NOVEL ASPECTS OF FLAGELLAR BIOGENESIS AND VIRULENCE IN *HELICOBACTER PYLORI*

by

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(Under The Direction of TIMOTHY R HOOVER)

ABSTRACT

The human gastric pathogen *Helicobacter pylori* utilizes all three sigma factors (σ^{80}, σ^{54} and σ^{28}) found in this bacterium for flagellar biogenesis. σ^{54}-dependent transcription has an absolute requirement for activator protein which usually binds to enhancer upstream of the promoter and contacts the σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) bound at the promoter through DNA looping. *H. pylori* FlgR is a σ^{54} -dependent activator that is required for expression of the σ^{54}-dependent flagellar genes in this bacterium and is part of a two-component regulatory system. FlgS is the cognate sensor kinase of FlgR and is also required for transcription of the σ^{54}-dependent flagellar genes. FlgR is unusual in that it lacks the DNA-binding domain found in most other σ^{54}-dependent activators. Studies using σ^{54}-dependent *flaB'-'xylE* reporter gene constructs in *H. pylori* demonstrated that 42 bp of DNA upstream of the *flaB* promoter was sufficient for efficient transcription. Purified FlgR activated transcription from the *Salmonella enterica* serovar Typhimurium *glnA* promoter in an *in vitro* transcription assay. Taken together, these data argue that FlgR does not activate transcription from an enhancer, but rather by contacting σ^{54}-holoenzyme directly. HP0137 interacts with FlgS in a yeast two-hybrid assay, and inactivation of the gene encoding this protein interfered with flagellar biogenesis. Expression of

flaB was unaffected in the *hp0137* mutant, suggesting that HP0137 is not required for FlgS function but instead is involved in flagellar assembly. A second project examined the function of a putative acetone carboxylase in *H. pylori*. Acetone enhanced the growth of a wild-type *H. pylori* strain but not that of an *acxB* (encodes α subunit of acetone carboxylase) mutant. Acetone was depleted over the course of several hours by cultures of wild-type *H. pylori*, but not by cultures of the *acxB* mutant. Taken together, these observations suggest that *H. pylori* has a functional acetone carboxylase. The *acxB* mutant was more sensitive to acetaldehyde, which is structurally similar to acetone, than the parental strain, suggesting that acetone carboxylase has a role in detoxification of acetaldehyde. The *acxB* mutant was defective in colonization of mice, indicating that acetone carboxylase has an important role in host colonization.

INDEX WORDS: *Helicobacter pylori* FlgR, Flagellar biosynthesis, σ^{54}-dependent activator, HP0137, FlgS, Flagellar export apparatus, Acetone carboxylase, Acetaldehyde, Virulence factor

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B.S., MUMBAI UNIVERSITY, INDIA, 1997

M.S., MUMBAI UNIVERSITY, INDIA, 1999

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

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December 2004

DEDICATION

This dissertation is dedicated to pappa and aai, who have played a pivotal role in my life and whom I shall never forget. I dedicate this work to my parents, whose loving support and encouragement has helped me throughout my life. Words cannot express my gratitude towards you. To maa and miku, who have kept my spirits high throughout my graduate research. I missed you all a lot. Last but not the least, I affectionately dedicate this work to my husband Rajesh who has been a strong pillar of love and support and whom I missed staying with all through my graduate studies. Thanks for your patience, this work would not have been possible without your encouragement!

ACKNOWLEDGEMENTS

I would like to thank Dr. Tim Hoover for his advice and encouragement during my research studies. Thanks to my committee members, Dr. Duncan Krause, Dr. Ellen Neidle, Dr. Rob Maier and Dr. Mark Farmer for their guidance and suggestions. Thanks to Hao and Lara, I have enjoyed working with you. To all the friends I have made in Athens, I will miss hanging out with you. Special thanks to Madhavi and Nandita for being great friends, encouraging me through the ups and downs of my research and most importantly Madhavi for sharing my passion for “Bachchan movies”!

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LIST OF ABBREVIATIONS

Abbreviation	**Full name**
AAA+	ATPases Associated with various cellular Actvities
CagA	Cytotoxin associated protein A
FIS	Factor for inversion stimulation
HTH	Helix-turn-helix
IHF	Integration host factor
LPS	Lipopolysaccharide
MALT	Mucosa associated lymphoid tissue lymphoma
MCP	Methyl accepting chemotaxis protein
PTS	Phosphotransferase system
PPI	Proton Pump Inhibitor
PBP	Penicillin binding protein
PAF	Platelet activating factor
SCOT	Succinyl CoA acetoacetate CoA transferase
TCA	Tricarboxylic acid
TTS	Type III secretion
UAS	Upstream activation sequence
VacA	Vacuolating toxin A

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

For more than two centuries, pathologists and clinicians speculated on the origins of gastric ulcers. Towards the end of the 19th century, as bacteria were shown to be the causative agents of many human diseases, the dogma that the human stomach was sterile still held its roots. It was not until 1982, when two Australians, Barry Marshall and Robyn Warren, first isolated *Helicobacter pylori* and showed the correlation between the pathogen and the disease. Originally named as *Campylobacter pyloridis*, *H. pylori* has been established as the etiological agent of chronic gastritis (17). A large body of evidence implicated chronic gastritis with the development of adenocarcinomas of the stomach, the most common gastric malignancy in the world. Colonization of the gastric mucosa by *H. pylori* has been associated with the development of peptic ulcers, gastric cancers, gastric non-Hodgkin's lymphomas (133) and with another lymphoproliferative disorder, gastric mucosa-associated lymphoid tissue (MALT) lymphoma (48). Thus, *H. pylori*, previously, an obscure organism, has now been associated with many of the most important diseases involving gastroduodenal tissue. Evidence indicates that, once acquired, *H. pylori* persists for life if left untreated (17).

H. pylori occurs in the stomachs of humans from all parts of the world. In developing countries, 70 to 90% of the population carries *H. pylori*; almost all carriers acquire the infection before the age of 10 years. In developed countries, the prevalence of infection is lower, ranging from 25 to 50% (174). In the United States, peptic ulcer disease results in an estimated 1 million

hospitalizations and 6500 deaths annually. The financial burden associated with peptic ulcer disease each year in the United States is approximately $6 billion in hospitalization costs, physician office visits, decreased productivity and days lost from work (165).

Mode of transmission

The human stomach is the only known reservoir for *H. pylori*, but animals and water sources have been thought to be potential sources for *H. pylori* infection (113). Although the mode of transmission remains unclear, three routes have been described. The first, and least common is iatrogenic which involves introduction of endoscopes in contact with the gastric mucosa of an infected individual to another individual. This risk is prevalent among gastroenterologists and endoscopists due to occupational exposure (29). A number of studies propose that *H. pylori* acquisition occurs via fecally contaminated water sources (43). However, the organism has not been isolated from water. Finally, there have been studies where *H. pylori* has been isolated from saliva and dental plaque (24, 90) suggesting oral-oral transmission as a possible source of infection. A lower economic status and low levels of sanitation have been reported to influence the rates of infection within a distinct population (29). Not many studies have been done to examine the role of genetic predisposition for a *H. pylori* infection. Although the pathology of the bacterium is widely studied, the mode of transmission remains highly speculative.

Although *H. pylori* is considered an extracellular pathogen, there is some evidence of the intracellular localization of the bacterium. In some studies, *H. pylori* has been identified in the cytoplasm of gastric epithelial cells (5). Although *H. pylori* has also been found within the mucous vacuoles or the lysosomes of epithelial cells, there does not seem to be lysis of these

internalized bacteria (26) which may contribute to their persistence and host tissue damage over a period of time.

Treatment

Currently the only approved anti-*H. pylori* therapies involve a combination of antimicrobials and acid suppression compounds. Antibiotics such as metronidazole, amoxicillin, tetracycline or clarithromycin are commonly administered over a period of two weeks (108). Other treatments include bismuth subsalycilate, rantidine bismuth citrate, quinolones and proton pump inhibitors (PPIs). H_2 blockers are also used that work by blocking histamine which stimulates acid secretion. Acid suppression by H_2 blockers or PPIs in conjunction with antibiotics help alleviate abdominal pain, nausea, help heal gastric mucosal inflammation and may enhance the efficacy of the antibiotics. According to the FDA approved treatment options, triple therapy regimen which includes a combination of two antibiotics with a PPI are more effective than dual therapy which includes a single antibiotic and a PPI. Also, longer length of treatment (14 days versus 10 days) results in better eradication rates.

Characterization of *H. pylori*

The genus *Helicobacter* contains many different species which are well adapted for colonization of the human or animal gastrointestinal tract. Table 1 lists the different species of *Helicobacter* colonizing their natural hosts. The stomach lies between the esophagus and the first part of the small intestine (the duodenum). Like the other parts of the gastrointestinal system, the stomach walls are made of a number of layers. Starting inside the stomach (the lumen) going out, the first main layer is the mucosa. This consists of an epithelium, the lamina propria underneath,

Table 1. Natural hosts and colonization sites for different species of *Helicobacter*

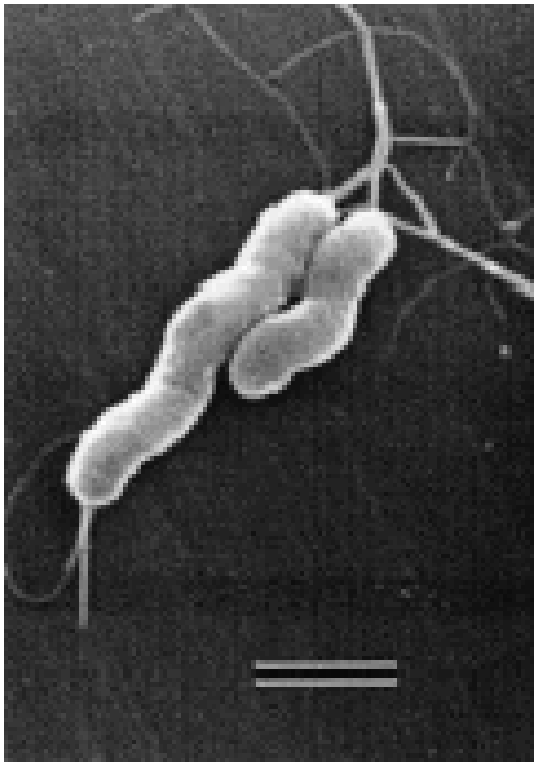
***Helicobacter* species**	**Natural Host**	**Site of Colonization**
H. acinonychis	Cheetah	Gastric antral mucosa
H. bizzozeronii	Dog	
H. felis	Cat, Dog	
H. mustelae	Ferret	
H. nemestrinae	Pigtailed macaque	
H. pylori	Human, Rhesus monkey	
H. suis	Pig	
H. bilis	Mouse, Dog, Human	Intestinal tract/hepatobiliary system
H. canadensis	Human	
H. canis	Dog, Human	
H. cholecystus	Hamster	
H. ganmani	Mouse	
H. hepaticus	Mouse	
H. pametenis	Bird, Swine	
H. pullorum	Chicken, Human	
H. rodentium	Mouse	

and a thin bit of smooth muscle called the muscularis mucosa. Underneath lies the submucosa which consists of fibrous connective tissue. The epithelium of the stomach forms deep pits which contain cells secreting either gastric enzymes or hydrochloric acid. Gastric helicobacters such as *H. pylori*, *H. felis* and *H. mustelae* inhabit the antral part of the gastric mucosa adjacent to the epithelial cells. To avoid being swept away by peristalsis or the continuous turnover of mucus and epithelial cells, *H. pylori* adheres to the gastric epithelium via a number of adhesins that are discussed later (176).

Helicobacters are gram-negative, microaerophilic, non-spore forming rods that undergo metamorphosis into coccoidal bodies upon prolonged culture (31). *H. pylori* is 2.5 to 5 μm long and 0.5 to 1.0 μm wide and belongs to the ε subdivision of Proteobacteria (Fig. 1). They possess four to six unipolar sheathed flagella which are essential for bacterial motility. The flagellum is approximately 30 μm long and 2.5 nm in width and ends in a terminal bulb which is an extension of the flagellar sheath (43). The flagellar sheath is continuous with the outer membrane and is believed to protect the acid-labile flagellar filament from gastric acidity (64).

At present, complete genome sequences have been published for two *H. pylori* strains, 26695 (177) and J99 (3). The genome sizes of *H. pylori* 26695 and J99 are 1.67 Mb and 1.64 Mb, respectively, with a G+C composition on average of 39 mol %. In both genomes, almost 60% of the open reading frames code for proteins with a predicted function, 24% are conserved in other bacterial species but are of unknown function, and 17 % are *H. pylori* specific with no known homologues in the databases. The two *H. pylori* genomes are highly conserved with respect to gene content (1,495 and 1,552 open reading frames in J99 and 26695 respectively), functional categorization and gene order. Sequence variation between the two strains is significantly greater at the nucleotide level than at the amino acid level. Because the nucleotide

Figure 1. Morphology of *Helicobacter*. Spiral-shaped *H. pylori* with 2 to 6 polar sheathed flagella as seen by scanning electron microscopy. Bar = 1 μm. Microbes & Infection Vol 2, p 55-60, 2000.



variation occurrs most commonly in the third position of the coding triplet, the primary sequence of the encoded protein is highly conserved (40). Eighty-nine genes are specific to strain J99 and 117 are specific to strain 26695. For example, genetic analysis indicates that strain J99 can ferment pyruvate to acetate but the strain 26695 cannot do so due to a frameshift in its phosphotransacetylase gene. Also, a homologue of alcohol dehydrogenase is present in both the strains. In addition, the single, identifiable strain-specific gene involved in energy metabolism in strain J99 (JHP1429)[1] is a second alcohol dehydrogenase homologue. In strains J99 and 26695, the flagellin FlaA protein sequences are identical and the FlaB protein sequences differ by only a single amino acid (40). Forty percent of *H. pylori* isolates harbor plasmids ranging in size from 1.5 to 23.3 kb. These plasmids, however, do not encode any known virulence factors (40). *H. pylori* strains are naturally competent for DNA uptake, a property which may account for the diversity among *H. pylori* strains (52).

Cell wall and lipopolysaccharides

The *H. pylori* cell surface is hydrophobic and negatively charged. Although the physical organization of the cell envelope is similar to other gram-negative bacteria, there do exist certain differences. The peptidoglycan composition of the *H. pylori* cell envelope differs significantly from that of *E. coli*. *H. pylori* has a high proportion of muropeptides, with a pentapeptide acetylmuramic acid. It also lacks murein-bound lipoprotein, trimeric mucopeptides, and (L-D) cross-linked muropeptides (53). This may result in the formation of a weak peptidoglycan enabling the bacterium to metamorphose from a spiral rod to a coccoid form upon prolonged

[1] JHP and HP refer to ORF numbers in the genome sequences of *H. pylori* strain J99 and 26695 respectively.

culture *in vitro*. The *H. pylori* genome has homologs of all the enzymes required for peptidoglycan synthesis. *H. pylori* also has penicillin-binding proteins (PBPs) (38) which are involved in peptidoglycan biosynthesis and modification (42, 113). A recent study using radio-labeled ampicillin identified *H. pylori* PBPs that did not share homology with known PBPs from other bacteria (68).

Lipopolysaccharides (LPS) form a key component of the outer membrane of *H. pylori. H. pylori* produces two types of LPS, a high-molecular weight smooth form (S-LPS) and a low-molecular weight rough form (R-LPS) (103). The smooth form consists of an O-side chain, a core oligosaccharide and lipid A. Several passages of the smooth form of *H. pylori* on solid media, however, gives rise to the rough form which lacks the O-side chain (130). The lipid A component of *H. pylori* is also unusual compared to other gram-negative bacteria as it has low mitogenic and pyrogenic activities. Also, lipid A from *H. pylori* has low lethal toxicity compared to lipid A from enterobacteria (116).

The O-antigen of *H. pylori* has extended chain with fucosylated and non-fucosylated N-acetyllactosamine units. These repeating units of the O-side chain have been shown to mimic type 2 Lewis blood group antigens (Le^x and Le^y) in structure (9). *H. pylori* LPS has been shown to display phase variation from a single strain, and various isolates possessing different LPS structures and glycosylation have been isolated explaining the heterogeneity of this bacterium (7). When grown at pH 5 or pH 7, qualitative differences are seen in the LPS profiles of the bacterium suggesting that *H. pylori* may alter its LPS structure in response to acidic pH (107). The cell surface of *H. pylori* is also unusual, in that it can incorporate proteins such as urease, catalase, HspA, HspB and superoxide dismutase, which occur intracellularly in other bacteria (18).

Flagellar apparatus

The ultrastructure of the *H. pylori* cell is characterized by the presence of unipolar sheathed flagella. Typically, there are 2-6 flagella with an average of 30 μm in length and 2.5 nm in width. The flagellar apparatus is composed of the basal body, the hook structure and the flagellar filament. The flagellar filament often terminates in a bulb-like structure. The flagella of *H. pylori* are surrounded by a sheath, a membranous layer continuous with the outer membrane of the bacterium. Details of *H. pylori* flagella structure and function are discussed later.

Respiration

Energy generation in *H. pylori* appears to be similar to that of other aerobically respiring bacteria with significant ATP yields resulting via oxidative phosphorylation. (83). ATP synthase converts proton electrochemical gradient across the bacterial cell membrane into ATP. ATP synthase consists of a hydrophilic cytoplasmic domain (F_1) and a membrane integrated hydrophobic F_0 domain. The F_0 proton channeling complex is made up of a, b, c subunits, while the F_1 ATPase consists of five different polypeptides with the compostion of α, β, γ, δ and ε subunits ($\alpha_3\beta_3\gamma\delta\varepsilon$). The genes encoding the subunits of F_0 and F_1 ATPase complex have been identified in both the sequenced strains (3, 177). In addition, a gene (HP1137/JHP1065) encoding a homologue of the b' subunit found in plants and photosynthetic bacteria is also present in *H. pylori*. Under acidic environmental conditions, *H. pylori* maintains a neutral intracellular pH and has a constant proton motive force at external pH values between 3 and 7. This proton motive force drives ATP synthesis and also flagellar rotation and solute uptake (83) (167).

Initial studies of respiration in *H. pylori* showed that intact cells were capable of oxidizing D-glucose, formate, DL-lactate, succinate and pyruvate. Although the rates of respiration were low for D-glucose and formate, DL-lactate, succinate and pyruvate were readily oxidized (83). From the genome sequences of both strains of *H. pylori* it is possible to predict the identity of the primary dehydrogenases responsible for feeding electrons from the above mentioned substrates into the quinone pool. A putative flavoprotein D-lactate dehydrogenase (HP1222/JHP1143) is present for lactate-dependent respiration, and so is a glycerol-3-phopshate dehydrogenase (HP0666/JHP611). Homologs of proline dehydrogenase (HP0056/JHP48), glycolate oxidase (HP0509/JHP459) and a D-amino acid dehydrogenase (HP0943/JHP8878) provide additional possibilities for substrate-derived electrons to be donated to the membrane-bound electron transport chain, however, these activities have not been demonstrated experimentally. In addition, a malate:quinone oxidoreductase (HP0086/JHP79) has been identified in *H. pylori* which would allow the use of L-malate as an electron donor (83).

In addition to molecular oxygen as a terminal electron acceptor, fumarate can also be used for the same purpose. The reduction of fumarate catalyzed by the enzyme fumarate reductase is an important energy transduction pathway in *H. pylori*. Mendz and co-workers originally identified a variety of products of fumarate metabolism in *H. pylori* cells or lysates using nuclear magnetic resonance (110). Under their experimental conditions, the primary product of fumarate catabolism was malate which was further converted to pyruvate. Upon further incubation, the end products were identified as succinate, acetate, lactate, alanine and formate. The production of succinate provided evidence of a fumarate reductase activity indicating that *H. pylori* generates ATP via anaerobic respiration (83).

Apart from fumarate, there is no direct evidence that *H. pylori* is able to use alternative electron acceptors other than oxygen.

H. pylori posseses a membrane-bound "uptake-type" hydrogenase involved in electron transfer and respiration (102) that could contribute to energy generation in the presence of H_2. Hydrogen is a by-product of colonic fermentation and is present in high amounts in the gastrointestinal tract of humans and rodents. However, molecular hydrogen is not an energy source for the host, and Olson and co-workers showed that *H. pylori* was capable of utilizing H_2 as an energy source (128) and that a mutant *H. pylori* strain unable to utilize H_2 was deficient in mice colonization (128).

Carbon Utilization in *H. pylori*

H. pylori strain 26695 and J99 have a limited ability to acquire and metabolize sugars. *H. pylori* cannot utilize oligosaccharides as it lacks the gene products required to transport and metabolize the complex sugars. A homologue of glucose and galactose transporter is the only identified sugar transporter in *H. pylori* (40, 109). The glucose transporter of *H. pylori* differs from the types found in other bacteria as the transport process is not inhibited by known glucose transport inhibitors such as cytochalasin B or Phloretin (10). Several homologues to organic acid transporters such as L-lactate permease, ketoglutarate permease, and a C_4-dicarboxylate transporter have been identified suggesting that organic acids also serve as important energy or carbon sources for *H. pylori*. Since homologues of the two nonreversible gluconeogenic enzymes (fructose-1, 6- bisphosphatase and pyruvate dikinase) are present and homologues of the two nonreversible glycolytic enzymes (phosphofructose kinase and pyruvate kinase) are absent, it appears that *H. pylori* uses the enzymes of the glycolytic/gluconeogenic pathway for anabolic

biosynthesis rather than for catabolic energy production (40).The Entner-Duodoroff pathway has been elucidated in *H. plyori* for glucose metabolism. *H. pylori* has homologues of genes that encode all the enzymes in the phosphopentose shunt except gluconate-6-phosphate dehydrogenase (43, 103).

Large amounts of amino acids, dipeptides and polypeptides are present in the gastric juice due to the gastric enzyme pepsin. Some of these molecules can be taken up by *H. pylori* through specific transport proteins. Genomic analysis has identified several orthologs of amino acid transporters for serine, proline, D-alanine and glutamate (34). Amino acid utilization by *H. pylori* cultured in a defined medium has been investigated by nuclear magnetic resonance spectroscopy and amino acid analysis. During microaerophilic growth in the absence of glucose, *H. pylori* can utilize arginine, aspartate, asparagines, glutamine and serine converting them to acetate, formate, succinate and lactate. These results indicate that in *H. pylori* fermentation of amino acids is an important mode of amino acid utilization (34).

Genomic analysis suggests that pyruvate is made from lactate, alanine and serine rather than glucose (43). Pyruvate is converted to acetyl coenzyme A by pyruvate oxidoreductase, as *H. pylori* lacks homolgues for pyruvate dehydrogenase, pyruvate formate-lyase and pyruvate oxidase (40, 152). *H. pylori* dissimilates pyruvate to acetate, formate, succinate and lactate. Acetyl-CoA generated by pyruvate oxidoreductase enters the tricarboxylic acid cycle (TCA) and is further metabolized for energy generation (164, 77). *H. pylori* can also ferment pyruvate to acetate. This reaction is predicted to occur in strain J99, but not in strain 26695 due to a potential frameshift mutation in the phosphotransacetylase gene. Hence, strain 26695 would not be able to convert acetate to acetyl-CoA by running the fermentation pathway in reverse.

However, the presence of a single strain specific acetyl CoA synthetase allows the conversion of acetate to acetyl-CoA in strain 26695 which can then enter the TCA cycle (40).

Both sequenced strains of *H. pylori* contain genes required for C_2 or short chain fatty acid transporter and their catabolism. No identifiable homologues were found for the genes involved in long chain fatty acid β-oxidation (40). Ketone bodies such as acetone, acetoacetate and 3-β hydroxybutyrate could result from the β-oxidation of short chain fatty acids. In healthy adult humans, upto 185 grams of ketone bodies are produced by the mitochondria of the liver and are used as an energy source when glucose is limiting (62, 93). Under acidic conditions such as those found in the stomach, acetone is produced by the spontaneous decarboxylation of acetoacetate.

Both *H. pylori* strains 26695 and J99 have a potential acetone carboxylase which can convert acetone to acetoacetate. Acetone utilization was first studied in *Rhodopseudomonas gelatinosa* and the enzyme was purified from (161) *Xanthobacter autotrophicus* strain Py2 (163). Genes encoding the *X. autotrophicus* acetone carboxylase, *acxA* (encodes β subunit), *acxB* (encodes α subunit) and *acxC* (encodes γ subunit), are organized in an operon (164). Analysis of both the *H. pylori* strains reveals an operon consisting of genes (*hp0695, hp0696* and *hp0697*) having homology to the *acx* genes. Also the genes in the operon are arranged similarly to those of *X. autotrophicus* and *R. capsulatus* (164). In the annotated *H. pylori* sequence, the products of *hp0695* and *hp0696* are indicated as hydantoin utilization protein A and N-methylhydantoinase, respectively. However, HP0695 and HP0696 share only 15-30% homology with known hydantoinases. Also, HP0697 does not share any homology to known hydantoinases. In contrast, HP0695, HP0696 and HP0697 share 50-63% amino acid identity with the acetone carboxylase β, α and γ subunits from *X. autotrophicus* and *R. capsulatus*. This strongly argues that the *hp0695, hp0696* and *hp0697* encode the subunits of the acetone carboxylase in *H. pylori.*

In *H. pylori*, acetone can be converted to acetoacetate by the enzyme acetone carboxylase. Acetoacetate can be further metabolized to acetoacetyl-CoA by the enzyme succinyl CoA: acetoacetate CoA transferase (SCOT). Acetoacetyl-CoA is subsequently metabolized to generate two molecules of acetyl-CoA by acetoacetyl-CoA thiolase which can then feed into the TCA cycle. It is interesting that the genes encoding the subunits of SCOT (*hp0691, scoA* and *hp0692, scoB*) and thiolase (*hp0690, fadA*) are clustered with the genes coding the acetone carboxylase. Also, one of two open reading frames between *scoA* and *hp0695* shares homology with a short chain fatty acid permease which could transport acetoacetate or 3-β hydroxybutyrate. It is quite possible that acetone obtained from the host or from the decarboxylation of acetoacetate can be metabolized by *H. pylori* to acetyl-CoA for subsequent energy generation. The role of acetone carboxylase in *H. pylori* is discussed in chapter IV.

Role of Virulence Factors in Colonization and Persistence

Gastric colonization by *H. pylori* is characterized by persistence of the bacterium in a highly hostile, challenging environment which is subjected to low pH values and fluctuating concentrations of nutrients and also by a host response leading to activation of immune defense mechanisms. In order to overcome these difficulties in its ecological niche, *H. pylori* has developed a wide array of virulence factors which help the bacterium for initial colonization and a successful long term infection. The use of animal models for experimental studies, has greatly expanded our understanding of the virulence factors of *H. pylori*. Studies in gnotobiotic piglets have demonstrated the importance of motility as a colonization factor for *H. pylori*, as non-motile mutants defective in flagellar production are unable to colonize the gastric mucosa (47). Similarly, mice and ferrets have been used to study *H. felis* and *H. mustalae,* respectively (55,

56). Mongolian gerbils have also been used extensively for studying *H. pylori* infections (183). With the availability of molecular techniques and use of animal models, we now have a better understanding of the role of different virulence factors of *H. pylori*, some of which are discussed below and summarized in Table 2.

(i) Urease

All *H. pylori* isolates produce large amounts of the enzyme urease. Urease catalyzes the hydrolysis of urea to yield ammonia and carbamate. The latter compound spontaneously decomposes to yield another molecule of ammonia and carbonic acid (44). The net effect of these reactions is an elevated pH around the bacterium, thereby protecting it from gastric acidity. The genes encoding *H. pylori* urease are located as a single 6.13 kb gene cluster designated *ureABIEFGH* (27). Each of the gene except *ureI*, share homology with the urease genes of other species including *Klebsiella aerogenes*, *Proteus mirabilis* and *Ureaplasma urealyticum*. The native urease of *H. pylori* has a molecular mass of 540 kDa and is a nickel containing hexameric molecule consisting of two subunits, UreA and UreB (30kDa and 62kDa respectively) in a 1:1 molar ratio (115). Expression of *ureA* and *ureB i*s sufficient to produce an apoenzyme that lacks nickel ions in the catalytic site and is therefore inactive. For synthesis of a catalytically active urease, the products of the accessory genes *ureI*, *ureE*, *ureF*, *ureG* and *ureH* are required. It is believed that these accessory proteins interact with the apoenzyme and deliver nickel ion to the active site in an energy-dependent process (27). In contrast to other bacterial species, urease from *H. pylori* is not strictly cytoplasmic, as it has been detected on the surface of old cultures of *H. pylori* cells due to cell autolysis (136). It is generally presumed that, urease activity is requiredfor the production of a neutral microenvironment for the organism within the gastric lumen. Isogenic

Table 2. Factors contributing to *H. pylori* pathogenesis

Factor	Suggested Function
Urease	Gastric acid neutralization Mucosal toxicity
Flagella	Bacterial motility
VacA	Cytotoxic for gastric epithelium
CagA	Interference with host signal transduction Rearrangement of actin cytoskeleton
Superoxide dismutase, Catalase	Resistance to phagocytic killing
Adhesins	Adherence to gastric epithelial cells
PAF	Induction of gastric inflammation
Phospholipase A	Disruption of gastric mucosal barrier

urease-negative mutants were unable to colonize gnotobiotic piglets (30). Also, isogenic urease-negative mutants of *H. mustelae* failed to colonize ferrets (6, 45). Although urease is not required for *in vitro* viability of *H. pylori*, it is clear that the enzyme is essential for gastric colonization and represents a critical virulence determinant.

(ii) Motility

H. pylori possesses two to six polar sheathed flagella that enable the bacterium to move in a highly viscous environment, penetrate the mucus layer and establish a successful infection. Motility is an important colonization factor, as studies have demonstrated that non-motile mutants do not establish a persistent infection in a gnotobiotic pig model (46). Structural components of the flagella, as well as secretory and regulatory proteins involved in flagellar biogenesis have been well studied in recent years expanding our knowledge of *H. pylori* motility. The structure and function of *H. pylori* flagellum and regulation of flagellar genes will be discussed in more detail in later sections.

(iii) Vacuolating Cytotoxin

The gene encoding the vacuolating cytotoxin (*vacA*) has been cloned from several cytotoxin producing strains of *H. pylori*. *vacA* encodes a potential 139 kDa protein that shows no sequence homology to other known bacterial toxins (32). This 139 kDa protoxin contains a leader sequence of 33 amino acids, the cytotoxin itself (VacA), and a carboxy terminal fragment of approximately 50 kDa that exhibits homology to a carboxy terminal fragment of the IgA protease precursor of *Neisseria gonorrhoeae*. The carboxy terminal fragment of *N. gonorrhoeae* IgA protease is involved in the translocation of the protease through the outer membrane and is

therefore essential for the cytotoxin secretion. Translocation through the cytoplasmic membrane, however, does not require any additional factors, and thus the VacA is considered a autotransporter (32, 43). The cytotoxin induces acidic vacuoles in the cytoplasm of eukaryotic cells. These vacuoles appear to be derived from the late endosomal compartments within eukaryotic cells (43). Although VacA causes epithelial cell vacuolation *in vitro*, this does not lead to rapid cell death. Studies have shown the association of s1 type of VacA (which causes vacuolation *in vitro*) with peptic ulceration and the s2 type (which is non vacuolating *in vitro*) with the absence of ulcers. Also, oral administration of VacA to mice causes damage to the gastroduodenal epithelium including superficial ulceration (32). Thus, several lines of evidence implicate that vacuolating cytotoxin of *H. pylori* is an important virulence factor in the pathogenesis of peptic ulceration.

(iv) *cag* Pathogenicity Island (*cag* PAI)

H. pylori cytotoxin-associated gene antigen CagA is a 128 kDa protein and its corresponding gene (*cagA*) is located in the pathogenicity island (PAI) on the *H. pylori* chromosome. Inspite of extensive studies, the exact mechanism of CagA-induced pathogenesis remains unclear. There are 31 open reading frames predicted within the *cag* region. Besides CagA, the *cag* PAI encodes a *Helicobacter* specific type-IV secretion system. There is evidence that adherent *H. pylori* cells translocate CagA into host cells via a functional type-IV secretion system (169). Inactivation of all *cag* genes tested, except *cagN*, abolishes CagA translocation. Inside the host cell, CagA becomes phosphorylated at a specific tyrosine residue, although, the host cell kinase responsible for phosphorylation of CagA remains to be identified. It has been suggested that following phosphorylation CagA becomes part of a membrane-associated

complex inside the host cell. This leads to rearrangement of the actin cytoskeleton affecting the plasticity of the cell. CagA may also interfere with host cellular signal transduction, affecting gastric epithelial cell proliferation, cell viability and attenuation of apoptosis. The observation that Cag proteins interfere with the cell cycle might explain the increased risk of gastric cancers in individuals having a history of *H. pylori* infections.

(v) Oxidative Stress Enzymes

Infection with *H. pylori* induces an inflammatory response recruiting polymorphonuclear leukocytes (PMNs) to the site of infection. This leads to the production of superoxide anion ($O_2^{\cdot -}$) a highly reactive and toxic oxygen species, formed as part of the oxidative burst of PMN and enzymatic activities of gastric epithelial cells. *H. pylori* neutralizes reactive oxygen species by mechanisms that include the enzymes superoxide dismutase (SOD), catalase (KatA), alkylhydroperoxide reductase (AhpC) and thioredoxin-linked thioperoxidase (3, 43, 69). SOD catalyzes the dismutation of superoxide ions to hydrogen peroxide which may be deactivated further by catalase or peroxidase. In *H. pylori*, SOD is an iron containing cytosolic enzyme (Fe-SOD) consisting of two identical subunits. Catalase protects the bacteria by breaking down hydrogen peroxide into water and oxygen. *H. pylori* catalase is homotetrameric, and unlike other bacterial catalases, is very stable at high hydrogen peroxide concentrations, which may reflect adaptation of *H. pylori* to an environment comparatively rich in reactive oxygen species.

Alkylhydroperoxide reductase catalyzes the reduction of alkyhydroperoxide to the corresponding alcohol. In *H. pylori*, the gene *ahpC* encodes alkylhydroperoxide reductase and it has been demonstrated that *ahpC* mutants are deficient in mice colonization (127). Thiol peroxidases (Tpx) prevent inactivation of enzymes, such as glutamine synthetase, which are

sensitive to oxidative stress by removing hydrogen peroxide in a metal catalyzed oxidation system. *H. pylori* Tpx is encoded by *tpx* (also called *tagD*), and *tpx* mutants have reduced ability to colonize mice (127).

(vi) Adhesins

It is widely accepted that *H. pylori* adheres to gastric epithelium receptors by means of specific adhesins. Outer membrane proteins, phospholipids, glyoclipids and the Lewis^b blood group antigen have been implicated in binding either mucin or gastric mucosal epithelial cells (130). *H. pylori* gene *hpaA* encodes the sialic acid adhesion HpaA which binds to sialic acid containing glycoconjugates on gastric epithelial cells and neutrophils. HpaA has also been observed as a component of the extracellular flagellar sheath. *H. pylori* BabA is a member of a large family of outer membrane proteins that bind fucosylated glycoproteins on gastric epithelial cells. *H. pylori* has also been shown to bind laminin through a laminin-binding protein. Evidence from several studies suggest that *H. pylori* lipopolysaccharide (LPS) is also involved in laminin binding (50, 78, 178).

(vii) Other Factors Involved in Pathogenesis

H. pylori possesses virulence factors that disrupt the gastric mucosal layer and causes inflammation. Phospholipase A attacks the protective phospholipid-rich layer at the apical membrane of mucus cells (131). *H. pylori* LPS disrupts the gastric mucus coat by interfering with the interaction between the mucin and its receptor (130). Platelet activating factor (PAF) is a phospholipid mediator that stimulates gastric acid secretion through specific parietal cell receptors. *H. pylori* metabolizes the nonulcerogenic precursor lyso-PAF into PAF which induces

mucosal injury through increased acid secretion (43). *H. pylori* also induces the secretion of interleukin-8 (IL-8), a potent inflammatory mediator that recruits neutrophils to the site of infection. High nitric oxide production by the inducible nitric oxide synthase of neutrophils is associated with immune activation and tissue injury. *H. pylori* also appears to induce programmed cell death or apoptosis of gastric epithelial cells and stimulates oxidative DNA damage in infected human gastric mucosa (43).

Flagellar Structure and Function

Ultrastructural, biochemical and genetic studies on *Escherichia coli* and *Salmonella enterica* serovar Typhimurium flagella have contributed to the understanding of structure-function relationships in the bacterial flagellum. In the case of *H. pylori*, motility via its flagella is essential for the bacterium to colonize the host and establish a persistent infection. The flagella of *H. pylori* are similar in structure to those of enteric bacteria except for the sheath that surrounds them in *H. pylori*. *H. pylori* usually possess four to six unipolar sheathed flagella, each of which are about 30 μm in length and 2.5 nm in width. The *H. pylori* flagellum exhibits a characteristic bulb-like structure at its distal end that represents a dilation of the flagellar sheath (167). The sheath is an extension of the outer membrane and is believed to protect the flagellar subunits from dissociating due to the surrounding acidic gastric environment. The bilayered flagellar sheath is composed of phospholipids, lipopolysaccharides, and a unique flagellar sheath protein, HpaA (43, 103, 176).

As in the case of enteric bacteria, *H. pylori* flagella are composed of three structural elements, the basal body, hook, and filament (Fig.2). The basal body is a cylindrical structure consisting of a rod and set of rings embedded in various layers of the cell envelope. The basal

body also contains proteins required for rotation, chemotaxis and flagellar protein export. The hook is short, curved structure that acts as a flexible linker and couples the torque from the basal body to the flagellar filament. The filament is a long, rigid, helical structure that performs hydrodynamic work on the surroundings and propels the cell (101, 167).

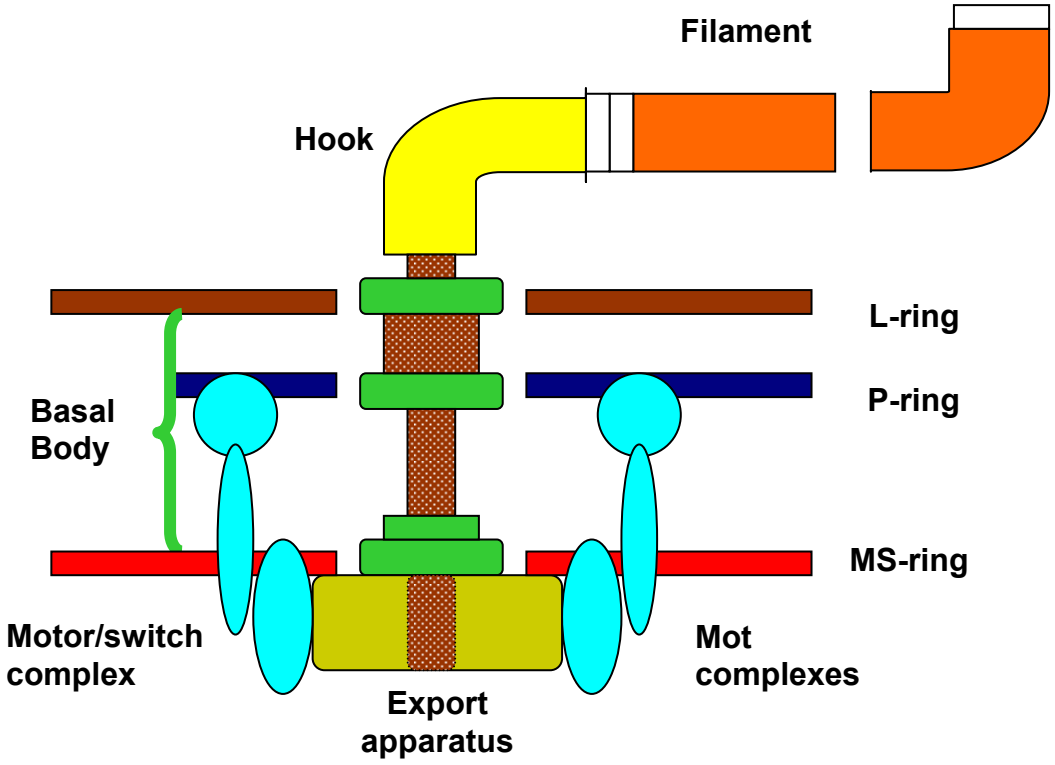
The MS ring encoded by *fliF*, is located in the cytoplasmic membrane and is the first flagellar structural component to be assembled (81). This ring acts as a mounting plate for the flagellar rod proteins FlgB, FlgC and FlgG (73). It also serves as an anchor for the motor/switch proteins FliM, FliN and FliG (59) and the motor rotation proteins MotA and MotB (36). The L ring is composed of the FlgH protein and is situated in the LPS layer, while the P ring consists of the FlgI protein and is located in the peptidoglycan or murein layer of the cell envelope (81). The hook is composed of a single type of subunit, the hook protein or FlgE. Located between the hook and filament, are two hook-associated proteins FlgK and FlgL (101). In *E. coli*, the filament is made up of a single filament protein FliC (101). Other bacteria have multiple flagellins, including *Caulobacter crescentus* (FljK, FljL and FljJ) (192) and *Vibrio cholerae* (FlaA, FlaC, FlaE, FlaD and FlaB). In *V. cholerae*, however, only one of these flagellins, FlaA, is essential for motility (142). The *H. pylori* flagellar filament is composed of two flagellins, FlaA and FlaB. FlaB, the minor flagellin is located proximal to the hook, while FlaA covers the bulk of the filament. Both the flagellins are essential for full motility and colonization (82, 95, 172). FliD is located at the tip of the flagellar filament and promotes filament elongation by preventing loss of flagellar subunits into the surrounding during flagellar biogenesis in enteric bacteria (87). Since the *H. pylori* flagella are surrounded by a sheath, it is not obvious if FliD would be required similarly for filament assembly.

Flagellar biogenesis involves the orderly transport of structural flagellar proteins and their assembly at the tip of the growing flagellum. Flagellar proteins that are localized external to the cytoplasmic membrane and include the rod, hook and filament proteins are secreted through a flagellar specific pathway which shares homology to the type III secretion (TTS) of virulence factors of gram-negative bacteria (167). TTS chaperones are found in all bacteria that use flagellar and/or virulence-associated TTS systems. In *H. pylori*, proteins constituting the flagellar export apparatus, include FliH, FliI, FliQ, FliL, FliP, FliR, FlhA and FlhB (57, 80, 140, 156). FliI is an ATPase which provides the energy for the translocation of export substrates across the cytoplasmic membrane (51). FliH is a regulatory protein which prevents FliI from hydrolyzing ATP until the flagellar export apparatus is competent to link this hydrolysis to the translocation of the substrate. Flagellar substrates associate with the FliI-FliH complex and are transported out of the cell in an energy dependent manner.

TTS chaperones that help in translocating flagellar substrates are small-size (12-18 kDa) proteins of an acidic pH and an overall α-helical structure (72). In *S. enterica* serovar Typhimurium, FliJ functions as a general chaperone to prevent the export of substrates from premature aggregation in the cytoplasm (72). In *H. pylori* there are no homologues of FliJ. However, *hp0245* appears to be in an operon with *flgI* (encodes P-ring protein) and codes for a small acidic protein that is largely α-helical in structure and could be a potential TTS chaperone in *H. pylori*. *H. pylori* does possess the TTS chaperone FliS, which assists in translocation of flagellin subunits. *H. pylori* mutants in *fliI*, *flhA*, *flhB* and *fliQ* are non-motile and aflagellated suggesting the importance of these proteins in flagellar export (140).

In *S. enterica* serovar Typhimurium, the flagellar export apparatus switches its substrate specificity upon completion of hook assembly. The hook length control protein (FliK) and the

Figure 2. Ultrastructure of bacterial flagellum. The flagellum consists of a basal body, the hook and the flagellar filament. The basal body is made up of a rod and a set of rings embedded in the various layers of the cell envelope. The hook is a flexible linker connecting the basal body to the filament which acts as a propeller to propel the cell.


Filament
Hook
L-ring
P-ring
Basal
Body
MS-ring
Motor/switch
complex
Mot
complexes
Export
apparatus

C-terminal domain of the export apparatus associated protein FlhB are involved in this process (72). FlhB presumably assists in the assembly and the export of the rod and hook protein components. Upon completion of the hook structure, the C-terminal domain of FliK interacts with the cytoplasmic domain of FlhB, resulting in a conformational change in FlhB. This results in the specific switching of the filament-type substrates from the previous rod and hook-type substrates (72). A *H. pylori* FliK homologue was not identified in the original annotation of the *H. pylori* genome sequences, but subsequent analysis using the Pfam collection of Hidden Markov models revealed HP0906 as member of the FliK family. Inactivation of *hp0906* resulted in a mutant with the expected phenotype of a *fliK* mutant (Lara Pereira, unpublished data). Based on the genome sequence of *H. pylori* strain 26695, a list of genes involved in flagellar biogenesis and regulation is listed in Table 3.

Chemotaxis and Motility

H. pylori colonizes the mucous layer overlaying the gastric epithelial cells. To avoid being subjected to the gastric acid or being washed away due to the continuous shedding of the mucosal layer *H. pylori* has to move towards the epithelial cell surface (114, 189). Experimental data shows that *H. pylori* exhibits a chemotactic activity towards compounds such as urea, sodium bicarbonate, sodium chloride (114) as well as amino acids such as glutamine, histidine, lysine and alanine (167). Mucin has also been postulated to be a chemoattractant for *H. pylori*, however, due to the large molecular size of the compound, it is possible that degradative products of mucin are likely to be chemoattractants rather than mucin itself (160). Urea as well as sodium and bicarbonate ions are abundant in the mucous layer of the epithelial cells, due to its passive diffusion from the bloodstream and active secretion from the mucus and parietal cells.

Movement of *H. pylori* towards urea and bicarbonate appeared to be enhanced in the presence of the enzyme urease and in a highly viscous medium (119), reflecting a potential adaptive existence in the ecological niche of the human gastric mucosa. The rotation of the *H. pylori* flagellum is dependent on a proton motive force. It has been suggested that hydrolysis of urea by the enzyme urease, contributes to the proton motive force and that chemotactic movement of the bacterium towards urea may provide substrate for the hydrolysis (119).

Chemotaxis and its regulation has been well documented in *E. coli* and *S. enterica* serovar *typhimurium* (1, 4). These bacteria possess methyl-accepting chemotaxis proteins (MCPs), which act as sensors of attractants or repellants. The MCP consists of a periplasmic ligand interaction domain linked to a cytoplasmic signaling and adaptation domain. In addition to MCPs, there are four different regulatory proteins, CheA, CheY, CheW and CheZ. These proteins transduce the signal from the receptors to the flagellar motor. CheA and CheY are part of a two component regulatory system, in which CheA is the sensor histidine kinase and CheY is the response regulator. CheW is a receptor-coupling factor while CheZ mediates the dephosphorylation of CheY-phosphate (4).

When a MCP binds a ligand, it undergoes a conformational change in the cytoplasmic domain. This change is recognized by a CheA-CheW complex, which is bound to the MCP via CheW. Binding of a repellant to the MCP results in a phosphorylation of CheA which then transfers the phosphate to its response regulator CheY. Phosphorylated CheY binds to FliM in the flagellar motor-switch complex resulting in a clockwise rotation of the flagellum and tumbling of the cell, resulting in the random reorientation of the cell (4). If an attractant binds to a MCP, autophosphorylation of CheA is suppressed which leads to lower levels of phosphorylated CheY and hence to less frequent tumbles and more frequent runs of the cell

Table 3. Flagellar genes in *H. pylori* strain 26695

Genes encoding structural proteins and the HP numbers of the ORFs		Function	Genes required for flagellar assembly and the HP numbers of the ORFs		Function
fliF	HP0351	MS ring protein	*flhA (flbA)*	HP1041	Export apparatus
flgI	HP0246	P ring protein	*flhF*	HP1035	Unknown
flgH	HP0325	L ring protein	*fliP*	HP0685	Export apparatus
fliE	HP1557	MS ring/rod adaptor	*fliQ*	HP1419	Export apparatus
flgC	HP1558	Proximal rod protein	*fliI*	HP1420	Export apparatus
flgB	HP1559	Proximal rod protein	*fliR*	HP0173	Export apparatus
flgG	HP1585	Distal rod protein	*fliT*	HP0754	Filament cap export
flgG'	HP1092	FlgG homolog	*fliH*	HP0353	Export apparatus
flgE	HP0870	Hook protein	*fliS*	HP0753	Flagellin export
flgD	HP0907	Hook capping protein	*fliY*	HP1030	Unknown
flgE'	HP0908	FlgE homolog	*flmH (flaG)*	HP0327	Flagellin modification
flgK	HP1119	Hook associated protein	*flhB*	HP0770	Export apparatus
fliD	HP0752	Filament cap	*flmA*	HP0840	Flagellin modification
flaA	HP0601	Flagellin A	*fliK*	HP0906	Hook length control protein
flaB	HP0115	Flagellin B	**Genes encoding regulatory proteins**		
flaB'	HP0295	Flagellin B homolog			
fliG	HP0352	Rotor component	*fliA*	HP1032	σ^{28}
fliM	HP1031	Motor/switch	*flgR*	HP0703	Activator of σ^{54}-holoenzyme
fliN	HP0584	Motor/switch	*flgS*	HP0244	Sensor kinase for FlgR
motA	HP0815	Motor rotation protein	*rpoN*	HP0714	σ^{54}
motB	HP0816	Motor rotation protein	*flgM*	HP1122	Anti- σ^{28}

towards increasing concentrations of the attractant. CheZ also stimulates the dephosphorylation of CheY-phosphate thereby counteracting the clockwise rotation of the flagellum. The chemotactic movement of the bacteria towards attractants involves two other proteins, CheR, a methyltransferase, and CheB, a methylesterase. In the absence of any chemotactic signal, the MCPs are continuously methylated and demethylated by CheR and CheB, leading to a basal level of receptor methylation which maintains an intermediate run-tumble behaviour (4).

Approximately nine genes have been identified in the *H. pylori* genome that are thought to be involved in chemotaxis and regulation of chemotactic signals. These include genes encoding CheY, CheW, three CheV proteins, which are homologues of *B. subtilis* CheV that possesses a N-terminal CheW domain linked to a response regulator domain of the CheY family, a bifunctional CheAY protein and three MCPs (*tlpA, tlpB, tlpC*) (12, 58, 177). The three MCPs of *H. pylori* are membrane-bound proteins, associated with two soluble proteins, CheA and CheW (12). Interestingly, homologues of CheR and CheB, the methyltransferase and methylesterase are not found in *H. pylori* (167). This suggests that the adaptive response of *H. pylori* to chemotactic signals differs from that of the well characterized chemotaxis pathways in *E.coli* and *S. enterica* serovar Typhimurium. Chemotaxis related genes and their functions are listed in Table 4.

Flagellar Genes in Enteric Bacteria

The motility regulon in enteric bacteria consists of genes whose products are structural components of the flagellum as well as those whose products regulate the expression of flagellar genes or the rotation of the completed flagellum. More than 50 genes are required for flagellar formation and function. The flagellar genes constitute at least fourteen different operons, most of

which are clustered in four regions on the chromosome (100). Transcription of these operons is part of an organized cascade called the flagellar regulon and is co-ordinated by the flagellar assembly hierarchy. In *E.coli* and *S. enterica* serovar Typhimurium this regulon is subject to complex regulation in which all genes at the first two levels of the three-tiered hierarchy must be functional for subsequent classes to be expressed. The four flagellar regions that form contiguous clusters on the chromosome are referred to as flagellar regions I, II, IIIa and IIIb (100). Within each region there are several operons consisting of one to nine genes.

Region I consists of genes encoding the flagellar structural proteins such as the basal body and hook structures. These include the genes encoding the rod protein (*flgB*, *flgC*), basal body outer ring proteins (*flgH*, *flgI*), hook protein (*flgE*) and the hook-filament junction proteins (*flgK* and *flgL*). Also present in this region is *flgM*, which codes for an anti-sigma 28 factor (100)

Region II contains genes dedicated to chemotaxis and motility. These include chemotaxis genes *cheA*, *cheB*, *cheR*, *cheW*, *cheY* and *cheZ*, chemotaxis receptor genes *tar* and *tap* and the motor rotation genes *motA* and *motB*. Also present in region II is the master regulator genes *flhD* and *flhC*, the products of which initiate the cascade of flagellar biosynthesis. The *flhE* operon includes *flhA* which encodes the protein FlhA which is part of the flagellar protein export apparatus (100).

Region IIIa is comparatively small and consists of the flagellar filament gene *fliC*. It also contains *fliA*, which encodes σ^{28} required for the transcription of the flagellin gene *fliC* and other late flagellar genes such as the filament cap gene *fliD* and the genes encoding the flagellin chaperones *fliS* and *fliT* which are required for export of flagellar filament-type proteins. All the three genes, *fliD, fliS* and *fliT* also belong to region III on the chromosome (100).

Table 4. Genes involved in chemotaxis and their functions in *H. pylori* strain 26695.

Annotated HP number	Gene Name	Putative Function
HP0392	*cheA*	Sensor histidine kinase
HP1067	*cheY*	Response regulator
HP0616, HP0393, HP0019	*cheV*	Chemotaxis protein
HP0391	*cheW*	Purine binding chemotaxis protein
HP0099	*tlpA*	Methyl-accepting chemotaxis protein
HP0103	*tlpB*	Methyl-accepting chemotaxis protein
HP0082	*tlpC*	Methyl-accepting chemotaxis protein

Region IIIb consists of genes needed in the early stage of flagellar assembly. These genes include *fliF* (encodes the MS ring), *fliE* (encodes MS-rod adaptor), *fliG*, *fliM* and *fliN* (encode the motor-switch complex), *fliI* and *fliH* (encode an ATPase which is part of the protein export apparatus and a regulator of FliI, respectively) and *fliK* (encodes hook length control protein) (100).

Flagellar Gene Regulation in Enteric Bacteria

In *E. coli* and *S. enterica* serovar Typhimurium flagellar gene transcription is controlled by the cell cycle. According to the relative positions in the transcriptional hierarchy, flagellar operons are classified into three classes. Class I contains the master operon, *flhCD*, consisting of two genes *flhD* and *flhC*, the products of which are required for Class II expression. Transcription of *flhCD* operon is dependent on cyclic AMP and catabolite activator protein (CAP), which binds upstream of the *flhCD* promoter. The FlhD and FlhC proteins of *E. coli* were purified and shown to be activators of σ^{70}-dependent expression of the Class II genes (98) (Fig 3).

Class II contains the genes responsible for the formation of the flagellar basal body structure and the basal body-hook complex. Class II also contains the *fliA* gene, which encodes the alternative sigma factor, σ^{28}, and is required for the expression of the Class III genes (126). One of the Class II genes, *flgM*, encodes the anti-sigma factor FlgM. FlgM binds to σ^{28} within the holoenzyme, interfering with promoter-binding activity and thereby blocking transcription of σ^{28}-dependent late flagellar genes. Upon completion of the basal body-hook complex, FlgM is translocated outside the cell through the flagellar hook structure. The onset of FlgM secretion results in a decrease in its cellular concentration and a derepression of σ^{28}-

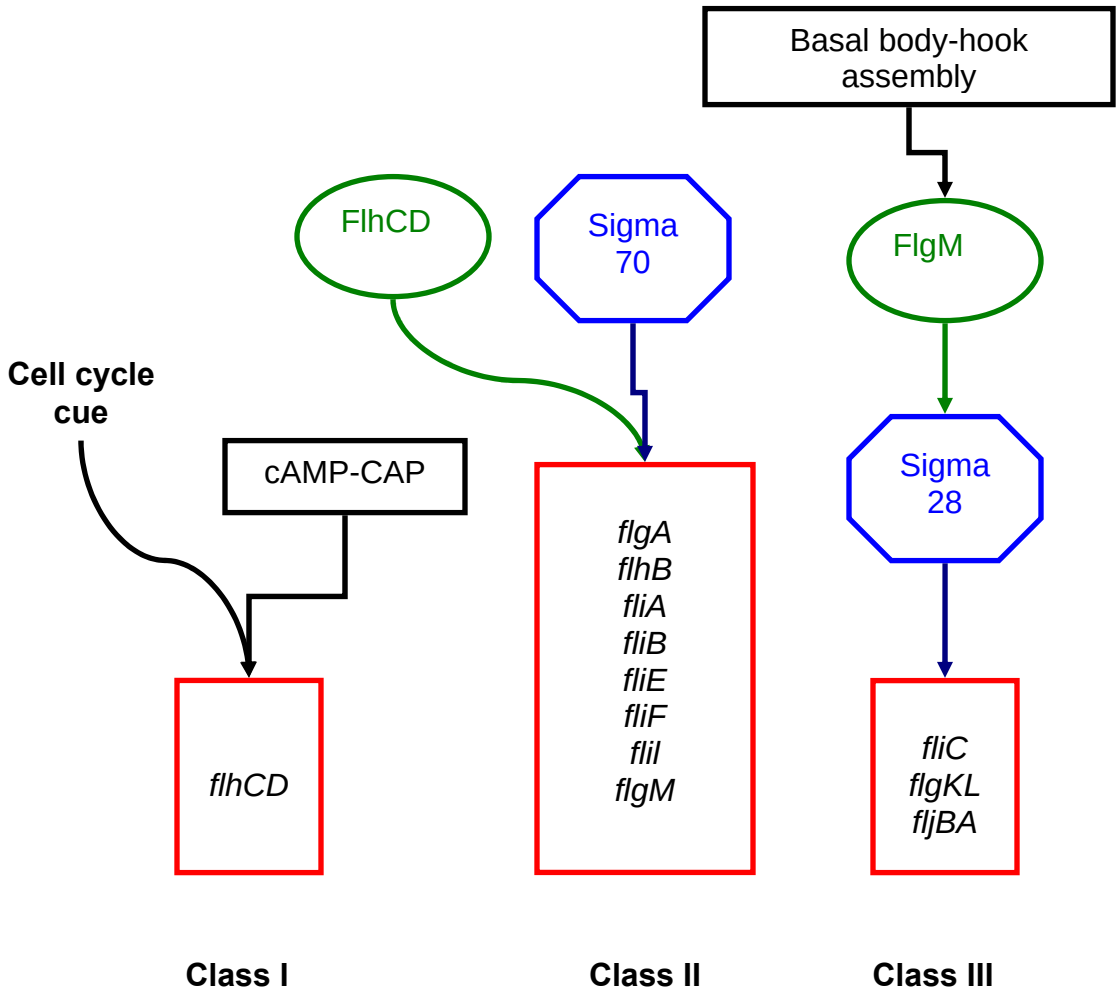
mediated expression of Class III flagellar genes (100). Hence, in enteric bacteria, completion of the hook structure is a major checkpoint in flagellar development as it signals FlgM secretion and initiation of expression of Class III genes.

The Class III gene (*fliC*) encodes the flagellar filament protein, FliC. Work in *E.coli* has shown that flagellar biosynthesis and cell division are co-regulated (143). Expression of the *flhCD* operon is highest during the exponential phase, decreases during the late exponential phase and then increases to half the maximal levels during the stationary phase, which demonstrates the temporal nature of flagellar gene expression in enteric bacteria.

Flagellar Gene Regulation in *C. crescentus*

C. crescentus differentiates from a motile swarmer cell that is not capable of cell division to a non-motile stalked cell which is able to reproduce. Synthesis and loss of its single polar flagellum have been demonstrated to be coupled to an asymmetric cell division (192). *C. crescentus* is generally regarded as a model developmental system, and the characterization of flagellar regulation has been used to elucidate the mechanism of cell cycle control in this bacterium. In *C. crescentus*, initiation of flagellar biosynthesis is triggered by DNA replication. This signal leads to the expression of Class I master regulator gene, *ctrA*, the product of which activates transcription of Class II genes in conjunction with σ^{73}-RNA polymerase holoenzyme, the major form of RNA polymerase in *C. crescentus* (Fig 4). In addition to its role in flagellar biogenesis, CtrA is also involved in regulation of DNA replication, initiation of cell division and synthesis of DNA methytransferase (147). CtrA, whose activity is regulated by phosphorylation, activates the flagellar transcription hierarchy in early pre-divisional cells.

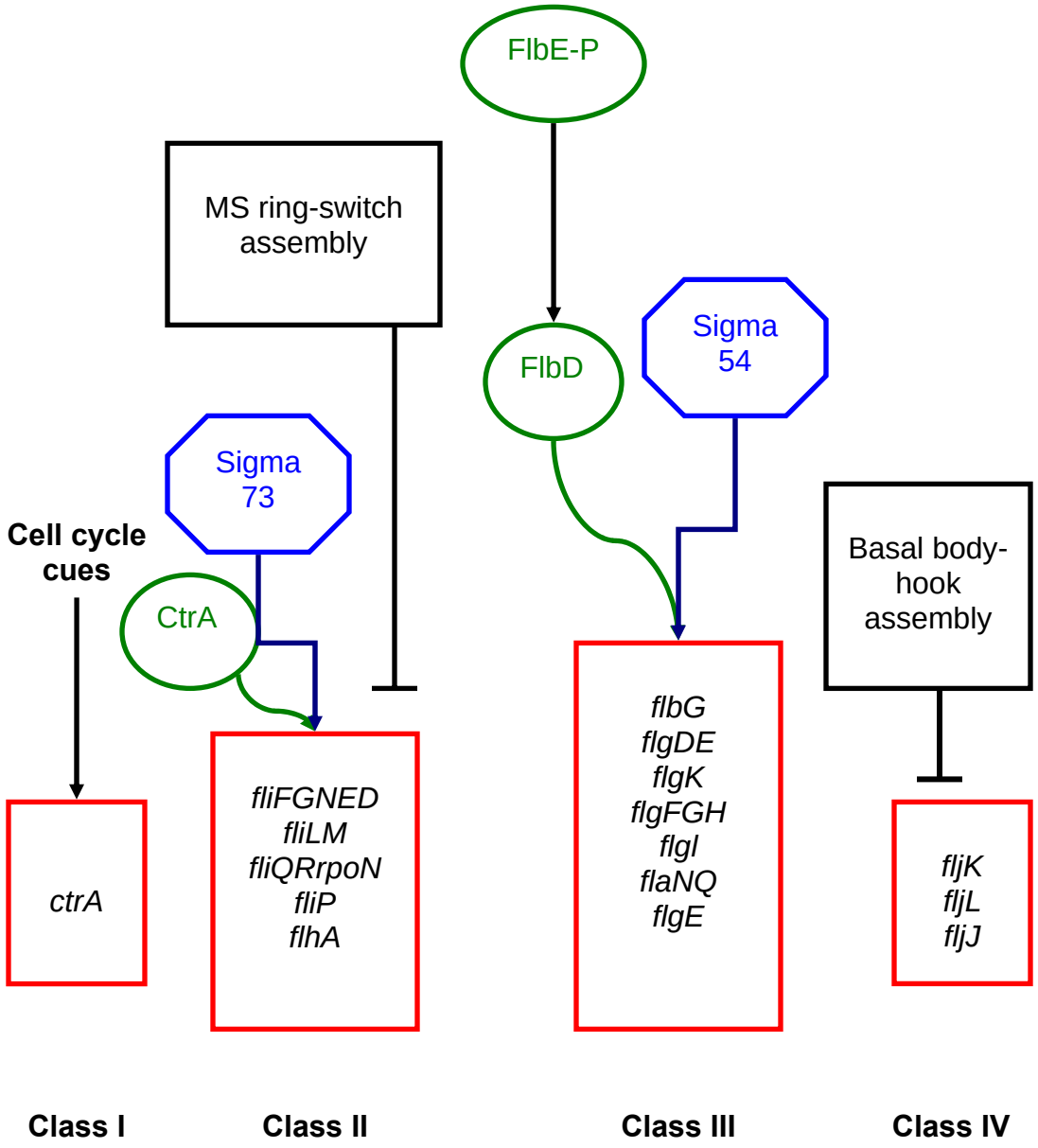
Figure 3. Flagellar gene regulation in enteric bacteria. Cell cycle cues trigger the expression of the Class I gene which encodes the master regulator proteins FlhCD. FlhCD activate transcription of the Class II genes which encode components of the basal body-hook complex with σ^{70}-RNA polymerase holoenzyme. Completion of the basal body-hook assembly acts as a signal for the export of anti- σ^{28} protein FlgM and activates transcription of σ^{28}-dependent Class III genes which encodes the flagellar filament protein.


Basal body-hook assembly
FlhCD
Sigma 70
FlgM
Cell cycle cue
cAMP-CAP
Sigma 28
flgA
flhB
fliA
fliB
fliE
fliF
fliI
flgM
flhCD
fliC
flgKL
fljBA
Class I
Class II
Class III

The Class II genes encode subunits of the basal body, protein export apparatus and the transcription factors RpoN (σ^{54}) and FlbD (192). FlbD is a response regulator of a two-component system and is phosphorylated by its sensor kinase FlbE. Phosphorylation of FlbD allows it to activate transcription of the σ^{54}-dependent Class III genes, which encode the basal body and hook proteins, as well as the Class IV genes *fljK*, *fljJ* and *fljL*, which encode the three subunits of the flagellar filament. The promoter regulatory regions of genes that are activated by FlbD have binding sites for FlbD and the integration host factor (IHF), which were shown to be required *in vivo* for full transcriptional activation of several Class III genes (192). The FlbD protein also represses transcription of the Class II *fliF* operon, which contains five genes including *flbD* (191), by binding to a flagellar transcription regulator (*ftr*) sequence element within the *fliF* promoter. Thus, FlbD is a key component of a genetic switch mediating the transition from Class II to Class III gene transcription as it represses Class II expression and stimulates transcription of Class III operons.

In addition to *rpoN* and *flbD*, the structural genes of Class II are also required for transcription of Class III genes, which in turn are required for transcription of Class IV genes. Flagellar gene expression in *C. crescentus* is coordinated with flagellum biosynthesis by two assembly checkpoints. The first involves the transcription of Class III genes and requires the completion of the MS ring-switch assembly. The second checkpoint occurs upon expression of Class IV genes which requires completion of the basal body-hook complex (145). Studies have shown that the *C. crescentus* MS ring-switch checkpoint is mediated by a negative regulatory gene *bfa*. Class II mutant strains, that have secondary mutations in the *bfa* gene can transcribe all the Class III flagellar genes, thereby bypassing the requirement of one of the checkpoints (192).

Figure 4. Flagellar gene regulation in *C. crescentus*. DNA replication acts as a trigger to activate the Class I gene which encodes the master regulator protein , CtrA. CtrA activates transcription with σ^{73}-RNA polymerase holoenzyme from the Class II genes which encode the export apparatus proteins and the MS ring of the basal body. Completion of the MS ring leads to the activation of σ^{54}-dependent Class III genes which encode the basal body and hook proteins. The Class IV genes encoding the flagellar filament proteins are expressed following the formation of the basal body-hook complex.


FlbE-P
MS ring-switch assembly
FlbD
Sigma 54
Sigma 73
Cell cycle cues
CtrA
Basal body-hook assembly
ctrA
fliFGNED
fliLM
fliQRrpoN
fliP
flhA
flbG
flgDE
flgK
flgFGH
flgI
flaNQ
flgE
fljK
fljL
fljJ
Class I
Class II
Class III
Class IV

In *C. crescentus* the flagellar filament is composed of three different flagellin subunits, FljK, FljJ and FljL. Expression of *fljL* is subject to completion of basal body-hook complex checkpoint as well as a post-transcriptional regulatory mechanism. *fljK* is the only σ^{54}-dependent flagellin gene which is not regulated by the MS ring-switch checkpoint. The mechanism of regulation of *fljJ* is not known (192).

The asymmetric pattern of cell division results in the compartmentalization of late flagellar gene expression in *C. crescentus*. *fljK* transcripts are made in the pre-divisional cells and then segregated exclusively to the progeny swarmer cell during cell division. Studies with a constitutively active form of FlbD indicate that FlbD activation is spatially restricted as the sensor kinase FlbE is localized only in the swarmer cell compartment of the pre-divisional cell (192).

Flagellar Gene Regulation in *H. pylori*

Complete genome sequences of *H. pylori* 26695 and J99 have identified approximately 40 genes involved in flagellar biogenesis and regulation. The flagellar genes of *H. pylori* are scattered throughout the genome in 25 or more transcriptional units in contrast to the flagellar genes of enteric bacteria and *C. crescentus* which are clustered in well defined genomic regions (100). Studies on *H. pylori* flagellar biosynthesis have shown that regulation of flagellar genes is complex and involves all three sigma factors found in this bacterium.

In *H. pylori*, σ^{80} is the primary sigma factor which is required for the expression of housekeeping genes as well as genes encoding flagellar basal body proteins, components of the flagellar protein export apparatus and chemotaxis proteins (12, 140, 166). The alternative sigma factor σ^{54} and the σ^{54} -dependent activator FlgR are responsible for the transcription of genes

encoding components of the rod, hook, hook-associated proteins and the minor flagellin FlaB (167, 168). Genes encoding the major flagellin (*flaA*), the filament capping protein *(fliD*), and proteins involved in flagellin modification and flagellin export (*flaG* and *fliS*) respectively are σ^{28} -dependent (87, 95, 177). The involvement of both σ^{54} and σ^{28} in the regulation of flagellar gene transcription in *H. pylori* is somewhat similar to that of the human pathogen *Vibrio cholerae,* which also uses both σ^{54} and σ^{28} (142).

Transcription of σ^{54} -dependent genes in *H. pylori* involves the activator protein FlgR. FlgR, like several other σ^{54} -dependent activators is a response regulator of a two- component system and shares homology to the NtrC family of transcriptional activators (168). The cognate sensor kinase of FlgR is encoded by *orf0244* (*flgS*) (11), appears to be in an operon with *flgI* and *orf0245*. This is in contrast to many other pairs of sensor kinases and their response regulators, whose genes are often part of the same operon. *flgR*, is the last gene within an operon that appears to be expressed constitutively. Interestingly, unlike most other σ^{54} -dependent activators, FlgR lacks a predicted DNA-binding domain (168).

Drawing parallels from other bacteria, I propose a model for flagellar gene regulation in *H. pylori* that is presented in Figure 5. Flagellar biogenesis in *H. pylori* is temporally regulated and is coordinated with the hierarchical expression of distinct classes of flagellar genes. As mentioned previously, in enteric bacteria and *C. crescentus*, the hierarchical expression of different classes of flagellar genes is initiated by the expression of the Class I gene encoding the master regulator, FlhCD and CtrA, respectively (101, 192). No homologues of known master regulators, however, have been found in *H. pylori* or in other closely related ε-Proteobacteria. It is possible that *H. pylori* possesses a unique master regulator that functions with σ^{80}-RNA polymerase holoenzyme to activate transcription from the Class II genes.

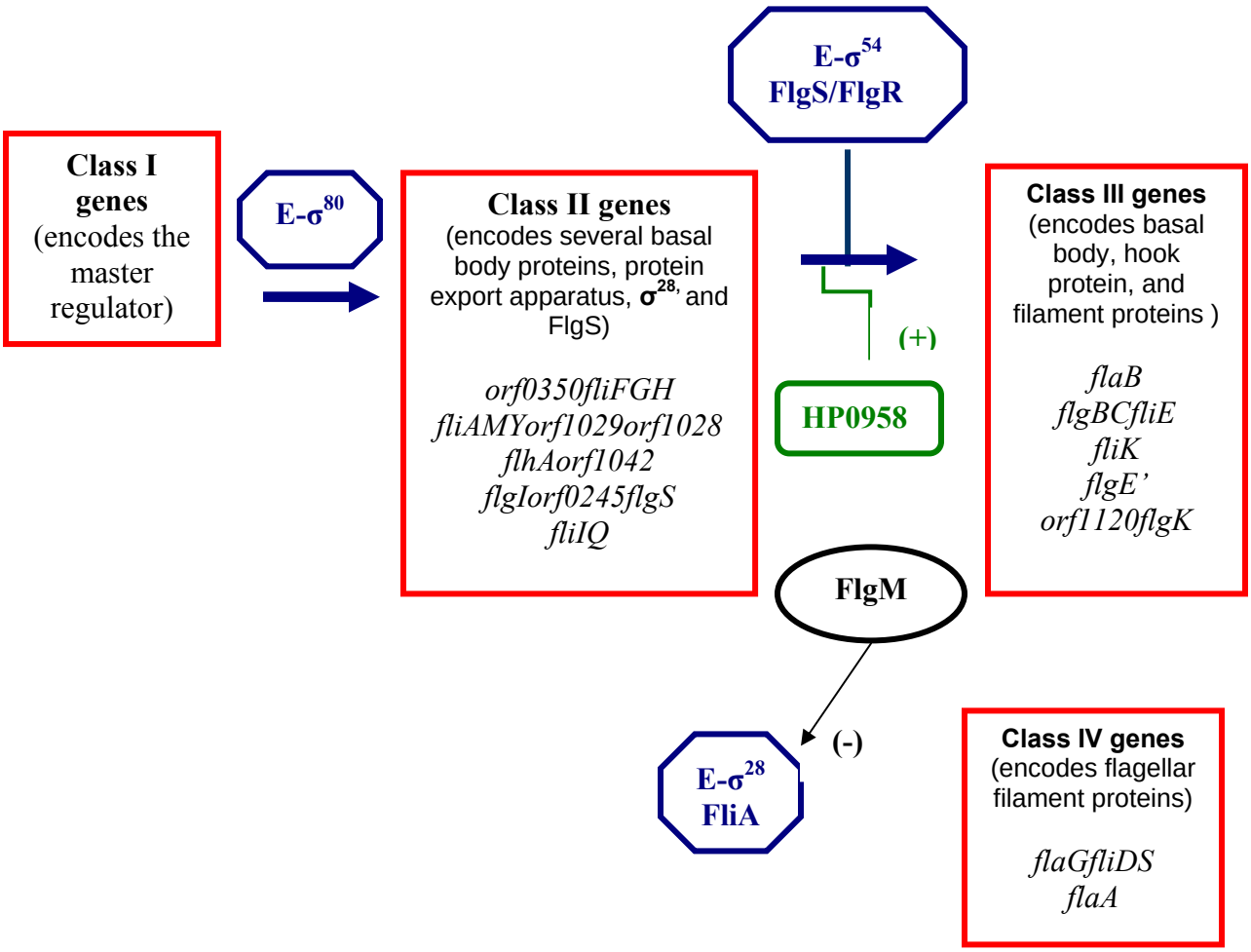
Predicted *H. pylori* Class II genes encode components of the basal body, motor-switch, protein export apparatus as well as the sensor kinase FlgS. Upon expression of *flgS* and other Class II genes, FlgS is able to phosphorylate FlgR, which can then activate transcription of the σ^{54} -dependent Class III flagellar genes. These Class III flagellar genes encode components of the rod and hook, as well as hook-associated proteins and the minor flagellin FlaB.

Class IV flagellar genes are transcribed by σ^{28} -RNA polymerase holoenzyme and encode components of the filament and are therefore the last structural components of the flagellum. Although *fliA* (encodes σ^{28}) is expressed early in the cascade, σ^{28}-dependent genes are not expressed until the end of the hierarchy. Colland and co-workers (28) identified HP1122 (FlgM) as an anti- σ^{28} factor in *H. pylori* which prevents σ^{28} from forming the holoenzyme with core RNA polymerase. It is not known at this time how the inhibitory effect of FlgM on σ^{28} is alleviated to allow expression of σ^{28}-dependent late flagellar genes.

Interestingly, unlike most other sensor proteins that reside in the cytoplasmic membrane, FlgS appears to be located in the cytoplasm. Environmental regulation of σ^{54} -dependent flagellar genes has been demonstrated in *Campylobacter coli* (2), which is closely related to *H. pylori*. Transcription of *C. coli flaB* is modulated by growth medium pH, temperature and the concentration of certain salts. It is possible that the modulation of the amount of FlaB in response to environmental stimuli allows *C. coli,* and possibly *H. pylori,* to produce flagella that are suited to its ecological niche.

As described earlier, in enteric bacteria and in *C. crescentus*, the hierarchical expression of flagellar genes is coordinated by flagellar assembly checkpoints. In *H. pylori*, formation of an early basal body complex may serve as a checkpoint for flagellar biosynthesis. One study showed that mutations in *fliF*, which encode the MS ring and several other genes encoding

Figure 5. Flagellar gene regulation in *H. pylori.* Unknown environmental signals or cellular cues trigger the expression of the Class I gene, the product of which activates transcription with σ^{80}-RNA polymerase holoenzyme of the Class II genes. The Class II genes encode the flagellar export apparatus proteins as well as the initial components of the basal body and the sensor kinase FlgS. Phosphorylated FlgS transfers the phosphate to its cognate response regulator FlgR, which then activates transcription from σ^{54}-dependent Class III genes. The Class III genes encode the basal body proteins, minor flagellin FlaB and the hook protein. The Class IV genes are σ^{28}-dependent and encode the major filament protein FlaA.


Class I genes
(encodes the master regulator)
E-σ80
Class II genes
(encodes several basal body proteins, protein export apparatus, σ28, and FlgS)
orf0350fliFGH
fliAMYorf1029orf1028
flhAorf1042
flgIorf0245flgS
fliIQ
E-σ54
FlgS/FlgR
(+)
HP0958
Class III genes
(encodes basal body, hook protein, and filament proteins)
flaB
flgBCfliE
fliK
flgE'
orf1120flgK
FlgM
(-)
E-σ28
FliA
Class IV genes
(encodes flagellar filament proteins)
flaGfliDS
flaA

components of the export apparatus, resulted in a two-to five-fold decrease in the levels of *flaA*, *flaB* and *flgE* transcripts, suggesting a checkpoint for the expression of late flagellar genes (140). Mutations in the flagellar protein export apparatus gene *flbA* (*flhA*) resulted in the decreased expression of *flgE* and no detectable expression of *flaA* or *flaB* (156). Obviously, more experimental data are necessary to delineate the regulation of flagellar gene expression in *H. pylori.* Data from protein-protein interaction map of *H. pylori* based on a high throughput screen of a yeast two-hybrid assay (144) have revealed two other conserved hypothetical proteins, HP0958 and HP0137 which interacted with σ^{54} and FlgS, respectively. Inactivation of either *hp0958* or *hp0137* resulted in a loss of flagellar synthesis, suggesting roles for the products of the genes in regulation of the RpoN regulon or flagellar assembly in *H. pylori*. The role of HP0137 in flagellar biogenesis in *H. pylori* will be discussed chapter III.

Sigma Factors

Regulation of gene expression is a crucial process in the conservation of cellular energy and resources for bacteria. The simplest form of regulation is at the transcriptional level involving the bacterial RNA polymerase, a multisubunit enzyme with the composition of $\alpha_2\beta\beta'$. This form of the RNA polymerase is referred to as the core RNA polymerase. Binding of an additional protein, the sigma factor (σ) to the core results in the formation of a complex called RNA polymerase holoenzyme which can initiate transcription from promoters. It is the sigma factor that confers specificity to the different classes of promoters, thereby playing an important role in the regulation of different genes (190).

There are two main families of sigma factors, σ^{70} family and the alternate sigma factor σ^{54} family (190). σ^{70} is responsible for the transcription of most of the genes in the bacterium,

while σ^{54} aids in transcription of genes expressed under certain conditions. The majority of the sigma factors in both gram-positive and gram-negative bacteria belong to the σ^{70} family and recognize promoters with consensus sequences at -35 and -10 to the transcriptional start site (99).

The σ^{70} family is further divided into three groups (190). Group 1 contains the primary sigma factor required for the transcription of housekeeping genes, which are essential for cell viability. Group 2 is composed of the non-essential primary-like sigma factors which include the stationary phase σ factors, cyanobacterial σ factors and σ factors from high GC content gram-positive bacteria (190). Group 3 sigma factors are the alternate σ factors required only under certain circumstances or for particular developmental phases in the bacterium. This group includes heat shock σ factors, flagellar σ factors, extracytoplasmic σ factors and σ factors involved in sporulation (190).

Three sigma factors have been identified in *H. pylori* : σ^{80} (RpoD), σ^{54} (RpoN) and σ^{28} (FliA) (16, 35, 167). Analysis of *H. pylori* 26695 and J99 failed to reveal homologs of the stationary phase sigma factor RpoS as well as the heat shock specific sigma factor RpoH (σ^{32}). In *H. pylori* σ^{80} is the primary sigma factor involved in transcription of most of the genes, while σ^{54} and σ^{28} appear to be dedicated for flagellar biogenesis (16). Functional analysis of σ^{80} showed that it is important for cell viability in *H. pylori* (13).

Sigma 70

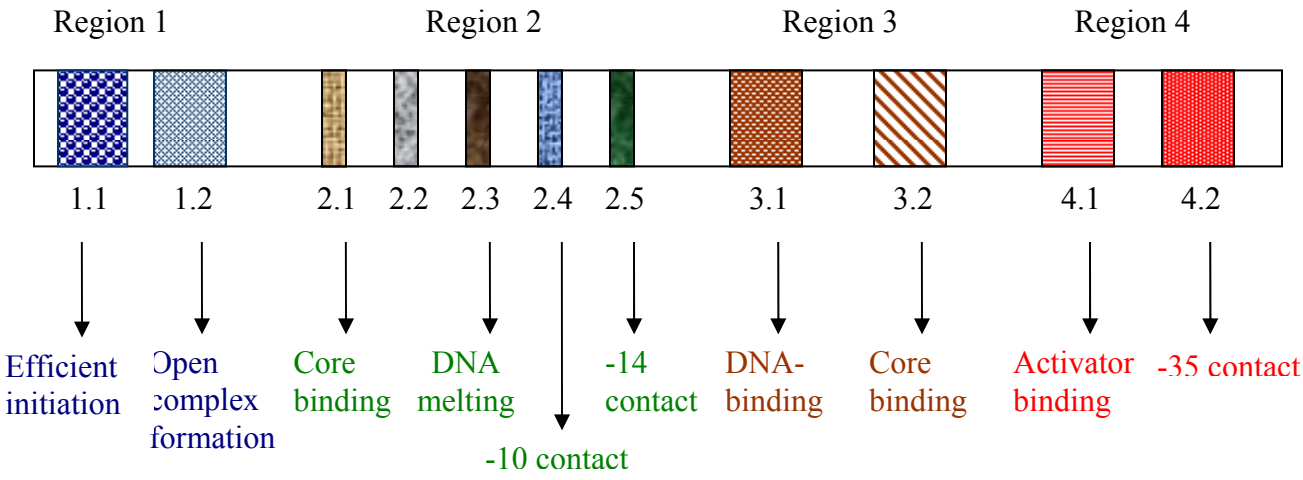
E. coli σ^{70} protein is composed of four independent compactly folded domains. The domains are connected to each other by flexible linkers and are further subdivided into regions (Fig 6). Region 1 is the least conserved and is divided into two subregions. Subregion 1.1 is present in only certain primary σ^{70} factors and modulates DNA binding activity by initiating

transcription only in the presence of the core (186). *H. pylori* σ^{80}, for example, lacks region 1.1 (13). Except for the extracytoplasmic sigma factor, all other σ factors contain region 1.2. Deletions in region 1.2 arrest transcription initiation, suggesting that this region is required for open complex formation (186).

Region 2 is the most conserved domain and is divided into five subregions. Region 2.1 interacts with subunits of the core RNA polymerase, region 2.3 is involved in DNA melting while region 2.4 recognizes the -10 consensus sequence, also called as the Pribnow box. Region 2.5 contacts nucleotides at positions -14 and -15 in *E.coli* promoters, similar to the -16 promoter consensus in *B. subtilis* (67, 162, 179). However, the function of region 2.2 is not known. Region 3 consists of 2 subunits, a well conserved helix-turn-helix (HTH) DNA-binding motif in region 3.1 and a less conserved region 3.2 involved in core binding (99). The C-terminal region 4 can be divided into two subregions. Region 4.1 binds transcriptional activators such as the λ phage C1 protein (97), whereas region 4.2 recognizes the -35 promoter consensus element (41, 63).

E. coli σ^{70} has the highest affinity for promoters with the consensus sequence TATAAT at -10 and TTGACA at -35 (66). The promoter binding specificity for *H. pylori* σ^{80} is less clear. Studies of alignment of σ^{80}-dependent *vacA* -10 regions of 12 different *H. pylori* strains revealed a TAAAAA consensus sequence which shows identity with only four of the six positions in the *E. coli* consensus sequence (54). When the -10 sequence of *vacA* was changed to the *E.coli* consensus sequence the mutation resulted in a 15-fold decrease in transcriptional activity (54). The alignment of *vacA* -35 regions of *H. pylori* 26695 with the corresponding domains of other bacterial species revealed a consensus sequence TTTATG which matched the consensus *E.coli* -35 hexamer TTGACA at only three of the six positions. These differences in consensus of -35 sequence suggests structural differences exist between *H. pylori* σ^{80} and *E.coli* σ^{70}.

Figure 6. Structure-function relationship of σ^{70} . The σ^{70} protein is divided into 4 regionsand each region is further divided into sub-regions. The function of each sub-region is indicated. Sub-region 2.2 has no assigned function.


Region 1
Region 2
Region 3
Region 4
1.1
1.2
2.1
2.2
2.3
2.4
2.5
3.1
3.2
4.1
4.2
Efficient initiation
Open complex formation
Core binding
DNA melting
-10 contact
-14 contact
DNA-binding
Core binding
Activator binding
-35 contact

Further evidence for this comes from the observation that RNA polymerase containing *H. pylori* σ^{80} recognized the *cagA* promoter more efficiently than polymerase containing the *E.coli* σ^{70} (166). This specificity of *H. pylori* σ^{80} can be traced to the spacer region between domains 1 and 2 of *H. pylori* σ^{80} (13). This region was named a spacer because it had no other known role than that of connecting the two functional domains. Hybrid σ factors carrying the spacer region of *H. pylori* σ^{80} cannot initiate transcription from σ^{70} -dependent promoters in *E. coli*. Replacement of the spacer region of σ^{80} with that from σ^{70} results in improved promoter recognition in *E. coli* (13). As reconstituted RNA polymerase containing σ^{70} , σ^{80} or a hybrid of these two proteins can bind both *E. coli* and *H. pylori* promoters to the same extent, the functional property of the spacer region does not interfere with DNA recognition and binding, suggesting a role for the spacer region in transcription initiation (13).

Sigma 28

FliA (σ^{28}) is a member of the alternative group of σ factors and is involved in flagellar biosynthesis in *E. coli* and *H. pylori* as well as several other bacteria. Sequence comparison of σ^{28} to σ^{70} reveals the absence of region 1, the activation response domain in FliA. FliA, however, does possess the highly conserved regions 2, 3 and 4 (65, 70). In *E. coli*, σ^{28} recognizes consensus sequences within the -10 and -35 regions of the promoter and initiates transcription from Class III late flagellar genes. In enteric bacteria, FliA is kept inactive by the anti-sigma factor FlgM until completion of the basal body-hook complex, at which time FlgM is exported from the cell via the flagellar protein export apparautus (100).

The σ^{D} of *B. subtilis* and WhiG of *Streptomyces coelicolor* are also members of the flagellar σ factors (190). The function and regulation σ^{D} of *B. subtilis* is similar to that of FliA of

enteric bacteria. *S. coelicolor* WhiG, however, is involved in sporulation. Although WhiG has a different function, *S. coeliocolor whiG* can complement a *fliA* null mutant of *S. typhimurium* to restore flagellar biosynthesis and motility (88).

As indicated previously *H. pylori* FliA is involved in the transcription of the Class IV late flagellar genes (87, 95, 177). HP1122 was identified in a high throughput screen of a yeast two hybrid assay as interacting with FliA, and was eventually shown to be an anti-σ^{28} factor and was designated FlgM (28). Inactivation of *flgM* resulted in greater than two-fold increase in the *flaA* transcription suggesting that FlgM negatively regulated *flaA* expression. As expected, overproduction of FlgM reduced the amount of *flaA* transcript compared to the wild-type (28). *H. pylori* FlgM inhibits FliA activity by preventing it from interacting with the β subunit of core RNA polymerase. This differs from *S. enterica* serovar Typhimurium FlgM which prevents σ^{28}-dependent gene expression by binding to σ^{28} within σ^{28}-RNA polymerase holoenzyme and preventing it from binding to the promoter (28).

Sigma 54

Sigma 54 (σ^{54}) encoded by the *rpoN* gene was originally found in *S. enterica* serovar Typhimurium where it was required for the transcription of genes involved in nitrogen assimilation (137). With the rise of genome sequencing, it is apparent that σ^{54} is widespread in the Domain Bacteria. Homologs of *rpoN* have been found in diverse bacteria such as *Aquifex aeolicus, Bacteroides thetaiotaomicron, Bacillus subtilis, Borrelia burgdorferi, Chlorobium tepidum* and *Chlamydia trachomatis* to name a few (171). Although originally shown to be required for nitrogen metabolism, σ^{54} is now known to be involved in various cellular activities such as C_4-dicarboxylic acid transport, pilin and flagellin synthesis, toluene and xylene

catabolism, hydrogen metabolism and fructose utilization (171, 168). Amino acid sequence analysis reveals a high degree of sequence conservation among σ^{54} proteins from gram-positive and gram-negative bacteria. A single copy of *rpoN* is usually present in a bacterial genome, but some bacteria such as *Bradyrhizobium japonicam* have two copies of *rpoN*. Genome sequence analysis has also revealed that many bacteria lack *rpoN,* including *Mycobacterium tuberculosis, Haemophilus influenzae, Mycoplasma genitalium, Mycoplasma pneumoniae* and the cyanobacterium *Synechocystis* sp strain PCC 6803.

σ^{54} -dependent transcription differs from that of σ^{70} in two main aspects. First, σ^{54} -holoenzyme recognizes promoters with the consensus sequence TGGCAC and TTGCA/T at -24 and -12, respectively, relative to the transcriptional start site. The spacing between the conserved elements is critical as deletion or addition of a single base pair results in inactivation of the promoter. Secondly, σ^{54} -dependent transcription has an absolute requirement for an activator protein which hydrolyzes ATP to activate transcription (138).

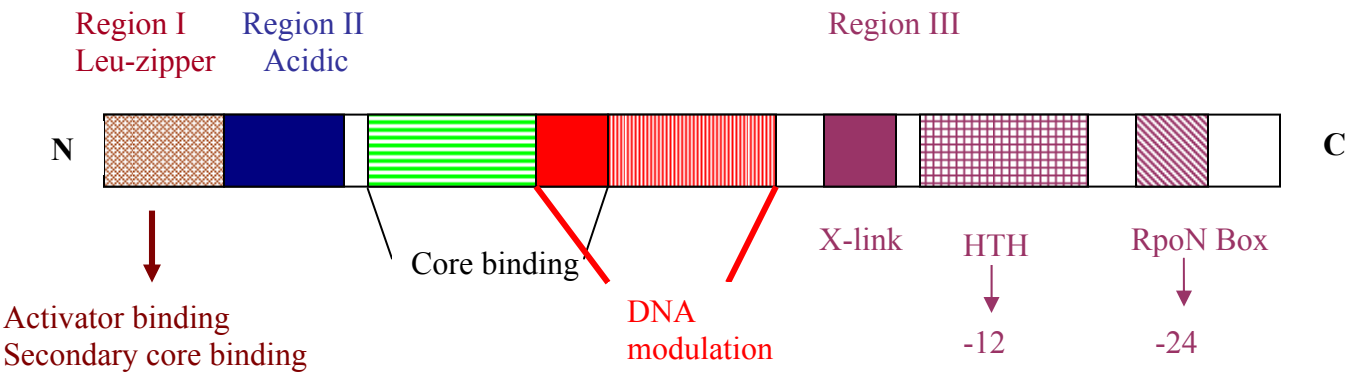
σ^{54} can be divided into three functional regions (Fig 7). Region I, a well conserved region comprises of about 50 amino acids and is often rich in glutamine and leucine. Region I of σ^{54} from *H. pylori,* however, has a low degree of homology with the region I of σ^{54} from other bacteria and has only a single glutamine residue (171). Region I plays an important role in transcriptional activation as deletion of this region bypasses the requirement for activator protein and nucleotide hydrolysis under solution conditions that permit transient DNA melting. Region I has been suggested as a secondary core binding site involving interactions between the core RNA polymerase and σ^{54} (22) and also for interactions with the activator protein (25).

Region II is an acidic region of 50-120 amino acids having no sequence conservation among members of the σ^{54} family. Although σ^{54} from many bacteria have an acidic region II,

this region of *B. subtilis* σ^{54} is not acidic and is completely absent in the proteins from *Rhodobacter capsulatus* and *H. pylori* (33). In enteric bacteria, the acidic residues can be divided into four contiguous subregions. Two of these subregions contain acidic trimer repeats wherein every third residue in an 18 residue sequence is acidic (187). Gralla and colleagues suggested that this region functions in DNA melting, since deletions within this region decreased the rate of open complex formation (155). Deletion of regions I and II of σ^{54} of *S. enterica* serovar Typhimurium resulted in a loss of interaction of σ^{54} with the activator protein, implying that region II is needed for interactions with the activator protein (84). Since region II is completely absent in some bacteria its exact function remains unclear.

Region III of σ^{54} is well conserved among bacteria and is responsible for interacting with the core RNA polymerase as well as contacting promoter DNA (188) (19). This region is about 550 amino acids in length, with residues Gln121 through Glu215 involved in minimal core binding (20, 112), residues Gln180- Met306 constituting a DNA-binding modulation domain that stimulates the promoter binding activity of σ^{54} (23), and residues Val329 to Val477 required for promoter binding. This C-terminal portion of region III contains a potential helix-turn-helix (HTH) motif which recognizes the -12 region of the promoter (111) and a highly conserved run of amino acids (ARRTVAKYRE) called the "RpoN box" which is implicated in binding to the -24 region. Substitutions within the RpoN box drastically reduce the DNA binding activity (175). Crosslinking studies indicate that a part of the protein upstream of the HTH motif which is termed as the X-linker region is closely associated with the promoter DNA in promoter complexes formed with both free σ^{54} and σ^{54} -holoenzyme (21). Region III also appears to play a role in transcriptional activation since certain substitutions in this region do not prevent the protein from binding core RNA polymerase or the promoter but do interfere with transcription

Figure 7. Structure-function relationship of σ^{54}. The three domains of σ^{54} are indicated with their corresponding functions.

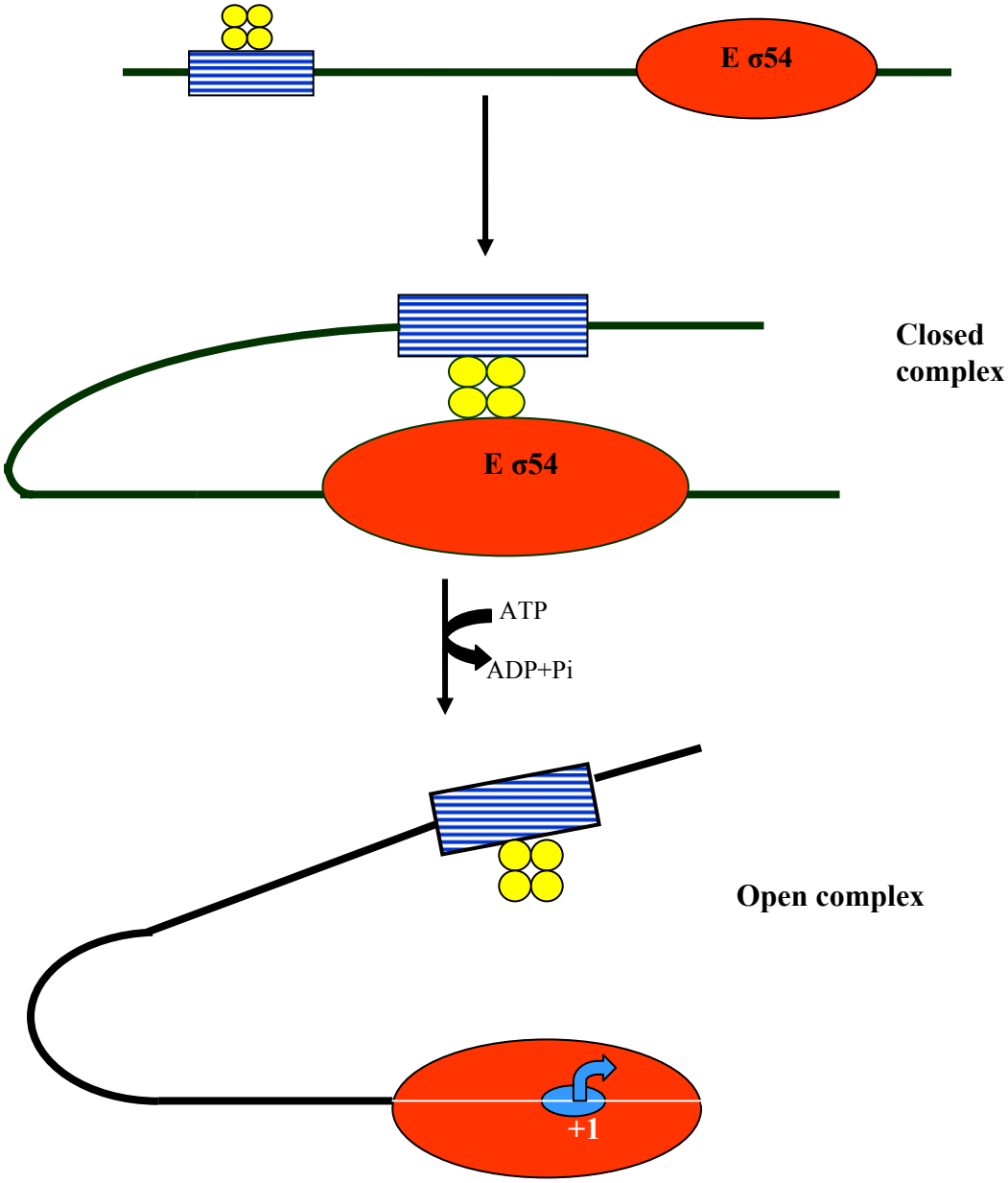

Region I
Leu-zipper
Region II
Acidic
Region III
N
C
Core binding
X-link
HTH
RpoN Box
Activator binding
Secondary core binding
DNA
modulation
-12
-24

initiation (86). As mentioned earlier, Region I of *H. pylori* σ^{54} is not glutamine-rich and the potein lacks the acidic region II found in many other σ^{54} proteins (60). *H. pylori* σ^{54} does possess, however, the conserved HTH motif and RpoN box in region III of the protein. Another difference between *H. pylori* σ^{54} and that of other bacteria lies in the recognition of consensus sequence TGGAAC at -24 of the promoter region which is different from that in other bacteria (TGGCAC) (16). Substitution of A at -23 with C drastically reduces the affinity of σ^{54}-holoenzyme for the promoter in enteric bacteria (8). Studies in our lab indicate that *H. pylori* σ^{54} has a higher affinity for promoters with *H. pylori* consensus sequence than that from other bacteria.

Transcriptional Activation with σ^{54}-RNA Polymerase Holoenzyme

σ^{54}-dependent transcriptional activation differs significantly from that of σ^{70}-dependent activation. σ^{54}-holoenzyme forms a stable closed complex with the promoter but is unable to form a transcriptionally competent open complex in the absence of an activator protein (139). The activator catalyzes the ATP-dependent isomerization of a closed complex to a transcriptionally active open complex in which the DNA around the transcriptional start site is melted (Fig 8). The activator protein, also called enhancer-binding protein, binds cooperatively to two or more sites called enhancers or upstream activation sequences (UAS) which are usually located 100-200 bp upstream of the transcriptional start site of the target gene. In the case of *S. enterica* serovar Typhimurium *glnA* the enhancer functions efficiently when placed more than 2 kb from the promoter (148). Although enhancers are usually located upstream of the promoter, a functional enhancer for *B. subtilis rocG* is located > 2 kb downstream of the promoter, a feature common of many eukaryotic enhancer sequences (14). Enhancers contain two or more binding

Figure 8. Mechanism of transcriptional activation with σ^{54}-holoenzyme. Activators of σ^{54}-holoenzyme (in yellow) bind to enhancers (in blue and white) upstream of the promoter DNA. The activators contact the σ^{54}-holoenzyme (in red) bound to the promoter through DNA looping and catalyzes the isomerization of the closed complex into a transcriptionally competent open complex which requires the hydrolysis of ATP.


E σ54
Closed complex
E σ54
ATP
ADP+Pi
Open complex
+1

sites for an activator. For *S. enterica* serovar Typhimurium *glnA*, 3 or 4 dimers of nitrogen regulatory protein C (NtrC) were reported to bind to the *glnA* enhancer as observed by scanning electron microscopy (149). Two dimers appeared to bind directly to the DNA, while the remaining dimers contacted the enhancer bound dimers through protein-protein interactions. The *glnA* enhancer appears to stimulate oligomerization of NtrC which is required for activity of the protein. NtrC mutants that fail to recognize the enhancer, however, can still activate transcription if present at high concentrations (124). Morever, *Sinorhizobium meliloti* dicarboxylic acid transport protein D, (DctD) and the *S. meliloti* nitrogen fixation protein A, (NifA) are capable of activating transcription when their C-terminal DNA binding domains are deleted (75, 76, 182). These mutant activators apparently oligomerize in solution and contact the closed promoter complex directly to activate transcription.

Enhancer-bound activator can contact the closed complex through random and transient conformational changes in the DNA (117, 139). Alternatively, auxillary proteins such as IHF stabilize the DNA loops formed from these transient conformational changes in the DNA (74, 153). Binding of IHF to a specific site located between the promoter and enhancer induces a sharp bend in the DNA which facilitates interaction between σ^{54} -holoenzyme and the enhancer-bound activator protein (74).

Enhancer-bound activator proteins must hydrolyze ATP or other nucleoside triphosphates to provide energy for open complex formation (184). Evidence that hydrolysis of the nucleotide is required for open complex formation comes from the observation that non-hydrolyzable forms of ATP, such as ATPγS, can bind to the activator but cannot replace ATP in these reactions. Morever, mutant activator proteins that fail to hydrolyze ATP are unable to activate transcription (61, 185). DctD and NtrC can hydrolyze ATP or GTP, but not CTP or UTP, to activate

transcription (94, 185). Rates of hydrolysis of nucleoside triphosphates are quite low with the highest reported turnover rates being 2-3 molecules of ATP hydrolyzed per second per dimer (94).

Structure-Function Relationships in σ^{54}-Dependent Activators

Activators of σ^{54}-holoenzyme are modular in structure and generally possess three functional domains which can fold correctly and function relatively independently of each other (118, 123). The N-terminal domain is involved in signal recognition and transcriptional regulation and is linked to the central domain through a flexible protease-sensitive linker. The central domain is required for ATP hydrolysis and transcriptional activation and the C-terminal domain is involved in binding to the enhancer element (129).

The N-terminal domain is the least conserved of all the three domains. In most cases, it is responsible for exerting a negative effect on the activity of the central domain. Removal of the N-terminal domain of DctD and *Pseudomonas putida* XylR produces a constitutively active protein (94, 135). Similarly, removal of the regulatory C-terminal domain of *B. subtilis* LevR produces a constitutive protein (104). In contrast, removal of N-terminal domain of NtrC does not result in a constitutive form of the protein (42). Some proteins such as *E.coli* protein PspF, lack the N-terminal regulatory domain and are constitutively active (85).

The central domain of σ^{54}-dependent activators is approximately 240 amino acid residues in length and is responsible for ATP hydrolysis and transcriptional activation. Homology between σ^{54} activators is highest in the central domain. In the absence of other domains, the central domains of DctD and NifA can activate transcription if present at high, non-physiological levels (75, 76). The central domain belongs to the AAA+ (ATPases Associated with diverse

cellular Activities) superfamily of ATPases (120). Members of AAA+ superfamily are found in all the three Domain of life and participate in diverse cellular processes such as membrane fusion, proteolysis, DNA replication and transcription and include the regulatory components of proteases like Lon and Clp, proteins involved in DNA replication, recombination, restriction (replication factor C proteins, bacterial DnaA, RuvB, McrB proteins, subunits of origin replication complex), *Bacillus* sporulation protein SpoVJ, metal chelatases, dynein motor proteins and prokaryotic σ^{54} -dependent transcriptional activators to name a few (120). The AAA family constitutes a subfamily of the Walker-type NTPases which is defined by conserved Walker A and B motifs that are responsible for NTP binding and hydrolysis. Our understanding of this region is based on the recent data obtained from the crystal structure of the σ^{54} -dependent activator NtrC1 from *A. aeolicus*, which like other AAA+ proteins forms a ring structure with a large central pore (194).

The central domain can be subdivided into seven well-conserved regions designated C1-C7. Subregion C1 contains a glycine-rich motif known as P-loop or Walker-type A sequence, GE(S/T)G(T/S/V)GK(E/D), found commonly in purine nucleotide-binding protein (154). In NtrC and XylR, substitutions of glycine at position 6 inhibits ATP binding and transcriptional activation (125, 134). Alanine scanning mutagenesis of the P-loop in DctD revealed that substitutions at positions 3, 4, 6, 7 and 8 also disrupted ATP hydrolysis and transcriptional activation (61). Subregion C4 contains a motif resembling a hydrophobic Walker-type B sequence, also common in purine nucleotide-binding proteins. This motif contains a conserved aspartate residue presumed to bind the Mg^{2+} in MgATP (180).

The C3 region is located between the Walker-type A and B motifs. The N-terminal part of this region forms an α-helix while the C-terminal part forms a predicted loop structure (129).

The consensus sequence ESELFGxxxGAFTGA within the C3 subregion is characteristic of σ^{54}-dependent activators. The ESELFG motif is located in the helix 8 of the N-terminal region in *A. aeolicus*. Mutations in this region inhibit transcriptional activation with little effect on ATPase activity implying the role of C3 subregion in contacting σ^{54}-holoenzyme (96, 125, 181, 185). The ESELFG motif is located near the edge of the central pore of the heptameric NtrC1. Since Region I of σ^{54} binds the central pore of the activator ring complex, it can effectively bind to the ESELFG motif. Similarly, mutant forms of DctD having single amino acid substitutions in ESELFG motif have reduced affinities for σ^{54} in chemical cross-linking assays suggesting a role for this motif in contacting σ^{54} (182). The C-terminal motif GAFTGA in the C3 region forms a loop that extends into the central pore of the NtrC1 ring structure (181, 182, 194). Substitutions in this motif of DctD, often, do not affect the ATPase activity of the protein but interfere with transcriptional activation (175, 176). Based on the NtrC1 structure, the ESELFG motif of DctD, is predicted to be a part of the helix which is involved in orienting and stabilizing the GAFTGA loop (194). Certain substitutions in this motif result in mutant proteins which have increased dependence on the upstream enhancer sequences (194). Other residues implicated in ATP binding and hydrolysis are the "Sensor II" region in the C7 subregion and an arginine residue in C6 subregion respectively (150).

The C-terminal domains of σ^{54} activators contain a putative helix-turn-helix (HTH) motif for DNA binding. An exception is LevR of *B. subtilis* which has an N-terminal DNA binding domain (118). Mutations within this motif abolish DNA-binding function of the activator indicating it recognizes the enhancer (30). The first helix and turn are conserved, whereas, the second helix is variable since it recognizes different enhancer sequences. In contrast to other activators which have their dimerization determinants in the central domain, NtrC contains its

dimerization determinant in the C-terminal DNA binding domain and this dimerization determinant share homology with *E. coli* factor for inversion stimulation (FIS) protein (123).

Regulation of Activators of σ^{54} -holoenzyme

Analysis of genome sequences have identified σ^{54} -dependent activators from a diverse group of bacteria such as those belonging to Proteobacteria, *Spirochaetes*, *Chlamydia, Planctomyces*, Hydrogenobacteria, Gram-positive bacteria and the Green sulphur bacteria (171). Detailed studies have been done with few activators such as *S. meliloti* DctD, NtrC from enteric bacteria, *K. pneumoniae* NifA, *E.coli* PspF and *P. putida* XylR. Although their mechanism of transcriptional activation is common, the mode of regulation of these activator proteins can vary. Table 5 summarizes the different classes of σ^{54} -dependent activators and their mode of regulation.

Activators such as NtrC, DctD and *C. crescentus* FlbD are members of the two-component regulatory system (92, 146). This system is made up of a sensor histidine kinase which senses environmental stimuli and alters the expression of target genes through its cognate response regulator. The sensor kinase transduces the signal by affecting the phosphorylation state of an aspartate residue within the response domain of the regulator (132, 170). The sensor kinase undergoes autophosphorylation in response to the signal, but the subsequent transfer of the phosphate to the regulator protein is catalyzed by the response regulator (132). Many of these response regulators can autophosphorylate in the presence of small phospho donors such as acetyl-phosphate or carbamyl-phosphate (193). Phosphorylation of the regulator stimulates oligomerization of the protein which activates the ATPase activity of the protein required for open complex formation. Some sensor kinases exhibit a regulated phosphatase activity which

stimulates the removal of phosphate from the phosphorylated regulator protein (170). In the case of *S. enterica* serovar Typhimurium NtrB, the phosphatase activity of this sensor kinase requires the PII protein which is responsible for sensing the levels of nitrogen in the cell (122). Although sensor kinases and response regulators are generally separate polypeptides, a notable exception is found in *Xanthomonas campestris* RpfC, wherein, the response regulator is joined to the C-terminus of the sensor kinase (173).

A second class of σ^{54}-dependent activators include XylR, FhlA, and DmpR which are stimulated by binding small effector molecules (135, 159). XylR and DmpR regulate the expression of genes involved in aromatic compounds degradation in Pseudomonads and aromatic compounds serve as effectors for those proteins (79). *E.coli* FhlA binds formate as an effector and activates genes involved in formate and hydrogen metabolism (106).

B. subtilis LevR is both positively and negatively regulated by phosphorylation, by the phosphotransferase system (PTS). PTS is involved in the phosphorylation of specific sugars for their transport across the cell membrane (89). LevR activates transcription of the *lev* operon that includes *levDEFG,* the products of which encode a PTS EII complex for the phosphorylation and transport of fructose (37). The EII complex positively and negatively regulates LevR, phosphorylation of His-585 of LevR in the presence of fructose stimulates its activity. In the absence of fructose, LevR is phosphorylated at His-869 by LevE which inactivates the protein (105).

In *S. meliloti*, *Rhizobium trifoli* and *B. japonicum*, NifA is under redox control (15, 91, 157). A conserved cysteine motif located between the central and C-terminal domains of NifA from these bacteria is thought to bind a metal cluster that is involved in oxygen or redox sensing.

Table 5. Classes of σ^{54}-dependent Activators and their Mode of Regulation

Mode of regulation	Activator	Organism	Physiological signal	Function of target gene
Two-component system	NtrC	Enteric bacteria	Nitrogen limitation	Nitrogen assimilation
	DctD	*S. meliloti*	C4-dicarboxylic acid	C4-dicarboxylic acid transport
	FlbD	*C. crescentus*	Cell cycle	Flagellum biosynthesis
Inducer binding	XylR	*P. putida*	Xylene	Xylene metabolism
	DmpR	*P. putida*	Phenol	Phenol catabolism
	FhlA	*E. coli*	Formate	Hydrogen metabolism
Phosphorylation	LevR	*B. subtilis*	Fructose	Fructose utilization
Redox control	NifA	*S. meliloti* *B. japonicum*	Oxygen	Nitrogen fixation
Protein:protein interaction	NifA	*K. pneumoniae* *A. vinelandii*	Oxygen, nitrogen status	Nitrogen fixation
Transcriptional control	PspF	*E. coli*	Stress	Stabilize cells under stress

Removal of metal ions from the growth media inactivates the NifA protein indicating that the redox state of metal clusters influences the activity of the protein in these bacteria (39).

In *K. pneumoniae* and *Azotobacter vinelandii*, the activity of NifA is negatively regulated by NifL in response to fixed nitrogen and molecular oxygen (71, 158). The NifA proteins from these bacteria lack the above mentioned conserved cysteine residues found in *S. meliloti* NifA. NifL contains a FAD-binding site and the oxidation state of FAD regulates NifA activity. In the presence of oxygen, FAD is oxidized and NifL inhibits the activity of NifA. Under nitrogen-limiting conditions, GlnK, a paralog of the PII protein relieves NifA inhibition by NifL (151).

PspF from *E. coli* represents yet another class of σ^{54}-dependent activators which possesses only the AAA+ domain and the DNA-binding domain. PspF is translated as a constitutively active protein, whose activity is negatively regulated by the PspA protein, the expression of which is negatively regulated by PspF. PspA binds directly to AAA+ domain of PspF and inhibits its ATPase activity (49). Other σ^{54}-dependent activators lacking the N-terminal regulatory domain are the HrpR/HrpS proteins from *Pseudomonas. syringae*, which like PspF are also negatively regulated by a protein (HrpV) whose expression they regulate (141).

H. pylori flagellar regulatory protein FlgR

Analysis of the *H. pylori* genome indicated presence of σ^{54} and a single activator protein of σ^{54}. Spohn and colleagues (168) identified the function of the σ^{54}-dependent activator in transcription of several flagellar genes and hence named it as FlgR for flagellar regulatory protein. *flgR* is part of a constitutively expressed operon transcribed by σ^{70}-RNA polymerase holoenzyme that includes *orf0698-orf0699-dgkA-gyrA-orf0702-flgR* (168). *dgkA* encodes a diacyl glycerolkinase, while *gyrA* encodes the subunit A of DNA gyrase. No function has been

assigned to the products of *orf0698*, *orf0699* and *orf0702*. *H. pylori* FlgR is part of a two-component regulatory system where the product of *hp0244 (flgS)* encodes a sensor histidine kinase that phosophorylates FlgR. *flgS* appears to be part of a operon that includes *flgI* (encodes the P-ring of the flagellar basal body) and *hp0245*. The product of *hp0245* is a small protein (12.1 kDa) with an acidic pI of 5.22 and is predominantly α-helical in structure, all of which are characteristic of TTS chaperones.

Transcriptional start sites have been mapped for five σ^{54}-dependent flagellar operons, *flaB, flgE, flgDE', flgBC* and *orf1120-flgK* (168). FlgR has been shown to be required for the transcription of these genes. Using whole genome microarray analysis, Niehus and co-workers identified seven additional genes which appear to be part of the RpoN regulon. These include flagellar associated genes *flgL*, *flgJ*, *hp1076, hp1233, hp1145, hp1155, hp0366, hp0367* , *hp0869* (encodes HypA, a nickel-binding protein involved in hydrogenase and urease maturation) and *murG* which encodes a glycosyltransferase active in peptidoglycan biosynthesis (121).

Comparison of the deduced amino acid sequences of FlgR with other known σ^{54}-dependent activators revealed that FlgR lacks a C-terminal DNA-binding domain. Activators from other bacteria such as *Chlamydia trachomatis* and other chlamydial species also lack a C-terminal DNA-binding domain. Part of my work was to determine if FlgR activates transcription without binding DNA or if it needs to bind DNA, perhaps through another protein, to activate transcription, the results of which are discussed in Chapter II.

Thesis Summary

Colonization of the gastric mucosa by *H. pylori* has been associated with the development of peptic ulcers, which can progress to more serious gastric diseases. *H. pylori* has

2-6 polar flagella which are required for host colonization. Regulation of flagellar genes in *H. pylori* is complex and involves all three σ factors found in this bacterium. My work focused on the σ^{54}-dependent transcriptional activator FlgR which activates transcription of several flagellar genes. Activators of σ^{54}-holoenzyme generally bind to enhancer sequences located upstream of the promoter and contact holoenzyme bound at the promoter to stimulate transcription initiation. FlgR lacks an apparent DNA-binding domain, and part of my work was to determine if FlgR activates transcription without binding DNA. Using reporter gene constructs in *H. pylori* and *in vitro* transcription assays, I demonstrated that FlgR does not bind an enhancer to activate transcription. The findings suggest that FlgR binds to σ^{54}-holoenzyme directly, either before or after it has engaged the promoter, to activate transcription. Like other σ^{54} -dependent activators, FlgR activates transcription by coupling ATP hydrolysis to open complex formation which is capable of initiating transcription. Consistent with this, I found that a truncated constitutive active form of FlgR hydrolyzes ATP and activates transcription with σ^{54} -holoenzyme *in vitro*. I also estimated the levels of FlgR in *H. pylori* by western blotting and found these to be slightly higher than those reported for other σ^{54} -dependent activators that have a C-terminal DNA binding domain. A slightly higher concentration of FlgR may be necessary for efficient transcriptional activation in *H. pylori*, since unlike other σ^{54} -dependent activators, the local concentration of FlgR at its target promoters cannot be increased by tethering of the protein to an enhancer or UAS.

The protein-protein interaction map of *H. pylori* showed that FlgS interacted specifically with HP0137, a conserved hypothetical protein of 211 amino acids with a predicted membrane spanning helix. HP0137 shares homology with other proteins, including those from *Campylobacter jejuni* and *Neisseria meningitidis*. The C-terminal region of HP0137 belongs to

the DUF162 Pfam protein family and the functions of these proteins are unknown. My purpose was to determine if HP0137 played a role in flagellar biogenesis in *H. pylori*, and in particular, if it influenced FlgS activity in *H. pylori.* I constructed a Δ *hp0137* deletion mutant and analyzed the phenotype of the resulting mutant. The Δ *hp0137* mutant was non-motile and when examined by electron microscopy was found to lack flagella. Expression of flagellar genes in the deletion mutant was assessed with *xylE* reporter gene constructs fused to the promoter region of various flagellar genes. I also performed western blot analysis to detect the presence of the minor flagellin FlaB, the expression of which is dependent on σ^{54}. Loss of HP0137 did not affect the transcription of the xylE reporter genes nor did it prevent accumulation of FlaB. These data suggest that HP0137 is required for flagellar assembly, but not flagellar gene expression in *H. pylori,* details of which are discussed in Chapter III.

A final goal of my research was to examine acetone metabolism in *H. pylori* and determine its importance in colonization. Acetone is a ketone body produced by the spontaneous decarboxylation of acetoacetate, which is significantly accelerated at low pH suggesting that the stomach may be a logical niche in which acetone concentrations are significant. Acetone carboxylase is a multimeric protein ($\alpha_2\beta_2\gamma_2$) that catalyzes the ATP-dependent carboxylation of acetone to acetoacetate. Analysis of the database revealed that *H. pylori* has ORFs that share homology with the acetone carboxylase (*acx*) genes.

Inactivation of the *H. pylori acxB* interfered with the ability of the bacterium to colonize the mouse stomach. The *acxB* mutant strain was also defective in its growth, having a slower generation time and reaching a lower cell density. The *acxB* mutant strain was also more sensitive to acetaldehyde than the wild-type strain. Acetaldehyde is structurally very similar to acetone and the *H. pylori* acetone carboxylase may recognize it as a substrate and detoxify it.

Acetaldehyde could be generated in *H. pylori* from ethanol by alcohol dehydrogenase, which could account for the poorer growth of the *acxB* mutant strain. My studies on acetone metabolism in *H. pylori* are discussed in chapter IV.

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CHAPTER II

HELICOBACTER PYLORI FLGR IS AN ENHANCER-INDEPENDENT ACTIVATOR OF σ^{54}-RNA POLYMERASE HOLOENZYME[2]

[2] Brahmachary, P., M. G. Dashti, J. W. Olson and T. R. Hoover. 2004. J. Bacteriol. 186 : 4535-4542. Reprinted here with permission of publisher.

Abstract

Helicobacter pylori FlgR activates transcription with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) from at least five flagellar operons. Activators of σ^{54}-holoenzyme generally bind enhancer sequences located >70 bp upstream of the promoter and contact σ^{54}-holoenzyme bound at the promoter through DNA looping to activate transcription. *H. pylori* FlgR lacks the carboxy-terminal DNA-binding domain present in most σ^{54}-dependent activators. As little as 42 bp of DNA upstream of the *flaB* promoter and 26 bp of DNA sequence downstream of the transcriptional start site were sufficient for FlgR-mediated expression from a *flaB'-'xylE* reporter gene in *H. pylori*, indicating that FlgR does not use an enhancer to activate transcription. Other examples of σ^{54}-dependent activators that lack a DNA-binding domain include *Chlamydia trachomatis* CtcC and activators from the other Chlamydia whose genomes have been sequenced. FlgR from *Helicobacter hepaticus* and *Campylobacter jejuni*, which are closely related to *H. pylori*, appear to have carboxy-terminal DNA-binding domains, suggesting that the loss of the DNA-binding domain from *H. pylori* FlgR occurred after the divergence of these bacterial species. Removal of the amino-terminal regulatory domain of FlgR resulted in a constitutively active form of the protein that activated transcription from σ^{54}-dependent genes in *Escherichia coli*. The truncated FlgR protein also activated transcription with *E. coli* σ^{54}-holoenzyme in an *in vitro* transcription assay.

Introduction

Helicobacter pylori is a microaerophilic, motile bacterium that is the etiological agent of chronic gastritis in humans (10, 11). Colonization of the gastric mucosa by *H. pylori* is associated with development of peptic ulcers, gastric non-Hodgkin's lymphomas and gastric mucosa-associated lymphoid tissue lymphoma (5). Motility in *H. pylori* is achieved through 2-6 polar flagella and is essential for colonization in gnotobiotic piglets (12, 13).

The *H. pylori* genome contains about 40 known flagellar genes scattered throughout the genome which are organized into 25 or more transcriptional units (1, 58). Where the regulation of flagellar biogenesis has been studied in other bacteria, flagellar gene expression is under control of a regulatory hierarchy in which genes encoding the basal body and protein export apparatus are expressed first, followed by the genes encoding components of the hook, and then finally the genes encoding filament proteins (31, 62).

Flagellar gene regulation in *H. pylori* is complex, involving all three sigma factors found in the bacterium. Genes encoding flagellar components required early in flagellar biogenesis are transcribed by σ^{80}-RNA polymerase holoenzyme, the primary form of RNA polymerase holoenzyme in *H. pylori*, and are equivalent to the class II flagellar genes in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (31). Class III flagellar genes in *H. pylori* are transcribed by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) and encode basal body rod proteins (*flgBC*), the hook protein (*flgE*), and a minor flagellin (*flaB*) (54, 56). Expression of the σ^{54}-dependent flagellar genes requires a two-component system consisting of the sensor kinase HP0244 (3), which we refer to as FlgS, and the response regulator FlgR (54). The gene encoding *flgS* appears to be within a class II operon (1, 58), which may provide a mechanism for controlling the hierarchical expression of the class III operons. The class IV flagellar genes in *H.*

pylori, which include the major flagellin gene *flaA,* are transcribed by σ^{28}-RNA polymerase holoenzyme (22, 29). Temporal control of class IV genes appears to be coordinated through the regulation of both the expression and activity of σ^{28}. The gene encoding σ^{28}, *fliA*, is part of a class II operon (1, 58), and the activity of σ^{28} is negatively regulated through interactions with the anti-σ^{28} factor FlgM (9, 19).

FlgR activates transcription with σ^{54}-holoenzyme and belongs to a large family of activators that are widespread in bacteria and are involved in regulation of diverse functions including nitrogen fixation, C_4-dicarboxylic acid transport, degradation of aromatic compounds, hydrogen metabolism, flagellar biogenesis and pilin formation (24, 64). To activate transcription, activators of σ^{54}-holoenzyme typically bind to enhancer-like sequences located upstream of the promoter and contact σ^{54} -holoenzyme bound at the promoter in a closed complex through DNA looping (7, 50, 51, 55). Productive interactions between the activators and σ^{54}-holonenzyme lead to conversion of the closed promoter complex to an open complex in a reaction that is coupled to ATP hydrolysis by the activator (38, 47, 52, 61).

Activators of σ^{54}-holoenzyme are modular in structure, generally consisting of an amino-terminal regulatory domain, a central domain responsible for transcriptional activation and ATP hydrolysis, and a carboxy-terminal DNA binding domain (41, 64). The central activation domain belongs to the AAA+ superfamily of ATPases (ATPases associated with various cellular activities), the members of which are involved in diverse functions including transcription, DNA replication, protein folding and unfolding, proteolysis, and membrane fusion (39, 43, 65).

FlgR is unusual in that it lacks the DNA-binding domain found in other activators (Fig. 1), consisting of only an amino-terminal response regulator domain and an AAA+ domain. *Chlamydia trachomatis* CtcC was reported recently to lack the carboxy-terminal DNA-binding

domain (23), indicating that FlgR is not unique in its unusual structural arrangement. Previous study on CtcC, however, did not address the issue of whether an enhancer is required for efficient CtcC-mediated transcriptional activation in *C. trachomatis*.

To address the possibility that an enhancer-binding activity needed for FlgR function resides on a separate polypeptide, *flaB'-'xylE* reporter genes that carried varying amounts of DNA sequence upstream of the promoter were constructed and FlgR-mediated transcriptional activation was monitored from these reporters in *H. pylori*. As little as 42 bp of sequence upstream of the *flaB* promoter and 26 bp of sequence downstream of the transcriptional start site were needed for efficient expression in *H. pylori*, indicating that FlgR does not use an enhancer to activate transcription. The levels of FlgR in *H. pylori* were estimated by western blotting and found to be somewhat higher than those reported for other σ^{54}-dependent activators that have a carboxy-terminal DNA-binding domain. The AAA+ domain of FlgR was expressed in *E. coli* and purified. This truncated FlgR protein was constitutively active and was able to function with *E. coli* σ^{54}-holoenzyme both *in vivo* and *in vitro*.

Materials and Methods

Bacterial strains and media. *E. coli* strain DH5α [Φ80d *lacZ* ΔM15 *recA1 gyrA96 thi-1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 deoR* Δ(*lacZYA-arg F*) U169] was used for cloning and was cultured in Luria-Bertani (LB) medium at 37˚C. *H. pylori* strains ATCC 43504 and 26695 were grown on tryptic soy agar (TSA) supplemented with 5% horse serum at 37˚C under microaerobic conditions which consisted of an atmosphere of 4% O_2, 5% CO_2, and 91% N_2. When included in the medium, antibiotics were used at the following concentrations: 100 μg/ml ampicillin, 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 primers were from Integrated DNA Technologies. PCR amplifications were done with either *Taq* DNA polymerase (Promega) or *Pfu* DNA polymerase (Stratagene). DNA was amplified in 30 cycles using the following temperature regimen: 94˚C for 2 min, 49˚C for 1.5 min and 72˚C for 3 min. PCR products were cloned into the cloning vector pGEM-T (Promega) and sequencing of the cloned PCR products was performed at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Transformation of *H. pylori*. Electrocompetent *H. pylori* cells were prepared and transformed as described previously (53). When transforming cells with derivatives of the shuttle vector pHel3, plasmid DNA was methylated using S-adenosylmethionine and *H. pylori* cell extract essentially as described previously to improve the transformation efficiency (44). Briefly, 25 μg of plasmid DNA was treated with *H. pylori* cell extract (3 mg/ml) in a 200 μl reaction containing 20 mM Tris-HCl, 50 mM KCl pH 7.9, 5 mM EDTA and 2 μM S-adenosylmethionine (Sigma). Samples were incubated at 37˚C for 30 min, after which time the DNA was purified using Qiagen gel extraction columns. Treated plasmids were introduced into *H. pylori* by electroporation and transformants were selected on TSA containing kanamycin.

Construction of *H. pylori* mutant strains with insertions in *flgR* or *flgS*. Plasmid pGHPAY91 carries the *flgR* gene from *H. pylori* strain 26695 and was obtained from the American Type Culture Collection. A 1.4 kb *Eco*RI fragment from plasmid pHP1 (34) that carries the *Campylobacter coli aphA3* cassette was cloned into a unique *Stu*I site within *flgR* in plasmid pGHPAY91 to create a suicide vector that was introduced into *H. pylori* strain ATCC 43504 by electroporation. Transformants were selected on TSA containing kanamycin and genomic DNA

from some of these colonies was analyzed by PCR to confirm that the chromosomal copy of *flgR* had been inactivated with the *aphA3* cassette. One of these strains was saved and named MGD1. An insertion mutant in *H. pylori flgR* was similarly generated using a cassette bearing a chloramphenicol transacetylase (*cat*) gene from *C. coli*. The entire *flgR* from *H. pylori* strain 26695 was amplified by PCR and cloned in pGEM-T. A 1.3 kb *Eco*RI fragment from plasmid pSKAT4 (59) that carried the *C. coli cat* cassette was introduced into a unique *Eco*47III site within *flgR* in this plasmid, which then was transformed into *H. pylori* strain ATCC 43504 by electroporation. Transformants were selected on TSA containing chloramphenicol and inactivation of the chromosomal copy of *flgR* by the *cat* cassette in these strains was confirmed by PCR. One *flgR:cat* mutant strain was saved and designated as HP31.

The entire *flgS* was amplified from *H. pylori* strain ATCC 43504 by PCR and cloned into pGEM-T. The 1.4 kb *Eco*RI fragment carrying the *C. coli aphA3* cassette was inserted into a unique *Hin*dIII site with *flgS* in this plasmid to create plasmid pMD5. Plasmid pMD5 was introduced into *H. pylori* strain ATCC 43504 by electroporation and transformants were selected on TSA containing kanamycin. Genomic DNA from kanamycin-resistant colonies was analyzed by PCR to confirm that the chromosomal copy of *flgS* had been inactivated with the *aphA3* cassette and one *flgS*:*aphA3* mutant strain was saved and designated as MGD2. The 1.3 kb *Eco*RI fragment bearing the *C. coli cat* cassette was also inserted into the *Hin*dIII site within *flgS* in pMD5. This plasmid was introduced into *H. pylori* strain ATCC 43504 by electroporation and transformants were selected on TSA containing chloramphenicol. Genomic DNA from chloramphenicol-resistant colonies was analyzed by PCR to confirm that the chromosomal copy of *flgS* had been inactivated with the *cat* cassette and one of the *flgS:cat* strains was saved and named strain HP22.

Construction of *xylE* reporter genes. For construction of all of the reporter genes described below, genomic DNA from *H. pylori* strain 26695 was used as a template. Cloned PCR products were sequenced to verify that no mutations had been introduced during DNA amplification. Reporter genes were constructed using a promoterless *Pseudomonas putida* catechol 2,3 dioxygenase (*xylE*) reporter (44). A region of DNA that corresponded to positions –67 to + 26 relative to the transcriptional start site of *H. pylori flaB*, which was determined previously (54), was amplified by PCR and cloned upstream of the promoterless *xylE* reporter gene. The resulting *flaB'-'xylE* reporter gene (designated *flaB1'-'xylE*) was moved into the shuttle vector pHel3 (16) to create plasmid pPBHP21, which was introduced into *H. pylori* strains by electroporation. Similarly, a region of DNA corresponding to positions –393 to + 26 relative to the transcriptional start site of *flaB* was amplified and cloned upstream of *xylE* to create the *flaB2'-'xylE* reporter gene, which was moved into the shuttle vector pHel3 to create plasmid pPBHP22.

For construction of the *flaA'-'xylE* reporter gene, DNA corresponding to positions -126 to + 47 relative to the transcriptional start site of *flaA* (37) was amplified by PCR and then introduced upstream of *xylE* . The resulting *flaA'-'xylE* reporter gene was cloned into pHel3 to create plasmid pPBHP24. For construction of the *flgI'-'xylE* reporter gene, a DNA fragment corresponding to positions –225 to +24 relative to the translational start site of *flgI* was amplified and cloned upstream of *xylE*. The resulting *flgI'-'xylE* reporter gene was moved into the shuttle vector pHel3 to create plasmid pPBHP23.

Plasmid constructions for expression of FlgR proteins. To produce a full-length histidine-tagged version of FlgR, *flgR* was amplified from pGHPAY91 and cloned into the expression vector pTrcHis-C (Invitrogen). The resulting plasmid pMD20 introduced a sequence coding for a

histidine-tag to the 5'-end of *flgR*. To express a maltose-binding protein-FlgR fusion protein (MBP-FlgR), a DNA fragment bearing *flgR* was moved from pMD20 into a derivative of pMAL-c (New England Biolabs) resulting in plasmid pPBHP12. A plasmid that expressed the FlgR AAA+ domain (residues His-131 to Arg-381) with a histidine-tag at the amino-terminus was constructed by amplifying a 750-bp DNA fragment corresponding to this region of *flgR* using *H. pylori* 26695 genomic DNA as a template and cloning the PCR product into the expression vector pTrcHis-C to create plasmid pPBHP80. Plasmid pHX182, which expresses the *Sinorhizobium meliloti* DctD AAA+ domain (residues Leu-141 to Ser-390) linked to a histidine-tag at the amino-terminus, is a derivative of pTrcHis-C and was provided by Hao Xu (63).

Measurement of XylE activity. XylE activities were measured in whole cells as described (44). *H. pylori* strains containing the *xylE* reporter plasmids were grown on TSA supplemented with kanamycin for 48 h then resuspended in 50 mM phosphate buffer, pH 7.4, to a cell density of 1 OD_{600} unit, which corresponded to $1x10^9$cfu/ml. Reactions were initiated by adding cells (50-100 μl) to reaction mixtures containing 10 mM catechol in 50 mM potassium phosphate, pH 7.4. Catechol oxidation to 2-hydroxymuconic semialdehyde was monitored continuously at 375 nm with a Beckman DU 640B recording spectrophotometer at room temperature. A unit of XylE activity corresponds to 1 μmole catechol oxidized/min and values were expressed as units/min/10^8 cells.

Purification of FlgR proteins. Cultures of E. coli DH5α bearing plasmid pPBHP12 were grown in LB at 37˚C to an OD_{650} of 0.5, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 mM to induce the expression of MBP-FlgR. After an additional 3-4 h incubation, cells were harvested by centrifugation and the resulting cell pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 5% (v/v)

glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA, 200 mM KCl and 0.5 mM phenylmethyl sulphonyl fluoride (PMSF) (Buffer C). Cells were lysed in a French pressure cell at 9000 p.s.i. and the resulting cell extract was clarified by centrifugation at 1000 x g for 50 min. The supernatant was loaded onto an amylose agarose (New England Biolabs) affinity column and MBP-FlgR was eluted with Buffer C plus 10 mM maltose. Protein fractions were pooled and dialyzed against 20 mM HEPES, pH 7.4, 5% (v/v) glycerol, 1 mM DTT, 100 mM potassium thiocyanate. Protein concentrations were determined by bichinchonic acid protein assay (Pierce) using bovine serum albumin as a standard.

The histidine-tagged FlgR AAA+ domain was expressed in E. coli DH5α from plasmid pPBHP80. Cells were grown at 37˚C to an OD_{650} of 0.8 at which point IPTG was added to the medium to a final concentration of 1 mM. Cultures were incubated for an additional 3-4 h. Cells were harvested by centrifugation, resuspended in 50 mM Tris- acetate, pH 8.2, 200 mM KCl, 1 mM EDTA and 0.5 mM PMSF, and lysed in a French pressure cell at 9000 p.s.i. The cell extract was clarified by centrifugation at 1000 x g for 50 min and the resulting supernatent was loaded onto a nickel-nitrilotriacetic acid resin column (Qiagen). Histidine-tagged FlgR AAA+ domain was eluted in a buffer containing 300 mM NaCl, 50 mM sodium phosphate, pH 7.8, 250 mM imidazole, and 5% (v/v) glycerol. Fractions containing FlgR AAA+ domain were pooled and dialyzed against 20 mM Tris-HCl, pH 8.8, 5% (v/v) glycerol, 100 mM potassium thiocyanate and 0.5 mM DTT (buffer A). The protein was then applied to a 5 ml HiTrapQ anion exchange column (Pharmacia) and eluted in a linear gradient to 1 mM KCl in Buffer A.

Assaying activity of FlgR proteins in *E. coli*. Plasmids pMD20, pPBHP80 and pHX182 were introduced into an *E. coli* DH5α strain that contained plasmid pRKMAZ: +UAS, which bears a *S. meliloti dctA'-'lacZ* reporter gene (25). Cultures were grown in LB at 37˚C to an OD_{650} of

0.8, at which time IPTG was added to a final concentration of 1 mM where indicated. Cultures were incubated an addition 7 h, at which point six independent sets of whole cell β-galactosidase assays were done in duplicate as described previously (2), with activities expressed as Miller units (36).

Western blot analysis. *H. pylori* cells were lysed in SDS loading buffer then 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 either full-length histidine-tagged FlgR (this study) or the *H. pylori* flagellum (kindly provided by Dr. Paul O'Toole, Massey University). 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).

***In vitro* transcription assay.** Single round transcription assays were performed as described previously (21). Plasmid pJES534 (48) yields a ~155 nucleotide uracil-less transcript from the *S. enterica* serovar *typhimurium glnA* promoter and was used as a DNA template. Reaction mixtures contained 0.1 μM to 10 μM FlgR AAA+ domain protein, 1 unit *E.coli* RNA polymerase (Epicentre), 200 nM *S. enterica* serovar Typhimurium σ^{54} , 10 nM plasmid DNA, 4 mM ATP, 400 μM GTP, 5 μM CTP and 7.5 μCi [^{32}P-α]-CTP (3000 Ci/mmole; Amersham). Proteins were incubated with the DNA template at 37˚C for 10 min after which time ATP was added to stimulate open complex formation. After 10 min, the remaining nucleotides were added to allow synthesis of the transcripts along with 0.1 mg/ml heparin to prevent further open complex formation. Reactions were stopped after 10 min and transcripts were visualized on a denaturing polyacrylamide gel followed by exposure to x-ray film.

Glutamine synthetase assays. Glutamine synthetase activities were determined by the γ-glutamyltransferase assay as described previously (4). Cultures of DH5α that carried plasmids pPBHP80 or pHX182 were grown in LB medium to mid-log phase and harvested by centrifugation. Cells were permeabilized by including hexadecyltrimethylammonium bromide in the assay buffer as described (4). Glutamine synthetase units were expressed as nanomoles of γ-glutamyl hydroxymate produced per minute and normalized to a cell density of 1 OD_{650} unit. All assays were done at least six times.

Results

FlgR and FlgS are required for motility and expression of σ^{54}-dependent flagellar genes. FlgR is an activator of σ^{54}-holoenzyme that was shown previously to be required for motility and transcription of five σ^{54}-dependent flagellar operons (*flaB*, *flgE*, *orf0906-flgDE'*, *flgBC*, and *orf1120-flgK*) in *H. pylori* strain G27 (54). The gene encoding FlgR is at the 3'-end of a putative operon that includes genes encoding diacylglycerol kinase (*dgkA*), the α subunit of DNA gyrase (*gyrA*), and three hypothetical proteins of unknown function (1, 58). Similar to the previous observations with *H. pylori* strain G27, inactivation of *flgR* in *H. pylori* strain ATCC 43504 with either an *aphA3* or *cat* cassette resulted in loss of motility and expression of FlgE and FlaB (Fig. 2; data only shown for the *flgR:aphA3* mutant strain).

The *flgR* mutant strain expressed the major flagellin, FlaA, which is dependent on σ^{28} for its expression (29). This observation was consistent with the previous report for *H. pylori* strain G27 that *flgR* was not required for expression of *flaA* (54). This finding also illustrates a difference in the mechanisms for controlling the expression of σ^{28}-dependent flagellar genes in *S. enterica* serovar Typhimurium and *H. pylori*. In *S. enterica* serovar Typhimurium, σ^{28} function is

inhibited by the anti-σ^{28} factor FlgM until the hook-basal body complex is completed, at which point FlgM is translocated out of the cell by the flagellar protein export apparatus (31). Although *H. pylori* possesses a FlgM homolog that negatively regulates the function of σ^{28} (9, 19), alleviation of the inhibitory effect of FlgM on σ^{28} does not involve formation of the hook-basal body complex since *H. pylori* strains with mutations in the hook protein gene do not produce flagellar filaments yet they still accumulate FlaA (46).

The *flgS* gene (*orf0244*) encodes the cognate sensor kinase of FlgR and is at the 3'-end of a potential operon that includes *flgI* and *orf0245*, which encode the flagellar basal body P-ring protein and a hypothetical protein of unknown function, respectively (1, 58). Inactivation of *flgS* in *H. pylori* strain G27 resulted in loss of motility and expression of σ^{54}-dependent flagellar genes, as assessed by two-dimensional gel electrophoresis analysis of cell extracts of the mutant strain (3). Like these earlier observations, inactivation of *flgS* in *H. pylori* strain ATCC 43504 resulted in loss of motility and expression of FlgE and FlaB (Fig. 2). In contrast to the previous report with *H. pylori* strain G27, however, inactivation of *flgS* in *H. pylori* strain ATCC 43504 did not eliminate expression of FlaA. This apparent discrepancy could be due to differences between the two *H. pylori* strains. Alternatively, since FlaA would not localize correctly in the absence of FlgE and FlgB it may not accumulate to wild-type levels in the *flgS* mutant strain. Therefore, our ability to detect FlaA in the *flgS* mutant strain may simply reflect the higher sensitivity of the western blotting procedure we used compared to the two-dimensional gel electrophoresis method used in the previous study for detecting expression of FlaA.

FlgR does not require upstream activation sequences for efficient transcriptional activation from the *flaB* promoter. Activators of σ^{54}-holoenzyme generally bind to DNA sequences located relatively far from the promoter and contact the closed promoter complex through DNA

looping to activate transcription. These activator binding sites are typically located >100 bp upstream of the transcriptional start site since shorter distances hinder the ability of the activator to interact with σ^{54}-holoenzyme through DNA looping due to constraints in DNA flexibility (64). As illustrated in Figure 1, *H. pylori* FlgR lacks the carboxy-terminal DNA-binding domain found in most other σ^{54}-dependent activators, suggesting that FlgR does not bind DNA to activate transcription. Consistent with the lack of a DNA-binding domain, purified full-length FlgR failed to bind a DNA fragment that bore the *flaB* promoter regulatory region in gel mobility shift assays (data not shown). We wanted to examine the possibility, however, that a DNA-binding activity required for FlgR function resides on a separate polypeptide.

To determine if upstream activation sequences are required for expression of σ^{54}-dependent flagellar genes in *H. pylori*, we constructed *flaB'-'xylE* reporter genes that had either 393 bp or 67 bp of DNA sequence upstream of the transcriptional start site of *flaB*, which we designated as *flaB2'-'xylE* and *flaB1'-'xylE*, respectively. Both reporter genes contained 26 bp of DNA sequence downstream of the transcriptional start site of *flaB* that corresponded to the untranslated region of the *flaB* transcript. These reporter genes were placed on the shuttle vector pHel3 and introduced into *H. pylori* strain ATCC 43504. Expression levels from the two *flaB'-'xylE* reporter genes were similar and dependent on both FlgR and FlgS (Table 1). Low level expression from the *flaB2'-'xylE* reporter gene was observed in the *flgR* and *flgS* mutant strains, suggesting that σ^{80}-holoenzyme or σ^{28}-holoenzyme might initiate transcription weakly from a sequence located upstream of the *flaB* promoter between position -67 and –393.

Expression from a *flaA'-'xylE* reporter gene, which is dependent on σ^{28}-holoenzyme, and a *flgI'-'xylE* reporter gene, which is dependent on σ^{80}-holoenzyme, was compared with that from the *flaB'-'xylE* reporter genes (Table 1). Expression from the *flaA'-'xylE* reporter gene was

independent of FlgR and FlgS, while expression from the *flgI'-'xylE* reporter gene was ~2-fold lower in the *flgR* mutant strain but not in the *flgS* mutant strain (Table 1). We do not understand why disruption of *flgR* resulted in lower expression from the *flgI'-'xylE* reporter gene but it does not seem likely that it was due to the failure of the mutant strain to express the σ^{54}-dependent flagellar genes since this would also require FlgS. The XylE activities observed for the flagellar reporter genes were consistent with the expected relative amounts of the products of these genes associated with the *H. pylori* flagellum (i.e., FlaA>FlaB>FlgI). Taken together, these data indicate that FlgR does not use an upstream activation sequence or enhancer to elicit its function on the *flaB* promoter.

FlgR levels in *H. pylori* are slightly higher than levels of σ^{54}-dependent activators in other bacteria. Transcriptional activation in the absence of DNA-binding has been reported previously for other σ^{54}-dependent activators, including NtrC (42), DctD (18, 60), NifA (17), and PspF (20). In these previous studies, the DNA-binding motifs of the activators were either deleted or mutated to eliminate DNA-binding activity, or the upstream activation sequence of the target gene was removed. Transcriptional activation under these conditions required that the activator be present at higher than normal levels. We wished to estimate the levels of FlgR in *H. pylori* to determine if it was expressed at levels that were higher than σ^{54}-dependent activators that function by binding to an upstream activation sequence or enhancer.

Levels of NtrC in *E. coli* range from about 10 molecules of monomeric protein per cell when cells are grown in a medium with excess nitrogen to approximately 140 molecules per cell following starvation of cells for nitrogen (32, 49). We estimated FlgR levels in *H. pylori* strain ATCC 43504 by western blotting using antiserum directed against the full-length histidine-tagged FlgR. For the western blot, purified MBP-FlgR was used as a standard to avoid

underestimating the FlgR concentration due to antibodies that may have recognized the histidine-tag. Varying amounts of *H. pylori* cells were lysed and loaded directly onto the SDS-polyacrylamide gel for the western blot assay. Under the assay conditions the detection limit for MBP-FlgR was ~12.5 ng, or approximately 0.14 pmols MBP-FlgR monomer (Fig. 3). FlgR could be detected by the western blot assay when as little as 1 x 10^8 cells were lysed and loaded on the gel, which corresponded to ~800 FlgR monomers per cell. Thus, assuming that the intracellular volumes of *H. pylori* and *E. coli* are similar, the level of FlgR in *H. pylori* appears to be about 6-fold higher than that of NtrC in *E. coli* under conditions where these proteins are activating transcription.

FlgR functions with *E. coli* σ^{54}-holoenzyme. We wished to examine the function of FlgR in an *in vitro* transcription assay. *H. pylori* σ^{54}-holoenzyme has not been purified in an active form, and so we planned to use *E. coli* σ^{54}-holoenzyme for these experiments. Previous work in the lab showed that *H. pylori rpoN*, which encodes σ^{54}, failed to complement a *S. enterica* serovar Typhimurium *rpoN* mutant strain (M. Dashti, unpublished data), and so it was unclear if FlgR could function with *E. coli* σ^{54}-holoenzyme.

To examine *H. pylori* FlgR function in *E. coli*, *flgR* was cloned into the expression vector pTrc-HisC, which introduced a sequence coding for a histidine-tag at the 5'-end of the gene. Removal of the amino-terminal receiver domain of *S. meliloti* DctD had been shown previously to result in a constitutively active form of the protein (26), and we wished to determine if removal of the receiver domain of FlgR would similarly result in constitutive activity. Therefore, a truncated *flgR* allele that encoded residues His-131 through Arg-381, which is the carboxy-terminal amino acid residue of native FlgR, was cloned into pTrc-HisC.

Expression of both the full-length and truncated FlgR proteins was inducible with IPTG to about the same level (data not shown). Activity of the FlgR proteins was monitored in *E. coli* using a *S. meliloti dctA'-'lacZ* reporter gene, the expression from which is dependent on σ^{54}-holoenzyme. This reporter gene was used because *S. enterica* serovar Typhimurium σ^{54} (and also presumably *E. coli* σ^{54}) has a low affinity for the *H. pylori flaB* promoter (L. Pereira and T. R. Hoover, unpublished data). Full-length FlgR activated transcription from the *dctA'-'lacZ* reporter gene very weakly (~3-fold above background) when its expression was induced with IPTG, but failed to activate transcription above background levels when its expression was not induced (Fig. 4). Since FlgR presumably needs to be phosphorylated to activate transcription, the low level activity observed with the full-length FlgR in *E. coli* suggested that the protein could be phosphorylated by another sensor kinase or a small phosphor-donor, as occurs with other response regulators (30, 40).

In contrast to the results with the full-length protein, the FlgR AAA+ domain activated transcription from the *dctA'-'lacZ* reporter gene >120-fold above background levels when its expression was not induced with IPTG. The activity of the FlgR AAA+ domain in *E. coli* compared favorably with that of the DctD AAA+ domain, indicating that the FlgR AAA+ domain was able to function effectively with *E. coli* σ^{54}-holoenzyme. The activities of both the FlgR AAA+ domain and the DctD AAA+ domain decreased upon induction of these proteins with IPTG. This decrease in activity was not likely due to aggregation since these proteins were in the soluble fraction when we overexpressed them for purification. Since σ^{54}-dependent activators can bind σ^{54} (8), the FlgR and DctD AAA+ domains may have sequestered σ^{54} and prevented it from binding core RNA polymerase to form the holoenzyme.

To determine if the FlgR and DctD AAA+ domains influenced expression of other σ^{54}-dependent genes in *E. coli*, we examined the effect of these proteins on expression of *glnA*, which encodes glutamine synthetase. Expression of *glnA* is dependent on σ^{54} and NtrC, which binds to several sites located upstream of *glnA*p2, the σ^{54}-dependent *glnA* promoter (50). The presence of the FlgR AAA+ domain or the DctD AAA+ domain resulted in ~3-fold increase in glutamine synthetase activity (Table 2), suggesting that these activators stimulated transcription from *glnA*p2. Overexpression of these proteins, however, did not inhibit *glnA* expression as observed with the *dctA'-'lacZ* reporter gene. We do not know the reason for this, but one possibility is that σ^{54}-holoenzyme has a higher affinity for *glnA*p2 than it does for the *dctA* promoter and therefore might be less sensitive to decreased levels of free σ^{54} in the cell.

The FlgR AAA+ domain was purified and its activity was examined *in vitro*. The purified FlgR AAA+ domain hydrolyzed ATP and activated transcription with *E. coli* RNA polymerase from a DNA template that carried *S. enterica* serovar Typhimurium *glnA*p2 in an *in vitro* transcription assay (Fig. 5). Transcripts were detected with as little as 0.5 μM FlgR AAA+ domain monomer. As observed with the *dctA'-'lacZ* reporter gene *in vivo*, but in contrast to the results for *glnA*p2, high concentrations of the FlgR AAA+ domain inhibited transcription initiation from *glnA*p2 in the *in vitro* transcription assay. The reason for the discrepancy in the *in vivo* and *in vitro* results with *glnA*p2 might be due to lower amounts of σ^{54}-holoenzyme in the *in vitro* system. Alternatively, conditions in the *in vitro* system may not be optimized for efficient transcription making the *in vitro* assay more sensitive to perturbations. Regardless of the reason, the results of the *in vivo* and *in vitro* transcription assays suggest further that FlgR does not bind DNA to activate transcription nor does it require another DNA-binding protein to do so since it is

unlikely that the promoter regulatory regions of *dctA*, *glnA* and *flaB* share a common upstream activation sequence.

Discussion

Most bacterial transcriptional activators bind to specific sites within the promoter regulator regions of their target genes to recruit RNA polymerase to the promoter or to stimulate a step in transcription initiation that occurs after the initial binding of RNA polymerase to the promoter. One notable exception is the bacteriophage N4 single-stranded DNA binding protein (N4SSB), which activates transcription with *E. coli* σ^{70}-holoenzyme at N4 late promoters without binding DNA (35). N4SSB interacts with the carboxy-terminus of the β' subunit of RNA polymerase and appears to stimulate a step that follows the initial binding of RNA polymerase to the promoter (35). *E. coli* MarA and the closely related SoxS protein are other examples of transcriptional activators that function somewhat differently than most bacterial activators. Although MarA and SoxS are DNA-binding proteins, they appear to bind DNA after interacting with RNA polymerase in solution (33). MarA and SoxS interact with the α-subunit of RNA polymerase in the absence of DNA to form binary complexes that are thought to scan chromosomal DNA for target promoters.

Activators of σ^{54}-holoenzyme generally bind to sites that are located relatively far from the promoter and contact the closed complex through DNA looping to activate transcription (64). *H. pylori* FlgR lacks the DNA-binding domain associated with most other σ^{54}-dependent activators, and the results presented here demonstrate that FlgR does not require an enhancer or upstream activation sequence to activate transcription. We infer that FlgR binds σ^{54}-holoenzyme directly, either before or after formation of the closed promoter complex, to activate transcription. While mutant forms of other σ^{54}-dependent activators have been reported to activate transcription in the

absence of DNA-binding (17, 18, 20, 42), FlgR is unusual in that it represents a naturally occurring enhancer-independent activator of σ^{54}-holoenzyme. FlgR appears to be present at concentrations in the cell that are higher than those of NtrC in *E. coli*. The *glnA* enhancer facilitates oligomerization of NtrC which is required for transcriptional activation from *glnA*p2 (48). Thus, higher concentrations of FlgR may be needed to compensate for the absence of enhancer-binding and allow oligomerization of the protein.

C. trachomatis CtcC was described recently as an activator of σ^{54}-holoenzyme that lacks a DNA-binding domain (23) and we infer that like FlgR, CtcC does not require sequences upstream of the promoter to activate transcription. Search of the databases for additional σ^{54}-dependent activators that lack the DNA-binding domain identified activators from *Chlamydia pneumoniae*, *Chlamydia muridarum*, and *Chlamydophila caviae* with this unusual structural property (Fig. 1). Two other potential σ^{54}-dependent activators that appear to lack the carboxy-terminal DNA-binding domain were found in database searches, one in *Xanthomonas campestris* pv. *campestris* ATCC33913 (gene designated as *pilR* in database) and another in *Pseudomonas putida* KT2440 (designated PP5166 in database). There is some doubt, however, as to whether the sequences of these activators are correct or if they encode σ^{54}-dependent activators. The carboxy-terminal end of the deduced amino acid sequence of *X. campestris* PilR corresponds to 28 amino acids before that of *H. pylori* FlgR, placing it within the conserved sensor II motif of the AAA+ domain. Since this motif is important for function of other σ^{54}-dependent activators, it is unlikely that such a truncation would result in an active protein. The carboxy-terminal end of the deduced amino acid sequence of *P. putida* PP5166 extends three residues beyond that of *H. pylori* FlgR. *P. putida* PP5166, however, has a poor match for the highly conserved GAFTGA motif in the C3 region of the protein (deduced amino acid sequence of this motif in PP5166 is

GSHGGT). The GAFTGA motif, which is diagnostic of σ^{54}-dependent activators (45) functions in contacting σ^{54} and coupling ATP hydrolysis to open complex formation, and substitutions within this motif often result in loss of activity (6, 8, 14, 28, 60, 61). Thus, PP5166 may not be an activator of σ^{54}-holoenzyme, but may function with another form of RNA polymerase holoenzyme.

A potential advantage to the cell for using an activator of σ^{54}-holoenzyme that does not bind DNA is that extensive regulatory regions upstream of target promoters which harbor binding sites for activators and auxiliary proteins involved in transcriptional activation, such as the integration host factor, are dispensable. A major drawback, however, is that the cell may be limited to a single σ^{54}-dependent activator dedicated for a particular cellular function since there is no obvious mechanism for preventing activation from all of the σ^{54}-dependent genes within the genome. Indeed, FlgR, CtcC, and the other chlamydial activators are the sole σ^{54}-dependent activators in their respective bacteria, and at least for FlgR, the activator appears to be dedicated for a specific cellular activity. The metabolic savings gained by employing activators that function without binding DNA seems small given the sacrifice in regulatory potential that accompanies the use of such activators. Therefore, we expect activators of σ^{54}-holoenzyme that do not bind DNA to be restricted to bacteria with relatively limited needs for regulatory potential. Consistent with this hypothesis, such σ^{54}-dependent activators have been found only in pathogens that have limited biosynthetic capability.

All of the chlamydial genomes that have been sequenced to date include an open reading frame that encodes a potential σ^{54}-dependent activator that lacks the DNA-binding domain. In contrast, *Helicobacter hepaticus* and *Campylobacter jejuni*, which are closely related to *H. pylori*, each have a single σ^{54}-dependent activator but these activators have a potential DNA-

binding domain (Fig. 1). This is somewhat surprising given that these activators appear to have the same role in flagellar biogenesis as *H. pylori* FlgR (15, 54, 57). We infer from this observation that loss of the DNA-binding domains in the chlamydial σ^{54}-dependent activators and *H. pylori* FlgR occurred independently of each other.

The crystal structure of the AAA+ domain of NtrC1, a σ^{54}-dependent activator from *Aquifex aeolicus*, was recently reported (27). Alignment of the FlgR, CtcC, and the chlamydial activator sequences with that of NtrC1 indicated that the carboxy-termini of these proteins correspond to residues within the last helix of the α-helical subdomain of the NtrC1 AAA+ domain. Thus, these σ^{54}-dependent activators appear to have lost the entire DNA-binding domain during the course of evolution. It is unclear if the loss of the entire DNA-binding domain reflects an economic benefit by removing as much of the protein as possible, or if there is a structural or functional basis for the proteins to terminate at this point.

Acknowledgements. This work was funded by award MCB-9974558 to T. R. H. from the National Science Foundation. We thank Dr. Paul O'Toole for providing antiserum directed against the *H. pylori* flagellum. M. G. Dashti constructed the *H. pylori flgR:aphA3* and *flgS:aphA3* mutants and has contributed to figure 2 in the manuscript. J. W. Olson helped with the transformation of the pHel3 shuttle plasmids into *H. pylori*.

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Table 1. Expression of *flaB'-'xylE*, *flaA'-'xylE*, and *flgI'-'xylE* reporter genes in various *H. pylori* strains.

***H. pylori* strain**	**Relevant genotype**	**XylE activity (units/min/10^8 cells) for the different reporter genes[a]**			
		flaB2'-'xylE	*flaB1'-'xylE*	*flaA'-'xylE*	*flgI'-'xylE*
ATCC 43504	wild-type	12.4 ± 4.9	9.0 ± 2.1	21.5 ± 10.9	5.2 ± 2.3
HP31	*flgR:cat*	2.6 ± 1.9	0.3 ± 0.3	17.2 ± 6.3	2.2 ± 0.4
HP22	*flgS:cat*	4.1 ± 1.9	0.1 ± 0.2	19.2 ± 7.9	5.6 ± 2.9

[a]Values represent the average of at least six replicates with the standard deviations indicated for each average value.

Table 2. Glutamine synthetase activities in *E. coli* DH5α strains that express the FlgR AAA+ domain or the DctD AAA+ domain.

Plasmid	**protein expressed**	**glutamine synthetase activity (nmols γ-glutamyl hydroxymate/min/A_{650} unit[a]**	
		-IPTG	+IPTG
none	none	5.46 ± 1.22	6.38 ± 2.00
pPBHP80	FlgR AAA+ domain	17.4 ± 5.42	15.4 ± 3.14
pHX182	DctD AAA+ domain	16.5 ± 5.07	9.62 ± 3.40

[a]Values represent the average of at least six replicates with the standard deviations indicated for each average value.

Figure 1. **Comparison of a partial sequence of *H. pylori* FlgR with that of other σ^{54}-dependent activators.** The conserved Walker A , Walker B, and sensor II motifs within the AAA+ domains of selected σ^{54}-dependent activators are indicated. "X" denotes any amino acid residue with the number that follows indicating the spacing between the motifs. The sequences from σ^{54}-dependent activators that are shown are *S. meliloti* DctD (Sm DctD); *H. pylori* FlgR (Hp FlgR); *H. hepaticus* FlgR (Hh FlgR); *C. jejuni* FlgR (Cj FlgR); *C. trachomatis* CtcC (Ct CtcC); and activators from *C. muridarum* (Cm act), *C. pneumoniae* (Cp act), and *C. caviae* (Cc act). Helix 4 indicates the last helix of the α-helical subdomain of the DctD AAA+ domain that was predicted by threading the DctD sequence onto the *A. aeolicus* NtrC1 AAA+ domain structure (27). The serine residue underlined in helix 4 of the DctD sequence is Ser-390, which is the carboxy-terminus of the DctD AAA+ domain used in the studies described here. The predicted helix-turn-helix motifs of DctD, *H. hepaticus* FlgR, and *C. jejuni* FlgR are indicated. The four amino acid residues of the carboxy-termini of the FlgR proteins are shown for comparison.

	Walker A	Walker B	Sensor II	helix 4	helix-turn-helix
Sm DctD	PL-X27-GETGSGKE-X58-GTLFLDE-X114-GNVRELSHFAE-X18-SSGATL-X22-VKETLQALGIPRKTFYDKLQR-X23				
Hp FlgR	SF-X28-GESGVGKE-X58-GTIFLDE-X114-GNVRELLGVVE-X16-FLER				
Hh FlgR	DF-X27-GQSGVGKD-X58-GSVFLDE-X114-GNVRELLSVIE-X16-FLES -X34- VQKASDILGMNLEVLRHKIAR-X3				
Cj FlgR	DF-X27-GESGVGKE-X58-GTLFLDE-X114-GNIRELISVVQ-X16-FLEA -X25- KDQASQILGMDIKILNEKIKK-X6				
Ct CtcC	PL-X27-GESGCGKE-X58-GTLLLED-X114-GNVRELSNVLE-X20				
Cm act	PL-X28-GESGCGKE-X58-GTLLLDE-X114-GNVRELSNVLE-X20				
Cp act	PL-X28-GESGCGKE-X58-GTLLLDE-X114-GNIRELSNVLE-X19				
Cc act	PL-X27-GESGCGKE-X58-GTLLLDE-X114-GNIRELSNVLE-X19				

Figure 2. Immunoblot of cell extracts of wild-type *H. pylori*, *flgR* and *flgS* mutant strains. Each lane contained cell extracts (50 μg protein) from one of the *H. pylori* strains. The blot was probed with antiserum directed against the *H. pylori* flagellum. Lane 1, wild-type *H. pylori* 43504 strain; lane 2, MGD1 (*flgR:aphA3* mutant strain); lane 3, MGD2 (*flgS:aphA3* mutant strain). FlgE, FlaB, and FlaA bands are indicated.

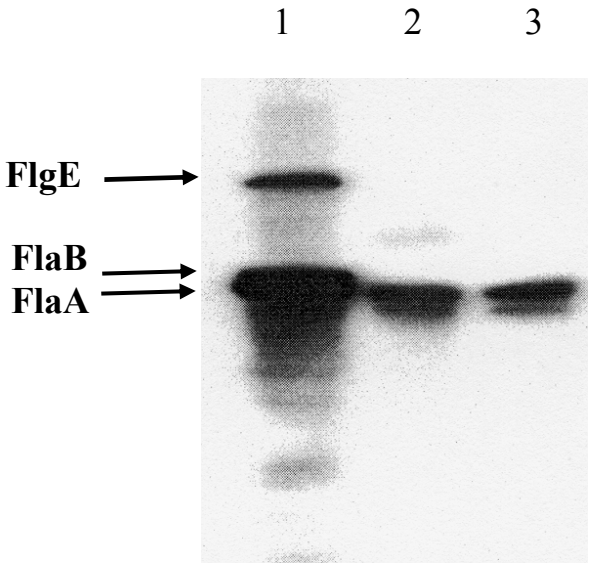

1
2
3
FlgE
FlaB
FlaA

Figure 3. Immunoblot of FlgR in *H. pylori* cell extracts. The immunoblot was probed with antibody directed against the histidine-tagged FlgR protein. The arrow indicates the band corresponding to FlgR. Different amounts of cell extract of wild-type *H. pylori* were loaded onto lanes 1-7 (lane 1, 1 x 10^9 cells; lane 2, 5 x 10^8 cells; lane 3, 2 x 10^8 cells; lane 4, 1 x 10^8 cells; lane 5, 5 x 10^7 cells; lane 6, 2 x 10^7 cells; and lane 7, 1 x 10^7 cells). In lane 8, 1 x 10^9 cells of the mutant *H. pylori flgR:cat* strain were loaded. Different amounts of purified MBP-FlgR were loaded onto lanes 9-14 (lane 9, 200 ng; lane 10, 100 ng; lane 11, 50 ng; lane 12, 25 ng; lane 13, 12.5 ng; lane 14, 6 ng).

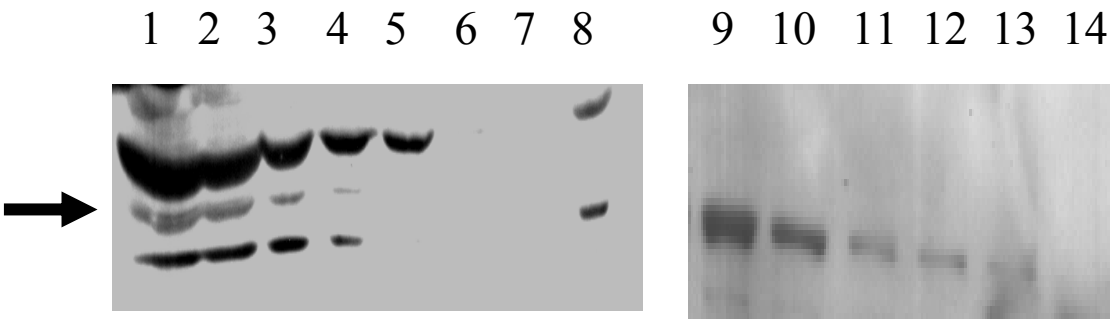

1 2 3 4 5 6 7 8
9 10 11 12 13 14

Figure 4. *In vivo* activity of AAA+ domains of FlgR and DctD. Activity of full-length FlgR, the FlgR AAA+ domain, and the DctD AAA+ domain on a *dctA'-'lacZ* reporter gene in *E. coli*. The gray bars indicate activity for culture in which the expression of the FlgR or DctD proteins was induced, while the black bars indicate activity for cultures in which the expression of the proteins was not induced with IPTG. Values on the Y-axis represent β-galactosidase activities in Miller Units. Lanes 1 and 2, activity for full-length FlgR; lanes 3 and 4, activity for FlgR AAA+ domain; lanes 5 and 6, activity for DctD AAA+ domain.

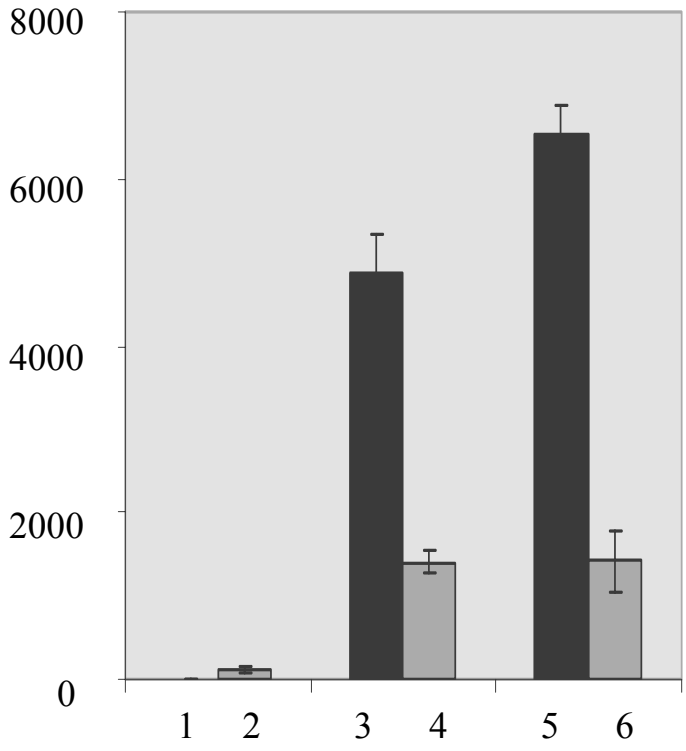
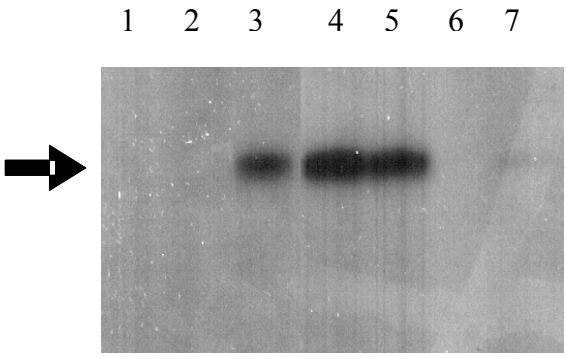

8000
6000
4000
2000
0
1
2
3
4
5
6

Figure 5. ***In vitro*** **transcriptional activation with the FlgR AAA+ domain.** Reaction mixtures contained FlgR AAA+ domain (monomer) at the following concentrations: lane 1, none; lane 2, 0.1 μM; lane 3, 0.5 μM; lane 4, 1 μM; lane 5, 2.5 μM; lane 6, 5 μM; and lane 7, 10 μM. The arrow indicates the transcript of the expected size (~155 nucleotides) from the *glnA* promoter.


1 2 3 4 5 6 7
0 0.1 0.5 1.0 2.5 5.0 10

CHAPER III

IDENTIFICATION OF A NOVEL PROTEIN REQUIRED FOR FLAGELLAR ASSEMBLY IN *HELICOBACTER PYLORI* [3]

[3] Brahmachary, P and T. R. Hoover 2004 Submitted to Helicobacter.

Abstract

Background. Flagellar biogenesis requires all three σ factors found in *Helicobacter pylori* (σ^{80}, σ^{54}, and σ^{28}). FlgS is a sensor kinase needed for expression of σ^{54}-dependent flagellar genes in *H. pylori*. Results from a previous high-throughput screen of a yeast two-hybrid assay showed that *H. pylori* HP0137, a protein of unknown function, interacts with FlgS and FlgE2 (HP0908), a protein that shares homology with the flagellar hook protein. Our purpose was to determine if HP0137 has a role in flagellar biogenesis.

Methods. An *hp0137* deletion mutant was made in *H. pylori* ATCC 43504 and examined by electron microscopy. The ability of the mutant to express three classes of flagellar genes was assessed with reporter genes in which the promoter regions of various flagellar genes were fused to *xylE* (encodes catechol 2,3-dioxygenase). Western blot analysis was performed to detect the presence of the minor flagellin FlaB.

Results. The Δ*hp0137* was non-motile and aflagellate. The mutant expressed *flgI'-'xylE* (σ^{80}-dependent), *flaA'-'xylE* (σ^{28}-dependent), and *flaB'-'xylE* (σ^{54}-dependent) reporter genes at wild-type levels. Despite the lack of flagella, levels of FlaB in the mutant strain were close to wild type.

Conclusions. HP0137 is required for flagellar biogenesis in *H. pylori*. Loss of HP0137 did not affect transcription of representatives from the three classes of flagellar genes, nor did it prevent accumulation of FlaB. These data suggest a role for HP0137 in flagellar assembly, perhaps by assisting the export of FlgE2 or other flagellar proteins.

Keywords. *Helicobacter pylori*, motility, flagellar biogenesis, FlgS, σ^{54}

Introduction

Helicobacter pylori is a microaerophilic, motile bacterium that colonizes the human gastric muscosa. A major human pathogen, it is the etiological agent of chronic gastritis [1, 2] and is associated with the development of peptic ulcers, gastric non-Hodgkin's lymphomas and gastric mucosa-associated lymphoid tissue lymphomas [3]. In addition to virulence factors such as urease [4], vacuolating toxin (VacA) [5], and the cytotoxin-associated protein [6, 7], motility is also an important colonization factor [8].

H. pylori possesses two to six polar, sheathed flagella that enable the bacterium to move through the highly viscous mucous layer of the gastric epithelium [9]. Analysis of the *H. pylori* 26695 and J99 genome sequences identified over 40 known flagellar genes scattered throughout the genome and organized into 25 or more transcriptional units [10, 11]. As in other bacteria, flagellar gene expression in *H. pylori* likely follows a hierarchy wherein the genes encoding the basal body and flagellar protein export apparatus are expressed initially, followed by genes encoding the hook and hook-associated proteins and then the genes encoding filament proteins [12, 13].

Flagellar gene expresson in *H. pylori* involves all three sigma factors found in this bacterium, σ^{80} (the primary σ factor), σ^{54} (RpoN), and σ^{28} (FliA). Flagellar genes expressed early in the transcriptional hierarchy are transcribed by σ^{80}-RNA polymerase holoenzyme. The σ^{54}-dependent flagellar genes are required midway through flagellar biogenesis and include genes coding for the minor flagellin (*flaB*), the proximal rod proteins (*flgBC*) and the hook protein (*flgE*) [14-16]. The flagellar genes required at the very end of flagellar biogenesis include those encoding the major flagellin (*flaA*), the filament cap protein (*fliD*) and flagellin chaperones (*fliST*), and are transcribed by σ^{28}-RNA polymerase holoenzyme [16-18].

In addition to σ^{54}, expression of genes within the RpoN regulon requires a two-component regulatory system consisting of the sensor kinase FlgS (also referred to as FleS) and the response regulator FlgR (FleR) [14, 16, 19, 20]. FlgR belongs to a large family of transcriptional activators that function with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme). Activators of σ^{54}-holoenzyme stimulate the conversion of a closed complex between σ^{54}-holoenzyme and the promoter to an open complex that is competent to initiate transcription [21]. FlgR presumably must be phosphorylated by FlgS-phosphate to activate transcription. Sensor kinases often are responsive to environmental or cellular signals, but it is not known if FlgS is responsive to such cues or if other factors in *H. pylori* regulate FlgS activity.

A protein-protein interaction map for *H. pylori* constructed from a high throughput screen of a yeast two-hybrid system [22] (Hybrigenics PimRider™ database; http://pim.hybrigenics.com/pimrider) indicated that FlgS interacted with the hypothetical protein HP0137. HP0137 also interacted with several other *H. pylori* proteins in the yeast two-hybrid assay, including the chaperone GroEL, VacA, and FlgE2 (HP0908). The annotated genome sequence of *H. pylori* 26695 indicates that FlgE2 is a flagellar hook protein homolog, but an exact function has not been assigned to this protein. We show here that FlgE2 is required for motility and flagellar biogenesis, suggesting that it is an essential component of the *H. pylori* flagellum.

To determine if HP0137 was involved in flagellar biogenesis, and in particular FlgS function, we deleted the corresponding gene (*hp0137*) in *H. pylori* ATCC 43504 and examined the effect of this mutation on expression of the σ^{54}-dependent *flaB* gene as well as other flagellar genes with *xylE* reporter genes. The Δ*hp0137* mutant was non-motile and lacked flagella, but expressed *flaB'-'xylE*, *flgI'-'xylE*, and *flaA'-'xylE* reporter genes normally. The Δ*hp0137* mutant

also expressed FlaB to near wild-type levels as assessed by western blotting. These data suggest that HP0137 does not influence flagellar gene expression but is required for flagellar assembly, perhaps by assisting in the export of FlgE2 or other flagellar genes products.

Material and Methods

Bacterial Strains and Culture Conditions

Unless indicated otherwise, *H. pylori* strains ATCC 43504 and 26695 were grown on tryptic soy agar (TSA) supplemented with 5 % horse serum at 37°C under microaerophilic conditions which consisted of 4 % O_2, 5 % CO_2 and 91 % N_2. *E.coli* strain DH5α [Φ80 *lacZ* ΔM15 *recA1 gyrA96 thi-1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 deoR* Δ(*lacZYA-arg F*) U169**]** was used for cloning and was cultured in Luria-Bertani medium at 37°C. When necessary, antibiotics were used at the following concentration : 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 30 μg/ml kanamycin and 200 μg/ml bacitracin. Soft agar plates for assessing motility of *H. pylori* strains contained TSA supplemented with 5% horse serum and 0.35% (w/v) agar.

Helicobacter pylori mutant strains

H. pylori 26695 chromosomal DNA was used as the template for polymerase chain reaction (PCR) amplification. The deletion of *hp0137* in *H. pylori* 43504 was carried out as follows. DNA sequences upstream and downstream of *hp0137* along with the 5'-end and 3'-end, respectively, of *hp0137* were amplified in two separate PCRs. *Eco*RI sites were introduced approximately 100 bp from the 5'- and 3'- ends of *hp0137* in the primers used for PCR. The two PCR products were ligated together using the *Eco*RI sites, which resulted in a deletion of about 430 bp of *hp0137* corresponding to codons 33 to 177 of the gene. A 1.3 kb *Eco*RI fragment from plasmid pSKAT4 [23] that carried the *Campylobacter coli* chloramphenicol resistance gene *(cat)* was introduced

into the *Eco*RI site in the truncated *hp0137* gene to create plasmid pPBHP89. This suicide vector was introduced into *H. pylori* strain ATCC 43504 by natural transformation and mutants resulting from homologous recombination between *hp0137* on the chromosome and the Δ*hp0137:cat* allele carried on the plasmid were selected on TSA supplemented with horse serum and chloramphenicol. Genomic DNA from chloramphenicol resistant colonies was analyzed by PCR to confirm that the Δ*hp0137:cat* allele had replaced the wild-type *hp0137* allele. One of the Δ*hp0137:cat* mutants were designated as strain HP63 and analyzed further.

A DNA fragment bearing *hp0908* from *H. pylori* strain 26695 was amplified by PCR and cloned in pGEM-T (Promega) to create plasmid pPBHP28. The 1.3 kb *Eco*RI fragment from plasmid pSKAT4 that carried the *C. coli cat* cassette was introduced into a unique *Eco*RI site within *hp0908* in plasmid pPBHP28. The *cat* cassette disrupted codon 86 of *hp0908* and the resulting suicide vector was introduced into *H. pylori* strain ATCC 43504 by natural transformation. Mutants resulting from homologous recombination between *hp0908* on the chromosome and the *hp0908:cat* allele were selected on TSA supplemented with horse serum and chloramphenicol. Genomic DNA from chloramphenicol resistant colonies was analyzed by PCR to confirm that the chromosomal copy of *hp0908* had been inactivated by the *cat* cassette. One of the *hp0908:cat* mutants was designated HP19 and analyzed further.

Construction of *H. pylori flgR:cat* and *flgS:cat* mutants were described previously [19]. A *rpoN:cat* mutant was kindly provided by Lara Periera and a *bcp:cat* mutant was kindly provided by Ge Wang.

Electron microscopy

H. pylori strains were grown on Brucella agar containing 10% sheep blood for 48 h. Cells were gently resuspended in phosphate buffered saline (PBS), pH 7.4. Samples were applied to formvar

coated grids and negatively stained with 2% phosphotungstic acid, then visualized by a JEOL 100CXII electron microscope (JEOL, Tokyo, Japan) at the Center for Advanced Ultrastructural Studies at the University of Georgia.

Measurement of XylE activity

Reporter genes in which a promoterless *Pseudomonas putida xylE* (encodes catechol 2,3-dioxygenase) was joined to the promoter regions of *H. pylori flaA*, *flaB* or *flgI* were as described previously [19]. These reporter genes were introduced into *H. pylori* strains on the shuttle vector pHel3 [24]. Whole cells XylE activities for strains bearing these reporter genes were measured as described previously [25]. *H. pylori* cells harboring the *xylE* reporter plasmids were adjusted to OD_{600} of 1. Reaction was started by adding 100 μl of the cell suspension to 10 mM catechol in 50 mM potassium phosphate, pH 7.4. Catechol oxidation to 2-hydroxymuconic semialdehyde was monitored at 375 nm with a Beckman DU 640 spectrophotometer at room temperature. One unit XylE activity corresponded to 1 μmol catechol oxidized/min and values were expressed as units per 10^8 cells.

Western blot analysis

H. pylori cells were lysed in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and subjected to SDS-PAGE on a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membrane. The membrane was probed with rabbit antiserum directed against a maltose-binding protein (MBP)-FlaB fusion protein. A peroxidase-conjugated goat antibody directed against rabbit IgG was used as the secondary antibody (ICN/Cappel). Protein bands were visualized by luminescence with an ECL kit (Amersham).

Results

***H. pylori* HP0137 is required for flagellar biogenesis.** Since HP0137 interacted with FlgS and HP0908 in the yeast two-hybrid assay, we wished to determine if it had a role in flagellar biogenesis. A *H. pylori* mutant in which most of *hp0137* was replaced with a *cat* cassette was constructed in *H. pylori* ATCC 43504. The Δ*hp0137:cat* mutant was non-motile when examined microscopically and on soft agar medium (data not shown). Disruption of the gene located immediately downstream of *hp0137*, (*bcp*; annotated as a bacterioferritin comigratory protein in the *H. pylori* 26695 genome sequence) with the *cat* cassette did not result in loss of motility (data not shown). This observation ruled out the possibility that the non-motile phenotype of the Δ*hp0137:cat* mutant was due to polar effects on downstream genes. The Δ *orf0137:cat* mutant was examined by transmission electron microscopy to see if it produced flagella. Sheathed polar flagella were clearly visible in the parental *H. pylori* strain, but were lacking in the Δ*hp0137:cat* mutant (Fig. 1B).

Disruption of *hp0137* does not appear to influence expression of flagellar genes. Since the protein-protein interaction map of *H. pylori* indicated interactions between HP0137 and FlgS, we reasoned that HP0137 might be required for FlgS function, which could account for the absence of flagella in the Δ*hp0137:cat* mutant. The effect of disruption of *hp0137* on the expression of genes in RpoN regulon was examined using a *flaB'-'xylE* reporter gene. Expression of the *flaB'-'xylE* reporter gene was shown previously to require both FlgS and FlgR [19]. As seen in Figure 2, levels of expression from *flaB'-'xylE* reporter gene were comparable in the Δ*hp0137:cat* mutant and its parental strain, indicating that HP0137 is not required for FlgS activity. Expression of *flaA'-'xylE* and *flgI'-'xylE* reporter genes, which are dependent on σ^{28}- and σ^{80}-RNA polymerase holoenzyme respectively, was also examined in the Δ*hp0137:cat* mutant. As

with the *flaB'-'xylE* reporter gene, expression of these two reporter genes was unaffected by disruption of *hp0137* (Fig. 2).

To determine if the FlaB protein was synthesized in the Δ*hp0137:cat* mutant, cell extracts of the strain were examined by western blotting. As a negative control for the western blot assay, cell extracts from a *H. pylori rpoN:cat* mutant, which fails to express *flaB*, were included in the blot. FlaB accumulated in the Δ*hp0137:cat* mutant to near wild-type levels (Fig. 3). Taken together, these data suggest that HP0137 has a role in flagellar assembly but is not required for the expression of genes in the RpoN regulon or the two other classes of flagellar genes.

FlgE2 is required for formation of a functional flagellum. In the *H. pylori* 26695 genome sequence, *hp0908* is annotated as a *flgE* homolog and appears to be in an operon with *flgD*, which encodes a hook-capping protein that facilitates the assembly of hook protein subunits. Another gene, *hp0870*, is also annotated as *flgE* in the *H. pylori* 26695 genome and has been shown to encode the hook protein [10]. *Campylobacter jejuni* has a similar arrangement of *flgD* and *flgE* genes in its genome, with the *Cj0042-Cj0043* operon containing *flgD* and a *flgE2* homolog, respectively, and *Cj1729c* corresponding to *flgE*. Deletion of *Cj1729c*, but not *Cj0043*, interferes with motility and flagellar assembly [26].

We wished to determine if *hp0870* (*flgE2*) is required for synthesis of functional flagella in *H. pylori* or if it is dispensable for flagellar biogenesis as is its counterpart in *C. jejuni*. A *cat* cassette was used to disrupt *flgE2* in *H. pylori* ATCC 43504. The resulting *flgE2:cat* mutant was non-motile and failed to produce flagella (Fig. 1), indicating that *flgE2* has an essential role in flagellar biogenesis in *H. pylori*. Expression levels of the *flaB'-'xylE* and *flaA'-'xylE* reporter genes in the *flgE2:cat* mutant were comparable to those in the parental strain (data not shown),

suggesting the disruption of *flgE2* does not affect expression of the RpoN or FliA regulons in *H. pylori*.

Discussion

We demonstrated here that HP0137 is required for flagellar assembly in *H. pylori*, although its exact role in flagellar biogenesis is not clear. The deduced amino acid sequence of HP0137 indicates a protein of 212 amino acid residues in length with a molecular weight of 23,610 Da. HP0137 is predicted to have a single membrane spanning region (amino acid residues 113 to 133), but its localization to the cytoplasmic membrane has not been confirmed. It seems unlikely that HP0137 is a structural component of the *H. pylori* flagellum, since homologs of the protein are not found in other bacteria, such as *Salmonella enterica* serovar Typhimurium, where flagellum structure and function have been extensively studied.

Our interest in HP0137 was piqued initially by the report of its interaction with FlgS in the yeast two-hybrid assay. We demonstrate here, however, that HP0137 is not required for FlgS function in *H. pylori*. At this point, we do not know if the interactions between FlgS and HP0137 in the yeast two-hybrid assay are coincidental or if HP0137 might have a role in negatively regulating FlgS activity. If HP0137 does negatively regulate FlgS activity, it may not have been readily apparent in the Δ*hp0137:cat* mutant since other regulatory mechanisms may be operating that down regulate the RpoN regulon and could mask any stimulatory effect that disruption of *hp0137* might have on FlgS activity. In support of this hypothesis, some mutations in *H. pylori* that interfere with flagellar protein export result in increased expression of the *flaB'-'xylE* reporter gene, suggesting that a positive regulator of the RpoN regulon is secreted from the cytoplasm through the flagellar protein export apparatus (L. Pereira and T. R. Hoover, unpublished data). Further studies are obviously needed to determine if HP0137 negatively

regulates FlgS activity. Even if HP0137 does inhibit FlgS activity, it seems unlikely that the lesion in flagellar assembly in the Δ*hp0137:cat* mutant would result from the loss of such a function.

HP0137 also interacts with FlgE2 in the yeast two-hybrid assay, and disruption of these interactions in the Δ*hp0137:cat* mutant may have accounted for the defect in flagellar assembly. Consistent with this hypothesis is our observation that FlgE2 is required for flagellar assembly, which is in contrast to what was observed for *C. jejuni* [26]. HP0137 homologs are found in other members of the ε-subdivision of *Proteobacteria*, including *C. jejuni*, *Helicobacter hepaticus*, and *Wolinella succinogenes*. Since FlgE2 is not required for flagellar assembly in *C. jejuni*, determining if HP0137 is needed for flagellar biogenesis in *C. jejuni* could shed light on how HP0137 exerts its effect on flagellar assembly in *H. pylori*.

It is possible that HP0137 assists in the export of FlgE2. The flagellar protein export apparatus is a type III secretion (TTS) system. In addition to the flagellar-associated TTS system, bacteria often have virulence-associated TTS systems that are used to target bacterial effector proteins into eukaryotic cells. The export of these effector proteins, as well as many flagellar proteins, is assisted by chaperones in the cytoplasm. TTS chaperones are generally small (12-18 kDa), acidic proteins that have an overall α-helical character [27]. *H. pylori* FliS, which is a flagellin chaperone, fits well with these criteria as it is a 14,542 Da protein with a pI of 4.77 and a predicted secondary structure that is largely α-helical. HP0137, however, does not meet the criteria for TTS chaperones very well with a molecular weight of 23.6 kDa, a pI of 7.66, and a predicted secondary structure consisting of ~50% α-helix. These properties of HP0137 argue against it being a TTS chaperone, but HP0137 could play other roles in the export of flagellar proteins.

Acknowledgements

This work was funded by awards MCB-9974588 from the National Science Foundation and AI056036 from the National Institute of Allergy and Infectious Diseases to T. R. H. We thank Lara Periera and Ge Wang for providing mutant *H. pylori* strains and Rainer Haas for providing plasmid pHel3. We also thank Drs. John Shields and Mark Farmer for technical assistance with electron microscopy.

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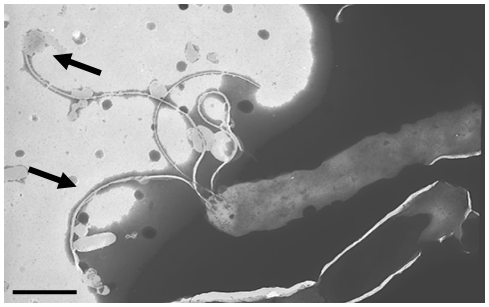
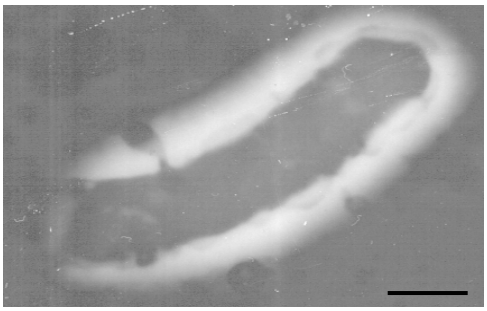
Figure 1. Electron micrographs of *Helicobacter pylori* strains. Cells were applied to formvar coated grids and negatively stained with 2 % phosphotungstic acid and visualized by transmission electron microscopy at 19,000 x magnification. Bar is equivalent to 1 μm. (A) Wild-type *H. pylori* ATCC 43504 are shown in which the polar sheathed flagella are clearly visible and indicated by arrows. Note the characteristic terminal swelling in the flagella (indicated by top arrow). (B) Electron micrograph of the Δ*hp0137:cat* mutant. No flagella were apparent in the > 50 cells that were examined. (C) Electron micrograph of *hp0908:cat* mutant. No flagella were visible in the >50 cells that were examined.

(A)



(B)



(C)

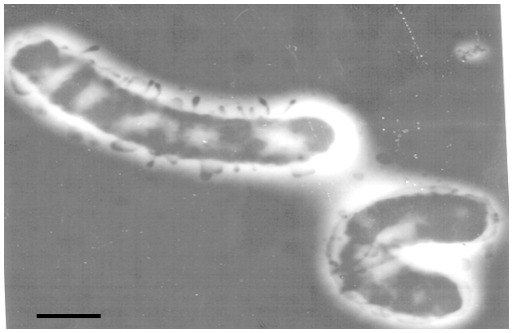


Figure 2. Expression of *flaB'-'xylE*, *flaA'-'xylE* and *flgI'-'xylE* reporter genes in wild-type *H. pylori* and Δ*hp0137:cat* mutant. Black bars indicate the activity of reporter genes for *H. pylori* ATCC 43504, while gray bars indicate the activity of reporter genes for Δ*hp0137:cat*. One unit of XylE activity corresponds to 1 μmol catechol oxidized / min. XylE activities are expressed as units/10^8cells and values represent the averages of at least six replicates. Error bars indicate the standard deviation in these replicates.

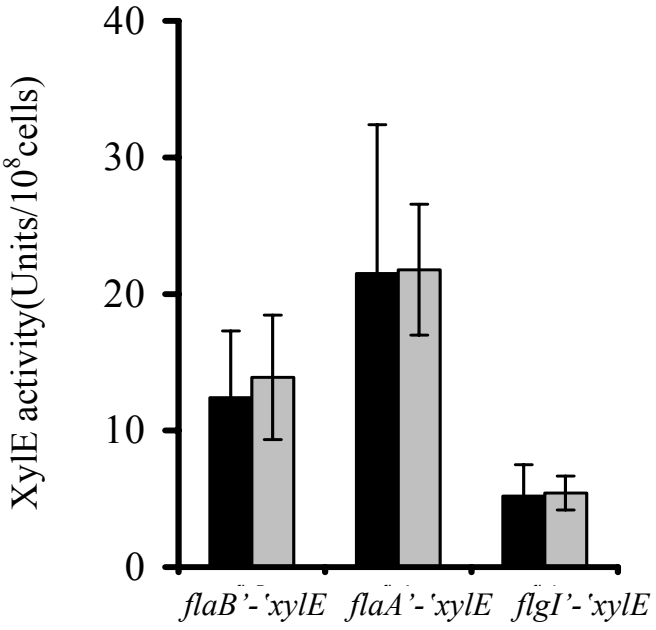
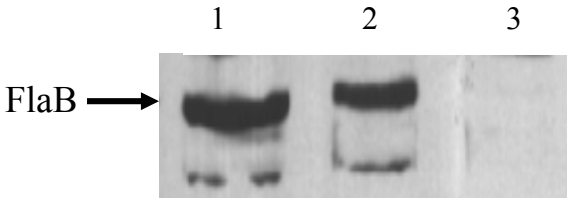

40
30
20
10
0
XylE activity(Units/10^8cells)
flaB'-'xylE
flaA'-'xylE
flgI'-'xylE

Figure 3. Immunoblot for FlaB in *H. pylori* cell extracts. The membrane was probed with antiserum directed against a MBP-FlaB fusion protein. Each lane contained extracts from $1x10^9$ cells. Lane 1, *H. pylori* ATCC 43504; lane 2, Δ*hp0137:cat* mutant; lane 3, *rpoN:cat* mutant.


1
2
3
FlaB

CHAPER IV

ACETONE CARBOXYLASE ENHANCES THE ABILITY OF THE HUMAN GASTRIC PATHOGEN *HELICOBACTER PYLORI* TO COLONIZE MICE[4]

[4] Brahmachary, P., Ge Wang, R. J. Maier and T. R. Hoover. 2004. To be submitted to Infection and Immunity

Abstract

Helicobacter pylori is the etiological agent of peptic ulcer disease in humans. Analysis of the genome sequences of *H. pylori* strains 26695 and J99 revealed a potential operon whose products share homology with the subunits of acetone carboxylase (encoded by *acxABC*) from *Xantobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10. The *acxB* homolog in *H. pylori* SS1 was inactivated with a chloramphenicol-resistance (*cat*) cassette and the resulting *acxB:cat* mutant was characterized. Growth of *H. pylori* SS1, but not the *acxB:cat* mutant, was enhanced by including acetone in the medium. Acetone was consumed in liquid cultures of the wild-type *H. pylori* strain, but not in cultures of the *acxB:cat* mutant, indicating that *H. pylori* SS1 possesses a *bona fide* acetone carboxylase. Relatively high levels of acetone were found to be associated with the gastric tissue of mice, indicating that acetone could be a significant energy source for *H. pylori* in the mouse. The *acxB:cat* mutant displayed an increased sensitivity to acetaldehyde suggesting a role for *H. pylori* acetone carboxylase in the detoxification of this reactive fermentation product. The *acxB:cat* mutant was also compromised in its ability to colonize mice. It is unclear if the colonization defect is due to the inability of the *acxB:cat* mutant to utilize acetone or if it results from the increased sensitivity of the mutant to acetaldehyde.

Introduction

Helicobacter pylori is a microaerophilic, gram-negative bacterium that is an important human pathogen of the gastric mucosa (1, 6). Colonization of the gastric mucosa by *H. pylori* leads to chronic inflammation that can progress to a variety of diseases, including chronic gastritis, peptic ulcer, gastric cancer and mucosal-associated lymphoma (2, 4, 18). In the absence of antimicrobial therapy, the host is likely to suffer a lifetime *H. pylori* infection of the gastric mucosa. The ability of *H. pylori* to persist in the human stomach for extended periods indicates that it is well adapted to acquire the nutrients it needs for growth in this unique niche.

H. pylori is capable of utilizing molecular hydrogen as an energy source (9), and a functional hydrogenase is important for successful colonization of mice by *H. pylori* (15). Several studies have examined the ability of *H. pylori* to utilize various carbon sources. *H. pylori* has a limited ability to acquire and metabolize sugars, an observation that is consistent with the analysis of the genomic sequences of *H. pylori* strains 22695 and J99 (5). Glucose is the only carbohydrate that *H. pylori* is capable of utilizing which it does via the Entner-Doudoroff pathway (12, 13). Pyruvate, a key intermediate in central metabolism, appears to be generated primarily from lactate, alanine and serine, rather than glucose in *H. pylori* (14, 26). Pyruvate is converted to acetyl-CoA by pyruvate oxidoreductase in *H. pylori*, which can then feed into the tricarboxylic acid cycle (8). Alternatively, *H. pylori* can dissimilate pyruvate to form acetate, formate, succinate and lactate (14).

Analysis of the genome sequences of *H. pylori* strains 26695 and J99 revealed a cluster of genes within a 10.2 kb DNA sequence whose products could be used to convert acetone to acetyl-CoA (Fig. 1). Three of these gene products in the *H. pylori* 26695 genome are HP0695, HP0696 and HP0697, and are annotated in The Institute for Genomic Research's database as hydantoin utilization protein A, N-methylhydantoinase, and a hypothetical protein, respectively (http://www.tigr.org/tigr-

scripts/CMR2/GenomePage3.spl?database=ghp). These proteins were subsequently found to share 50-63% amino acid identity with the β, α, and γ subunits, respectively, of acetone carboxylase from *Xanthobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10 (25), enzyme that catalyzes the ATP-dependent carboxylation of acetone to acetoacetate (24). Two other genes within this 10.2 kb DNA cluster in *H. pylori*, *scoA* (*hp0691*) and *scoB* (*hp0692*), encode succinyl CoA:acetoacetate CoA-transferase (SCOT), which converts acetoacetate plus succinyl-CoA to acetoacetyl-CoA plus succinate (3). Acetoacetyl-CoA produced by SCOT can be metabolized further by acetoacetyl-CoA thiolase, which is encoded by *fadA* (*hp0690*), to generate two molecules of acetyl-CoA from acetoacetyl-CoA and CoA (5). Thus, the gene cluster appears to encode all of the enzymes required to convert acetone to acetyl-CoA.

Acetone is one of three ketone bodies produced in humans that result from fatty acid degradation, the other two being acetoacetate and 3-β-hydroxybutyrate. Ketone bodies are produced in the mitochondria of perivenous hepatocytes in the liver and are used as an energy source when carbohydrates are not readily available (7). Acetoacetate and 3-β-hydroxybutryate are produced from the β-oxidation of long chain fatty acids, while acetone is generated by the spontaneous decarboxylation of acetoacetate (7). Thus, acetone should be a readily available carbon or energy source for *H. pylori* in the gastric mucosa.

We sought to determine if the *hp0695-hp0696-hp0697* operon encodes acetone carboxylase and if these genes are important for host colonization by *H. pylori*. Therefore, we inactivated the *acxB* homolog (*hp0696*) in *H. pylori* SS1, a strain which is adapted for colonization of mice, by insertion of a cassette bearing a chloramphenicol transacetylase gene (*cat*) and examined the phenotype of the resulting mutant. Acetone enhanced the growth of cultures of *H. pylori* SS1 and was consumed by the bacterium, but not with the *acxB:cat* mutant, indicating that *H. pylori* SS1 possesses a functional acetone

carboxylase. The *acxB:cat* mutant displayed an increased sensitivity to acetaldehyde, a compound that is structurally similar to acetone, suggesting that *H. pylori* acetone carboxylase recognizes both acetone and acetaldehyde as substrates. The *acxB:cat* mutant was compromised significantly in its ability to colonize mice, which may result from the inability of the mutant strain to utilize acetone or the increased sensitivity of the mutant to acetaldehyde.

Materials and Methods

Bacterial strains and media. Plasmid construction and cloning was done in *E. coli* strain DH5α [Φ80 *lacZ* ΔM15 *recA1gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *supE44 relA1 deoR* Δ(*lacZYA-arg F*) U169] which was cultured in Luria-Bertani medium at 37°C. *H. pylori* strain 26695 was used as the template for polymerase chain reaction (PCR). *H. pylori* strains 43504 and SS1 were used as the wild-type strains for all experiments and were cultured on either blood agar or tryptic soy agar supplemented with 5 % horse serum (TSA-serum) at 37°C under an atmosphere of 4 % O_2, 5 % CO_2 and 91 % N_2. When cultured in liquid medium, *H. pylori* cultures were grown in Mueller-Hinton broth supplemented with 5% horse serum and 30 μg/ml bacitracin in 150-ml serum vials sealed with 20 mm Teflon/silicone discs and aluminum caps and under an atmosphere of 4 % O_2, 5 % CO_2, 10 % H_2, 81 % N_2. Unless indicated otherwise, when antibiotics were included in the medium they were added to the following concentrations: 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 200 μg/ml bacitracin 10 μg/ml vancomycin, and 10 μg/ml amphotericin B.

Inactivation of *acxB* (*hp0696*) in *H. pylori*. A 2.3-kb DNA fragment that carried *acxB* was amplified by PCR from *H. pylori* strain 22695 and cloned into pGEM-T (Promega). A *cat* cassette was introduced into this plasmid at an *Eco*47III site located approximately in the middle of *acxB*. The resulting plasmid was used as a suicide vector for inactivating the chromosomal copy of *acxB* in two different *H. pylori*

strains. The suicide vector was introduced into *H. pylori* strains ATCC 43504 and SS1, a strain that can colonize mice. For both strains, we confirmed by PCR that the chromosomal copy of *acxB* was disrupted by allelic exchange with the plasmid-borne copy of the gene. Because repeated passage of *H. pylori* strain SS1 on medium has been reported to result in loss of infectivity in mice, the *acxB* mutant was constructed in a fresh isolate of strain SS1 recovered from an infected mouse. The number of passages of *acxB* mutant in the SS1 strain was limited and recorded, and the mutant was stored frozen at -80^{o}C. The parental SS1 strain was maintained and stored frozen in an identical manner.

Growth curves for *H. pylori* strains. *H. pylori* cells from TSA-serum plates on which the strains had been streaked on the previous day were suspended in phosphate buffered saline (PBS) and used to inoculate liquid medium at an OD_{600} of 0.03. Where indicated, acetone was added aseptically to the medium. Samples were taken at various times and cell densities were measured by light scattering at OD_{600}. Alternatively, viable cell counts were determined following serial dilution of the samples and plating on TSA-serum. Following 4 to 5 days incubation, the numbers of colony forming units (cfu) were determined for the plates.

Acetaldehyde inhibition assay. *H. pylori* cells from TSA-serum plates were suspended in PBS and used to inoculate Mueller-Hinton broth supplemented with bacitracin in 150-ml serum vials under an an atmosphere of 4 % O_2, 5 % CO_2, 10 % H_2, 81 % N_2 to give cell counts of ~10^8 cfu/ml. Acetaldehyde was added to the cultures at various concentrations and samples were removed at different times following the addition of acetaldehyde. Serial dilutions of the samples were made and plated on TSA-serum. The plates were incubated for 4 to 5 days at which time the number of cfu were determined for each sample. Mean $\log_{10}$ cfu/ml values from three independent assays were calculated to determine the rate of killing versus time. For the filter disc inhibition assay, ~10^9 *H. pylori* cells were plated on TSA-serum and incubated for 1 h, at which time sterile 7.5 mm filter paper discs saturated with neat

acetaldehyde were placed in the centers of the plates. The plates were incubated for 3 days and the zones of inhibition around the discs were recorded.

Acetone utilization assay. *H. pylori* cells from TSA-serum plates were suspended in PBS and used to inoculate Mueller-Hinton broth supplemented with 5 % serum and 6.7 mM acetone to an OD_{600} of 0.1. Cultures were incubated on a rotary shaker at 37°C and samples from the gas phase were taken every 30 min and analyzed with a gas chromatograph equipped with a flame ionization detector (Model GC-8A, Shimadzu). Samples were removed from the head space with 1 ml VICI Pressure-Lok Precision analytical syringe (Precision Sampling) and injected into a DB624 column (30 m x 0.53 mm, 3μm mesh). The injector and the detector temperatures were 250°C and air was used as the carrier at 0.4 kg/cm^2. Retention times were recorded with a Chromatopac (Model CR601, Shimadzu), and the retention time for acetone under these conditions was ~0.61 min. A standard curve was generated by plotting the area of the acetone peak versus concentration for a set of acetone standards prepared in Mueller-Hinton broth supplemented with serum and acetone levels in test samples were estimated from this standard curve.

Measuring acetone levels in mouse stomachs. Stomachs were surgically removed from pre-mortem and post-mortem C57BL/6J mice (Jackson Labs) and placed in small glass vials which were half-filled with glass beads to minimize head space. The vials were sealed with 20 mm Teflon/silicone discs and aluminum caps and placed on ice. Samples from the head space were analyzed by gas chromatography as described above. For each sample a separate acetone standard curve was prepared as follows. The mouse stomach was removed from the vial and washed extensively in ice cold PBS. The stomach was placed back in the vial and 100 μl of a known amount of acetone was applied to the tissue. The vial was resealed and placed on ice, and samples from the head space were analyzed by gas chromatography.

The stomach was removed from the vial and the procedure was repeated with a different known amount of acetone.

Mouse colonization. Mouse colonization assays were performed essentially as described earlier (23). Briefly, *H. pylori* cells were harvested after 48 h of growth on blood agar plates and suspended in PBS to an OD_{600} of 1.7. Headspace in the tube was sparged with argon to minimize oxygen exposure. These suspensions were administered to C57BL/6J mice via oral gavage. The mice were inoculated with *H. pylori* two times (two days apart) with a dose of 1.5×10^8 bacterial cells / mouse. The inoculum dose was determined from reproducible standard curves of OD_{600} versus viable cell number from plate counts. Three weeks after the first injection, the mice were sacrificed and the stomachs were removed, weighed, and homogenized in argon-sparged PBS. Homogenates were diluted serially and the dilutions were plated on blood agar supplemented with vancomycin, amphotericin B and bacitracin (100 μg/ml). The plates were incubated for 5-7 days then examined for *H. pylori* colonies.

Results

Acetone enhances the growth of *H. pylori* SS1. HP0695, HP0696 and HP0697 are predicted to be the β, α and γ subunits, respectively, of acetone carboxylase in *H. pylori* 26695 (25). Based on the predicted assigned function of these proteins, we wished to determine if acetone were utilized by *H. pylori* and if so, did it require the putative acetone carboxylase. We initially examined the growth of *H. pylori* SS1 in medium that was supplemented with acetone to see if acetone stimulated the growth of this strain. *H. pylori* SS1 was chosen for these studies since we ultimately wished to determine if the putative *H. pylori* acetone carboxylase played a role in host colonization, and *H. pylori* SS1 is a mouse-adapted strain that we could use for such studies.

H. pylori SS1 was grown under microaerobic conditions in sealed serum vials in Mueller-Hinton broth supplemented with horse serum and varying amounts of acetone ranging from 1.3 mM to 134 mM. Growth of *H. pylori* SS1 under these conditions was monitored initially by determining viable cell counts at various times. Inclusion of acetone in the growth medium enhanced slightly the final cell yield of the cultures of *H. pylori* SS1 (Fig. 2). Maximal stimulation of cell yield occurred at ~67 mM acetone, but stimulatory effects on cell yield were observed for all of the acetone concentrations that were tested (data not shown). The stimulatory effect of acetone on cell yield required the putative acetone carboxylase since acetone did not enhance the growth yield of a mutant in which the *acxB* homolog had been inactivated (Fig. 2).

We next examined if acetone stimulated the growth rate of *H. pylori* SS1. Growth rates of *H. pylori* cultures were monitored by light scattering during the log growth phase in the absence or presence of 67 mM acetone, the concentration of acetone that gave the highest stimulatory effect on growth yield. Inclusion of acetone in the growth medium stimulated the growth rate of the wild-type *H. pylori* SS1 strain, reducing the generation time from 3.5 h to 2.9 h. In contrast, acetone had no effect on the growth rate of the *acxB:cat* mutant. Unexpectedly, the generation time of the *acxB:cat* mutant was somewhat slower (~4.1 h) than that of the wild-type *H. pylori* SS1 strain. The reason for the slower growth rate of the *acxB:cat* mutant is unclear at this time.

The stimulatory effect of acetone on the growth of *H. pylori* SS1 suggested that this bacterium was able to metabolize acetone as an energy or carbon source. Moreover, the failure of the acetone to enhance growth of the *acxB:cat* mutant indicated that the ability of *H. pylori* SS1 to metabolize acetone was dependent upon a functional acetone carboxylase. To obtain additional evidence that *H. pylori* SS1 could metabolize acetone, we sought to verify that acetone was consumed by cultures of the bacterium. Gas chromatography was used to measure acetone levels in the headspace of the serum vials in which *H.*

pylori SS1 cultures were grown in medium supplemented with acetone. Acetone levels decreased over time for the *H. pylori* SS1 cultures, but not for cultures of the *acxB:cat* mutant (Fig. 3). Taken together, these observations provide strong evidence that *H. pylori* SS1 utilizes acetone as an energy or carbon source and that HP0695, HP0696 and HP0697 constitute a functional acetone carboxylase.

The *H. pylori acxB:cat* mutant has increased sensitivity to acetaldehyde. We reasoned that the growth defect of the *acxB:cat* mutant may have resulted from the inability of the strain to degrade a toxic metabolic intermediate. To test this hypothesis, we compared the sensitivities of the wild-type and mutant *H. pylori* SS1 strains to various compounds, including acetone, ethanol and acetaldehyde. Sterile filter discs were soaked in these compounds and placed in the center of agar plates on which the *H. pylori* strains had been spread previously. After incubating the plates for 3 days, zones of inhibition around the filter discs were measured. No differences in the zones of inhibition for the wild-type and mutant strains were observed for acetone and ethanol (data not shown). With acetaldehyde, however, the zones of inhibition were significantly larger for the *acxB:cat* mutant (Table 1). One reason for the variability seen in the data from the filter disc assays may have been due to the volatility of acetaldehyde, which has a boiling point of 21^{o}C. Because of this variability, we compared the sensitivities of the wild-type and *acxB:cat* mutant strains in liquid cultures.

H. pylori cells were exposed to various concentrations of acetaldehyde and the number of surviving cells was determined in these cultures at different times. Acetaldehyde reduced the viability of both the wild-type *H. pylori* SS1 strain and the *acxB:cat* mutant at micromolar concentrations (Fig. 4). The effective killing of the *acxB:cat* mutant strain by acetaldehyde, however, was at least 5- to 10-fold greater than that of the wild-type strain, consistent with the increased sensitivity of the mutant strain in the filter disc assays.

The *H. pylori acxB:cat* mutant is deficient in its ability to colonize mice. The ability of the *acxB:cat* mutant to colonize mice was compared with that of the parental *H. pylori* SS1 strain in two separate trials. In each trial, eleven mice were inoculated with the wild-type strain and eleven were inoculated with the *acxB:cat* mutant. Three weeks post-inoculation of the mice with the *H. pylori* strains, the mice were sacrificed and the numbers of *H. pylori* in the stomachs of the animals were determined. For the mice that had been inoculated with the wild-type strain, most of the animals (19/22 animals) had *H. pylori* counts that were well above the detection limit, which was 500 cfu per gram stomach (Fig. 5). The number of *H. pylori* in samples that were above the detection limit ranged from $10^4 – 10^6$ cfu per gram stomach. Most of the mice inoculated with the *acxB:cat* mutant also had measurable levels of *H. pylori* (15/22 animals), but the numbers of *H. pylori* associated with these mice were generally one to two orders of magnitude lower than those in mice that had been inoculated with the wild-type strain. A statistical analysis of the data using a Wilcoxin Rank test verified that the differences in the numbers of *H. pylori* isolated from mice inoculated with the two strains were significant at the 99% confidence level. Thus, these data clearly demonstrated that the presence of a functional acetone carboxylase enhanced the ability of *H. pylori* SS1 to colonize the mouse stomach.

Since these data suggested that acetone utilization by *H. pylori* was important for effective host colonization, we wished to determine if *H. pylori* normally encountered acetone levels in the mouse stomach that were high enough for it to be a significant energy source. Therefore, we measured acetone levels associated with mouse gastric tissue after removing the stomachs from mice and placing the stomachs in sealed serum vials. These vials were incubated on ice to allow the acetone associated with the gastric tissue to equilibrate with the gas phase in the vials, after which time gas phase samples were analyzed by gas chromatography for acetone. This procedure was done initially by sacrificing the animals and then removing their stomachs. Similar results, however, were obtained by removing the

stomachs from live animals which had been anesthetized. The amount of acetone associated with gastric tissue for each individual mice varied greatly, ranging from ~10 to 110 μmols acetone per gram wet weight tissue (Fig. 6). Most of the values (6/7), however, were closely clustered and fell between the range of 10 and 35 μmols acetone per gram wet weight tissue. These data clearly indicate that significant amounts of acetone are associated with mouse gastric tissue and could be available as a potential energy source for *H. pylori*.

Discussion

We show here that the growth of *H. pylori* SS1 is enhanced by acetone and that acetone is consumed by cultures of this bacterium. In contrast, acetone did not stimulate the growth of a *H. pylori acxB:cat* mutant nor was acetone consumed by cultures of this strain. These data provide strong evidence that *H. pylori* possess a functional acetone carboxylase as postulated previously by Ensign and his colleagues (25). The *H. pylori acxABC* operon is located within a cluster of genes that encode two enzymes, SCOT and acetoacetyl-CoA thiolase, which are capable of metabolizing acetoacetate formed from the carboxylation of acetone to acetyl-CoA. Functions for two of genes within this cluster, *hp0693* (*jhp0635*) and *hp0694* (*jhp0634*), have not been assigned. The predicted product of *hp0693* shares homology with short chain fatty acid permeases, and so it may function in the transport acetoacetate for catabolism.

H. pylori is a member of the ε-subdivision of Proteobacteria. The genes within the acetone metabolism gene cluster are not found in other ε-Proteobacteria whose genomes have been sequenced so far, which includes *Helicobacter hepaticus*, *Campylobacter jejuni* and *Wolinella succinogenes*. Interestingly, the acetone metabolism gene clusters in *H. pylori* 26695 and J99 are within the same locus (i.e., located between *feoB* and *dgkA*) but are oriented in opposite directions (Fig. 1). This suggests that

the gene cluster was at one time part of a mobile DNA element that was integrated into the genomes of these bacterial strains. Two genes coding for a predicted type II DNA methyltransferase and type II restriction enzyme are juxtaposed to the acetone metabolism gene cluster in *H. pylori* J99 but not in *H. pylori* 26695. Transposable elements often carry genes coding for DNA restriction/modification enzymes, and so the genes encoding these enzymes in *H. pylori* J99 may have been incorporated into the genome of this strain along with the genes involved in acetone metabolism. We were unable to identify direct repeats flanking the acetone metabolism gene cluster that might have resulted from duplication of the integration site during transposition of a mobile DNA element into the *H. pylori* genome, but these repeats could have been lost or obscured through accumulated mutations.

The absence of the acetone metabolism gene cluster in other ε-Proteobacteria suggests that *H. pylori* acquired these genes after it diverged as a separate species. One reason why *H. pylori* gained the capacity to metabolize acetone whereas other closely related pathogens did not may be related to the unique niche occupied by this bacterium. The low pH inside the stomach may favor the accumulation of acetone. To our knowledge acetone concentrations associated with gastric tissue have not been reported. The acetone levels associate with mouse gastric tissue that we estimated were much higher (>10-fold) than what we had expected based on the reported plasma acetone concentrations in humans. Acetoacetate is always present in the blood at low levels with normal serum levels usually <1 mM, and plasma acetone concentrations are generally slightly greater than this value (10, 16, 17). It is possible that acetone accumulates in the stomach due to the gastric acidity. Regardless of the reason, our data suggest that acetone represents a significant energy source for *H. pylori* in the mouse stomach, and by inference also in the human host.

An unexpected phenotype of the *acxB:cat* mutant was its increased sensitivity to acetaldehyde, which is a highly reactive and toxic fermentation product. Acetaldehyde is very similar to acetone in

structure, having a hydrogen atom in place of a methyl group. We postulate that in addition to using acetone as a substrate, *H. pylori* acetone carboxylase can also use acetaldehyde as a substrate, which would help to detoxify this fermentation by-product. This would distinguish the *H. pylori* enzyme from the *X. autotrophicus* acetone carboxylase which does not recognize acetaldehyde as a substrate (24). *H. pylori* could encounter significant levels of acetaldehyde in the stomach as a result of oxidation of ethanol by microbes in the normal gut flora or somatic cells (20). Alternatively, acetaldehyde could be generated endogenously by *H. pylori* since it possesses an alcohol dehydrogenase that catalyzes the oxidation of ethanol to acetaldehyde but lacks an aldehyde dehydrogenase that could catalyze the further oxidation of acetaldehyde to acetate (5, 21, 22). Acetaldehyde inhibits gastric mucosal regeneration and forms stable adducts with mucosal proteins (11), and localized production of acetaldehyde by *H. pylori* has been suggested to contribute to the pathogen-gastroduodenal injury associated with *H. pylori* infection (19, 22).

Carboxylation of acetaldehyde by *H. pylori* acetone carboxylase would yield the predicted product malonate semialdehyde, which being an aldehyde would be very reactive. Thus, *H. pylori* likely requires additional enzymes for the further metabolism of malonate semialdehyde. Consistent with this hypothesis, inactivation of *scoB* (encodes the β-subunit of SCOT) results in *H. pylori* being even more sensitive to acetaldehyde (P. Brahmachary, unpublished data), suggesting that SCOT metabolizes the malonate semialdehyde resulting from the carboxylation of acetaldehyde. Further investigation is required to determine if *H. pylori* acetone carboxylase is capable of carboxylating acetaldehyde and how the product of this reaction is metabolized further.

The *acxB:cat* mutant was significantly reduced in its ability to colonize the mouse stomach. At present, we do not know if the defect in colonization of the *acxB:cat* mutant is due to the inability of the mutant to utilize acetone, the increased sensitivity of the mutant to acetaldehyde, or some other reason.

Further characterization of *H. pylori* acetone carboxylase and the functions of the genes that are in the acetone metabolism cluster, however, should provide insight into how acetone carboxylase enhances the ability of *H. pylori* to colonize its host.

Acknowledgements: This work was supported by award AI056036 to T. R. H. from the National Institutes of Health.

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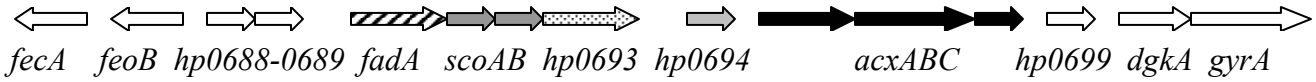
Table 1. Inhibition of *H. pylori* growth by acetaldehyde as assessed in filter disc assays.

	zones of inhibition (mm)[1]	
	H. pylori SS1 (wild-type)	*acxB:cat* mutant
Experiment #1	29.3 ±6.6	65.1 ± 25
Experiment #2	11.5 ± 2.3	43.2 ± 15

[1]Values represent the averages of four separate measurements for each experiment, with the standard deviations indicated for each mean value.

Figure 1. Organization of the genes involved in acetone metabolism in *H. pylori* strains 26695 and J99. The filled arrows designate genes within the acetone metabolism cluster while the open arrows designate the surrounding genes. The figure is not drawn to scale. Gene designations are indicated under each arrow. Note that the acetone metabolism clusters are in opposite orientations in the two strains. Genes that were not assigned a gene designation in the annotated genome sequences are indicated with either an *hp* designation (for the *H. pylori* 26695 genome) or a *jhp* designation (for the *H. pylori* J99 genome). Gene designations in parenthesis that appear under the *H. pylori* J99 sequence indicate the corresponding gene in *H. pylori* 26695. In *H. pylori* J99, *jhp0628* is a fusion of *hp0688* and *hp0689*. *H. pylori* J99 has two genes within this region, *jhp0628* and *jhp0629*, that encode a type II DNA methyltransferase and a type II restriction enzyme, respectively, and are not found in *H. pylori* 26695. Functions of the products of the genes within the acetone metabolism cluster are described in the text. The proposed functions of the products of the surrounding genes are: *fecA*, iron(III) dicitrate transport protein; *feoB*, iron(II) transport protein; *dgkA*, diacylglycerol kinase; and *gyrA*, subunit A of DNA gyrase.

***H. pylori* 26695**



***H. pylori* J99**

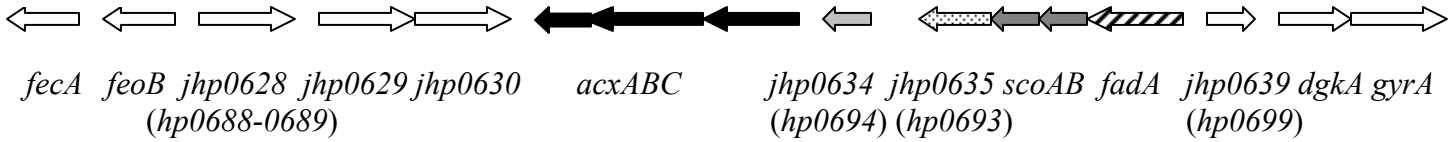


Figure 2. Effect of acetone on growth of *H. pylori* SS1 and an isogenic *acxB:cat* mutant. Wild-type *H. pylori* SS1 and the *acxB:cat* mutant strain were grown in Mueller-Hinton broth supplemented with 5% horse serum in the presence or absence of 67 mM acetone. Samples were taken at various times and the number of cfu/ml was determined for each sample. Sample points for constructing the growth curves are labeled as follows: wild-type *H. pylori* SS1 in absence of added acetone (diamonds); wild-type *H. pylori* SS1 in the presence of acetone (squares); *acxB:cat* mutant in absence of added acetone (circles); *acxB:cat* mutant in the presence of acetone (triangles).

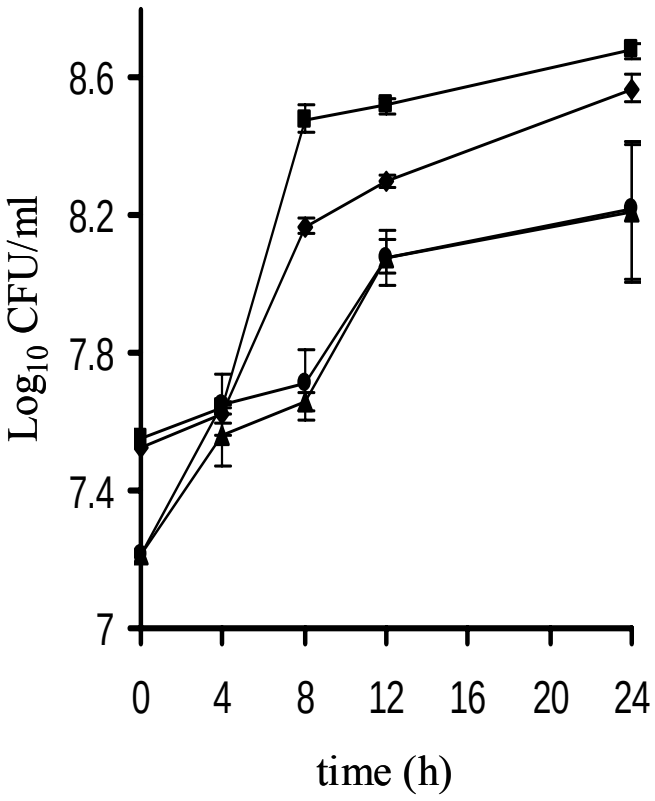

8.6
8.2
7.8
7.4
7
Log10 CFU/ml
0
4
8
12
16
20
24
time (h)

Figure 3. Consumption of acetone by *H. pylori* SS1. Mueller-Hinton broth supplemented with 5 % horse serum and 6.7 mM acetone was inoculated with either *H. pylori* SS1 (diamonds) or the *acxB:cat* mutant (squares) to give an OD_{600} of 0.1 in 150-ml sealed serum vials. Gas samples were removed from the head space at various times and assayed for acetone by gas chromatography to estimate the amount of acetone remaining in each culture vessel. At least three readings were determined for each time point and averaged. Error bars indicate the standard deviations for the mean values for each time point.

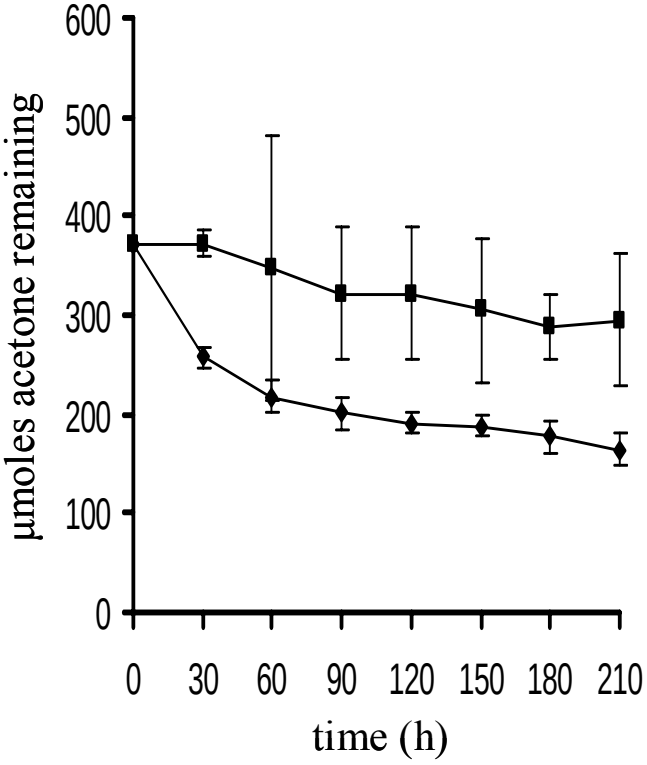

600
500
400
300
200
100
0
µmoles acetone remaining
0
30
60
90
120
150
180
210
time (h)

Figure 4. Sensitivity of *H. pylori* SS1 and an isogenic *acxB:cat* mutant strain to acetaldehyde. Wild-type *H. pylori* SS1 and the *acxB* mutant were inoculated in Mueller-Hinton broth supplemented with bacitracin to give cell count of ~ 10^8 cfu/ml. Acetaldehyde at different concentrations was added to the medium. Samples were taken at various times and the number of cfu/ml was determined for each sample. Mean and standard deviation from three independent observations is plotted. Sample points are as follows: *H. pylori* SS1 in presence of 87.5 μM acetaldehyde (squares); *H.pylori* SS1 in presence of 175 μM acetaldehyde (diamonds); *acxB:cat* mutant in presence of 87.5 μM acetaldehyde (circles); *acxB:cat* mutant in presence of 175 μM acetaldehyde (triangles).

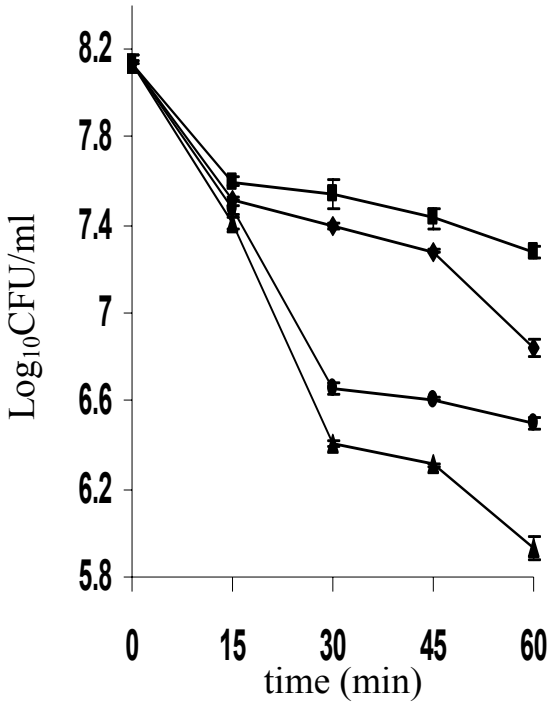

8.2
7.8
7.4
7
6.6
6.2
5.8
Log10CFU/ml
0
15
30
45
60
time (min)

Figure 5. Mouse colonization assay of *H. pylori* SS1 and an isogenic *acxB:cat* mutant strain. Data are presented as a scatter plot of colony forming units per gram of stomach as determined by plate counts. Each spot represents the cfu count from one mouse, expressed as the value of $\log_{10}$ (cfu/g stomach) in the Y-axis. The base line [$\log_{10}$ (cfu/g stomach) =2.7] is the detection limit of the assay, which represents the count below 500 cfu/g stomach.

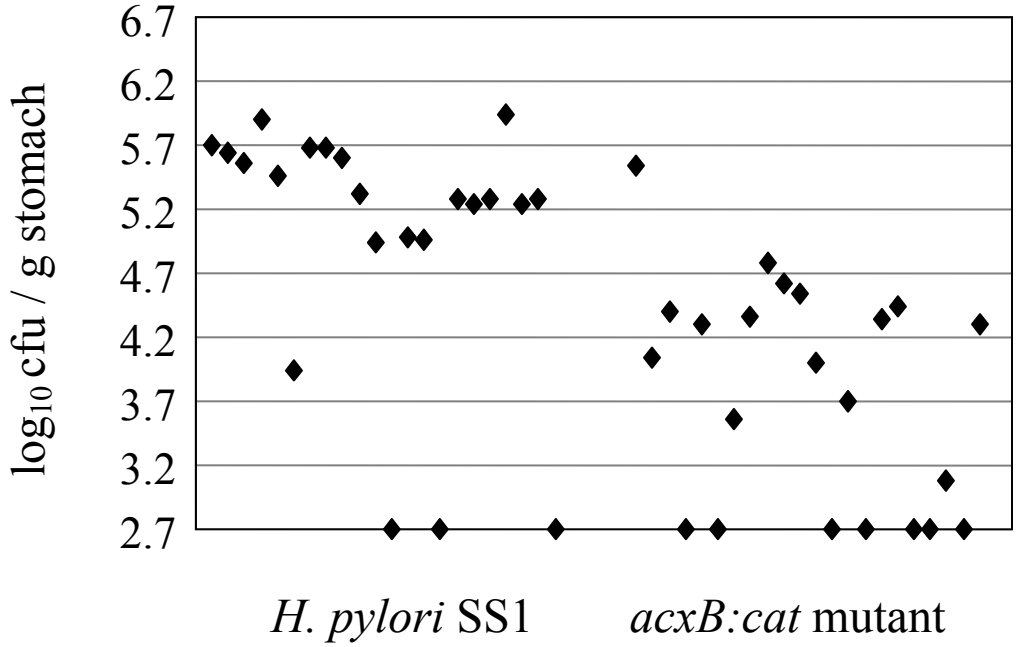
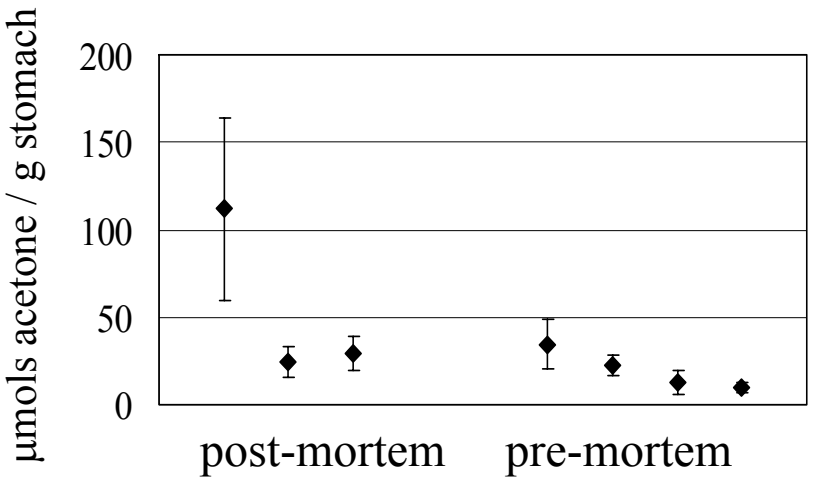

6.7
6.2
5.7
5.2
4.7
4.2
3.7
3.2
2.7
log10 cfu / g stomach
H. pylori SS1
acxB:cat mutant

Figure 6. Acetone levels associated with mouse gastric tissue. Levels of acetone in the stomachs were determined for several uninfected mice. For sample numbers 1 through 3, acetone levels associated with mouse gastric tissue were determined after sacrificing the animals and immediately removing their stomachs. For samples 4-7, the mice were anesthetized and then their stomachs were removed. Mouse stomachs were placed in sealed vials and the amounts of acetone in the gas phases in the vials were measured by gas chromatography after allowing the acetone associated with the gastric tissue to equilibrate with the gas phase. Each value represents an average of at least three measurements and error bars indicate the standard deviations for each sample.


µmols acetone / g stomach
200
150
100
50
0
post-mortem
pre-mortem

CHAPTER V

SUMMARY

Helicobacter pylori, is an important human pathogen in terms of suffering and financial burden associated with the diseases for which it is responsible. Identifying new targets for the control of *H. pylori* is essential to begin to reduce the morbidity and mortality associated with *H. pylori* infections. As has been shown in various studies, motility is an important virulence factor in *H. pylori* (2). Characterization of the mechanisms that govern flagellar gene regulation in *H. pylori* can lead to a better understanding of how this pathogen establishes and maintains a chronic infection of the gastric mucosa and may lead to the identification of novel targets for the control and detection of *H. pylori*.

As indicated previously, flagellar gene regulation in *H. pylori* is complex and involves all the three σ factors found in this bacterium. Genes encoding some components of the basal body, the hook protein and the minor flagellin are dependent on σ^{54} as well as a two-component system consisting of a sensor kinase, FlgS and its cognate response regulator FlgR (5). An unusual feature of FlgR is that it lacks the DNA-binding domain found in most other σ^{54}-activators. Using reporter gene constructs in *H. pylori*, I demonstrated that as little as 42 bp of DNA upstream of the promoter was sufficient for efficient transcription of the σ^{54}-dependent *flaB* gene. I also showed that the purified AAA+ domain of FlgR could function with σ^{54}-holoenzyme from enteric bacteria, both *in vivo* and *in vitro*, to activate transcription from the *S. enterica* serovar Typhimurium σ^{54}-dependent *glnA* promoter. Since the length of DNA sequence upstream of the *flaB* promoter is too short to accommodate an enhancer and the *S. enterica* serovar

Typhimurium *glnA* promoter regulatory region is not likely to harbor a binding site for *H. pylori* FlgR, these findings suggest that FlgR does not bind enhancer to activate transcription. Rather, FlgR likely binds to σ^{54}-holoenzyme directly, either before or after formation of the closed promoter complex, to activate transcription. I estimated the levels of FlgR in *H. pylori* by western blotting and found these to be slightly higher than those reported for other σ^{54}-dependent activators that possess C-terminal DNA-binding domains. It is possible that higher concentrations of FlgR in *H. pylori* are needed to compensate for the absence of enhancer binding and allow efficient oligomerization of the protein to activate transcription.

Lack of DNA-binding domain is not restricted to *H. pylori* FlgR. All of the chlamydia species that have been sequenced to date, have a σ^{54}-dependent activator that lacks a C-terminal DNA-binding domain. Further characterization of *H. pylori* FlgR and how it functions in the absence of enhancer binding is likely to yield insights into how these chalmydial activators function. Morever, the absence of DNA-binding activities in these proteins may make them more susceptible to factors that interfere with transcriptional activation (e.g., by inhibiting oligomerization of the activator or interactions with σ^{54}-holoenzyme), and so these activators represent potential new targets in these pathogens.

An important tool developed for regulation studies in *H. pylori* is a protein-protein interaction map constructed from a high-throughput screen of a yeast two-hybrid system (3). Although interactions observed in the yeast-two hybrid assay are not always physiologically relevant, such interactions can often lead to the identification of novel regulatory proteins. An example of this is the identification of *H. pylori* FlgM based on its interaction with σ^{28} in the yeast two-hybrid assay. FlgS interacted with the hypothetical protein HP0137 in the protein-protein interaction map. In order to determine if HP0137 was essential in flagellar biosynthesis, I

constructed a *hp0137* deletion mutant. The resulting mutant was non-motile on soft agar motility plates and lacked flagella when observed by electron microscopy. Expression of the σ^{54} (and FlgS)-dependent *flaB* was unaffected in the mutant, however, as assessed by western blotting and with a *flaB'-'xylE* reporter gene. Thus, HP0137 is not required for FlgS function. HP0137 was not required for expression of a *flaA'-'xylE* reporter gene, which is σ^{28} -dependent, or a *flgI'-'xylE* reporter gene, which is σ^{80} -dependent.

These data suggest that HP0137, a novel protein is required for flagellar assembly but is not essential for flagellar gene expression. It is possible that HP0137 plays a role in the export of one or more flagellar subunits. HP0137 possesses a potential single membrane spanning region and it will be interesting to determine if HP0137 is located to the flagellated pole of *H. pylori*. The interactions between HP0137 and FlgS in the yeast two-hybrid may be coincidental. Alternatively, HP0137 may have a role in switching off expression of the genes within the RpoN regulon through its interactions with FlgS, but this effect could be obscured by other factors regulating the RpoN regulon. Further studies on the role of HP0137 in flagellar biogenesis and regulation of the RpoN regulon in *H. pylori* should help resolve these issues.

Another novel aspect about *H. pylori* physiology which I examined that could lead to new strategies for the control of this pathogen is acetone utilization achieved via a putative acetone carboxylase. It is likely that *H. pylori* experiences significant levels of acetone in the human stomach as it is one of the ketone bodies and is produced by the spontaneous decarboxylation of acetoacetate under acidic conditions. Consistent with this hypothesis, I was able to detect substantial levels of acetone associated with the gastric tissue of mice. Analysis of the database revealed that *H. pylori* has genes that share homology with the acetone carboxylase (*acxABC*) genes. A couple of lines of evidence argue strongly that these *H. pylori* genes encode a

functional acetone carboxylase. First, acetone stimulated the growth of wild-type *H. pylori* but not that of an *acxB* mutant strain. Second, acetone was consumed by cultures of wild-type *H. pylori* but not by cultures of the *acxB* mutant. I postulate that acetone is converted to acetoacetate by acetone carboxylase, but is metabolized further to acetyl-CoA by enzymes encoded by genes located near the *acx* locus. The acetyl-CoA generated by these reactions could be used for biosynthesis and energy production throughout the tricarboxylic acid cycle, which may account for the enhanced growth of wild-type *H. pylori* in the presence of acetone.

An unexpected finding for the *acxB* mutant was the increased sensitivity to acetaldehyde, a compound that is structurally similar to acetone. Aldehydes are very reactive and I postulate that *H. pylori* acetone carboxylase plays a role in detoxification of acetaldehyde, which is a common fermentation product. Based on the carboxylation of acetone, I predict that the product resulting from the carboxylation of acetaldehyde would be malonate semialdehyde. If this is indeed how acetone carboxylase contributes to the detoxification of acetaldehyde then there must be other enzymes that further metabolize the malonate semialdehyde since this compound is an aldehyde and would be expected to be highly reactive. Consistent with this hypothesis, I found that inactivation of *scoB* (encodes one of the subunits of SCOT-succinyl CoA:acetoacetate CoA transferase) in *H. pylori* 43504 increased the sensitivity of *H. pylori* to acetaldehyde even further (unpublished data). These data suggest that SCOT is also a part of the pathway for acetaldehyde detoxification. CoA transferases such as SCOT often have broad substrate specificities (1), and so it is not unusual to speculate possible roles for SCOT in both acetone and acetaldehyde metabolism. In contrast, acetone carboxylase from *Xanthobacter* strain Py2 was shown to be specific for acetone (4). Thus, if *H. pylori* acetone carboxylase is able to utilize both acetone and acetaldehyde as substrates, this may be distinguish it from other acetone carboxylases.

The *acxB* mutant was deficient in its ability to colonize mice. At this point, it is unclear if the colonization defect was due to the inability of the mutant to utilize acetone or if it was related to the increased sensitivity of the mutant to acetaldehyde. Regardless of which reasons accounted for the colonization defect, acetone carboxylase and SCOT appear to be excellent targets for new therapeutics for controlling *H. pylori* since these enzymes are relatively unique to this pathogen.

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