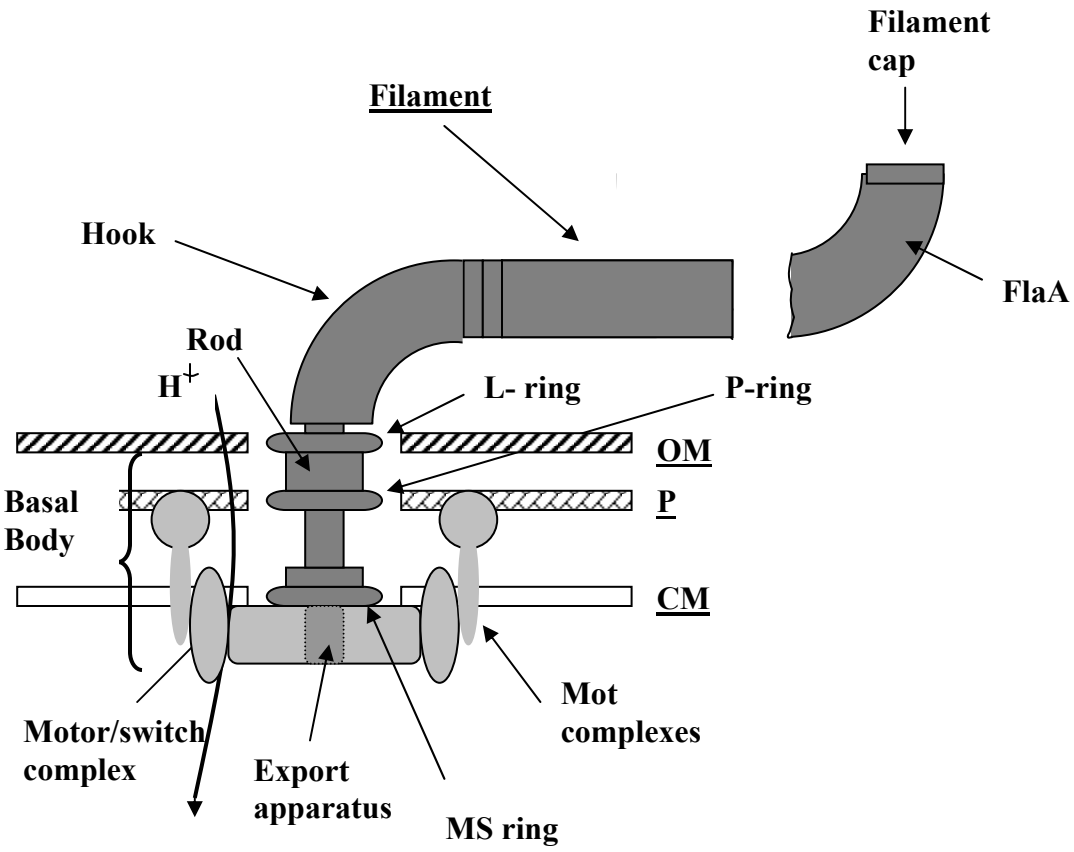

Filament cap
Filament
Hook
FlaA
Rod
H+
L- ring
P-ring
OM
P
Basal Body
CM
Motor/switch complex
Export apparatus
MS ring
Mot complexes

Figure 3. Comparison of flagellar regulatory hierarchies in *S. typhymurium* and *C. crescentus*.

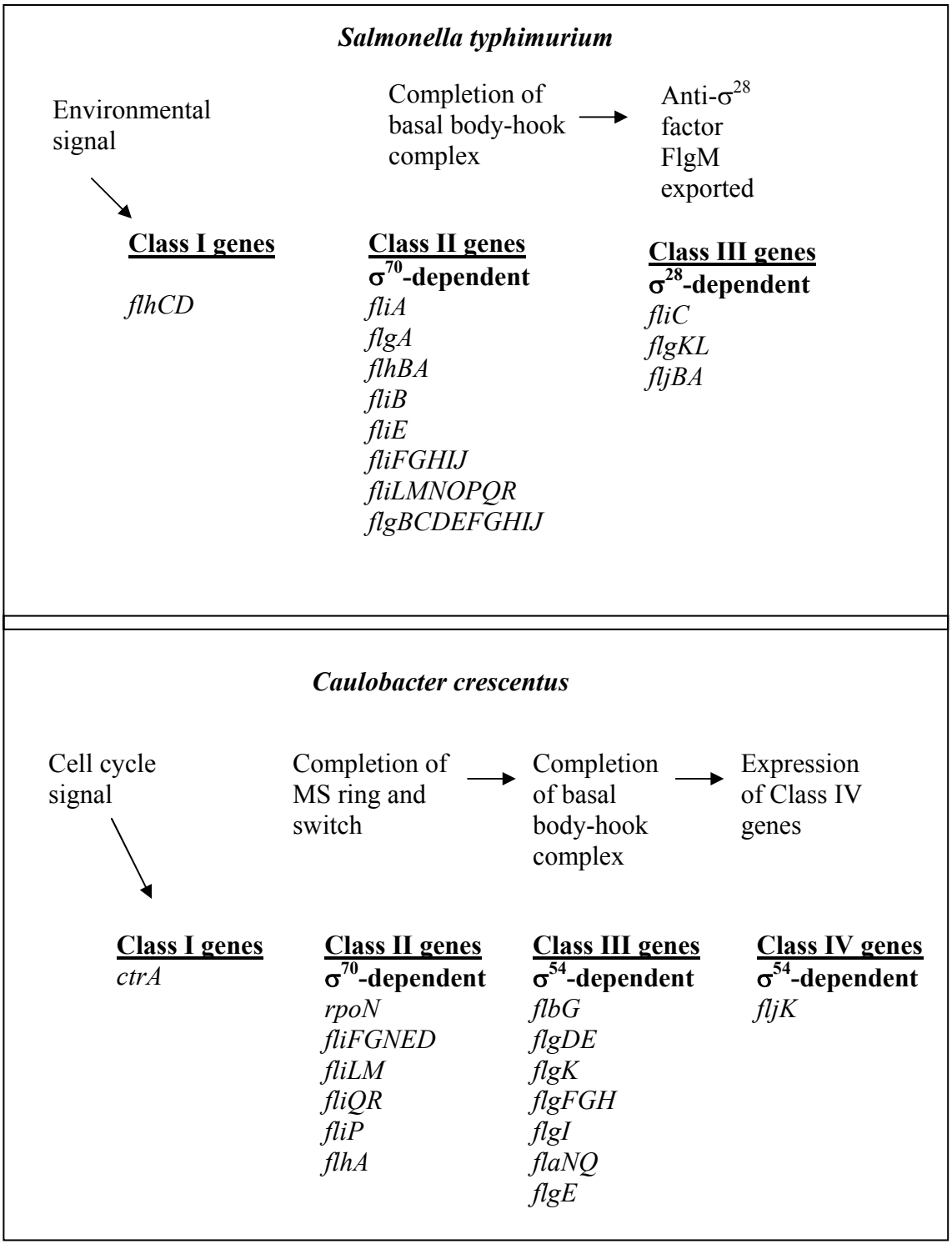

Salmonella typhimurium
Environmental signal
Completion of basal body-hook complex
Anti-σ28 factor FlgM exported
Class I genes
flhCD
Class II genes
σ70-dependent
fliA
flgA
flhBA
fliB
fliE
fliFGHIJ
fliLMNOPQR
flgBCDEFGHIJ
Class III genes
σ28-dependent
fliC
flgKL
fljBA
Caulobacter crescentus
Cell cycle signal
Completion of MS ring and switch
Completion of basal body-hook complex
Expression of Class IV genes
Class I genes
ctrA
Class II genes
σ70-dependent
rpoN
fliFGNED
fliLM
fliQR
fliP
flhA
Class III genes
σ54-dependent
flbG
flgDE
flgK
flgFGH
flgI
flaNQ
flgE
Class IV genes
σ54-dependent
fljK

Figure 4. Proposed model for flagellar gene regulation in *Helicobacter pylori.* The flagellar operons in each class of genes are indicated, as well as the form of RNA polymerase holoenzyme required for transcription of each set of genes. The expression of each class of genes requires expression of the previous class of genes to occur first. HP0958, the focus of this study, is required for stable accumulation of σ^{54} suggesting a role for this protein in positive regulation of class III operons. FlgM is an anti- σ^{28} factor that negatively regulates expression of class IV operons.

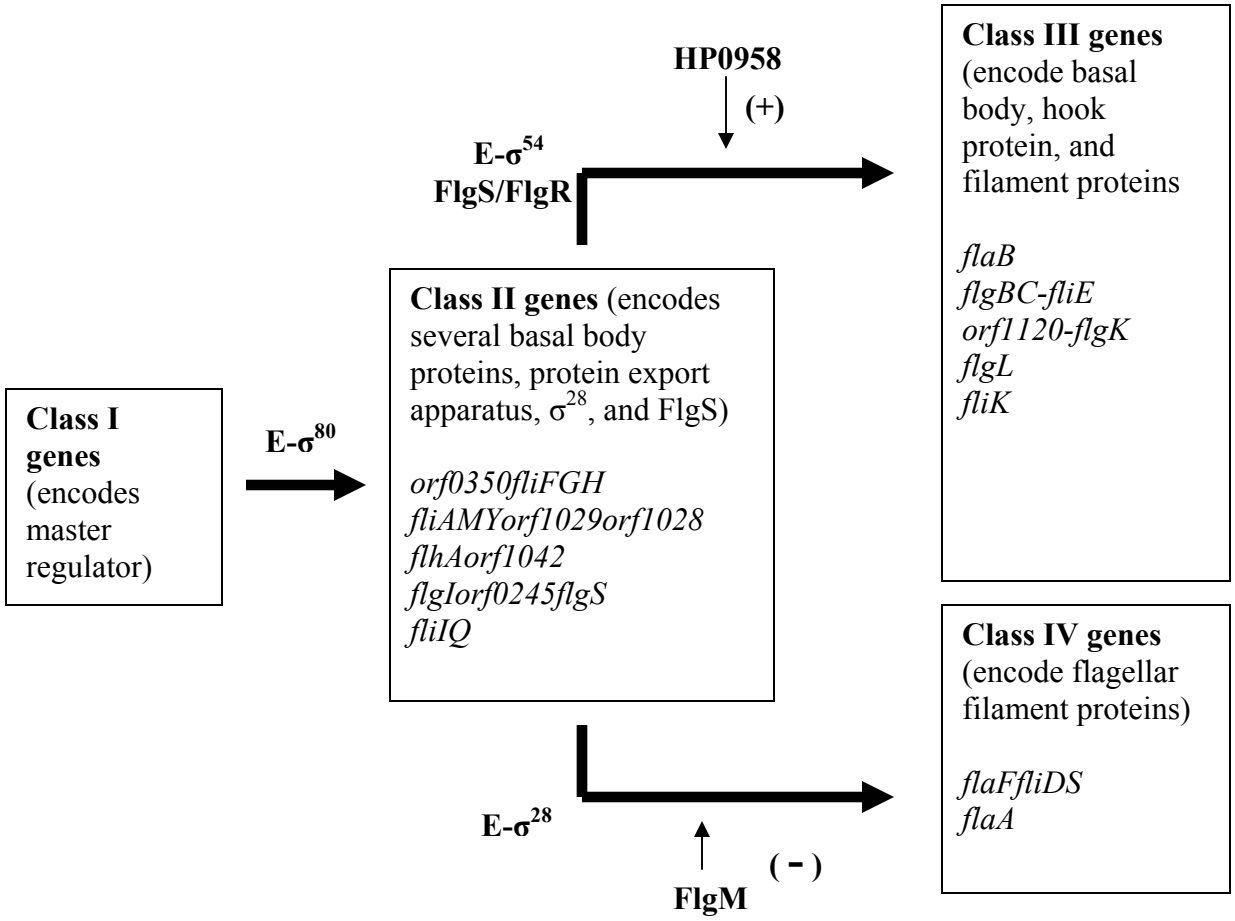

HP0958
(+)
E-σ54
FlgS/FlgR
Class III genes
(encode basal body, hook protein, and filament proteins
flaB
flgBC-fliE
orf1120-flgK
flgL
fliK
Class I genes
(encodes master regulator)
E-σ80
Class II genes (encodes several basal body proteins, protein export apparatus, σ28, and FlgS)
orf0350fliFGH
fliAMYorf1029orf1028
flhAorf1042
flgIorf0245flgS
fliIQ
Class IV genes
(encode flagellar filament proteins)
flaFfliDS
flaA
E-σ28
(-)
FlgM

Figure 5. Primary prokaryotic sigma factor - σ^{70}. The shaded regions are conserved among σ^{70} proteins from a wide variety of bacterial species. Regions 2, 3, and 4 are also conserved in most of the alternative sigma factors, such as σ^{28}.

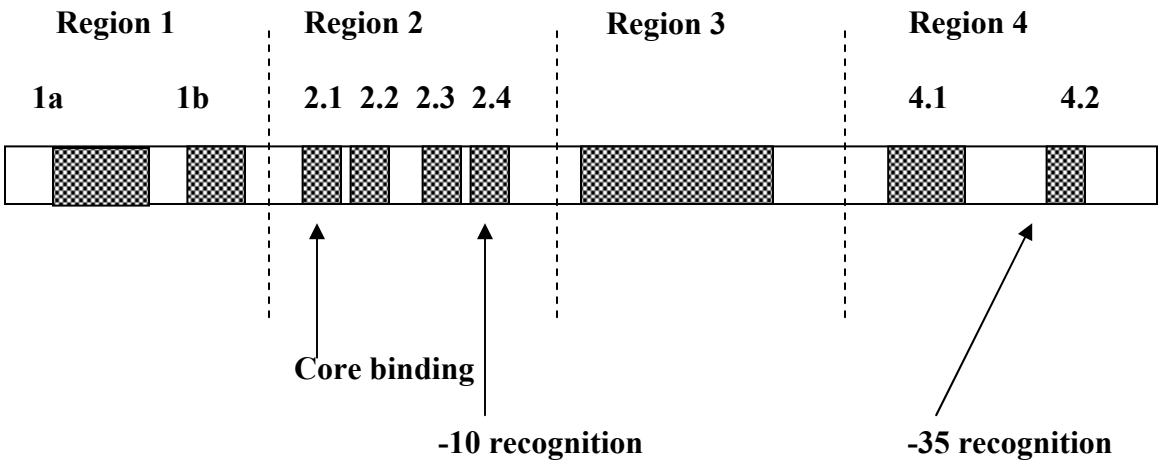

Region 1
Region 2
Region 3
Region 4
1a
1b
2.1
2.2
2.3
2.4
4.1
4.2
Core binding
-10 recognition
-35 recognition

Fig. 6. Structure of σ^{54}. Regions I, II and III are the three functional regions of σ^{54}. HTH is a DNA-binding helix-turn-helix motif; RpoN is the signature motif of σ^{54} proteins and has the consensus sequence ARRTVAKYRE.

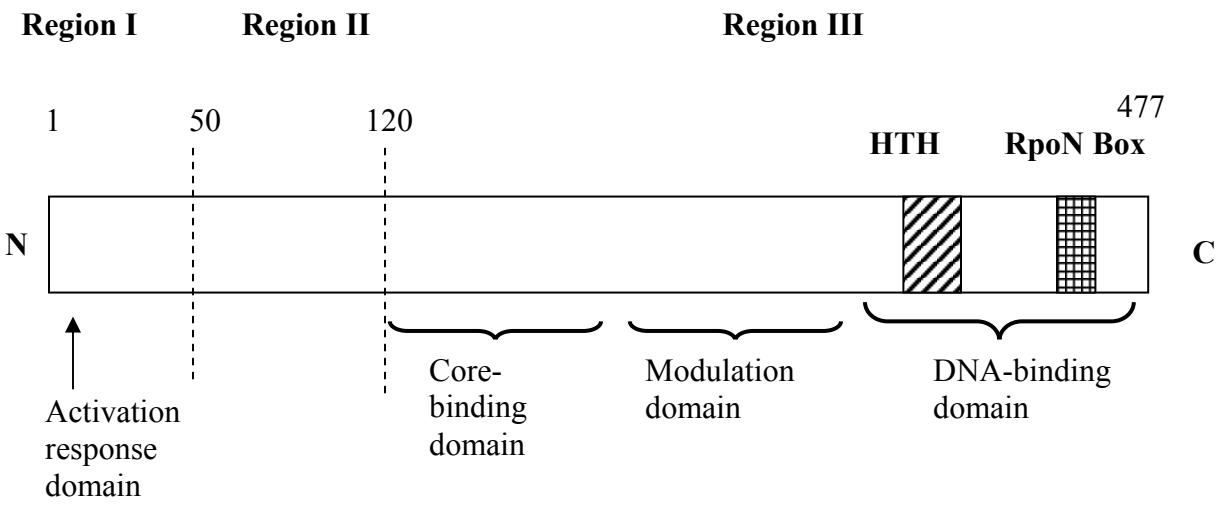
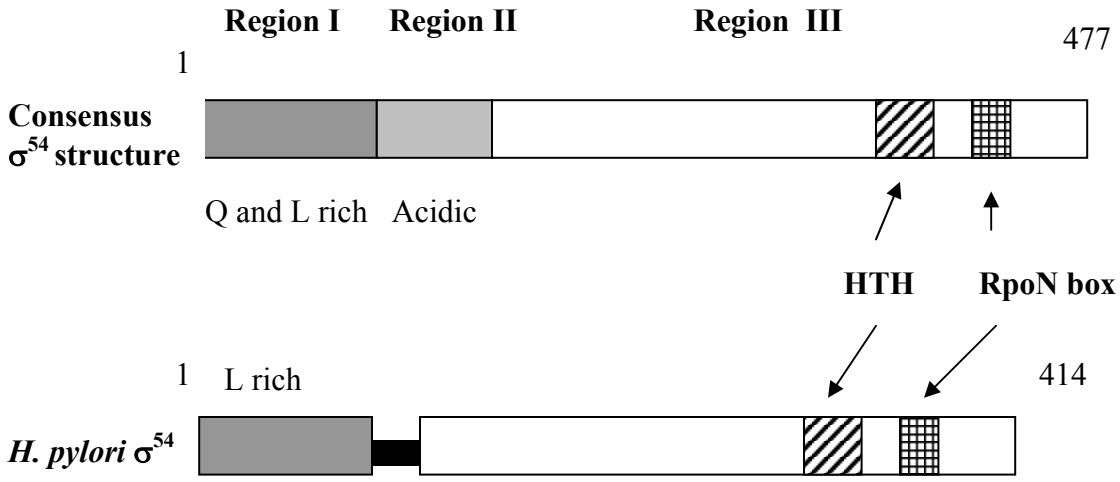

Region I
Region II
Region III
1
50
120
477
HTH
RpoN Box
N
C
Activation response domain
Core-binding domain
Modulation domain
DNA-binding domain

Figure 7. Comparison of *Helicobacter pylori* σ^{54} with σ^{54} from enteric bacteria. *H. pylori* σ^{54} has a conserved leucine (L)-rich region, helix-turn-helix (HTH), and an RpoN box, but the Q-rich and acidic regions are absent.


Region I
Region II
Region III
1
477
Consensus
σ54 structure
Q and L rich
Acidic
HTH
RpoN box
1
L rich
414
H. pylori σ54

Chapter 2

Stable Accumulation of σ^{54} in *Helicobacter pylori* requires the novel protein HP0958[1]

[1] Pereira, L., and T. R. Hoover. Accepted by *Journal of Bacteriology*.
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Abstract

Several flagellar genes in *Helicobacter pylori* are dependent on σ^{54} (RpoN) for their expression. These genes encode components of the basal body, the hook protein and a minor flagellin, FlaB. A protein-protein interaction map for *H. pylori* constructed from a high-throughput screen of a yeast two-hybrid assay (http://pim.hybrigenics.com/pimrider) revealed interactions between σ^{54} and the conserved hypothetical protein HP0958. To see if HP0958 influences σ^{54} function, the corresponding gene was disrupted with a kanamycin-resistance gene (*aphA3*) in *H. pylori* ATCC 43504 and the resulting mutant was analyzed. The *hp0958:aphA3* mutant was non-motile and failed to produce flagella. Introduction of a functional copy of *hp0958* into the genome of the *hp0958:aphA3* mutant restored flagellar biogenesis and motility. The *hp0958:aphA3* mutant was deficient in expressing two σ^{54}-dependent reporter genes, *flaB'-'xylE* and *hp1120'-'xylE*. Levels of σ^{54} in the *hp0958* mutant were substantially lower than those in the parental strain suggesting that the failure of the mutant to express the genes in the RpoN regulon and produce flagella was due to reduced σ^{54} levels. Expressing σ^{54} at high levels by putting *rpoN* under control of the *ureA* promoter restored flagellar biogenesis and motility in the *hp0958:aphA3* mutant. Turnover of σ^{54} was more rapid in the *hp0958:aphA3* mutant than it was in the wild-type strain suggesting that HP0958 supports wild-type σ^{54} levels in *H. pylori* by protecting it from proteolysis.

Introduction

Helicobacter pylori, a member of the ε-subdivision of Proteobacteria, colonizes the human gastric epithelium which leads to a gastric inflammation that can progress to chronic

gastritis, peptic ulcer, gastric cancer or mucosal-associated lymphoma (6, 10, 12). *H. pylori* must be motile to colonize the gastric epithelium (13, 14), and motility by the bacterium occurs through the action of two to six polar flagella.

Flagellar biogenesis in *H. pylori* involves the coordinated expression of over 40 flagellar genes scattered throughout the genome and organized into 25 or more transcriptional units (1, 33). Transcriptional regulation of these flagellar operons in *H. pylori* is complex, involving all three σ factors found in the bacterium, σ^{80} (the primary σ factor in *H. pylori*), σ^{54} (RpoN) and σ^{28} (FliA) (7, 15, 19, 29, 30, 32). In this regard regulation of flagellar biogenesis in *H. pylori* is similar to that of *Vibrio cholerae* and *Pseudomonas aeruginosa* which also require both σ^{54} and σ^{28} for expression of different classes of flagellar genes (11, 23, 34).

In *H. pylori*, σ^{80} is required for transcription of flagellar genes whose products are needed early in flagellar biogenesis and include basal body proteins and components of the flagellar protein export apparatus (30). *H. pylori* flagellar genes whose products are required midway through flagellar assembly are dependent on σ^{54} for their expression and include genes that encode the proximal rod proteins of the basal body (*flgBC*), the hook protein (*flgE*), hook-associated proteins (*flgK* and *flgL*) and a minor flagellin (*flaB*) (19, 29, 32). Finally, genes whose products are needed at the end of flagellar biogenesis are transcribed by σ^{28}-RNA polymerase holoenzyme and include genes encoding the major flagellin (*flaA*), the filament cap protein (*fliD*) and flagellar protein chaperones (*fliS* and *fliT*) (15, 16, 19).

Transcription of σ^{54}-dependent genes in *H. pylori* requires the activator FlgR which belongs to the NtrC family of transcriptional activators (7, 29). FlgR is a response regulator of a two-component signal transduction system and must be phosphorylated by its cognate sensor kinase, FlgS, to activate transcription (2, 7, 19). Sensor kinases are often responsive to

environmental or cellular signals, but it is not known if FlgS responds to such cues. Activators of σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) typically bind enhancers located upstream of the promoters of the genes they activate (8, 25). After binding to the enhancer, the activator contacts σ^{54}-holoenzyme bound at the promoter in a closed complex via looping of the DNA between the enhancer and promoter (26, 31). The activator hydrolyzes ATP and couples energy released from hydrolysis to stimulate the isomerization of the closed promoter complex to an open complex that is competent to initiate transcription (22, 35). Unlike most σ^{54}-dependent activators, *H. pylori* FlgR lacks a DNA-binding domain and apparently contacts σ^{54}-holoenzyme directly rather than through DNA looping to activate transcription (7).

In addition to the involvement of FlgS/FlgR system in the regulation of σ^{54}-dependent genes, other factors may regulate expression of genes of the RpoN regulon. A protein-protein interaction map for *H. pylori* was constructed using a high-throughput screen of a yeast two-hybrid system (24). The protein-protein interaction map indicated interactions between σ^{54} and the conserved hypothetical protein HP0958 (Hybrigenics PimRider™ database; http://pim.hybrigenics.com/pimrider). To determine if HP0958 influences σ^{54} function in *H. pylori*, we disrupted its corresponding gene with a cassette bearing a kanamycin-resistance gene (*aphA3*) in *H. pylori* ATCC 43504 and analyzed the phenotype of the resulting mutant. The *hp0958:aphA3* mutant was non-motile and aflagellated, which appeared to result from reduced levels of σ^{54}. The turnover rate of σ^{54} in the *hp0958:aphA3* mutant was significantly higher than that in the parental strain suggesting that HP0958 protects σ^{54} from proteolysis.

Materials and Methods

Bacterial strains and media. *Escherichia coli* DH5α [ϕ80 *lacZ* ΔM15 *recA*1 e*ndA*1 *gyrA*96 *thi*- 1 *hsdR*17 (r_k^-, m_k^+) *supE*44 *relA*1 *deoR* Δ(*lacZYA-argF*) U169) was grown in Luria- Burtani broth at 37°C. *H. pylori* ATCC 43504 was grown on blood agar supplemented with 10% sheep blood or tryptic soy agar supplemented with 5% horse serum (TSA-serum) and grown at 37°C under an atmosphere of 4% oxygen, 5% carbon dioxide and 91% nitrogen. Serum-free media was used to grow liquid cultures of *H. pylori* in brain-heart infusion broth supplemented with 0.1% β-cyclodextrin (Sigma) as described (9). Motility agar plates consisted of Mueller-Hinton broth supplemented with 5% horse serum and contained 0.35% agar. Sterile toothpicks were used to inoculate the motility agar with *H. pylori* strains, and motility was scored after incubating the plates at 37°C under an atmosphere of 4% oxygen, 5% carbon dioxide and 91% nitrogen for 4 to 5 days. When required, media was supplemented with 30 μg/ml chloramphenicol, kanamycin or tetracycline.

Protein purification. Details for construction of plasmids used for overproduction of *H. pylori* proteins are available upon request. PCR products used to construct the expression vectors were sequenced at the Integrated Biotechnology Laboratories at the University of Georgia to verify that no errors had been introduced during amplification.

A DNA fragment bearing *hp0958* was amplified by PCR from *H. pylori* 26695 genomic DNA and cloned into plasmid pJES489, which is a derivative of pMAL-c (New England Biolabs), to create a fusion of *hp0958* and *E. coli malE* (encodes the maltose-binding protein (MBP)). MBP-HP0958 was expressed from this plasmid in *E. coli* DH5α by growing a 1-liter culture to an O.D. of 0.3 then adding isopropyl-β-D-thiogalactoside (1 mM final concentration). After 3.5 h, cells were harvested, resuspended in 50 mM Tris-acetate, pH 8.2, 200 mM KCl, 1

mM EDTA and 1 mM dithiothreitol (DTT), and lysed with a French pressure cell at 7000 p.s.i. The crude cell extract was centrifuged at 17,000 x g for 45 minutes and the resulting supernatant liquid was applied to an amylose-agarose (New England Biolabs) affinity column that had been equilibrated previously with 20 mM Tris-HCl, pH 7.4, 5% (w/v) glycerol, 1 mM EDTA, 1 mM DTT and 200 mM KCl (Buffer A). MBP-HP0958 was eluted from the column with Buffer A plus 10 mM maltose, dialyzed against 20 mM Tris-HCl, pH 8.8, 0.5 mM DTT and 5% (w/v) glycerol (Buffer C), then applied to a High TrapQ anion-exchange column (5 ml; Amersham Biosciences) that had been equilibrated previously with Buffer C. MBP-HP0958 was eluted from the column with a gradient to Buffer C plus 1 M KCl at a salt concentration of ~0.2 M.

H. pylori rpoN was amplified by PCR from *H. pylori* strain 26695 and cloned into pJES489 to create a chimeric *malE-rpoN* gene. MBP-σ^{54} protein was overproduced in *E. coli* DH5α and purified through the amylose-agarose affinity chromatography step as described for MBP-HP0958. Fractions containing the protein were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mM maltose and 5% (w/v) glycerol (buffer B). The dialyzed fractions were applied to a heparin-agarose column which was equilibrated with buffer B. After washing with buffer B, MBP-σ^{54} was eluted from the column at ~0.2 M KCl with a linear gradient to buffer plus 1M KCl.

H. pylori flaB was amplified by PCR from *H. pylori* strain 26695 and cloned into pJES489 to create a chimeric *malE-flaB* gene. MBP-FlaB protein was overproduced in *E. coli* DH5α and purified as described for MBP-HP0958. Antisera directed against MBP-HP0958, MBP-σ^{54} or MBP-FlaB were raised in New Zealand white rabbits by Cocalico Labs, Reamstown, PA.

Construction of *H. pylori* mutant strains. Mutant strains were constructed using suicide vectors derived from pGEM-T (Promega) and contained fragments of the genes targeted for mutagenesis. Targetted genes were amplified from *H. pylori* 26695 genomic DNA by PCR, cloned into pGEM-T, and disrupted with a 1.4 kb *Eco*RI fragment bearing a *Campylobacter coli aph*A3 (confers resistance to kanamycin) cassette from plasmid pHP1 (17). Suicide vectors were introduced into *H. pylori* ATCC 43504 by natural transformation and recombinants in which the chromosomal copy of the targeted gene had been replaced by the disrupted copy of the gene were selected on TSA-serum supplemented with kanamycin. Insertion of the *aphA3* cassette in the targeted gene was verified by PCR using a set of primers that flanked the site of insertion. An *H. pylori rpoN* mutant was constructed by introducing an *Eco*RI site ~370 bp from the start codon of *H. pylori rpoN* using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) and cloning the *aphA3* cassette into this site. An *H. pylori hp0958* mutant was constructed following introduction of the *aphA3* cassette within a *Nhe*I site which resulted in disruption of the gene at codon 76. An *H. pylori hp0959* mutant was generated by inserting the *aphA3* cassette into a *Bam*HI site located ~450 bp downstream of the start codon of *hp0959*.

Complementation of *hp0958* mutant. A ~ 1.5 kb PCR product that carried most of *hp0959* and all of *hp0958* was cloned into pGEM-T and sequenced. The cloned DNA fragment was introduced into the *Eco*RV site of plasmid pEU39Cm (21), which carries a copy of *H. pylori hp0405* disrupted with a cassette containing *C. coli* chloramphenicol transacetylase (*cat*). The *Eco*RV site is present in *hp0405* and is adjacent to the *cat* cassette. The resulting suicide vector was transformed into the *hp0958:aphA3* mutant and recombinants in which the chromosomal copy of *hp0405* had been replaced with the disrupted gene carrying *hp0959-hp0958* along with the *cat* cassette were selected on TSA-serum supplemented with chloramphenicol. Introduction

of *hp0959-hp0958* into the *hp0405* locus was verified by PCR using a set of primers in which one was internal to *cat* and the other was within *hp0405*. PCR was also used to confirm that this strain retained the *aphA3* cassette in the *hp0958* locus.

Overproduction of σ^{54} and HP0958 in *H. pylori*. *H. pylori rpoN* was introduced into plasmid pPA (5) to place it under control of the *H. pylori ure*A promoter. A ~1.4 kb DNA fragment bearing the *ure*A promoter plus *rpoN* from the resulting plasmid was cloned into the *Eco*RV site of plasmid pEU39Cm. This plasmid was transformed into the *hp0958:aphA3* mutant and into wild-type *H. pylori* to introduce the *rpoN* allele under control of the *ureA* promoter into the *hp0405* locus as described above.

Similarly, *H. pylori hp0958* was placed under control of the *ureA* promoter by introducing it into plasmid pPA. A ~ 975 bp DNA fragment bearing the P_{ureA}-*hp0958* allele was cloned into pEU39Cm, and the resulting plasmid was transformed into the wild-type or *hp0958:aphA3* mutant strains to introduce the P_{ureA}-*hp0958* allele into the *hp0405* locus.

Construction of *H. pylori* reporter strains. Reporter genes in which the *H. pylori flaB* promoter region (positions -67 to +26 relative to the transcriptional start site) or the *H. pylori flaA* promoter region (positions -126 to +47 relative to the transcriptional start site) were joined to promoterless *Pseudomonas putida xylE* (encodes catechol 2,3-dioxygenase) have been described previously (7). An *orf1120'-'xylE* reporter gene was constructed by cloning a DNA fragment corresponding to positions -70 to +44 relative to the transcriptional start site of the *hp1120-flgK* operon upstream of *xylE*. The *flaB'-'xylE*, *hp1120'-'xylE* and *flaA'-'xylE* reporter genes were cloned into the *Eco*RV site of plasmid pEU39Cm to create suicide vectors that were used to introduce the reporter genes into the *hp0405* locus of various *H. pylori* strains as described above.

To construct a strain with a *rpoN'-'xylE* reporter gene, the start codon of *P. putida xylE* gene was joined in-frame to codon 61 of *H. pylori rpoN* that had been cloned previously in pGEM-T. A 1.3 kb fragment bearing *C. coli cat* was cloned immediately downstream of *xylE* in the *Eco*RI site that had been introduced previously in *rpoN*. The resulting suicide vector was transformed into wild-type *H. pylori* and the *hp0958:aphA3* mutant. Recombinants in which the chromosomal copy of *rpoN* was replaced with the *rpoN'-'xylE* reporter and the *cat* cassette were selected on TSA-serum supplemented with chloramphenicol and verified by PCR.

XylE assays. Whole cell XylE assays were carried out essentially as described previously (7). Rate of product (2-hydroxymuconic semialdehyde) formation was determined and activities were expressed as μmoles product formed/ min/ 10^8 *H. pylori* cells from at least 10 independent assays for each sample.

Electron microscopy. *H. pylori* cells were grown on blood agar for 48 h at 37^{o}C and then gently resuspended in phosphate buffered saline, pH 7.4. Samples were negatively stained with 2% phosphotungstic acid (pH 7.0) on formvar-coated copper grids and observed with a JEOL 100CK electron microscope (JEOL USA, Peabody, MA 01960) at the Center for Advanced Ultrastructural Research at the University of Georgia. Micrographs were taken at an accelerating voltage of 80 kV.

Western Blot Analysis. Immunoblotting with primary antibodies directed against MBP-HP0958, MBP-σ^{54}, MBP-FlaB or VacA (Austral Biologicals, San Ramon, CA) was done as described previously (7). The bound antibody was detected by enhanced chemiluminescence using peroxidase-coupled goat anti-rabbit antibody as the secondary antibody (MP Biomedicals Inc., Aurora, ID). Antibody directed against MBP-σ^{54} was affinity purified prior to use as follows. Purified histidine-tagged σ^{54} was subjected to SDS-PAGE, transferred to a

nitrocellulose membrane and visualized by staining with Ponceau red. The band was excised and the membrane strip was rinsed in distilled water, blocked with a 2% non-fat dry milk solution, washed with Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated overnight with 20 ml of a 1:5 dilution of antiserum directed against MBP-σ^{54}. The membrane strip was washed five times with TBST and incubated with an acidic buffer (0.2 M glycine, 0.2 M sodium chloride, 1% gelatin, pH 2.8) for 15 min to elute the antibody. The eluate was dialyzed overnight against a buffer containing 50 mM citric acid and 50 mM Na_2HPO_4, pH 5.5, followed by overnight dialysis against Tris-buffered saline.

σ^{54} protein stability assay. *H. pylori* cultures were grown to mid-log phase in serum-free medium as described (9), at which point tetracycline was added to stop protein synthesis. Aliquots were removed at various times following tetracycline addition. Cells were recovered by centrifugation and resuspended in phosphate-buffered saline. Approximately 10^8 cells from each sample were lysed and analyzed by western blotting.

To assay for secreted proteins, *H. pylori* cultures were grown to mid-log phase in serum-free medium as described (9). Cells were removed by centrifugation for 15 min at 20,000 x g at 4^{o}C. The resulting supernatant liquids were filtered through a 0.22 μM-pore size membrane filter to remove any residual bacteria. Extracellular proteins were precipitated using a modified trichloroacetic acid method as described (9), and approximately 20 μg of precipitated protein was analyzed by western blotting.

Results

Inactivation of *hp0958* interferes with flagellar biogenesis. *H. pylori hp0958* encodes a protein of unknown function that is predicted to be 254 amino acid residues in length. To

determine if the interactions between HP0958 and σ^{54} observed in the yeast two-hybrid assay were of physiological relevance, *hp0958* was disrupted by introducing a cassette bearing *aphA3* into codon 76 of the gene in *H. pylori* ATCC 43504. The *hp0958:aphA3* mutant was non-motile when tested on motility agar (Fig. 1) as well as when examined microscopically. Since loss of motility could have resulted from defects of either flagellar assembly or function the *hp0958* mutant was examined by transmission electron microscopy for the presence of flagella. Sheathed polar flagella were readily apparent in the wild-type *H. pylori* strain, but were absent in the *hp0958* mutant (data not shown).

The gene immediately downstream of *hp0958* is *kdtA*, which encodes 3-deoxy-D-manno-octulosonic acid transferase, an enzyme that is essential for lipopolysaccharide (LPS) biosynthesis (3, 4). Mutations in *H. pylori* that interfere with both LPS biosynthesis and flagellar biogenesis have been described previously (18) and so we wished to verify that the effect on flagellar biogenesis that we observed in the *hp0958:aphA3* mutant was not due to polar effects on *kdtA*. Mass spectrometry analysis of purified LPS from the *hp0958* mutant and wild-type strains revealed no differences between the two strains (data not shown), which argued strongly against the *aphA3* cassette in *hp0958* interfering with expression of *kdtA* or other genes downstream of *hp0958*. To confirm that disruption of *hp0958* was responsible for the phenotype of the mutant strain, we complemented the *hp0958:aphA3* mutant by introducing a functional copy of *hp0958* in the mutant strain. The *hp0958* gene along with most of the gene immediately upstream of *hp0958* (*hp0959*) was introduced into the *hp0958:aphA3* mutant at the *hp0405* locus. *H. pylori hp0405* encodes a predicted NifS-like protein of unknown function and this locus was used previously to construct *H. pylori* merodiploid strains (20, 21). Disruption of *hp0405* does not affect motility. Introduction of a functional copy of *hp0958* into the

hp0958:aphA3 mutant allowed expression of HP0958 at wild-type level (Fig. 2A) and restored flagellar synthesis and motility (Fig. 1). These results demonstrated that disruption of *hp0958* was responsible for the lesion in flagellar biogenesis in the original mutant. Since *hp0958* and *hp0959* are separated by only 10 bp of DNA, the restoration of HP0958 expression to wild-type level in the complemented strain suggests that the promoter for *hp0958* is in *hp0959*.

Homologs of HP0958 are found in several bacteria, including all of the ε-Proteobacteria whose genomes have been sequenced to date (*Helicobacter hepaticus*, *Campylobacter jejuni*, and *Wolinella succinogenes*). In all of these representatives of ε-Proteobacteria, the gene located immediately upstream of the *hp0958* homolog shares homology with *hp0959*, and in *W. succinogenes* the two genes appear to be fused. This prompted us to investigate whether HP0959 also had a role in flagellar biogenesis. The *aphA3* cassette was used to disrupt *hp0959* in *H. pylori* ATCC 43504 and the resulting mutant expressed wild-type levels of HP0958 and was flagellated and motile (data not shown). These data indicate that despite the apparent fusion of HP0958 and HP0959 homologs in *W. succinogenes*, HP0959 is not required for HP0958 function in *H. pylori* flagellar biogenesis.

Inactivation of HP0958 interferes with expression of flagellar genes in the RpoN regulon. Given the interaction between σ^{54} and HP0958 in the yeast two-hybrid assay and our observation that HP0958 is required for flagellar biogenesis, we reasoned that HP0958 may influence σ^{54} function in *H. pylori*. To test this hypothesis, FlaB levels were assessed by western blotting in *H. pylori* ATCC 43504 and the mutant strains. The *flaB* gene is part of the RpoN flagellar regulon (19, 29), and we observed that a *rpoN* mutant failed to accumulate FlaB (Fig. 2B). A faint cross-reacting protein band was seen just below where FlaB was expected to migrate in the lane with cell lysate from the *rpoN* mutant. FlaA shares 58.4% amino acid identity with FlaB

and is slightly smaller than FlaB (53.3 kDal versus 53.9 kDal), and we infer that this cross-reacting band is FlaA. Consistent with this hypothesis, the FlaB antiserum cross-reacted weakly with purified histidine-tagged FlaA (data not shown). The level of FlaB in the *hp0958:aphA3* mutant was dramatically reduced, but was restored to wild-type level in the *hp0958*:*aphA3* mutant carrying a functional copy of *hp0958* in the *hp0405* locus as expected from the restoration of flagellar synthesis and motility (Fig. 2B).

To determine if the reduced levels of FlaB in the *hp0958:aphA3* mutant were due to decreased transcription of *flaB*, we introduced a *flaB'-'xylE* reporter gene into the *hp0405* locus of the mutant. The *flaB'-'xylE* reporter gene was also introduced into the *hp0405* locus of the *rpoN:aphA3* and wild-type strains. Expression of the *flaB'-'xylE* reporter gene was reduced in the *hp0958*:*aphA3* mutant to a level that was comparable to that in the *rpoN:aphA3* mutant (Fig. 3), indicating that the reduced level of FlaB in the *hp0958:aphA3* mutant was due to decreased transcription of *flaB*. Expression of another RpoN-dependent reporter gene, *hp1120'-'xylE,* was reduced in the *hp0958* mutant (Fig. 3), suggesting that HP0958 is required for transcription of genes of the RpoN flagellar regulon. A σ^{28}-dependent *flaA'-'xylE* reporter gene was introduced into the *H. pylori* strain to determine if HP0958 was required for expression of flagellar genes outside the RpoN regulon. Expression of the *flaA'-'xylE* reporter gene in the *hp0958:aphA3* mutant was ~ 3-fold higher compared to that in the wild-type and *rpoN:aphA3* mutant strains (Fig. 3), indicating that HP0958 is not required for expression of flagellar genes in the FliA regulon.

Inactivation of HP0958 results in a decreased level of σ^{54}. To determine if the decreased expression of *flaB* in the *hp0958:aphA3* mutant was due to decreased activity or levels of σ^{54}, σ^{54} levels in the *hp0958:aphA3* mutant and its parental strain were compared. As shown in

Figure 2C, the level of σ^{54} in the *hp0958:aphA3* mutant was significantly reduced compared to the wild-type strain. Introduction of a functional copy of *hp0958* in the *hp0405* locus of the *hp0958:aphA3* mutant restored the level of σ^{54} to close to wild-type level, demonstrating that HP0958 is required for the accumulation of wild-type level of σ^{54}.

To test if overproduction of σ^{54} in the *hp0958:aphA3* mutant would restore flagellar synthesis and motility, *rpoN* was placed under control of the *ureA* promoter and introduced into the *hp0405* locus of the *hp0958:aphA3* mutant. The level of σ^{54} in this strain exceeded that of the wild-type strain (Fig. 2C) and restored motility and expression of *flaB* (Fig. 4). Taken together, these data suggest that HP0958 is required for accumulation, but not function, of σ^{54}.

Overproduction of HP0958 interferes with its function. In the initial attempt to complement the *hp0958:aphA3* mutation an *hp0958* allele under control of the *ureA* promoter (P_{ureA}-*hp0958*) was introduced into the *hp0405* locus of the mutant, which failed to restore motility (data not shown). Introduction of the P_{ureA}-*hp0958* allele into wild-type *H. pylori* interfered with motility (Fig. 1), and as with inactivation of *hp0958*, it resulted in decreased levels of FlaB and σ^{54} (Figs. 2B and 2C). The level of HP0958 in the strain with the P_{ureA}-*hp0958* allele was ~10-fold higher than wild-type level, which was estimated by determining the least number of cells needed to visualize the protein by western blotting (data not shown). We do not know why overproduction of HP0958 interfered with its function. Behavior of HP0958 overproduced in *H. pylori* in the SDS-polyacrylamide gel was unusual in that much of the protein migrated with a reduced mobility (Fig. 2A). This may have resulted from cross-linking or other stable interactions between HP0958 monomers which could have interfered with activity of the protein.

HP0958 prevents the rapid turnover of σ^{54}. To address whether HP0958 exerts its control over σ^{54} accumulation post-translationally, we introduced a *rpoN'-'xylE* reporter gene into the

hp0958:aphA3 mutant and its parental strain. This *rpoN'-'xylE* reporter gene encoded a translational fusion in which *xylE* was joined in-frame with codon 61 of *H. pylori rpoN*. Expression of the *rpoN'-'xylE* reporter gene was indistinguishable in the two strains (0.47 ± 0.10 units XylE activity/10^8 cells for the parental strain versus 0.51 ± 0.07 units XylE activity/10^8 cells for the *hp0958:aphA3* mutant), which argued that HP0958 affects σ^{54} levels in *H. pylori* at a post-translational step.

To determine if HP0958 influences σ^{54} stability, σ^{54} levels were monitored in wild-type and *hp0958:aphA3* mutant strains where *rpoN* was under control of the *ureA* promoter. Strains were grown in serum-free medium to mid-log phase, at which time tetracycline was added to block translation. Samples were analyzed for σ^{54} at various times following the addition of tetracycline. Levels of σ^{54} in the *hp0958:aphA3* mutant decreased with an apparent half-life of ~33 min, whereas σ^{54} levels remained constant for at least 4 h in the wild-type strain (Fig. 5). In the absence of tetracycline, σ^{54} levels remained constant in the wild-type and the *hp0958:aphA3* mutant strains over the entire course of the assay (data not shown). We were unable to detect σ^{54} in the extracellular proteins of the *hp0958:aphA3* mutant or its parental strain, but in a positive control, the vacuolating cytotoxin VacA was detected in the extracellular proteins of both strains (data not shown). Taken together, these data suggest that the rapid decrease in σ^{54} levels in the *hp0958:aphA3* mutant does not result from secretion but rather from protein degradation.

Discussion

We demonstrate here that HP0958 is required for the normal accumulation of σ^{54} in *H. pylori*, indicating that it may play a regulatory role in modulating σ^{54} levels under different growth or environmental conditions. HP0958 appears to influence σ^{54} levels by protecting it

from proteolysis. Regulated turnover is a mechanism by which the levels of other σ factors are sometimes controlled. *E. coli* σ^S, for example, is degraded by ClpXP protease but is protected from proteolysis under certain conditions by the chaperone DnaK (27, 28). Like DnaK, HP0958 may function as a chaperone to protect σ^{54} from proteolysis, which would explain the interactions between HP0958 and σ^{54} in the yeast two-hybrid assay. Suppression of the *hp0958:aphA3* mutation by overexpressing *rpoN* may be explained by high levels of σ^{54} overwhelming the proteolysis of the protein. Alternatively, HP0958 may modify σ^{54} to protect it from proteolysis. The suppression of the *hp0958* mutation by overproducing σ^{54}, however, suggests that any such potential modification of σ^{54} is not needed to convert it from an inactive to active form. Thus, the only essential role HP0958 appears to play in flagellar biogenesis is in maintaining σ^{54} levels capable of supporting efficient expression of the RpoN regulon. HP0958 may have an additional, non-essential role in regulating the FliA flagellar regulon, however, since expression of the *flaA'-'xylE* reporter gene was elevated in the *hp0958* mutant but not in the *rpoN* mutant. HP0958 may affect *flaA* expression by influencing the level or activity of the anti-σ^{28} factor FlgM or another factor that inhibits expression of *flaA*.

We have not observed any other phenotypes for the *hp0958:aphA3* mutant. HP0958 interacts with other *H. pylori* proteins in the yeast two-hybrid assay, including FliH (a regulator of the flagellar protein export apparatus), HP1462 (a putative secreted protein involved in motility) and TonB1 (a putative siderophore-mediated iron transport protein). Thus, HP0958 may have other roles in addition to maintaining a wild-type level of σ^{54}. Since σ^{54} is required for flagellar biogenesis, the interactions between HP0958 and FliH are intriguing. Inactivation of *fliH*, however, does not affect σ^{54} levels in *H. pylori* (L. Pereira, unpublished data). Despite

several attempts, we were unable to construct an *hp0958*/*fliH* double mutant suggesting that this combination of mutations is lethal.

Comparison of the genome sequences of *H. pylori*, *H. hepaticus*, *C. jejuni* and *W. succinogenes* reveals similar organization of flagellar genes and potential regulatory mechanisms controlling their expression. All of these ε-Proteobacteria possess HP0958 homologs and we anticipate that these proteins have roles in maintaining wild-type σ^{54} levels in these bacteria. FlgR is the only σ^{54}-dependent activator present in *H. pylori*, *H. hepaticus* and *C. jejuni*, suggesting that σ^{54} is dedicated for flagellar biogenesis in these bacteria. *W. succinogenes*, however, possesses two σ^{54}-dependent activators, FlgR and NifA, which appear to be required for the expression of flagellar and nitrogen fixation genes, respectively. Thus, the HP0958 homolog in *W. succinogenes* may be required for flagellar biogenesis and nitrogen fixation.

In addition to the ε-Proteobacteria, homologs of HP0958 are present in a number of bacteria from diverse phylogenic groups, including *Aquifex aeolicus*, *Bacteroides thetaiotaomicron*, *Chlamydia trachomatis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Thermoanaerobacter tengcongensis*, *Chlorobium tepidum*, *Borrelia burgdorferi* and *Treponema pallidum*. Many of these bacteria possess σ^{54} and the HP0958 homologs in these bacteria may have roles in modulating σ^{54} levels. Elucidating the mechanism by which HP0958 influences σ^{54} levels in *H. pylori* is certain to lead to a better understanding of the function of its homologs in other bacteria.

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Figure 1. Motility of *H. pylori* 43504 and various mutant derivatives. Cells were inoculated on semisolid motility agar plates with a sterile toothpick and incubated for 4 to 5 days at 37^{o}C under microaerophilic conditions. Strains that were tested for motility were *H. pylori* 43504 (wild-type), a *rpoN:aphA3* mutant, a *hp0958:aphA3* mutant, a *hp0958:aphA3* mutant in which a functional copy of *hp0958* was introduced into the *hp0405* locus (*hp0958:aphA3*/*hp0405:hp0958*), and *H. pylori* 43504 bearing P_{ureA}-*hp0958* allele in the *hp0405* locus (*hp0405:*P_{ureA}-*hp0958*).

Wild-type
rpoN:aphA3
hp0958:aphA3
hp0958:aphA3/
hp0405:hp0958
hp0405:PureA-hp0958

Figure 2. Western blot analysis of HP0958, FlaB and σ^{54} in various *H. pylori* strains. For panels A and B, approximately 10^8 cells were lysed and loaded in each lane, while ~4 x 10^8 cells were lysed and loaded in each lane in panel C. Membranes were probed with antiserum directed against MBP-HP0958 (panel A), MBP-FlaB (panel B) or MBP-σ^{54} (panel C). Relevant genotypes of the strains that were analyzed are indicated above the lanes and include *H. pylori* 43504 (wild-type), a *rpoN:aphA3* mutant, a *hp0958:aphA3* mutant, a *hp0958:aphA3* mutant complemented with a copy of *hp0958* in the *hp0405* locus (*hp0958:aphA3*/*hp0405:hp0958*), a *hp0958:aphA3* mutant in which σ^{54} was overproduced (*hp0958*:*aphA3*/*hp0405*:P_{ureA}-*rpoN*), and *H. pylori* 43504 bearing a P_{ureA}-*hp0958* allele in the *hp0405* locus (*hp0405*:P_{ureA}-*hp0958*). Arrows indicate the positions of the proteins analyzed in each western blot.

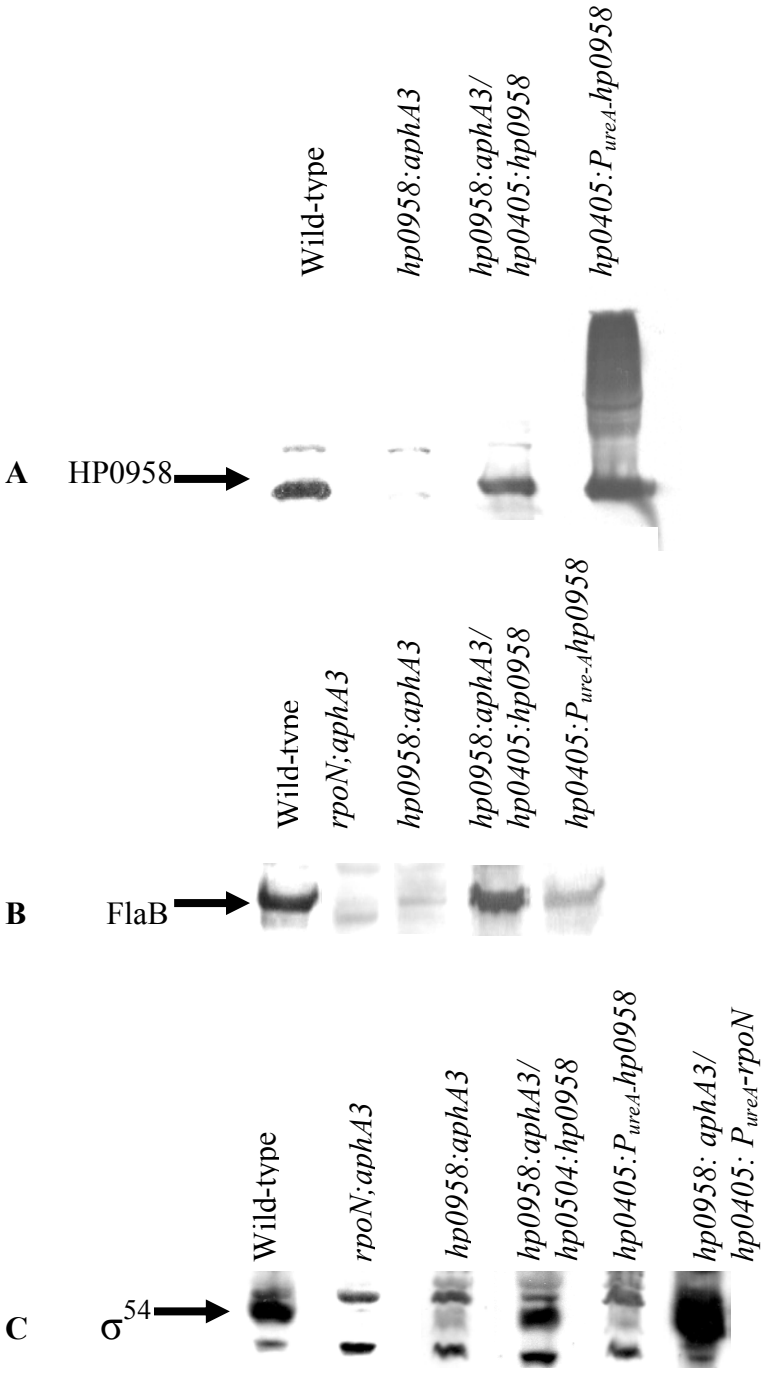

A
HP0958
Wild-type
hp0958:aphA3
hp0958:aphA3/
hp0405:hp0958
hp0405:PureA-hp0958
B
FlaB
Wild-tvne
rpoN;aphA3
hp0958:aphA3
hp0958:aphA3/
hp0405:hp0958
hp0405:Pure-Ahp0958
C
σ54
Wild-type
rpoN;aphA3
hp0958:aphA3
hp0958:aphA3/
hp0504:hp0958
hp0405:PureAhp0958
hp0958: aphA3/
hp0405: PureA-rpoN

Figure 3. Expression of *flaB'-'xylE*, *hp1120'-'xylE* and *flaA'-'xylE* reporter genes in various *H. pylori* strains. XylE activities were measured for the reporter genes indicated to the right of the graphs in *H. pylori* 43504 (wild-type; open bars), a *hp0958:aphA3* mutant (filled bars) and a *rpoN:aphA3* mutant (striped bars). Values represent the averages of at least ten assays and standard deviations for these values are indicated by the error bars.

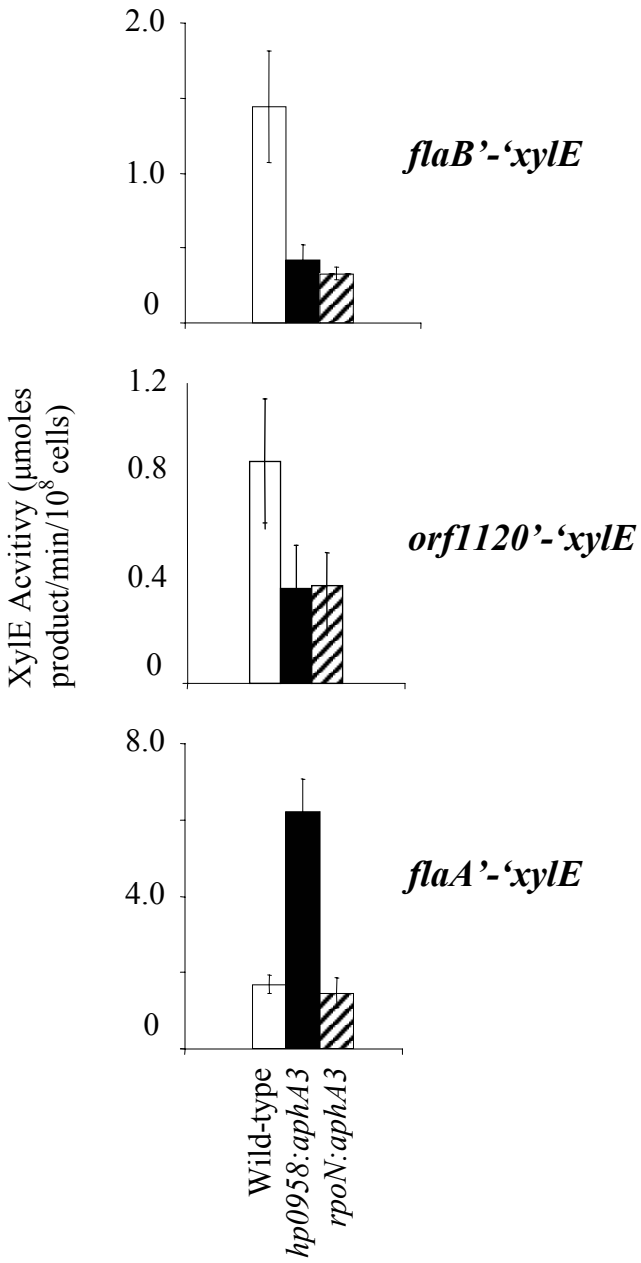

2.0
1.0
0
flaB'-'xylE
1.2
0.8
0.4
0
orf1120'-'xylE
XylE Acvitivy (µmoles product/min/10^8 cells)
8.0
4.0
0
flaA'-'xylE
Wild-type
hp0958:aphA3
rpoN:aphA3

Figure 4. Overproduction of σ^{54} in the *hp0958:aphA3* mutant restores motility and FlaB synthesis. (**A**) Motility of *H. pylori* 43504 (wild-type), a *hp0958:aphA3* mutant and a *hp0958:aphA3* mutant in which σ^{54} was overproduced from the *ureA* promoter was assessed in semisolid agar. (**B**) *H. pylori* strains (~10^8 cells) were analyzed by western blotting for FlaB (indicated by arrow). Lane 1, *H. pylori* 43504; lane 2, *hp0958:aphA3* mutant; lane 3, *hp0958:aphA3* mutant bearing a P_{ureA}-*rpoN* allele in the *hp0405* locus (*hp0958:aphA3*/*hp0405:*P_{ureA}-*rpoN*).

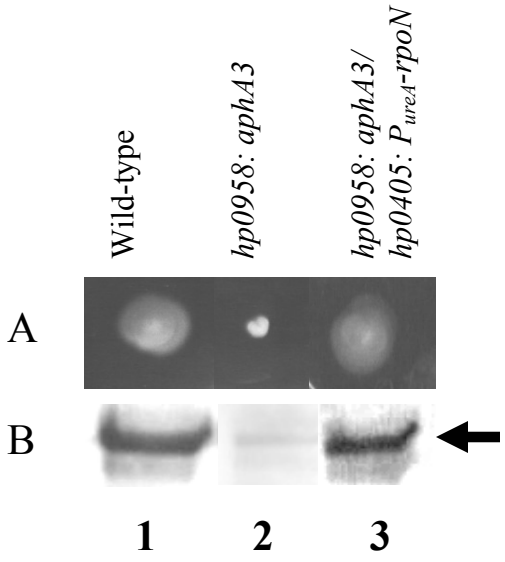
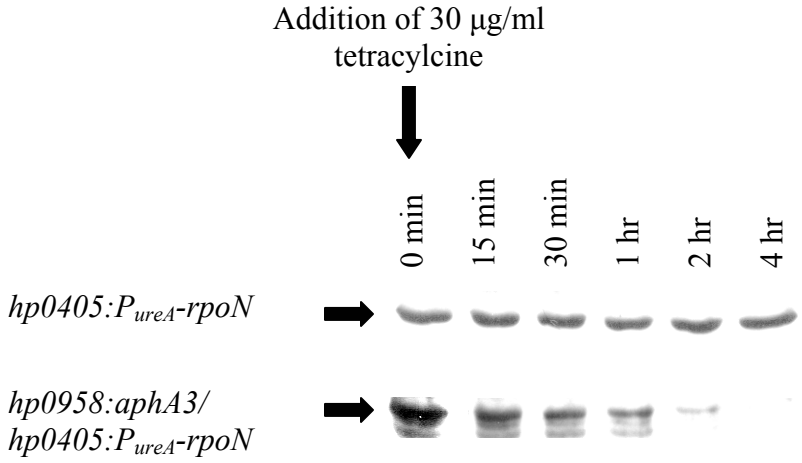

Wild-type
hp0958: aphA3
hp0958: aphA3/
hp0405: P_{ureA}-rpoN
A
B
1
2
3

Figure 5. Comparison of σ^{54} stability in a *hp0958:aphA3* mutant and its parental strain.

Levels of σ^{54} in a *hp0958:aphA3* mutant that overproduced σ^{54} from a P_{ureA}-*rpoN* allele (*hp0958:aphA3/hp0405*: P_{ureA}-*rpoN*) and its parental strain (*hp0405*:P_{ureA}-*rpoN*) were analyzed by western blotting at various times following the addition of tetracycline. Approximately 10^8 cells were lysed and loaded in each lane. σ^{54} is indicated by the arrows.


Addition of 30 µg/ml
tetracylcine
0 min
15 min
30 min
1 hr
2 hr
4 hr
hp0405:PureA-rpoN
hp0958:aphA3/
hp0405:PureA-rpoN

Chapter 3

Control of the *Helicobacter pylori* RpoN and FliA Flagellar Regulons by the Flagellar Protein Export Apparatus[1]

[1] Pereira, L., M. Aldrich and T. R. Hoover. To be submitted to *Journal of Bacteriology*.

Abstract

Flagellar biogenesis in *Helicobacter pylori* involves all three σ factors (σ^{80}, σ^{54} and σ^{28}) found in the bacterium. The *H. pylori* RpoN (σ^{54}) regulon includes genes encoding components of the basal body, hook, a minor flagellin (*flaB*), and several genes of unknown function. Our mutational analysis of one such gene, *hp0906*, revealed it was equivalent to *fliK*, which encodes a hook length control protein required for switching substrate specificity of the flagellar protein export apparatus from rod/hook-type to filament-type substrates. Expression of two RpoN-dependent reporter genes, *flaB'-'xylE* and *hp1120'-'xylE*, was stimulated ~7-fold in the *H. pylori fliK* mutant. Expression of a σ^{28} (FliA)-dependent *flaA'-'xylE* reporter gene was also enhanced in the *fliK* mutant, but to a lesser extent (<2-fold). Inactivation of *flhA*, which encodes a membrane component of the export apparatus, resulted in different outcomes with regard to expression of the RpoN-dependent reporter genes depending on the point at which *flhA* was disrupted. Disruption within a region encoding a predicted C-terminal cytoplasmic domain of FlhA resulted in decreased expression of the RpoN-dependent reporter genes, whereas an interruption near the 5'-end of the gene resulted in a ~15-fold stimulation in expression of these reporter genes. Expression of the *flaA'-'xylE* reporter gene was inhibited in both *flhA* mutant strains. These data suggest that expression of the RpoN and FliA regulons is coupled with the function of the flagellar protein export apparatus in *H. pylori*, and a model for transcriptional control of these regulons by the export apparatus is proposed.

Introduction

Helicobacter pylori is a microaerophilic, Gram-type negative bacterium that belongs to the ε-subdivision of Proteobacteria and is a a significant human pathogen of the gastric mucosa

(6, 10). Colonization of the gastric epithelium by *H. pylori* leads to a gastric inflammation that can progress to chronic gastritis, peptic ulcer, gastric cancer or mucosal-associated lymphoma (6, 7, 10, 39). Successful colonization of the gastric epithelium requires *H. pylori* to be motile, which is achieved through two to six polar, sheathed flagella (11, 12).

The bacterial flagellum consists of a basal body, hook and filament. During flagellar biogenesis the basal body is the first structure to be assembled, followed by the hook and finally the filament (28). Where it has been examined, the expression of flagellar genes is controlled by a transcriptional regulatory hierarchy that results in the synthesis of the flagellar components as they are needed for assembly of the flagellum (28, 51). The *H. pylori* genome contains over 40 flagellar genes that are scattered throughout the genome and organized into 25 or more transcriptional units (2, 49). Transcriptional regulation of these flagellar operons in *H. pylori* is complex, involving all three sigma factors found in the bacterium, σ^{80}, σ^{54} (RpoN), and σ^{28} (FliA).

Flagellar genes in the *H. pylori* RpoN regulon are required midway through the flagellar assembly process and encode components of the basal body, hook and filament. The *H. pylori* RpoN regulon consists of at least nine operons. Transcriptional start sites were mapped for five σ^{54}-dependent flagellar operons in *H. pylori*, *flaB* (encodes a minor flagellin), *flgE* (encodes hook protein), *hp0906* (the *hp* designation refers to the open reading frame number assigned in the annotated genome of *H. pylori* strain 22695), *flgBC* (encode proximal rod proteins) and *hp1120-flgK* (encodes hook-associated protein 1) (44, 46). A subsequent study using DNA microarrays identified *flgL* (encodes hook-associated protein 3), *hp1076*, *hp1154-murG* (encodes a glycosyltransferase for murein biosynthesis) and *flgJ* (encodes a binfunctional rod capping protein and muraminidase) as additional genes within the RpoN regulon (37). In addition to σ^{54},

expression of the genes in the *H. pylori* RpoN regulon requires a two-component system consisting of the response regulator FlgR (FleR) and the sensor kinase FlgS (FleS) (5, 44).

H. pylori flagellar genes in the FliA regulon are required at the end of flagellar biogenesis and code for components of the filament or factors required for assembly of these components. These genes include *flaA* (encodes the major flagellin), *fliD* (encodes filament cap protein), *fliS* and *fliT* (encode flagellar chaperones) (23, 27, 37). Expression of the FliA flagellar regulon is negatively regulated by the anti-σ^{28} factor FlgM (9, 21). In *Salmonella enterica* serovar Typhimurium the inhibitory effect of FlgM on σ^{28} activity is alleviated upon export of FlgM out of the cell via the flagellar protein export apparatus following completion of the hook-basal body complex (18, 22). Expression of *H. pylori flaA* does not require a functional *flgE* (38), indicating that relief of the inhibitory effect of FlgM on σ^{28} activity in *H. pylori* is not linked to formation of the hook-basal body complex as it is in *S. enterica* serovar Typhimurium.

Most of the flagellar proteins that are localized outside the cell membrane are exported from the cytoplasm by a type III flagellar export pathway (32). These proteins are translocated across the cytoplasmic membrane by an ATP-dependent mechanism into the central channel of the nascent flagellar structure where they are then able to diffuse to the distal end of the growing flagellum for assembly (28, 32). The flagellar protein export apparatus of *Escherichia coli* and *S. enterica* serovar Typhimurium consists of six membrane proteins, FlhA, FlhB, FliO, FliP, FliQ, and FliR, and three cytoplasmic proteins, FliH, FliI, and FliJ (28, 32). Homologs of all of these proteins have been identified in *H. pylori*, with the exception of FliO and FliJ (1, 2, 19, 20, 41, 43, 49). Components of the flagellar export apparatus and proteins associated with`` flagellar export are listed in Table 1. In the early stages of flagellar biogenesis the flagellar protein export apparatus preferentially exports rod and hook-type substrates. Upon completion

of the hook structure, the export apparatus switches specificity to filament-type substrates (16, 17, 26). This switching of substrate specificity involves the C-terminal cytoplasmic domain of FlhB ($FlhB_C$) and the hook length control protein FliK (13, 33, 35). Mutations in *fliK* or *flhB* that prevent switching in substrate specificity result in abnormally long hook structures called polyhooks (13, 33).

The initial annotations of the *H. pylori* 22695 and J99 genome sequences failed to identify *fliK*. Subsequent analysis of the deduced amino acid sequence of HP0906 from *H. pylori* 22695 revealed a region in this protein predicted by the Pfam collection of hidden Markov models to belong to a family that includes FliK. We inactivated the *hp0906* in *H. pylori* 43504 and examined the phenotype of the resulting mutant. The mutant was non-motile and some of the cells had the polyhooks characteristic of *S. enterica* serovar Typhimurium *fliK* mutants indicating that *hp0906* is equivalent to *fliK*. Expression of flagellar genes in the *fliK* mutant, as well as in *flhA* and *fliH* mutant strains, was examined using a set of reporter genes in which the promoter regions of various *H. pylori* flagellar genes were joined to *xylE* (encodes catechol 2,3-dioxygenase). Expression of the σ^{54}-dependent *flaB'-'xylE* and *hp1120'-'xylE* reporter genes was stimulated in the *fliK* mutant and in a *flhA* mutant in which the gene was disrupted near its 5'-end. In contrast, expression of the σ^{54}-dependent reporter genes was diminished in the *fliH* mutant and in a *flhA* mutant in which the gene was disrupted closer to its 3'-end. Expression of the σ^{28}-dependent *flaA'-'xylE* reporter gene was diminished substantially in both of the *flhA* mutants but was slightly higher in the *fliK* and *fliH* mutants. We propose a model in which the flagellar protein export apparatus controls expression of the RpoN regulon through interactions with the FlgS/FlgR two-component system and controls expression of the FliA regulon by secreting a negative regulator, probably FlgM, of this regulon.

Materials and Methods

Bacterial strains and media. *H. pylori* strains ATCC 43504 and 26695 were grown at 37˚C under an atmosphere of 4% O_2, 5% CO_2, and 91% N_2 on blood agar or tryptic soy agar supplemented with 5% horse serum (TSA-serum). When included in the medium, antibiotics were used at the following concentrations: 30 μg/ml chloramphenicol, 30 μg/ml kanamycin, 200 μg/ml bacitracin, and 15 μg/ml colistin.

Polymerase chain reactions. Genomic DNA used for polymerase chain reaction (PCR) was isolated from bacterial strains using the Wizard Genomic DNA Purification Kit (Promega). PCR amplifications were done with *Taq* DNA polymerase (Promega) using genomic DNA from *H. pylori* 26695 as a template. PCR products were cloned into the vector pGEM-T (Promega) and sequenced at the University of Georgia's Integrated Biotechnology Laboratories to confirm their identities and verify that no mutations had been introduced during amplification.

Construction of *H. pylori* mutant strains. A ~1.3 kb PCR product corresponding to ~550 bp from the 5'-end of *fliK* (*hp0906*) to ~210 bp of downstream of the 3'-end of the gene was amplified and cloned into pGEM-T. A ~1.3 kb *Eco*RI fragment from plasmid pSKAT4 (50) bearing the *Campylobacter coli* chloramphenical transacetylase (*cat*) gene was introduced into a unique *Eco*47III site in the cloned fragment of *fliK*, which inserted the *cat* cassette immediately following codon 367 of *fliK*. The resulting plasmid was introduced into *H. pylori* 43504 by natural transformation and mutants that resulted from allelic exchange between the chromosomal copy of *fliK* and the plasmid-borne copy disrupted with the *cat* cassette were selected on TSA-serum supplemented with chloramphenicol.

To construct a *H. pylori fliH* mutant, a ~ 770 bp PCR product bearing *fliH* was cloned into pGEM-T. A ~1.3 kb *Bam*HI fragment that contained the *cat* cassette from plasmid pSKAT4

was introduced into a unique *Bcl*I site within the cloned *fliH* gene, which disrupted the plasmid-borne copy of *fliH* within codon 228. The resulting plasmid was transformed into *H. pylori* 43504 and mutant strains in which the chromosomal copy of *fliH* had been disrupted were selected as described above.

Two different *H. pylori flhA* mutant strains were constructed as follows. For the first strain, ~1 kb of DNA from the 5'-end of *flhA* was amplied by PCR and cloned into plasmid pGEM-T. A ~1.3 kb *Sma*I fragment that contained the *cat* cassette from plasmid pSKAT4 was used to replace a 498 bp *Eco*47III fragment (corresponded to codons 77 to 242 of *flhA*) within the cloned *flhA* fragment. For the second *flhA* mutant strain, ~1 kb of DNA within *flhA* was amplified by PCR and cloned into pGEM-T. The QuickChange II Site-Directed Mutagenesis kit (Stratagene) was used to introduce an *Eco*RI site at codons 453 and 454 of *flhA*. Following site-directed mutagenesis, the cloned *flhA* fragment was sequenced to verify that the *Eco*RI site was present and that no other mutations had been introduced. A ~1.3 kb *Eco*RI fragment bearing the cat cassette from pSKAT4 was cloned into the engineered *Eco*RI site. The *flhA* suicide vectors were introduced into *H. pylori* 43504 and mutants were selected as described above. For all of the mutant strains, inactivation of the chromosomal copy of the target gene by the *cat* cassette was confirmed by PCR.

Transmission electron microscopy. *H. pylori* cells were grown on blood agar for 48 h at 37^{o}C and then gently resuspended in phosphate buffered saline, pH 7.4. Samples were negatively stained with 2% phosphotungstic acid (pH 7.0) and observed with a JEOL 100CK electron microscope (JEOL USA, Peabody, MA 01960) at the Center for Advanced Ultrastructural Research at the University of Georgia. Micrographs were taken at an accelerating voltage of 80 kV.

Measurement of XylE activity. Derivatives of the shuttle vector pHel3 (15) bearing *flaB'-'xylE*, *flaA'-'xylE*, or *flgI'-'xylE* reporter genes were described previously (8). For this study, a *hp1120'-'xylE* reporter gene was constructed in which a DNA fragment corresponding to positions –70 to + 44 relative to the transcriptional start site of the *hp*1120-*flgK* operon was amplified by PCR from *H. pylori* strain 43504 and introduced upstream of a promoter-less *Pseudomonas putida xylE*. The resulting *orf1120'-'xylE* fusion was cloned into the shuttle vector pHel3 to create pLPK. Reporter plasmids were introduced in *H. pylori* strains by natural transformation. XylE activities in these strains were measured in whole cells as described (8). One unit of XylE activity was defined as 1 μmole catechol oxidized/min and values were expressed as units/10^8 cells. Values were reported as averages for at least five independent assays for each sample and standard deviations were calculated.

Western blot analysis. *H. pylori* cells were lysed in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and applied to a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes which were probed with antiserum prepared in New Zealand white rabbits and directed against purified *H. pylori* FlaB which had a maltose-binding protein-tag linked to its N-terminus. Peroxidase-conjugated goat affinity purified antibody to rabbit IgG was used as the secondary antibody (ICN/Cappel). Cross-reactive protein bands were visualized by luminescence using an ECL kit (Amersham).

Results

***H. pylori hp0906* encodes a flagellar hook length control protein.** The *H. pylori* RpoN flagellar regulon includes a gene designated *hp0906*, the deduced amino acid sequence of which is 528 amino acid residues in length. The region corresponding to amino acid residues 336 to 477 of HP0906 is predicted by the Pfam collection of hidden Markov models to belong to a

family that includes the C-terminal domain of *S. enterica* serovar Typhimurium FliK, the flagellar hook length control protein. To determine if HP0906 were functionally equivalent to FliK, we inactivated *hp0906* in *H. pylori* 43504 and examined the phenotype of the resulting mutant. A cassette bearing the selectable *cat* marker was introduced into *hp0906* to disrupt the gene after codon 367. The *H. pylori hp0906* mutant was non-motile as assessed on soft agar plates and by microcinematography (data not shown). Disruption of *fliK* in *S. enterica* serovar Typhimurium results in the formation of abnormally long flagellar hooks referred to as polyhooks (40, 47). Most of the *hp0906* mutant cells lacked flagella when examined by electron microscopy, but some had the characteristic polyhooks observed with *S. enterica* serovar Typhimurium *fliK* mutants (Fig. 1). As with the normal *H. pylori* flagellum, the polyhook appeared to be surrounded by a sheath with an enlarged distal tip. The presence of polyhooks in the *H. pylori hp0906* mutant along with the assignment of HP0906 to the FliK family by the Pfam analysis argues strongly that HP0906 is the hook length control protein in *H. pylori*. The reason for the low frequency with which we observed polyhooks associated with the *H. pylori fliK* mutant is not known. One possible explanation is that FliK is required to initiate hook assembly as has been reported for *Caulobacter crescentus* (36).

Inactivation of *H. pylori fliK* stimulates expression of flagellar genes in the RpoN regulon. The effect of the *fliK* mutation on the expression of the different classes of flagellar genes was examined using reporter genes in which *xylE* was linked to the promoter regions of various flagellar genes. Two of these flagellar genes, *flaB* and *hp1120*, are within the RpoN regulon, one (*flaA*) is within the FliA (σ^{28}) regulon, and one (*flgI*) is apparently dependent on the primary σ factor of *H. pylori*, σ^{80}, for its transcription (37). As seen in figure 2, expression of the *flaB'-'xylE* and *hp1120'-'xylE* reporter genes required σ^{54}. Expression of these two σ^{54}-dependent

reporter genes was 5- to 7-fold higher in the *fliK* mutant compared to the wild-type strain (Fig. 2). Expression of the σ^{28}-dependent *flaA'-'xylE* reporter gene was also stimulated in the *fliK* mutant, but to a lesser extent (only ~1.6-fold) than the σ^{54}-dependent reporter genes. In contrast, expression of the σ^{80}-dependent *flgI'-'xylE* reporter gene was reduced ~2-fold in the *fliK* mutant.

The enhanced expression of the σ^{54}-dependent reporter genes in the *fliK* mutant did not appear to be due to elevated levels of σ^{54} or FlgR since western blot analysis indicated that these regulatory proteins were present at wild-type levels in the *fliK* mutant (data not shown). We did not have antiserum directed against FlgS to examine the level of this regulatory protein in the *fliK* mutant. The *flgS* gene is potentially part of an operon with *flgI*, however, and since expression of the *flgI'-'xylE* reporter gene was slightly diminished in the *fliK* mutant we do not expect FlgS levels to be elevated in this mutant. Thus, the enhanced expression of the σ^{54}-dependent reporter genes in the *fliK* mutant does not appear to result from elevated levels of any of the regulatory proteins known to be required for transcription of genes within the RpoN regulon.

Inactivation of *fliH* results in decreased expression of the flagellar genes in the RpoN regulon. Given the dramatic effect that disruption of *fliK* had on expression of the σ^{54}-dependent reporter genes, we wished to examine how inactivating genes encoding components of the flagellar protein export apparatus would impact expression of the RpoN regulon. We began this study by inactivating *fliH*, which encodes a regulatory protein that forms a complex with FliI and is thought to prevent FliI from hydrolyzing ATP until the energy released upon hydrolysis can be coupled to protein export (3, 34). FlilH is not essential for flagellar biogenesis in *S. enterica* serovar Typhimurium since a *fliH* null mutant in this bacterium is slightly motile, and motility in a *fliH* null mutant is improved upon overproduction of FliI or mutations in the

cytoplasmic domains of FlhA or FlhB (30). Disruption of *fliH* in *H. pylori* resulted in loss of motility as assessed on soft agar, and the *fliH* mutant strain lacked flagella when examined by transmission electron microscopy (data not shown). Expression of the *flaB'-'xylE* and *hp1120'-'xylE* reporter genes was 2- to 3-fold lower in the *fliH* mutant compared to the wild-type strain (Fig. 2). Wild-type levels of σ^{54} and FlgR were present in the *fliH* mutant as assessed by western blotting (data not shown), suggesting that the decreased expression of the σ^{54}-dependent reporter genes in this mutant were not due to reduced levels of these regulatory proteins. Expression of the *flgI'-'xylE* reporter gene was also reduced in the *fliH* mutant, but to a lesser extent than the RpoN-dependent reporter genes. In contrast, expression of the *flaA'-'xylE* reporter gene was slightly higher (~26%) in the *fliH* mutant versus the wild-type strain.

Depending on the site of disruption, inactivation of *H. pylori flhA* has distinct effects on the expression of genes in the RpoN regulon. A second component of the flagellar protein export apparatus that we examined for its influence on expression of the RpoN regulon was FlhA, which is a membrane component of the export apparatus. *H. pylori* FlhA contains seven predicted transmembrane helices within its N-terminal ~330 amino acid residues, while the ~400 residues at its C-terminus are predicted to constitute a large cytoplasmic domain referred to as $FlhA_C$ (43). Inactivation of *H. pylori flhA* had been reported previously to interfere with transcription of flagellar genes in the RpoN and FliA regulons (37, 43). In these previous studies, *flhA* was interrupted by inserting a kanamycin-resistance cassette in a *Hin*dIII site corresponding to codons 456 and 457, which are located within the region encoding $FlhA_C$. *H. pylori* 26695 *flhA* lacks this *Hin*dIII site, and so we used two *Eco*47III sites within this gene to construct a *flhA* mutant strain. In the resulting *flhA* allele, which we refer to as *flhA77*, codons 77 to 243 were replaced with a *cat* cassette.

As expected, the *flhA77* mutant strain was non-motile and aflagellated (data not shown). Expression of the *flaA'-'xylE* reporter gene was about ~6-fold lower in the *flhA77* mutant compared to the wild-type strain (Fig. 3), in agreement with the previous report from the DNA microarray studies on *flaA* transcript levels in a *H. pylori flhA* mutant (37). Also consistent with the results from the previous related study (37), expression of the *flgI'-'xylE* reporter gene was not affected in the *flhA77* mutant. Although expression of the *flgI'-'xylE* reporter gene appeared to be slightly reduced in the *flhA77* mutant, this reduction was not statistically significant. In contrast to the results from the previous DNA microarray studies in which *flaB* and *hp1120* transcript levels were reported to be reduced in a *H. pylori flhA* mutant (37), expression of the *flaB'-'xylE* and *hp1120'-'xylE* reporter genes was stimulated 14- and 18-fold, respectively, in the *flhA77* mutant strain (Fig. 3; data not shown for the *hp1120'-'xylE* reporter gene).

We postulated that the differences between our results and those of the previous related studies might reflect distinct consequences of *flhA* disruption depending on the size of the resulting truncated FlhA proteins. To address this hypothesis, we constructed a *flhA* allele that could yield a truncated FlhA protein of similar size to that in the previous related studies. This was done by introducing an *Eco*RI site at codons 454 and 455 of *flhA* from *H. pylori* 26695 by site-directed mutagenesis and then disrupting the gene at this position with a *cat* cassette. The resulting *flhA* allele, which we designated as *flhA454*, had the capacity of expressing a truncated FlhA protein that was only two amino acid residues shorter than that of the *flhA* allele used in previous related studies (37, 43). As observed with the *flhA77* mutant, expression of the *flaA'-'xylE* reporter gene was significantly reduced and expression of the *flgI'-'xylE* reporter gene was lowered slightly (~40%) in the *H. pylori* mutant with the *flhA454* allele (Fig. 3). In contrast to what we observed with the *flhA77* mutant, however, expression of the *flaB'-'xylE* and *hp1120'-*

'xylE reporter genes was inhibited in the strain with the *flhA454* allele (Fig. 3). As with the *fliK* and *fliH* mutants, the altered expression levels of the σ^{54}-dependent reporter genes in the strains with the two mutant *flhA* alleles did not appear to be associated with altered levels of σ^{54} or FlgR (data not shown).

Enhanced transcriptional activation of *flaB* in the *fliK* and *flhA77* mutant strains does not result in increased levels of FlaB. Whole cell extracts of the *H. pylori* mutants were analyzed by western blotting to determine if the results obtained with the *flaB'-'xylE* reporter gene mirrored changes in FlaB levels. Consistent with the requirement of σ^{54} for expression of *flaB*, western blot analysis of the *rpoN* mutant revealed only a very faint band that cross-reacted with the antiserum directed against FlaB (Fig. 4). FlaA and FlaB share 58% amino acid identity and are very similar in size (predicted molecular weights for FlaA and FlaB are 53,284 and 53,882 Dal, respectively). Thus, the faint cross-reacting band seen with cell extracts from the *rpoN* mutant may be FlaA. FlaB levels were reduced in the *fliH* mutant, consistent with the decreased expression of the *flaB'-'xy*lE reporter gene in this mutant. Despite the substantial increase in expression of the *flaB'-'xylE* reporter gene in the *fliK* and *flhA77* mutant strains, the levels of FlaB in these strains were not above wild-type levels (Fig. 4). This may have been due to decreased stability of FlaB in these mutant strains since the protein is presumably not exported from the cytoplasm in these strains. Alternatively, it could have resulted from additional levels of control (e.g., transcript stability or translational control) that may operate in *H. pylori* to modulate the expression of *flaB*. We were unable to analyze FlaA levels by western blotting in the mutant strains since the antiserum that we generated against FlaA does not discriminate between FlaA and FlaB.

Discussion

The regulation of flagellar biogenesis is a complex and highly coordinated process. Where it has been examined in detail, expression of flagellar genes involves a regulatory hierarchy in which a master regulator controls the earliest set of genes needed for flagellar assembly. In *E. coli* and *S. enterica* serovar Typhimurium the master regulator is FlhCD (4, 24, 25), while in *C. cresentus* it is CtrA (42). Although a 'classical' master regulator of flagellar biogenesis has not been identified in *H. pylori*, transcription of the flagellar genes in this bacterium does appear to be subject to a complex regulatory hierarchy. DNA microarray studies on growth-phase-dependent gene expression in *H. pylori* revealed temporal changes in flagellar gene transcription, and these patterns of gene expression suggested that the *H. pylori* flagellar genes were expressed in groups that roughly correspond to flagellar gene classes in other bacteria (48).

To elucidate the mechanisms used by *H. pylori* to accomplish the temporal regulation of genes within the RpoN and FliA flagellar regulons, it is important to know the functions of the genes within these regulons. Functions have not been assigned for the products of several of the genes within the RpoN regulon, including *hp0906*. Based on the Pfam prediction that HP0906 belongs to the FliK family and our observation that at least some of the *hp0906* mutant cells displayed polyhooks, HP0906 is functionally equivalent to FliK. The predicted size of *H. pylori* FliK is 58,161 Da, making it somewhat larger than the FliK proteins from *E. coli* and *S. enterica* serovar Typhimurium which are 39,246 and 41,748 Da, respectively. HP0906 may have other roles besides functioning as a hook length control protein, which could be the reason for its larger size and may also account for our inability to clone the full-length *hp0906* despite several attempts.

A recent model was proposed for the domain organization and function of *S. enterica* serovar Typhimurium FliK (35). FliK is a highly elongated molecule and is predicted to consist of two domains, $FliK_N$ and $FliK_C$, connected by a flexible linker, along with a short stretch of unstable C-terminal chain ($FliK_{CT}$) attached to $FliK_C$ (35). FliK is exported from the cytoplasm via the flagellar protein export apparatus during hook assembly (31). Within the central channel of the nascent flagellar structure, $FliK_N$ is thought to act as a sensor and transmitter of hook length information (35). This information is communicated to $FliK_C$ and $FliK_{CT}$ to alter their structure and allow $FliK_{CT}$ to interact with $FlhB_C$, triggering a conformational change in $FlhB_C$ that results in the export apparatus switching substrate specificity from rod/hook-type to filament-type (13, 33, 35).

Several studies have provided evidence that links the expression of the RpoN and FliA flagellar regulons with the flagellar protein export apparatus in *H. pylori* and the closely related *Campylobacter jejuni*. Porwollik and co-workers showed that inactivation of *fliI* or *fliQ* in *H. pylori* resulted in reduced levels of both flagellins and the hook protein (41). Allan and colleagues subsequently showed that in addition to *fliI* and *fliQ*, mutations in *flhB* also resulted in reduced levels of flagellins and hook protein (1). This latter study demonstrated further that *flaA* transcript levels were reduced in the *fliI*, *fliQ* and *flhB* mutants (1). Since *flaB* and *flgE* transcript levels were not examined in either of these previous studies, it is not clear if the reduced amounts of FlaB and hook protein in the mutant strains resulted from decreased transcription of genes in the RpoN regulon or interference at some other step in gene expression. Recent DNA microarray studies by Niehus and co-workers showed that disruption of *flhA* resulted in decreased expression of both the RpoN and FliA flagellar regulons (37). Finally, inactivation of genes encoding several components of the flagellar protein export apparatus in *C. jejuni*,

including *flhA*, *flhB*, *fliP*, and *fliR*, inhibited transcription of two different RpoN-dependent flagellar genes, but not the expression of a FliA-dependent reporter gene (14).

We show here that mutations in *flhA* result in dramatic differences with regard to expression of the RpoN regulon, and these differences reflect distinct consequences of *flhA* disruption depending on the potential size of the truncated FlhA protein. Alleles of *flhA* such as *flhA454* and that used by Niehus and co-workers (37) which have the potential to produce a truncated protein consisting of the membrane-associated domain and a significant portion of $FlhA_C$ inhibit expression of the RpoN regulon. McMurry and co-workers analyzed *S. enterica* serovar Typhimurium *flhA* by constructing a series of deletion mutations of 20 codons each that together comprised a systematic partial deletion of the entire $FlhA_C$ (29). Most of these FlhA variants allowed residual activity of the export apparatus, suggesting that $FlhA_C$ is not required for proper localization of the protein. Thus, the truncated FlhA protein produced from the *flhA454* allele may have localized correctly to the export apparatus even though it lacked 279 amino acid residues from its C-terminus. In contrast to *flhA454*, the *flhA77* allele, which has the potential to produce only the N-terminal 76 amino acid residues of FlhA, stimulated expression of the RpoN regulon.

The inhibitory effect of larger truncated FlhA proteins on expression of the RpoN regulon appears to be dependent on FlgM, since Niehus and co-workers observed that levels of the σ^{54}-dependent transcripts in a *flhA*/*flgM* double mutant were substantially higher than wild-type levels (37). Inactivation of *flgM* in a *flhF* mutant background did not similarly stimulate expression of the RpoN regulon (37), suggesting that the suppression of the *flhA* mutation by disruption of *flgM* cannot be explained by FlgM interfering directly with σ^{54}-RNA polymerase holoenzyme function. FlgM may inhibit expression of the RpoN regulon in some *flhA* mutants

by binding the flagellar protein export apparatus and interfering with its normal signaling to the RpoN regulon.

We suggest a model for the temporal transcriptional control of the RpoN and FliA regulons in *H. pylori* by the flagellar protein export apparatus (Fig. 5). In the case of the FliA regulon, we propose that the export apparatus secretes a factor which inhibits expression of σ^{28}-dependent flagellar genes, the most likely candidate being FlgM. This would explain the decreased expression of genes in the FliA regulon in the *flhA*, *fliQ* and *fliI* mutants (1, 37) since the export apparatus in these mutant strains is likely defective in exporting substrates. This implies, however, that FliH is not required for the export of FlgM since disruption of *fliH* did not interfere with transcription of the *flaA'-'xylE* reporter gene (Fig 2). Precedence exists for the export of flagellar substrates in the absence of FliH since a *S. enterica* serovar Typhimurium *fliH* null mutant is slightly motile and motility is improved in the *fliH* null mutant by overproduction of FliI (30). FlgM from *H. pylori* 26695 lacks the N-terminal 20 amino acid residues found in *S. enterica* serovar Typhimurium FlgM, but it does have the second region (IIa in *S. enterica* serovar Typhimurium FlgM) that is involved in protein secretion (9, 21). *S. enterica* serovar Typhimurium FlgM is exported from the cell upon completion of the hook-basal body complex (18). We postulate that *H. pylori* FlgM is exported prior to completion of the hook-basal body complex since expression of the *flaA'-'xylE* reporter gene was enhanced slightly in the *fliK* mutant (Fig. 2) and we expect the flagellar protein export apparatus of this mutant to be locked in the conformation that exports rod/hook-type substrates. Thus, expression of the σ^{28}-dependent flagellar genes in *H. pylori* may occur at an earlier stage in the flagellar assembly pathway than it does in *S. enterica* serovar Typhimurium. The products of the flagellar genes in the FliA regulon

would presumably accumulate in the cytoplasm until the flagellar protein export apparatus switched its substrate specificity to filament-type.

We postulate that the flagellar protein export apparatus mediates its effects on expression of the RpoN regulon through the FlgS/FlgR two-component system. In particular, the export apparatus may control expression of the RpoN regulon by modulating the activity of FlgS. Unlike many sensor kinases, FlgS lacks predicted transmembrane helices and appears to be a cytoplasmic protein. Upon assembly of flagellar protein export apparatus, interactions between FlgS and specific components of the export apparatus may modulate the autokinase activity of FlgS to influence phosphorylation of FlgR which is required for transcriptional activation of genes in the RpoN regulon (5, 8). This model is analogous to the chemotaxis system in which the activity of a cytoplasmic sensor kinase, CheA, is controlled by interactions with a membrane-bound methyl-accepting chemotaxis protein (45). The enhanced expression of the σ^{54}-dependent reporter genes in the mutant strain with the *flhA77* allele indicates that a functional export apparatus is not required for transcriptional activation of genes within the RpoN regulon. This observation argues against alternative models for control of the RpoN regulon by the export apparatus, such as the export apparatus secreting a factor that inhibits expression of the σ^{54}-dependent flagellar genes. Based on the enhanced expression of the σ^{54}-dependent reporter genes in the *fliK* mutant, we postulate that the ability of the flagellar protein export apparatus to interact productively with FlgS is dictated by the conformation of the export apparatus. The flagellar protein export apparatus in the *fliK* mutant is presumably locked in the conformation that secretes rod/hook-type substrates. This conformation may interact productively with FlgS whereas the conformation that exports filament-type substrates is unable to do so, which could explain the increased expression of the σ^{54}-dependent reporter genes in the *fliK* mutant. Since

fliK is part of the RpoN regulon, this could serve as a transcriptional feedback inhibition mechanism that shuts down expression of the RpoN regulon once the flagellar protein export apparatus switches specificity to filament-type substrates.

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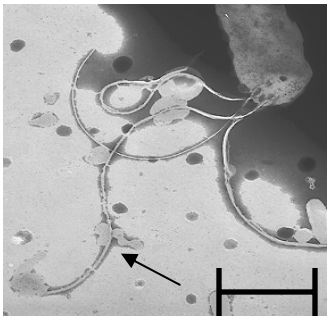
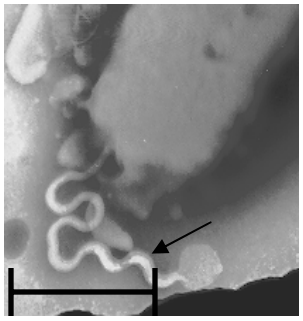
Table 1: Components associated with the flagellar assembly and the flagellar export apparatus.

Protein	Function
FlhA	Integral membrane protein - substrate translocation
FlhB	Integral membrane protein - substrate recognition
FliO	Integral membrane protein
FliP	Integral membrane protein
FliQ	Integral membrane protein
FliR	Integral membrane protein
FliH	Docking protein, regulator of FliI activity
FliI	ATPase
FliJ	General chaperone
FliK	Hook-length control
FlhF	Flagellar number and placement

Figure 1. Transmission electron micrographs of wild-type *H. pylori* and *hp0906:cat* mutant. Arrows indicate the normal flagellar filament in the wild-type strain and the polyhook structure in the *hp0906:cat* mutant. Note the shorter wavelength of the polyhook compared to the wild-type flagellar filament. Also note the terminal bulbs present in both flagellar structures. Bar = 1 μm.



wild-type



hp0906:cat

Figure 2. Expression of flagellar reporter genes in wild-type, *rpoN:cat*, *fliK:cat* and *fliH:cat* strains of *H. pylori*. The reporter genes assayed in the strains are indicated above each graph. Bar graphs represent the average values from at least 10 independent assays, with error bars indicating the standard deviations for these averages.

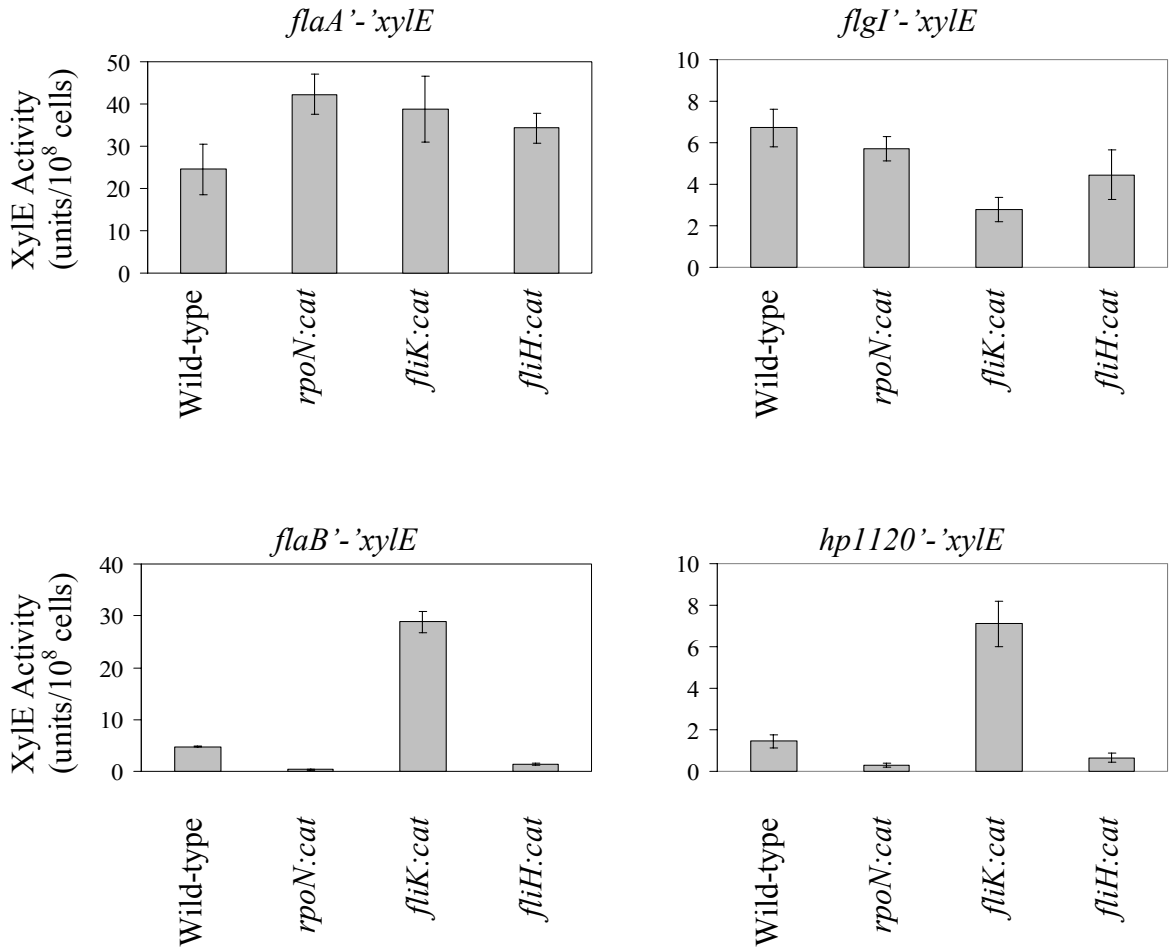

flaA'-'xylE
XylE Activity (units/10^8 cells)
50
40
30
20
10
0
Wild-type
rpoN:cat
fliK:cat
fliH:cat
flgI'-'xylE
10
8
6
4
2
0
Wild-type
rpoN:cat
fliK:cat
fliH:cat
flaB'-'xylE
XylE Activity (units/10^8 cells)
40
30
20
10
0
Wild-type
rpoN:cat
fliK:cat
fliH:cat
hp1120'-'xylE
10
8
6
4
2
0
Wild-type
rpoN:cat
fliK:cat
fliH:cat

Figure 3. Expression of flagellar reporter genes in *H. pylori* strains having the wild-type, *flhA77* or *flhA454* alleles. The reporter genes that were assayed in the strains are indicated above each graph. Bar graphs represent the average values from at least 5 independent assays, with error bars indicating the standard deviations for these averages.

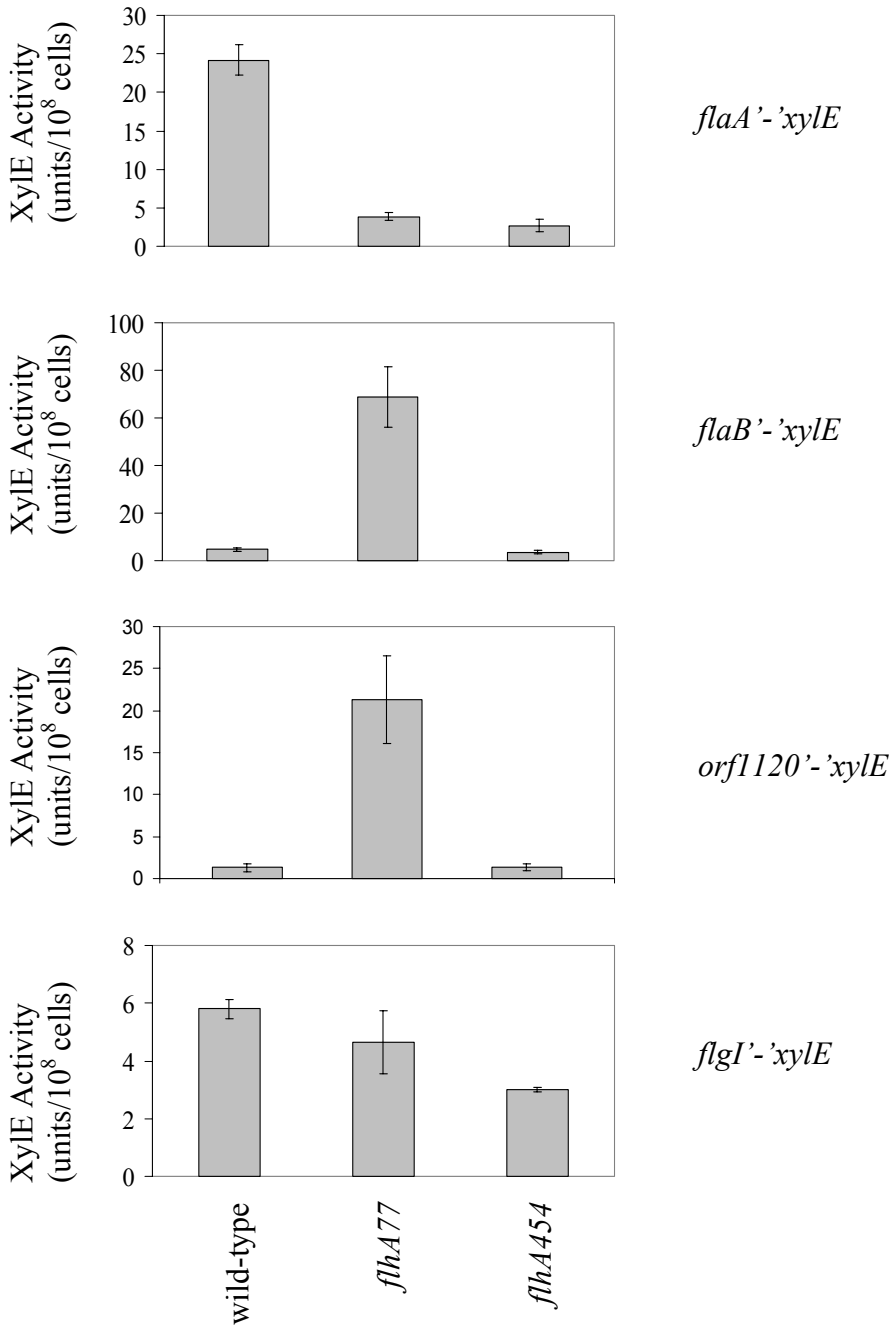

XylE Activity (units/10^8 cells)
30
25
20
15
10
5
0
flaA'-'xylE
XylE Activity (units/10^8 cells)
100
80
60
40
20
0
flaB'-'xylE
XylE Activity (units/10^8 cells)
30
25
20
15
10
5
0
orf1120'-'xylE
XylE Activity (units/10^8 cells)
8
6
4
2
0
flgI'-'xylE
wild-type
flhA77
flhA454

Figure 4. Western blot analysis for FlaB in cell extracts of various *H. pylori* strains. Approximately 10^8 cells of each strain were lysed in SDS-PAGE loading buffer and then loaded onto a SDS-PAGE gel. Antiserum directed against histidine-tagged FlaB was raised in a rabbit and used for detection of FlaB. Cross-reacting protein bands were visualized using peroxidase-conjugated goat antibody to rabbit IgG and an ECL (Amersham) luminescence kit.

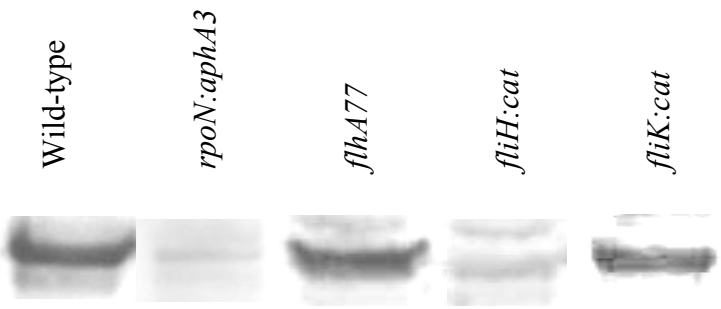
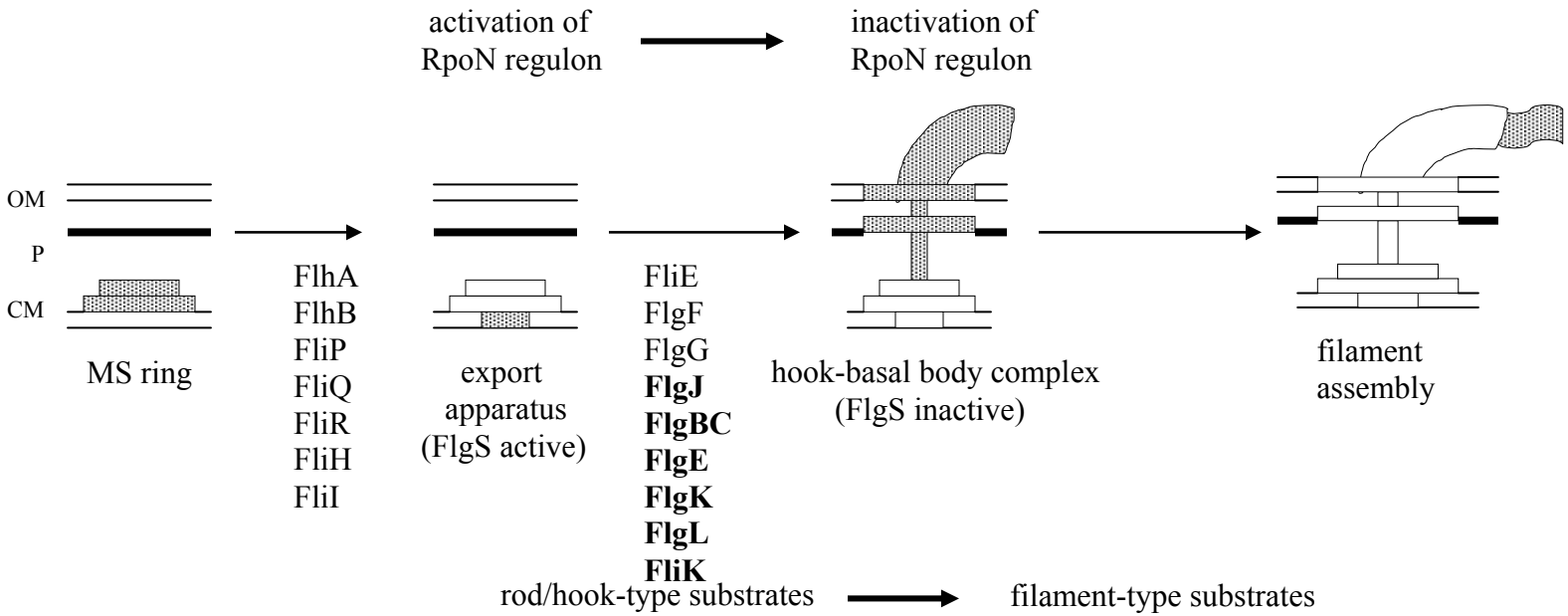

Wild-type
rpoN:aphA3
flhA77
fliH:cat
fliK:cat

Figure 5. Model for transcriptional control of the *H. pylori* RpoN regulon by the flagellar protein export apparatus. For each step of the model, newly assembled structures are indicated as stippled figures. As occurs in *S. enterica* serovar Typhimurium, the flagellar protein export apparatus is assembled following formation of the MS ring. Membrane components of the export apparatus are indicated within the brackets. Cytoplasmic components of the export apparatus that have been identified in *H. pylori* are FliH and FliI. The general chaperone of the export apparatus, FliJ, has not yet been identified in *H. pylori*. Following its assembly, we postulate that the flagellar protein export apparatus interacts with FlgS to stimulate the autokinase activity of this sensor kinase. FlgS-phosphate is able to donate its phosphate to FlgR, which results in the transcriptional activation of the σ^{54}-dependent flagellar genes. Rod/hook-type substrates that are products of the σ^{54}-dependent flagellar genes are indicated in bold. One of these products is FliK, which is required for switching substrate specificity of the export apparatus from rod/hook-type to filament-type. We postulate that following this conformation change, the export apparatus is no longer able to interact productively with FlgS to stimulate its autokinase activity. Thus, the switch in substrate specificity acts as a feedback mechanisms to shut down transcriptional activation of the RpoN regulon. CM, P and OM refer to the cytoplasmic membrane, peptidoglycan layer and outer membrane, respectively.


activation of
RpoN regulon
inactivation of
RpoN regulon
OM
P
CM
MS ring
FlhA
FlhB
FliP
FliQ
FliR
FliH
FliI
export
apparatus
(FlgS active)
FliE
FlgF
FlgG
FlgJ
FlgBC
FlgE
FlgK
FlgL
FliK
hook-basal body complex
(FlgS inactive)
filament
assembly
rod/hook-type substrates
filament-type substrates

Chapter 4

The promoter binding activity of *Helicobacter pylori* σ^{54} differs from that of other σ^{54} proteins[1]

[1] Pereira, L., Brahmachary, P., and T. R. Hoover. To be submitted to *Journal of Bacteriology.*

Abstract

Several genes encoding components of the *Helicobacter pylori* flagellum require σ^{54} for their expression. Promoter sequences were previously identified for five σ^{54}-dependent flagellar operons in *H. pylori* by primer extension assays. A striking feature of these sequences and potential σ^{54}-dependent promoters from other closely related ε-Proteobacteria is the presence of an A at position -23 instead of C or T since such a substitution drastically reduces the affinity of σ^{54}-RNA polymerase holoenzyme for the promoter in enteric bacteria. To determine if the base preference at position -23 for *H. pylori* σ^{54} differed from that of other σ^{54} proteins, the promoter binding affinity of the protein was examined *in vitro*. The *Escherichia coli* maltose-binding protein (MBP) was fused to the amino-terminus of *H. pylori* σ^{54} to facilitate its purification. The MBP-σ^{54} protein bound DNA probes bearing putative σ^{54}-dependent promoters from *H. pylori*. Substitution of the A at -23 of the *H. pylori flaB* promoter with a C reduced the apparent affinity of MBP-σ^{54} for the promoter sequence in the gel mobility shift assay. This substitution did not effect expression of a *flaB'-'xylE* reporter gene in *H. pylori*, but substitutions of A to G or A to T at -23 of the *flaB* promoter drastically reduced expression. Taken together, these findings confirm that the sequences identified previously are *bona fide* σ^{54}-dependent promoters and indicate that the specificity of *H. pylori* σ^{54} differs somewhat from that of σ^{54} proteins from many other bacteria.

Introduction

Association of core RNA polymerase with the σ subunit results in formation of RNA polymerase holoenzyme that recognizes specific promoter sequences (16). Bacteria often contain multiple σ factors that allow RNA polymerase holoenzyme to recognize different classes of

promoters (16). Primary σ factors are responsible for transcription of most of the genes in the cell, while others are alternative σ factors that regulate expression of specific sets of genes. An alternative σ factor found in many bacterial species is σ^{54} (RpoN), which is involved in diverse metabolic processes such as nitrogen assimilation and fixation, C_4-dicarboxylate transport, degradation of aromatic compounds, hydrogen metabolism, and flagellar biosynthesis (30).

σ^{54} differs from other σ factors both in its primary structure and its mode of action (7). Promoters recognized by σ^{54} from a variety of bacterial species have conserved elements located –12 and –24 relative to the transcriptional start site with the consensus sequences 5'-TTTGCW-3' (where W is A or T) and 5'-YTGGCACG-3' (where Y is C or T), respectively (4). σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) binds to the promoter to form a stable closed complex but isomerization of this closed complex to an open complex competent to initiate transcription requires an activator (34, 43). The activator binds to specific sites, known as upstream activation sequences (UAS) or enhancers (8, 36). These sites are sufficiently far enough from the promoter that the activator can bind to them and contact σ^{54}-holoenzyme in the closed complex through DNA looping (37, 40). Productive contacts between the activator and σ^{54}-holoenzyme lead to the isomerization of the closed complex to the open complex in a reaction that requires ATP hydrolysis by the activator (27, 34).

In the gastric pathogen *Helicobacter pylori*, σ^{54} is required for the transcription of several operons whose genes encode components of the flagellum (31, 38, 41). Transcription of the σ^{54}-dependent flagellar genes requires the activator FlgR, which is also a response regulator of a two-component system (5, 38). Unlike most σ^{54}-dependent activators, FlgR lacks the C-terminal DNA-binding domain that is responsible for UAS recognition and does not require DNA sequences upstream of the promoter to activate transcription, but rather appears to contact σ^{54}-

holoenzyme directly to activate transcription (6). Examination of the predicted promoters of the *H. pylori* σ^{54}-dependent flagellar genes reveals that most have an A at position -23 instead of C as occurs in the consensus sequence from σ^{54}-dependent promoters from other bacteria (31, 38). *H. pylori* is a member of the ε-subdivision of the phylum Proteobacteria, and potential σ^{54}-dependent promoters are located upstream of most of the orthologs of the *H. pylori* σ^{54}-dependent flagellar genes found in other ε-Proteobacteria whose genomes have been sequenced, which includes *Helicobacter hepaticus*, *Campylobacter jejuni* and *Wolinella succinogenes* (3, 33, 42). In the case of *C. jejuni*, many of these genes have been shown to be dependent on σ^{54} for their expression (12, 18, 19, 22). *W. succinogenes* genome contains an ortholog of the σ^{54}-dependent activator NifA within the cluster of nitrogen-fixation (*nif*) genes, and potential σ^{54}-dependent promoters are also located upstream of some of the *W. succinogenes nif* genes. As with the promoters of the *H. pylori* σ^{54}-dependent genes, the potential σ^{54}-dependent promoters from *H. hepaticus*, *C. jejuni* and *W. succinogenes* have a strong preference for A at position -23 (Fig. 1). This suggests that the specificity of σ^{54} proteins from *H. pylori* and other ε-Proteobacteria for the base at position –23 differs from that of other σ^{54} proteins.

To address this issue, *H. pylori* σ^{54} was purified and its affinity for various promoters was examined *in vitro*. *H. pylori* σ^{54} was fused to the maltose-binding protein (MBP) to facilitate its expression and purification. Results from an *in vitro* gel mobility shift assay suggest that the promoter recognition specificity of *H. pylori* σ^{54} differs from that of other σ^{54} proteins. Expression of a *flaB'-'xylE* reporter gene with various base substitutions at -23 further demonstrated the distinct promoter specificity of *H. pylori*. .

Materials and Methods

Bacterial strains. *Escherichia coli* strains used were BL21 (DE3) [F^- *E. coli* B *gal*$^-$ *hsds ompT lon* λDE3] and DH5α [ϕ80d *lacZ* ΔM15 *recA*1 e*ndA*1 *gyrA*96 *thi*$^-$1 *hsdR*17 (r_k^-, m_k^+) *supE*44 *relA*1 *deoR* Δ(*lacZYA-argF*) U169) and were grown in Luria- Bertani (LB) broth at 37°C. *H. pylori* strains 26695 and ATCC 43504 were grown on blood agar or tryptic soy agar supplemented with 5% horse serum (TSA-serum) and grown at 37°C under an atmosphere of 4% oxygen, 5% carbon dioxide, and 91% nitrogen. Plasmids were introduced into *H. pylori* by natural transformation as follows. *H. pylori* cells were patched onto TSA-serum and allowed to grow for 5 to 7 h. Plasmids (10 μl) were then spotted onto the cells which were transferred to TSA-serum supplemented with the appropriate antibiotics after 24 h to select for cells that had acquired the desired plasmid.

Polymerase chain reactions. Genomic DNA used for polymerase chain reaction (PCR) template was prepared using the Wizard Genomic DNA Purification Kit (Promega). All oligonucleotides used in the study for PCR primers were purchased from Integrated DNA Technologies. Amplification of *H. pylori* DNA sequence by PCR was done using *Taq* DNA polymerase. PCR products were cloned into the vector pGEM-T (Promega) and the sequences of the cloned PCR products were confirmed by the Integrated Biotechnology Laboratories at the University of Georgia.

Expression and purification of MBP-σ^{54}. *H. pylori rpoN* was amplified by PCR from *H. pylori* 43504 genomic DNA. *Nde*I and *Hin*dIII sites were introduced via the PCR primers at either end of the amplified DNA which was cloned into pGEM-T (Promega) and sequenced to verify that no mutations had been introduced during amplification. To construct an expression vector for the maltose-binding protein (MBP)-σ^{54}, the resulting plasmid was digested with *Nde*I and the 5'-

overhang was filled-in with T4 DNA polymerase. The plasmid DNA was then digested with *Hin*dIII and a 1.24 kb DNA fragment bearing *rpoN* was cloned into pJES489 which had been digested previously with *Not*I, filled-in with T4 DNA polymerase and then digested with *Hin*dIII. The plasmid pJES489 is a derivative of pMAL-c (New England Biolabs) that has a *Not*I linker inserted in the *Stu*I site and carries a fragment of *E. coli malE* which encodes the maltose-binding domain of MBP but not the signal peptide. The *malE-rpoN* chimera was under control of the *lac* promoter and LacI repressor expressed from $lacI^q$ on the same vector (pLEP1).

MBP-σ^{54} was expressed from plasmid pLEP1 in *E. coli* DH5α by growing 1 liter of cells at 37^{o}C to an OD_{600} of about 0.3. Expression of MBP-σ^{54} was induced by adding isopropyl β-D-1-thiogalactopyranoside to the growth medium to a final concentration of 1 mM and incubating the culture for an additional 3.5 hours. Cells were harvested, resuspended in 50 mM Tris-acetate, pH 8.2, 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and lysed in a French pressure cell at 7000 p.s.i. The cell extract was centrifuged at 17000 x g for 45 minutes and the supernatant containing MBP-σ^{54} was applied to an amylose-agarose affinity column (New England Biolabs, ~3 ml bed volume) that had been equilibrated previously with 20 mM Tris-HCl, pH 7.4, 5% (w/v) glycerol, 1 mM DTT, 1 mM EDTA and 200 mM KCl (buffer A). After washing the column with buffer A, MBP-σ^{54} was eluted with buffer A plus 10 mM maltose. Peak fractions containing MBP-σ^{54} were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mM maltose and 5% (w/v) glycerol (buffer B). The dialyzed fractions were applied to a Hi-Trap heparin-agarose (Pharmacia) column which had been equilibrated previously with buffer B. After washing with buffer B, MBP-σ^{54} was eluted from the column with a linear gradient to buffer B plus 1M KCl. The peak fractions of MBP-σ^{54}, which eluted at ~ 0.2 M KCl, were pooled, concentrated and stored at –

80°C in aliquots. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce). Antiserum against purified MBP-σ^{54} was raised in a New Zealand white rabbit (Cocalico Labs, Reamstown, PA).

***H. pylori flgR* and *rpoN* mutant strains.** An *H. pylori* mutant strain in which *flgR* gene was disrupted with the *Campylobacter coli* chloramphenicol transacetylase (*cat*) gene (strain BP2) was constructed previously [Brahmachary, 2004 #165]. An *H. pylori rpoN* mutant (strain LP1) was constructed by introducing an *Eco*RI site ~370 bp from the start codon of *H. pylori rpoN* using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) and a ~1.3 kb *Eco*RI fragment from plasmid pSKAT4 (49) that contained the *C. coli cat* cassette was cloned into this *Eco*RI site to create plasmid pLEP2. The plasmid was transformed into *H. pylori* 43504 and mutants in which the chromosomal copy of *rpoN* has been inactivated with the *cat* cassette were selected as described previously (6). Disruption of the chromosomal copy of *rpoN* in *H. pylori* strain LP1 was verified by PCR using genomic DNA from the mutant.

DNA-binding assays. Gel mobility shift assays were done as described previously (24). Binding reaction mixtures contained 25 mM Tris-acetate, pH 8.0, 8 mM magnesium acetate, 10 mM KCl, 1 mM DTT, 3.5% (w/v) PEG 8000, and 8 μg/ml sonicated calf thymus DNA in a final volume of 20 μL. Synthetic oligonucleotides used in gel mobility shift assays are indicated in Table 1 and were purchased from Integrated DNA Technologies. For each DNA probe, one of the oligonucleotide strands was end-labeled with ^{32}P-(γ)-ATP (3000 Ci/mmole, Amersham Biosciences) and polynucleotide kinase. Reactions contained 1-10 μM MBP-σ^{54} or histidine-tagged *Salmonella enterica* serovar Typhimurium σ^{54} as indicated. Purified *S. enterica* serovar Typhimurium σ^{54} was a gift from Hao Xu. *E. coli* RNA polymerase used for some binding assays was purchased from Epicentre. Binding reactions were initiated by adding ~0.25 nM

labeled DNA probe (~30,000 dpm) to the mixture. After incubating reaction mixtures at 30^{o}C for 10 min, protein-DNA complexes were resolved on a native polyacrylamide gel at 4^{o}C with 45 mM Tris-borate, pH 8.0, and 1 mM EDTA as the running buffer. Gels were dried and bands were visualized by exposing x-ray film to the dried gels.

Measurement of XylE activity. Derivatives of the shuttle vector pHel3 (20) that carried *flaB'-'xylE* or *hp1120'-'xylE* reporter genes were introduced in *H. pylori* by natural transformation. A *flaB'-'xylE* reporter gene was constructed initially by cloning a DNA fragment corresponding to positions –34 to + 44 relative to the transcriptional start site of *flaB* (38) upstream of a promoter-less *Pseudomonas putida xylE*. Substitutions of A to C, A to T and A to G at position -23 of the *flaB* promoter were introduced into this reporter plasmid with the Quick-Change II Site-Directed Mutagenesis kit. Mutant *flaB'-'xylE* reporter genes were sequenced following site-directed mutagenesis to confirm that the appropriate substitutions had been made and that no other mutations had been introduced. The *hp1120'-'xylE* reporter gene consisted of a DNA fragment corresponding to positions –70 to + 44 relative to the transcriptional start site of the *hp*1120-*flgK* operon (38) upstream of the promoter-less *P. putida xylE*. *H. pylori* strains bearing reporter plasmids were streaked onto TSA-serum agar supplemented with 30μg/ml kanamycin and incubated at 37^{o}C for 48 h. Cells were resuspended in phosphate buffered saline, pH 7.4 and the OD_{600} of each cell suspension was adjusted to approximately 1.0 unit which corresponded to ~1 x 10^{9} cells/ml. XylE activities were determined for the cells at room temperature spectrophotometrically at 375 nm as described previously (32). Values reported were the averages of at least ten independent assays.

Derivates of the shuttle vector pHEL3 that carried wild-type and mutant *flaB'-'xylE* reporter genes were transformed into *E. coli* that carries *H. pylori* FlgR central domain on the

vector pTrcHisC (Invitrogen) (6). *E. coli* strains bearing reporter plasmids were streaked on LB agar supplemented with 30μg/ml kanamycin and 50μg/ml ampicillin and grown at 37°C for 24 h. XylE assays were performed as described above for *H. pylori*.

Results

The promoter recognition specificity of *H. pylori* σ^{54} differs from that of other σ^{54} proteins.

In *H. pylori*, at least nine flagellar operons are transcribed by σ^{54}-holoenzyme. Some of the genes in these operons are *flaB* (encodes a minor flagellin), *flgE* (hook protein), *flgBC* (proximal rod proteins) *flgK* (hook-associated protein 1), *flgL* (hook-associated protein 3), *flgJ* (a bifunctional rod-capping protein and muraminidase) and *fliK* (hp0906; hook length control protein) (31, 38). Potential promoters for five σ^{54}-dependent operons were identified by primer extension analysis (38).

A sequence comparison of 186 σ^{54}-dependent promoters from a number of bacterial species resulted in the consensus DNA sequence 5'-YTGGCACGRNNNTTGCW-3' in which the conserved GG and GC doublets that are underlined occur at –24 and –12 relative to the transcriptional start site, respectively (4). Alignment of the putative *H. pylori* σ^{54}-dependent promoters and potential σ^{54}-dependent promoters from other ε-Proteobacteria shows a slightly different consensus (Fig. 1), with the most notable difference being the A at –23 instead of C in the ε-Proteobacteria consensus. Since this change in the *S. meliloti nifH* promoter severely diminishes the affinity of σ^{54}-holoenzyme for the promoter in enteric bacteria (1), we reasoned that the promoter specificity of *H. pylori* σ^{54} differs from that of many other σ^{54} proteins at the -

23 position. To test this hypothesis the binding of *H. pylori* σ^{54} to various promoter sequences *in vitro* was examined.

To facilitate the purification of *H. pylori* σ^{54}, we initially introduced a histidine-tag at the amino-terminus of the protein and expressed it in *E. coli*. The bulk of the histidine-tagged σ^{54} was insoluble, and we were unsuccessful in solubilizing and purifying the protein. Therefore, we constructed a chimeric MBP-σ^{54} protein since MBP has been reported to enhance the solubility of proteins to which it is fused (23). The MBP-σ^{54} was soluble and readily purified in two affinity chromatography steps.

Binding of MBP-σ^{54} protein to promoter DNA was examined initially in a gel mobility shift assay using 21 bp probes that spanned residues –29 to –9 of the *S. meliloti nifH* promoter or the *H. pylori flaB* promoter. The sequences of DNA probes used in this study are indicated in Table 1. The *nifH* promoter was used for these experiments since it matches the published consensus sequence and σ^{54} has a high affinity for it (4, 17, 24, 25). Binding activities of the MBP-σ^{54} protein to the two promoter sequences were compared with the activities of purified histidine-tagged σ^{54} from *S. enterica* serovar Typhimurium. As seen in the gel mobility shift assay, the MBP-σ^{54} protein bound the *nifH* promoter poorly, with <1% of the DNA probe shifted by MBP-σ^{54}; while *S. enterica* serovar Typhimurium σ^{54} bound the *nifH* promoter very effectively, shifting the mobility of >50% of the DNA probe (Fig. 2A).

Binding of the σ^{54} proteins to a *nifH* promoter fork junction DNA probe was also examined in the gel mobility shift assay. The fork junction DNA probe was double-stranded from –29 to –9 with a single-stranded overhang of the 5'-end of the template strand from –9 to –11 (see Table 1). The fork junction mimics DNA in the open complex, and σ^{54}-holoenzyme binds the *nifH* fork junction probe with higher affinity than the DNA probe that is double-stranded over its entire

length (17, 24, 25). Introduction of the fork junction did not improve the binding of *H. pylori* MBP-σ^{54} to *nifH* promoter (Fig. 2B). A second shifted species was observed above the complex formed with MBP-σ^{54} (Fig. 2B, lanes 3 and 4). This shifted species may have resulted from contaminating *E. coli* core RNA polymerase since purified *E. coli* core RNA polymerase resulted in a shifted species with the same mobility in the gel mobility shift assay (data not shown). In contrast to the results with MBP-σ^{54}, binding of *S. enterica* serovar Typhimurium σ^{54} to the *nifH* fork junction DNA probe was improved, which was especially evident at the lower protein concentrations (compare lanes 5 and 6 in Figs. 2A and 2B). At the highest concentration of *S. enterica* serovar Typhimurium σ^{54} tested >80% of the *nifH* fork junction probe was shifted.

Although σ^{54} binds promoter DNA by itself, σ^{54}-holoenzyme has a significantly higher affinity for promoter DNA than does free σ^{54} (10). *H. pylori* core RNA polymerase has not been purified in an active form and so we could not include it in the gel mobility shift assay to determine if it assisted MBP-σ^{54} in binding the *nifH* fork junction promoter. We determined, however, if *E. coli* core RNA polymerase could assist MBP- σ^{54} in the binding of the *nifH* promoter. When purified *E. coli* core RNA polymerase was included in the fork junction binding assay along with the MBP-σ^{54} protein there was no evidence of a holoenzyme-shifted species (data not shown), suggesting that MBP-σ^{54} does not bind effectively to *E. coli* core RNA polymerase.

Binding of the σ^{54} proteins to the *flaB* promoter was examined next. The *H. pylori* MBP-σ^{54} bound weakly to the *flaB* promoter double-stranded probe with ~1% of the DNA probe shifted by the protein. In contrast to the results with the *nifH* promoter probe, *S. enterica* serovar Typhimurium σ^{54} bound poorly to the *flaB* double-stranded probe (Fig. 3A). Binding of *H. pylori* MBP-σ^{54} to the *flaB* promoter fork junction probe was improved compared

to the double-stranded *flaB* promoter probe, with ~10% of the DNA probe shifted at the highest protein concentration and a shifted DNA species readily apparent at even the lowest protein concentration tested (Fig. 3B). Binding of *S. enterica* serovar Typhimurium σ^{54} to the *flaB* fork junction probe was better than that to the double-stranded probe, but was still significantly lower than that of the *nifH* promoter probes. As in the binding assays with the *nifH* fork junction probe, including *E. coli* core RNA polymerase in the assay did not improve the binding of MBP-σ^{54} to the *flaB* fork junction probe (data not shown).

The results of the gel mobility shift assay with the *nifH* and *flaB* promoters suggested that the residue at -23 was an important determinant for recognition of the promoter by *H. pylori* MBP-σ^{54}. However, the sequences of the *nifH* and *flaB* promoters differ at other positions which also could have contributed to the low affinity of *H. pylori* MBP-σ^{54} for the *nifH* promoter. Therefore, binding of *H. pylori* MBP-σ^{54} to a mutant *flaB* promoter in which the A at the -23 position was replaced with C was examined. *H. pylori* MBP-σ^{54} bound the mutant *flaB* fork junction probe, but the affinity of the protein for this mutant *flaB* promoter was clearly diminished (Fig.4). Taken together, these data indicate that the sequence recognition specificity for *H. pylori* σ^{54} at position -23 of the promoter differs from that of *S. enterica* serovar Typhimurium σ^{54}.

Binding of MBP-σ^{54} to the promoters of other σ^{54}-dependent flagellar operons from *H. pylori*. The putative promoter sequences of the *H. pylori* flagellar operons that are expressed by σ^{54}-holoenzyme differ with respect to each other. The relative affinity of MBP-σ^{54} for the five promoter sequences identified by Spohn and Scarlato from primer extension assays (38) was examined in a gel mobility shift assay. As with the *nifH* and *flaB* promoters, we compared binding of MBP-σ^{54} to double-stranded and fork junction DNA probes that corresponded to the –29 to –9 regions of each promoter (see Table 1). In general, MBP-σ^{54} bound the double-stranded

probes of the other flagellar gene promoters (*flgB, fliK, flgE* and *orf1120-flgK*) better than it did the *flaB* double-stranded probe (Fig. 5). MBP-σ^{54} appeared to have the highest affinity for the *flgE* and *fliK* double-stranded DNA probes, with ~2 to 3% of these DNA probes shifted at the lowest MBP-σ^{54} concentration tested (2.5 μM). These results clearly demonstrate that the sequences upstream of the flagellar genes that were previously identified as σ^{54}-dependent promoters are *bona fide* σ^{54}-dependent promoters.

In contrast to the result with the double-stranded DNA probes, *H. pylori* MBP-σ^{54} bound to the *flaB* promoter fork junction better than most of the other fork junction DNA probes (Fig. 5). Only the *flgE* promoter fork junction appeared to have a higher affinity for MBP-σ^{54} than the *flaB* promoter fork junction. For some of the flagellar promoters such as *fliK* and *flgE*, MBP-σ^{54} did not discriminate between the fork junction and double-stranded DNA probes in the gel mobility shift assay. Interestingly, the affinity of *H. pylori* MBP-σ^{54} for the fork junction DNA of the *orf1120-flgK* promoter was less than that for the double-stranded DNA of this promoter. This observation was reminiscent of activator-independent mutants of σ^{54} that form a σ^{54}-holoenzyme that has a higher affinity for the double-stranded than fork-junction DNA and are capable of initiating transcription in the absence of an activator (11, 13, 25, 46, 47). Certain promoter mutations in the –12 region also allow for activator-independent expression (48). These previous observations prompted us to determine if the promoter of the *orf1120-flgK* operon were a naturally occurring example of an activator-independent promoter. The same low level expression of the *orf1120-xylE* reporter gene was seen in *H. pylori rpoN* and *flgR* mutant backgrounds (Table 2), arguing that activator-independent expression does not occur in *H. pylori* from the *orf1120-flgK* operon under the growth conditions of our assay.

***H. pylori* σ^{54}-RNA polymerase holoenzyme tolerates an A to C substitution, but not T or G substitutions, at position -23 of the *flaB* promoter.** Since replacing the A at position -23 of the *flaB* promoter with a C decreased the affinity of MBP-σ^{54} for this promoter sequence, we determined if this substitution also reduced transcription initiation in *H. pylori*. In addition, we examined the effect of other substitutions at this position on transcription from the *flaB* promoter. A *flaB'-'xylE* reporter gene was constructed by cloning a DNA fragment corresponding to positions –34 to + 44 relative to the transcriptional start site of *flaB* upstream of a promoter-less *Pseudomonas putida xylE*. Expression of the resulting *flaB'-'xylE* reporter gene in *H. pylori* was dependent on both σ^{54} and FlgR (data not shown). The A to C substitution at -23 of the *flaB* promoter had no effect on expression of the *flaB'-'xylE* reporter gene (Fig. 6). In contrast, replacing the A at -23 with either T or G resulted in background levels of expression of the *flaB'-'xylE* reporter genes.

The expression of these reporter genes was also tested in *E. coli*. *H. pylori* FlgR central domain has been shown to function with *E. coli* σ^{54}–holoenzyme (6). Since the *flaB'-'xylE* reporter genes lack an enhancer sequence that would otherwise be recognized by the σ^{54}–dependent activator in *E. coli*, the *H. pylori* σ^{54}–dependent activator FlgR was introduced to ensure that reporter gene expression was the result of productive interactions between *E. coli* σ^{54}-holoenzyme and *H. pylori* FlgR. In *E. coli*, the A to C or T substitution at -23 resulted in ~ four-fold increase in *flaB'-'xylE* expression. However, replacing A with G at position -23 did not significantly affect *flaB'-'xylE* expression in *E. coli*.

Discussion

Flagellar biogenesis in *H. pylori* involves all three of the σ factors found in this bacterium. Transcription of genes encoding components of the basal body and flagellar protein export apparatus is dependent on σ^{80} (31, 39), the primary σ factor of *H. pylori*. Flagellar genes transcribed by σ^{54}-holoenzyme encode components of the basal body, hook and filament, as well as the hook length control protein, FliK (31, 38). The last set of flagellar genes require σ^{28} for their expression and encode the major flagellin, filament cap and enzymes that modify flagellin (26, 28, 31). Flagellar biogenesis in bacteria occurs in a stepwise manner starting with the synthesis of the basal body and protein export apparatus, followed by the hook, and then finally the filament (29). In other bacteria, flagellar assembly is coordinated with a transcriptional hierarchy of the flagellar genes (15, 29, 35, 51). The organization of the *H. pylori* flagellar genes according to the σ factor required for their expression suggests the same holds true for *H. pylori*. Consistent with this hypothesis, DNA microarray studies showed that the different classes of flagellar genes, organized according to the σ factor required for their expression, are transcribed maximally at different growth phases in *H. pylori* cultures (45).

Dissecting the molecular mechanisms involved in the temporal regulation of the flagellar gene expression in *H. pylori* requires an understanding of the function of σ^{54} in this bacterium. The data presented here show that the sequences identified previously as σ^{54}-dependent promoters based on primer extension assays (38) are indeed recognized by *H. pylori* σ^{54}. The affinity of MBP-σ^{54} for the flagellar promoter DNA probes in the gel mobility shift assay was significantly less than that of *S. enterica* serovar Typhimurium σ^{54} for the *nifH* promoter. It is unclear if the relatively poor promoter binding activity of MBP-σ^{54} was due to the MBP or if this is an intrinsic property of *H. pylori* σ^{54}.

A notable feature of most of the *H. pylori* σ^{54}-dependent promoter sequences is the A at the -23 position instead of C, since a C to A substitution in the *S. meliloti nifH* promoter drastically reduces the affinity of *S. enterica* serovar Typhimurium σ^{54} for the promoter (1). Sequence comparisions of the σ^{54}-dependent promoters from *H. pylori* and other ε-Proteobacteria (Fig. 1), along with the results of the fork junction binding assays with *H. pylori* MBP-σ^{54} (Figs. 4 and 5) suggested that σ^{54} from these bacteria has a preference for promoters with an A at -23. The RpoN box is a highly conserved motif near the C-terminus of σ^{54} and has been implicated in recognition of the -24 region of the promoter (9, 44). The consensus sequence of the RpoN box generated from σ^{54} proteins from a wide variety of bacterial species is RRTVAKYR (30), while the sequence of the RpoN box of σ^{54} from *H. pylori*, *H. hepaticus*, *C. jejuni* and *W. succinogenes* is slightly different (RRTITKYR) which could account for the different base specificity at position -23 of the promoter. We attempted to test this hypothesis by changing the sequence of the RpoN box of MBP-σ^{54} to the consensus sequence RRTVAKYR, but the resulting mutant protein failed to bind the fork junction DNA probes of the *nifH*, *flaB* or mutant *flaB* promoters (data not shown). Since the substitutions in the RpoN box may have influenced the folding or structure of the DNA-binding domain of σ^{54} we cannot draw any conclusion at this time about a potential relationship between the RpoN box and the -24 region sequences.

Unlike most other σ^{54}-dependent activators, *H. pylori* FlgR activates transcription without first binding an enhancer (6). FlgR proteins from *H. hepaticus*, *C. jejuni* and *W. succinogenes* have predicted C-terminal DNA-binding domains. While some of these predicted domains may not function in enhancer-binding, this does not seem likely for *W. succinogenes* FlgR since this bacterium has a second σ^{54}-dependent activator, NifA, and enhancer-binding is needed to prevent these FlgR and NifA from activating transcription from non-cognate promoters. Thus,

the preference for A at position -23 in the *H. pylori* σ^{54}-dependent promoters does not appear to be related to the mechanism by which FlgR activates transcription in this bacterium.

Despite the reduced affinity of MBP-σ^{54} for the *flaB* promoter probe in which the A at -23 was changed to C, expression from this mutant promoter was unaffected in *H. pylori* (Fig. 6). It is possible that sequences flanking the core promoter region assist binding of *H. pylori* σ^{54} to the mutant *flaB* promoter. Alternatively, *H. pylori* core RNA polymerase and/or FlgR may compensate for the reduced affinity of σ^{54} for the mutant *flaB* promoter. It will be of interest to test this latter hypothesis *in vitro* once we have obtained active preparations of *H. pylori* core RNA polymerase. In *E. coli*, an A to C or T change at -23 resulted in substantially higher expression levels suggesting that *E. coli* σ^{54}-holoenzyme has a poor affinity for promoters that have the *H. pylori* consensus sequence. This is in agreement with the *in vitro* gel shift assay, where *S. enterica* serovar Typhymurium displayed higher affinity for the *nifH* promoter which has a C at -23.

The products of the σ^{54}-dependent flagellar genes in *H. pylori* are required at different levels (29). For example, the *S. enterica* serovar Typhimurium flagellum contains about 130 subunits of the hook protein but only 6 subunits of the proximal rod proteins. In other bacteria, rates of transcription initiation from σ^{54}-dependent promoters can be influenced a number of factors, including the affinity of σ^{54}-holoenzyme for the promoter, the affinity of the activator for the UAS or enhancer, the position of the UAS or enhancer relative to the promoter, and the use of auxiliary DNA-binding proteins such as integration host factor which stabilize the DNA loop that brings the activator into contact with σ^{54}-holoenzyme (2, 14, 21, 50). Since *H. pylori* FlgR lacks the DNA-binding domain found in other activators of σ^{54}-holoenzyme, the affinity of σ^{54}-holoenzyme for the various flagellar gene promoters may be the primary means by which *H.*

pylori achieves different levels of flagellar gene transcripts. Consistent with this hypothesis, MBP-σ^{54} appeared to have the highest affinity for the promoters of *flaB* and *flgE*, the products of which are required at higher levels that those of the other σ^{54}-dependent operons.

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Table 1: Sequences of oligonucleotides used for gel mobility shift assays.

Promoter	Sequence
S. meliloti nifH double-stranded probe	5′-GGCTGGCACGACTTTTGCACG-3′ 3′-CCGACCGTGCTGAAAACGTGC-5′
S. meliloti nifH fork junction probe	5′-GGCTGGCACGACTTTTGC-3′ 3′-CCGACCGTGCTGAAAACGTGC-3′
H. pylori flaB double-stranded probe	5′-AGTTGGAACACCCTTTGCTTG-3′ 3′-TCAACCTTGTGGGAAACGAAC-5′
H. pylori flaB fork junction probe	5′-AGTTGGAACACCCTTTGC-3′ 3′-TCAACCTTGTGGGAAACGAAC-5′
H. pylori mutant *flaB* double-stranded probe	5′-AGTTGGCACACCCTTTGCTTG-3′ 3′-TCAACCGTGTGGGAAACGAAC-5′
H. pylori mutant *flaB* fork junction probe	5′-AGTTGGCACACCCTTTGC-3′ 3′-TCAACCGTGTGGGAAACGAAC-5′
H. pylori flgBC double-stranded probe	5′-TTTTGGAATGTTTTTTGCATA-3′ 3′-AAAACCTTACAAAAAACGTAT-5′
H. pylori flgBC fork junction probe	5′-TTTTGGAATGTTTTTTGC-3′ 3′-AAAACCTTACAAAAAACGTAT-5′
H. pylori fliK double-stranded probe	5′-TTAAGGAACTCTTTTTGCTTA-3′ 3′-AATTCCTTGAGAAAAACGAAT-5′
H. pylori fliK fork junction probe	5′-TTAAGGAACTCTTTTTGC-3′ 3′-AATTCCTTGAGAAAAACGAAT-5′
H. pylori flgE double-stranded probe	5′-TTTAGGAACACCTTTTGCTTT-3′ 3′-AAATCCTTGTGGAAAACGAAA-5′
H. pylori flgE fork junction probe	5′-TTTAGGAACACCTTTTGC-3′ 3′-AAATCCTTGTGGAAAACGAAA-3′
H. pylori orf1120-flgK double-stranded probe	5′-AAATGGCATAGTATTTGCTTG-3′ 3′-TTTACCGTATCATAAACGAAC-5′
H. pylori orf1120-flgK fork junction probe	5′-AAATGGCATAGTATTTGC-3′ 3′-TTTACCGTATCATAAACGAAC-5′

Table 2: Expression of the *orf1120-xylE* reporter gene in wild-type and mutant *H. pylori* strains.

***H. pylori* strain**	**Relevant genotype**	**XylE activity (µmoles product/min/10^8 cells)**
ATCC 43504	wild-type	2.25 ± 1.06
PB2	*flgR*$^-$	0.31 ± 0.15
LP1	*rpoN*$^-$	0.29 ± 0.18

Figure 1. Consensus sequence of σ^{54}-dependent promoters from *H. pylori* and other ε-Proteobacteria. Sequences of nine σ^{54}-dependent promoters from *H. pylori* and twenty-one potential promoters from *H. hepaticus*, *C. jejuni* and *W. succinogenes* were aligned and the frequency with which each of the four nucleotides appeared at specific positions was calculated. Positions are relative to the conserved GG and GC doublets, which occur approximately at positions -24 and -12, respectively, relative to the transcriptional start site (4). The sequences used to generate the consensus are found upstream of the following genes: *H. pylori flaB*, *flgL*, *flgE*, *fliK*, *flgJ*, *flgB*, *murG*, *hp1076* and *hp1120*; *H. hepaticus flaB*, *flgL*, *flgE*, *flgB*, *murG*, *Hh0583* (equivalent to *hp1076*) and *Hh0875* (equivalent to *hp1120*); *C. jejuni flaB*, *flgL* (*flaD*), *flgE*, *flgB* and *Cj1650* (equivalent to hp1076); *W. succinogenes flaB*, *flgL*, *flgE*, *flgB*, *Ws0228* (equivalent to *hp1076*), *Ws0260* (equivalent to *hp1120*), *nifH* and *nifB*. The sequences used for the alignment were within 60 bp of the predicted translational start sites of these genes. The frequency at which a given nucleotide occurred at each position is indicated by the different bars, with the black bar indicating A residues, the white bar indicating T residues, the light gray bar indicating G residues and the dark gray bar indicating C residues. Conserved nucleotides are those that occurred in at least 70% (21 of 30) of the sequences. W (A or T) and Y (C or T) are indicated in the consensus sequence if they were present in at least 90% (27 of 30) of the sequences. The general consensus sequence present in other bacteria is indicated below the chart.

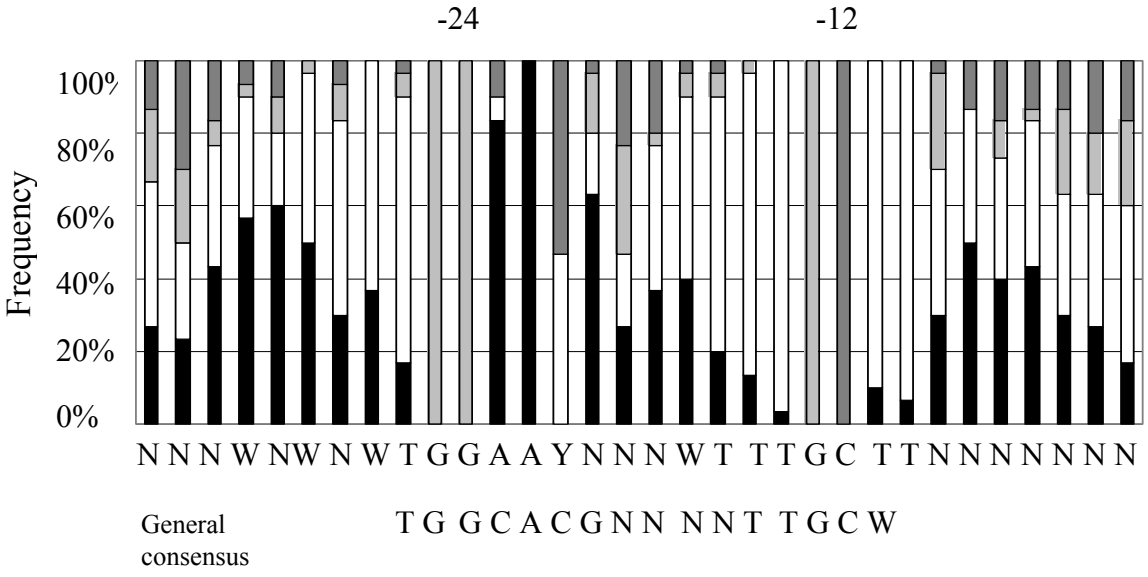

-24
-12
Frequency
100%
80%
60%
40%
20%
0%
N N N W NW N W T G G A A Y N N N W T T T G C T T N N N N N N N
General consensus
T G G C A C G N N N N T T G C W

Figure 2. Binding of σ^{54} proteins to the *S. meliloti nifH* promoter. Binding reactions contained 1 μM, 2.5 μM, 5 μM or 10 μM *H. pylori* MBP-σ^{54} in lanes 1, 2, 3 and 4, respectively; and 1 μM, 2.5 μM, 5 μM or 10 μM *S. enterica* serovar Typhimurium his-tagged σ^{54} in lanes 5, 6, 7 and 8, respectively. The DNA probes used for the assays were *S. meliloti nifH* double-stranded probe (panel A) and the *S. meliloti nifH* fork junction (panel B). Binding reactions in the panels A and B contained the same amount of labeled DNA probe (~30,000 dpm). After separating the protein-DNA complexes from free DNA by electrophoresis the gels represented in the two panels were exposed to x-ray film for the same length of time. The arrows indicate the species shifted by *H. pylori* MBP-σ^{54} (Hp) and *S. enterica* serovar Typhimurium his-tagged σ^{54} (Se). The free DNA probe is not shown. A shifted species above the MBP-σ^{54}-shifted species appears in panel B. This second shifted specific may be due to *E. coli* core RNA polymerase as indicated in the text.

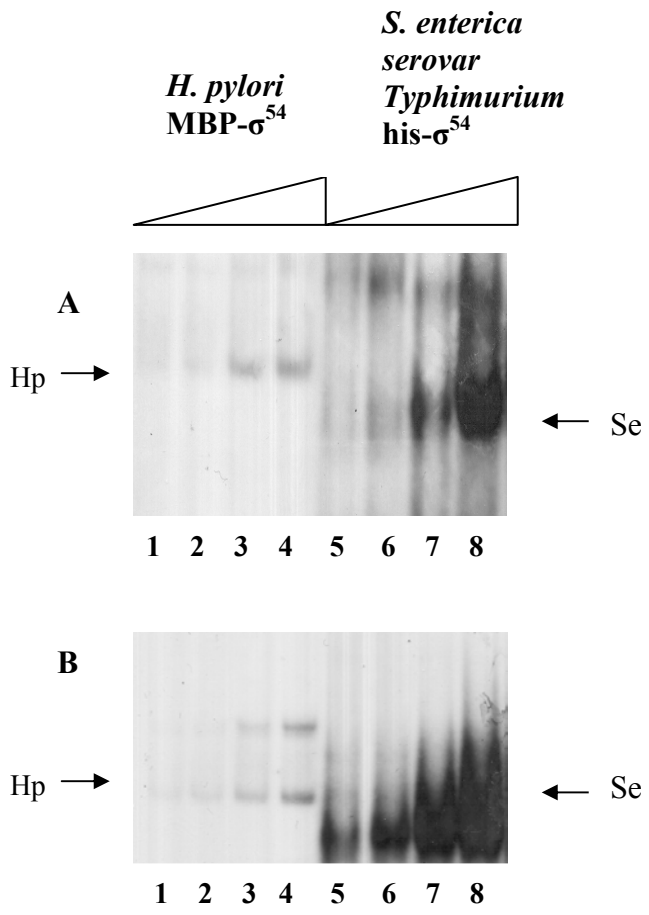

S. enterica serovar Typhimurium his-σ54
H. pylori MBP-σ54
A
Hp
Se
1 2 3 4 5 6 7 8
B
Hp
Se
1 2 3 4 5 6 7 8

Figure 3. Binding of σ^{54} proteins to the *H. pylori flaB* promoter. Binding reactions contained 1 μM, 2.5 μM, 5 μM or 10 μM *H. pylori* MBP-σ^{54} in lanes 1, 2, 3 and 4, respectively; and 1 μM, 2.5 μM, 5 μM or 10 μM *S. enterica* serovar Typhimurium his-tagged σ^{54} in lanes 5, 6, 7 and 8, respectively. The DNA probes used for the assays were *H. pylori flaB* double-stranded probe (panel A), and *H. pylori flaB* fork junction (panel B). Binding reactions in both panels contained the same amount of labeled DNA probe (~30,000 dpm). Only the shifted species are shown and are indicated by the arrows. The resolution of the species shifted by *H. pylori* MBP-σ^{54} (Hp) and *S. enterica* serovar Typhimurium his-tagged σ^{54} (Se) is greater here than it was in figure 3 because the electrophoresis time was increased.

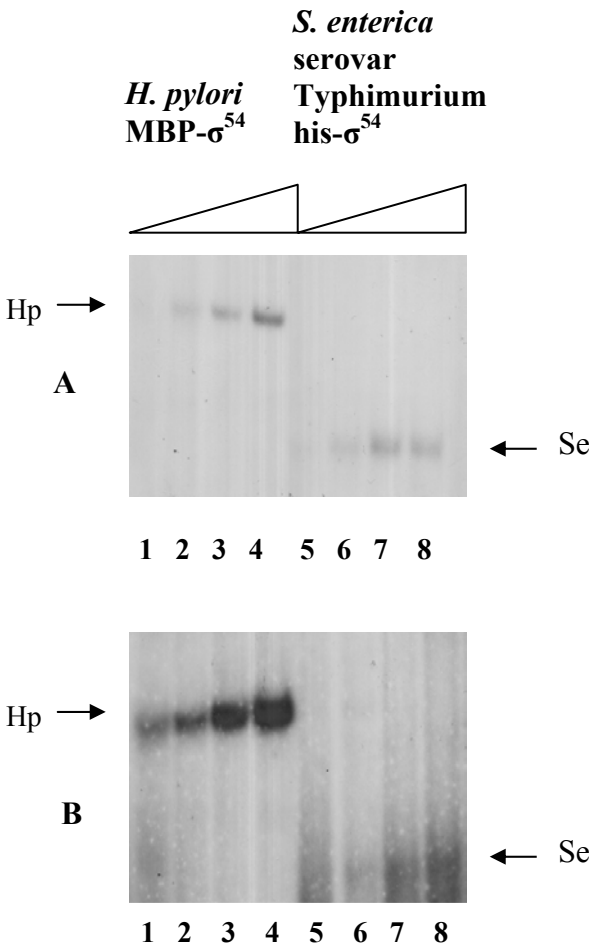

S. enterica
serovar
Typhimurium
his-σ54
H. pylori
MBP-σ54
Hp
A
Se
1 2 3 4 5 6 7 8
Hp
B
Se
1 2 3 4 5 6 7 8

Figure 4. Binding of *H. pylori* MBP-σ^{54} to wild-type and mutant *flaB* promoters. The DNA probes used in the gel mobility shift assay were the wild-type *flaB* fork junction probe (lanes 1-3) and a mutant *flaB* fork junction probe (lanes 4-5) in which the A at the –23 position was replaced with C. Binding reactions contained the same amount of labeled DNA probe (~30,000 dpm). *H. pylori* MBP-σ^{54} concentrations in the binding assays were 1 μM (lanes 1 and 4), 2.5 μM (lanes 2 and 5), or 5 μM (lanes 3 and 6). Only the shifted DNA species are shown.

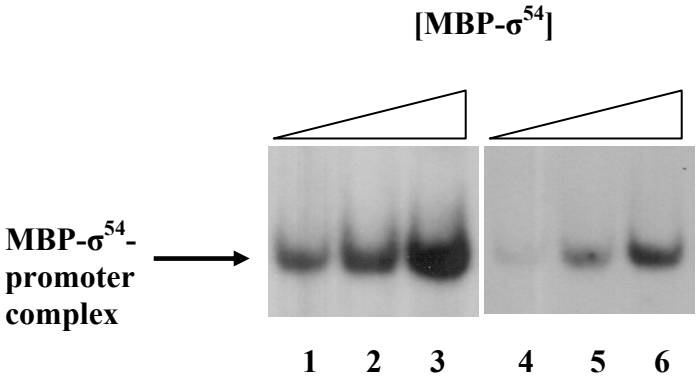

[MBP-σ54]
MBP-σ54-promoter complex
1 2 3 4 5 6

Figure 5. Binding of *H. pylori* MBP-σ^{54} to different flagellar promoters. The DNA probes used in the gel mobility shift assay were either fork junction (lanes 1-3) or double-stranded oligonucleotides (lanes 4-6). The sequences of the oligonucleotides are listed in Table 1. The promoter sequences examined were from *flaB*, *flgBC*, *fliK*, *flgE* and *orf1120-flgK* operons. *H. pylori* MBP-σ^{54} concentrations in the binding assays were 2.5 μM (lanes 1 and 4), 5 μM (lanes 2 and 5) or 10 μM (lanes 3 and 6). Only the shifted DNA species are shown.

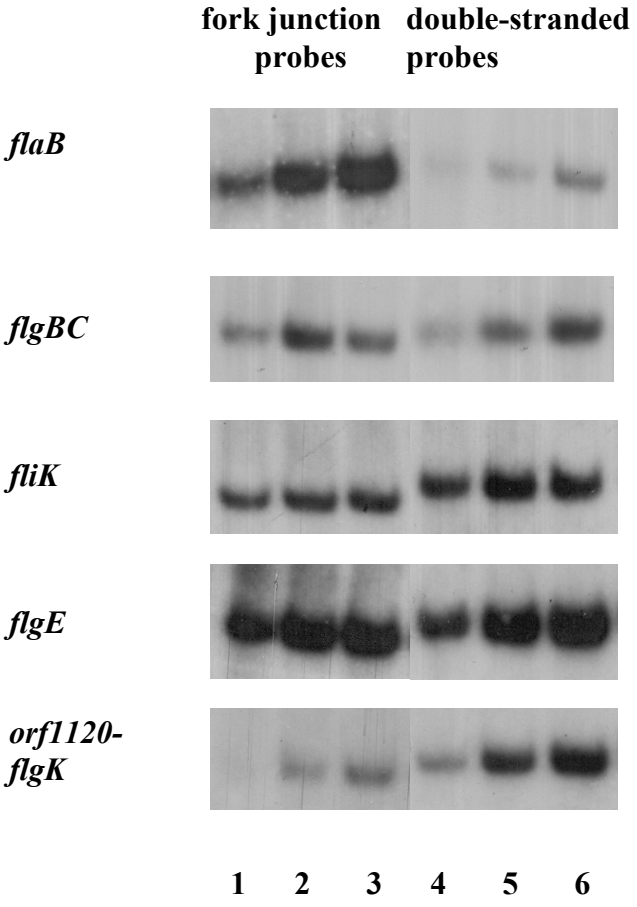
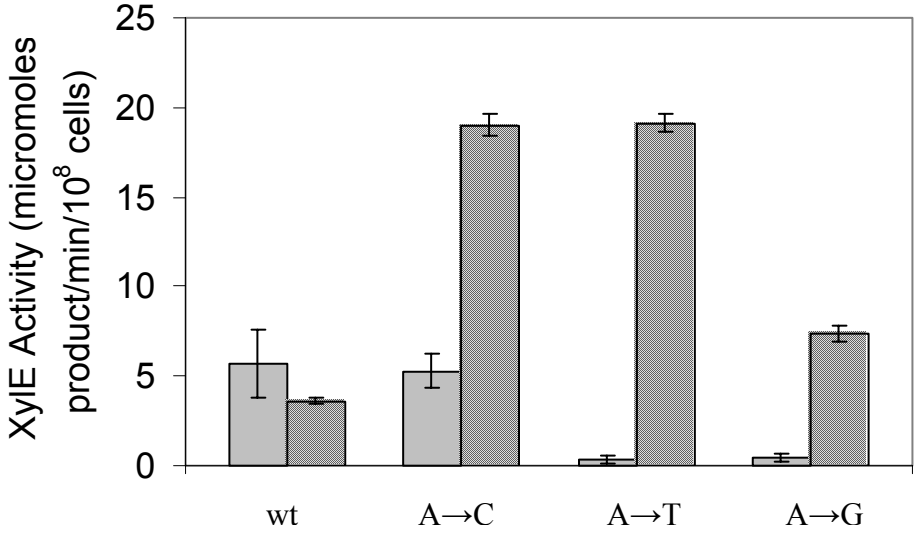

fork junction probes
double-stranded probes
flaB
flgBC
fliK
flgE
orf1120-flgK
1 2 3 4 5 6

Figure 6. Transcriptional activation from wild-type and mutant *flaB* promoters in *H. pylori* and *E. coli*. Expression levels from *flaB'-'xylE* reporter genes that had either the wild-type *flaB* promoter (wt) or mutant *flaB* promoters with an A to C, A to T or A to G change at -23 were monitored in *H. pylori* (light gray bars) and *E. coli* (dark gray bars). The bars indicate the average values for at least ten independent XylE assays. The error bars show the standard deviations for the averages. One unit of XylE activity is μmol catechol oxidized/min.


XylE Activity (micromoles product/min/10^8 cells)
25
20
15
10
5
0
wt
A→C
A→T
A→G

Dissertation Summary and Future Directions

Helicobacter pylori is the causative agent of numerous stomach ailments including acute gastritis and peptic ulcers (2, 3). If left untreated, these symptoms can progress to serious diseases such as gastric cancer and mucosal associated lymphoma (4, 11). The human gastric environment is the only known reservoir for *H. pylori* and its mode of transmission remains a subject of debate.

The genome sequence of *H. pylori* was made available in 1997 (14). This combined with the development of mutagenesis techniques and *H. pylori* shuttle vectors has greatly facilitated the study of this important human pathogen (5, 6, 10, 12). Several virulence factors of this organism have been established. One in particular, motility, was the focus of this study. Although a considerable amount of research has demonstrated that motility is essential for colonization of the gastric mucosa, many aspects of flagellar gene regulation still remain unclear in *H. pylori*.

All three sigma factors identified in *H. pylori* are involved in flagellar synthesis, with σ^{80}, σ^{54} and σ^{28} regulating the expression of class I, II and III genes, respectively (1, 13, 14). This particular study focused on the modulation of the RpoN regulon, and the data presented here suggest that control of the RpoN regulon occurs on multiple levels.

HP0958 prevents σ^{54} turnover in *H. pylori*. Sufficient levels of σ^{54} ensure the efficient expression of σ^{54}-dependent genes. The novel *H. pylori* protein HP0958, which was initially

identified as a protein interacting with σ^{54} in a yeast two-hybrid assay, was shown to be required for the stable accumulation of σ^{54}. An *hp0958* mutant has significantly lower levels of σ^{54}, resulting in a reduction of σ^{54}-dependent flagellar gene expression and a non-motile, non-flagellate phenotype. A *rpoN'-'xylE* translational fusion demonstrated that *rpoN* expression was unaffected in the mutant and suggested that HP0958 influences σ^{54} levels at a post-translational step. Examination of σ^{54} protein stability revealed that this regulatory protein is less stable in the *hp0958* mutant compared to the wild-type. I found no evidence for secretion of σ^{54}, suggesting that σ^{54} levels are reduced through proteolysis in the *hp0958* mutant. HP0958 may promote σ^{54} stability by acting as a chaperone, modifying σ^{54} to a protease-resistant form, rendering the protease(s) inactive, or facilitating the export of the protease that targets σ^{54}. Future research that would be of interest to pursue would include identifying the protease(s) involved in σ^{54} turnover and elucidating the mechanism by which HP0958 protects σ^{54} from degradation. In addition, it would be of interest to determine if HP0958 is required for the stable accumulation of other proteins in *H. pylori*.

HP0958 and its homologs, some of which are listed in Table 1, have four conserved cysteine residues near their C-termini and are within the motif CXGC-X_{19-21}-CXXCXRILY (Fig. 1A). To determine if these cysteine residues are important for HP0958 function, two of these residues were altered by site directed mutagenesis. A derivative of plasmid pEU39Cm that carries *hp0959-hp0958* was used as the template to create mutant alleles of *hp0958*. The Quick Change (II) Site-directed Mutagenesis kit (Stratagene) was used to substitute the second (Cys-202) or third (Cys-223) cysteine residue of the conserved motif with serine. The alleles were sequenced to ensure that no other mutations were introduced. Introduction of these mutant *hp0958* alleles failed to complement the defects in motility, FlaB expression and σ^{54}

accumulation in the *hp0958:aphA3* mutant (Figs. 2A, B and D). In the case of the mutant with the cys-223 to serine substitution ($HP0958_{C223S}$), the level of the protein was barely detectable by western blotting (Fig. 2C). The level of the other mutant protein, $HP0958_{C202S}$, was also reduced, but only ~two-fold lower than wild-type level. These data suggest that Cys-202 and Cys-223 are important for maintaining the stability of HP0958. The data also suggest that at least Cys-202 has a functional role in HP0958, but we cannot rule out the possibility that $HP0958_{C202S}$ failed to fold properly.

Functions of genes in an operon can sometimes be deduced from knowing the functions of the other genes in the operon. The organization of genes surrounding *hp0958* and its homologs is similar among the four ε–Proteobacteria whose genomes have been sequenced to date (Fig. 1B). These genes include *kdtA* (encodes the enzyme 3-deoxy-d-manno-octulosonic acid transferase needed for lipopolysaccharide synthesis) and a gene encoding a putative ribosomal pseudouridine synthase, both of which are downstream of the *hp0958* homolog. Upstream of *H. pylori hp0958* is *hp0959*, which encodes a gene of unknown function. Homologs of *hp0959* occur upstream of the *hp0958* homologs in other ε–Proteobacteria, but in *W. succinogenes* the two genes are fused. Homologs of *hp0959* occur upstream of *hp0958* homologs in more distantly related bacteria, including *Porphyromonas gingivalis, Bacteroides thetaiotaomicron, Clostridium tetani* and *Prevotella intermedia* (Fig. 1C). The fusion of *hp0958* and *hp0959* homologs in *W. succinogenes*, along with the observation that these genes are often closely associated in diverse bacteria, suggests that the products of these genes are involved in a common function. Disruption of *hp0959* in *H. pylori* however, did not affect HP0958 expression or function since the *hp0959:aphA3* mutant was motile and accumulated wild-type levels of HP0958, σ^{54} and FlaB

(Fig. 3). Thus, if HP0959 functions with HP0958 in modulating σ^{54} levels in *H. pylori*, its role is not essential.

With regard to the organization of genes surrounding *hp0958* homologs in other bacteria, there does not appear to be any strong consensus (Fig. 1C). In some cases genes involved in RNA degradation are linked to *hp0958* homologs, including *Chlamydophila caviae, Chlorobium tepidum* and *Borrelia burgdorferi*, which have a gene coding for the RNA subunit of RNase P downstream of the *hp0958* homolog; and *Myxococcoccus xanthus* which has a gene encoding a putative RNase H downstream of the *hp0958* homolog (Fig. 1C). Therefore, it does not seem prudent to speculate on potential functions of HP0958 homologs in other bacteria based solely on the context of their gene arrangement.

The flagellar protein export apparatus may function as a checkpoint for transcriptional activiation of the RpoN regulon. Although the different classes of flagellar genes have been established in *H. pylori*, it is unclear what check points, if any, exist to maintain the ordered expression of these genes. Work presented here suggests a link between the flagellar export apparatus and the RpoN regulon. A previous study by Niehus and coworkers showed by microarray analysis that a mutation in *flhA*, an essential component of the export apparatus, resulted in down-regulation of the RpoN regulon (9). In my study, however, *in vivo* reporter gene assays revealed a considerable stimulation of the RpoN regulon upon inactivation of *flhA*. This discrepancy appears to result from how the *flhA* mutations were constructed. Disruption of FlhA in the cytoplasmic C-terminal domain down-regulates the RpoN regulon, whereas an interruption in the membrane-integrated N-terminal domain stimulates expression of σ^{54}–dependent flagellar genes.

The export apparatus proteins FlhA and FlhB, have been shown to interact with each other suggesting that alterations in FlhA may have an impact on the conformation of the FlhB cytoplasmic domain, which has a role in determining substrate specificity of the export apparatus (7, 8). Based on my results with the *flhA* and *fliK* mutants, I propose a model in which the conformation of the export apparatus is sensed by FlgS. In this model, the autophosphorylation of FlgS is stimulated when the export system is competent for the recognition and export of rod/hook-type proteins (Chapter 3, Fig. 5). The docking protein FliH may mediate the interaction between FlgS and the export apparatus which would explain the decreased expression of the *flaB'-'xylE* and *orf1120'-'xylE* reporter genes in the *fliH* mutant. The change in substrate specificity from rod/hook-type substrates to filament-type substrates that is executed by the hook length control protein FliK may serve as the switch that 'turns off' FlgS activity.

Future research to address the proposed model should include characterization of the different *flhA* alleles to see if they produce truncated proteins that localize correctly to the membrane in *H. pylori.* In addition, it is important to determine if specific components of the export apparatus interact with FlgS and if these interactions stimulate autophosphorylation of FlgS. Finally, efforts should be made to isolate mutant forms of the export apparatus that are locked in the filament-type substrate conformation to test the hypothesis that FlgS does not interact productively with the export apparatus when it is in this conformation.

The promoter specificity of *H. pylori* σ^{54} differs from that of many other σ^{54} proteins. In contrast to *S. enterica* serovar Typhimurium σ^{54}, a *H. pylori* MBP- σ^{54} chimera bound very poorly to the *nifH* promoter (Chapter 4, Fig. 2), which matches the consensus sequence for σ^{54}-dependent promoters from a wide variety of bacteria. The *H. pylori* MBP- σ^{54} protein, however, bound more stably to the *flaB* promoter than the *S. enterica* serovar Typhimurium σ^{54}. The *flaB*

promoter differs from the consensus sequence derived from σ^{54}–dependent promoters from other bacteria in that it has an A at the -23 position instead of a C or T. Binding of MBP- σ^{54} to the *flaB* promoter was significantly lower when this base was changed from A to C. Taken together, these findings suggest a preference of *H. pylori* σ^{54} for A instead of C at position -23. However, *in vivo* reporter gene assays showed that a base change in the *H. pylori* conserved -23 position caused a reduction in the *flaB'-'xylE* expression only when the change involved in a G or a T. The base change to C did not drastically affect *flaB* expression suggesting that even though the affinity of σ^{54} itself is considerably decreased it is able to overcome this inhibitory effect *in vivo* when it is associated with core RNA polymerase and/or FlgR. Given that the *H. pylori orf1120-flgK* promoter naturally possesses a C instead of A at -23, tolerance of this base change was not completely unexpected. Expression of these reporter genes in *E. coli* was high only when the -24 promoter element of *H. pylori flaB* was altered to match the general consensus sequence which demonstrates further that the promoter specificity of *H. pylori* σ^{54} differs from that of σ^{54} from other bacteria.

Future work that would improve our understanding of *H. pylori* σ^{54} properties would include purifying RNA polymerase from *H. pylori*. The effect of core enzyme and/or the activator FlgR on σ^{54} promoter affinity could be examined. The sequence of the highly conserved RpoN box in *E. coli* σ^{54}, which is involved in recognition of the -24 promoter element, could be changed to match the RpoN box sequence in *H. pylori* σ^{54} which differs by two amino acids. The promoter affinity of the mutant *E. coli* σ^{54} could be examined *in vivo* using the reporter gene assays. This would help decipher if the difference in the RpoN box sequence is responsible for the distinct promoter affinities exhibited by these σ^{54} proteins.

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Table 1: Select homologs of HP0958 in the Comprehensive Microbial Resource database of The Institute for Genomic Research.

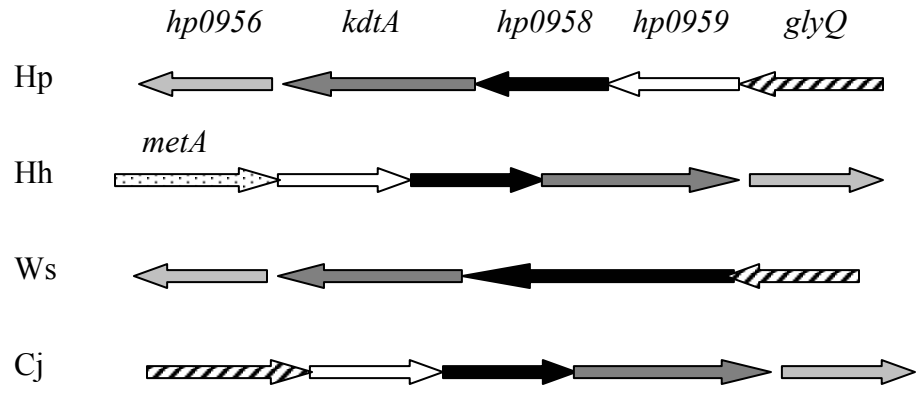
Organism	Accession	Identity	Similarity	P. Value
Helicobacter hepaticus ATCC 51449	HH0639	44.2 %	69.7 %	$4.6\ e^{-58}$
Wolinella succinogenes DSMZ 1740	WS2117	46.2 %	70.3 %	$7.2e^{-53}$
Campylobacter jejuni NCTC 11168	Cj0706	37.4 %	64.3 %	$1.9e^{-43}$
Chlamydophila caviae GPIC	CCA00200	26.4 %	48.9 %	$6.5e^{-20}$
Chlamydia trachomatis Serovar D	CT398	24.5 %	47.8 %	$2.8e^{-19}$
Porphyromonas gingivalis W83	PG2044	23.2 %	50.9 %	$9.8e^{-17}$
Aquifex aeolicus VF5	Aq_1223	24.0 %	52.4 %	$1.6e^{-16}$
Bacteroides thetaiotamicron VPI-5482	BT0883	24.8 %	51.7 %	$1.1e^{-15}$
Chlorobium tepidum TLS	CT1375	23.2 %	51.0 %	$2.1e^{-14}$
Clostridium tetani E88	CTC02006	26.9 %	51.2 %	$1.2e^{-13}$
Myxococcus xanthus DK1622	MXAN5727	25.6 %	45.0 %	$3.0e^{-13}$
Borrelia burgdorferi B31	BB0713	25.0 %	50.9 %	$2.0e^{-11}$

Figure 1. (A) Multiple sequence alignment of *H. pylori* HP0958 and its homologs from *H. hepaticus* (Hh), *W.succinogenes* (Ws), *C. jejuni* (Cj), *C. trachomatis* (Ctr), *B.burgdorferi* (Bb), *Myxococcus xanthus* (Mx), *Clostridium tetani* (Cte), *Chlamydphila caviae* (Cc), *Porphyromonas gingivalis* (Pg), *Aquifex aeolicus* (Aa), *Bacteroides thetaiotaomicron* (Bt), and *Chlorobium tepidum* (Ctp). The C-terminal region of the protein bearing the conserved cysteine motif is shown, with the underlined sequences indicating the motifs. (*) Identical residues, (+) conserved substitutions, (♦) semi-conserved substitutions. (B) Gene arrangement of *hp0958* and its homologs in the ε-Proteobacteria *H. pylori, H. hepaticus, W. succinogenes and C. jejuni*. Open arrows indicates a gene encoding a conserved hypothetical protein; the dark gray arrow indicates a gene encoding 3-deoxy-d-manno-octulosonic-acid transferase (*kdtA*); the light gray arrow indicates a putative ribosomal pseudouridine synthase; the striped arrow indicates a gene encoding the α-subunit of glycine tRNA synthetase (*glyQ*); and the stippled arrow encodes homoserine O-succinyl transferase (*metA*). The *H. pylori hp0959* and *hp0958* homologs are fused in *W. succinogenes*. (C) The organization of genes surrounding *hp0958* homologs (indicated by black arrows) is shown for a variety of distantly related bacteria. The *hp0958* and *hp0959* homologs are indicated above the potential open readings, as are the gene designations for the surrounding genes. Potential functions for the products of these genes are indicated below each open reading frame. The *rnpB* gene codes for the RNA subunit of RNase P.

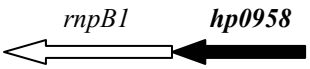
A

```
Hp    KQACGGCFIRLNDKIYTEVLTS-GDMITCPYCGRILYAEGAYENSAQPPKESQEESQELV 254
Hh    KQACGGCFIKLNDTIYSEILKG-NDIINCPHCGRILYANPSSKSTQTPTNTAEEKPQSKK 254
Ws    KQACGGCFIKINDKIYAEVIKS-EDIVTCPHCGRILYAEIQA------------------ 457
Cj    KQACYGCFMKIYDKTYLSVVKG-EEIVTCPHCGRILYKEQEEQN---------------- 238
Ctr   NRVCSGCHIALTPQHENLVRKQ-DHLVFCEHCSRILYWQELQSPSAEGATTKRRRRRTAV 254
Bb    NNVCKGCHMILPIEFANKVRREPNDIKFCPYCSRILYYQDKVQISDEIIPGSLADLVE-- 253
Mx    AGTCQGCNMNVPPQLYNQLRTG-LGTDICPSCNRIIYAVEALQETPAASK---------- 243
Cte   DGVCDGCKIRVAYEIKSELNKN--NIAYCDNCGRILFSNNIKKDKNEK------------ 245
Cc    NRVCSGCHIVLTPQHENLVRKK-DRLIFCEHCSRILYWREPDALTADSSAAKRRRRRAAI 254
Pg    RDACGGCFNKIPPQKQLDVKLR-KKIIVCEYCGRIMVDPEMGA----------------- 251
Aa    DEACAGCGIKIPSVLLSKMIKE-DSIEQCPSCGRFVYYKL-------------------- 235
Bt    RDACGGCFNKIPPQRQLDIRSR-KKVIVCEYCGRIMIDPELAGVQIEHKVEEAPATTTKR 249
Ctp   RQACSGCNTRVPTNR-HTLIVQ-GGFYVCESCGRIVVHERLFDEAAASGQQ--------- 253
        ♦* ** + +       +         *  *♦*++
```

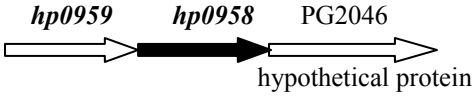
B



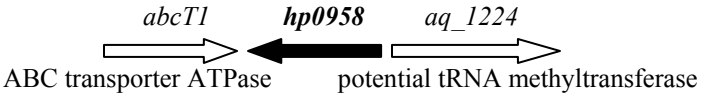
C *Chlamydophila caviae*



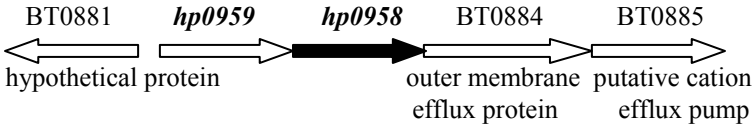
Porphyromonas gingivalis



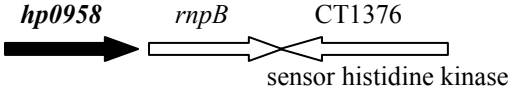
Aquifex aoelicus



Bacteroides thetaiotaomicron



Chlorobium tepidum



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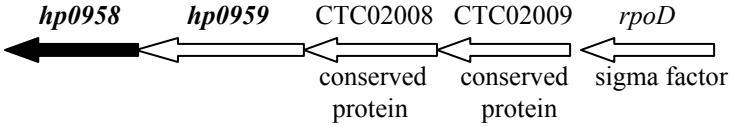

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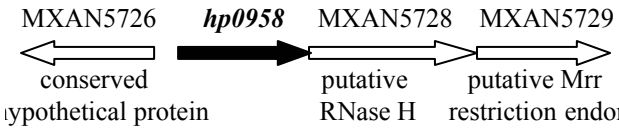
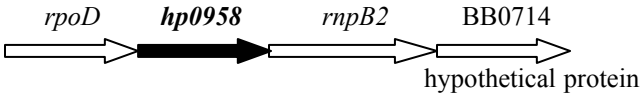


Borrelia burgdorferi



Prevotella intermedia

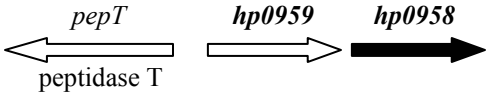


Figure 2. Amino acid substitutions in conserved cysteine residues of HP0958 interfere with its function or accumulation. For panels A, B and C, approximately 10^8 cells were lysed and loaded in each lane. Following the transfer of proteins in these samples to nitrocellulose membranes, the membranes were probed with antiserum directed against MBP-σ^{54} (panel A), MBP-FlaB (panel B), or MBP-HP0958 (panel C). Strains analyzed were *H. pylori* 43504 (wild-type) and the *hp0958:aphA3* mutant in which mutant alleles of *hp0958* were introduced into the *hp0405* locus. In these mutant alleles, cysteine residues 202 or 223 were changed to serine. The strains were also tested for motility by inoculating cells on semisolid motility agar plates with a sterile toothpick and incubated for 4 to 5 days at 37^{o}C under microaerophilic conditions (panel D).

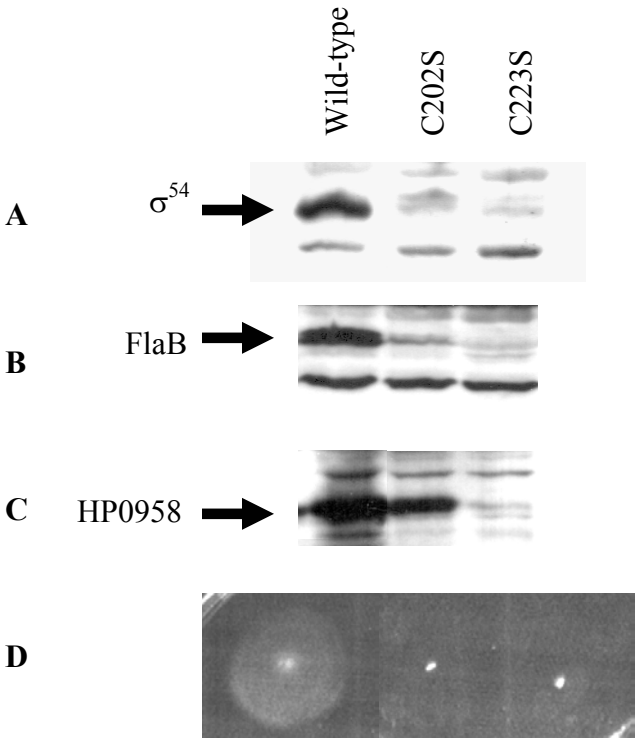
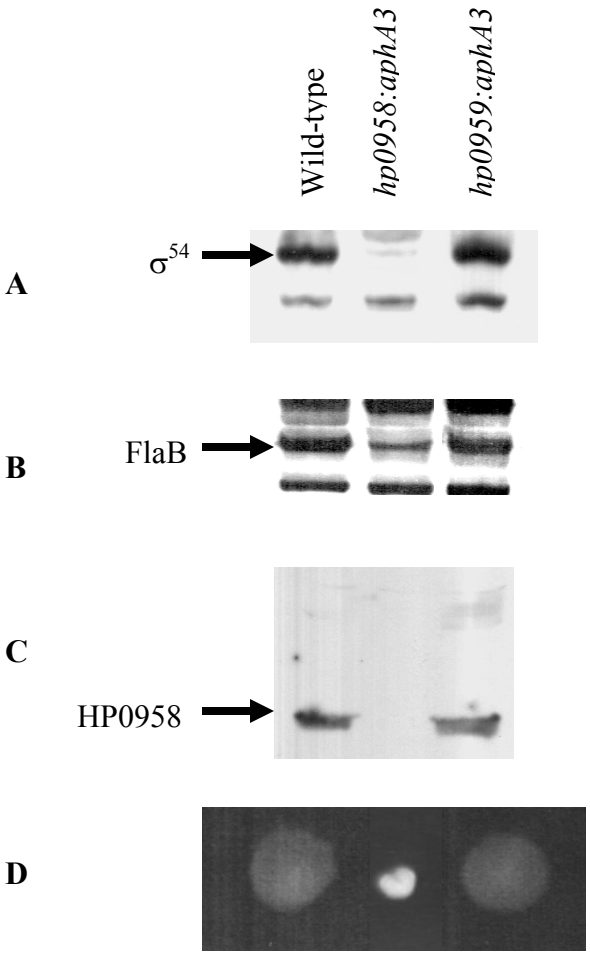

Wild-type
C202S
C223S
A
σ54
B
FlaB
C
HP0958
D

Figure 3. Disruption of *H. pylori hp0959* does not affect *hp0958* function. For panels A, B and C, approximately 10^8 cells were lysed and loaded in each lane. Following the transfer of proteins in these samples to nitrocellulose membranes, the membranes were probed with antiserum directed against MBP-σ^{54} (panel A), MBP-FlaB (panel B), or MBP-HP0958 (panel C). Panel D indicates semisolid motility agar plates that were inoculated with each strain using a sterile toothpick and incubated for 4 to 5 days at 37^{o}C under microaerophilic conditions. Strains analyzed were *H. pylori* wild-type, *hp0958:aphA3* mutant and *hp0959:aphA3* mutant.


Wild-type
hp0958:aphA3
hp0959:aphA3
A
σ54
B
FlaB
C
HP0958
D