Functional Analysis of the Helicobacter pylori Flagellar Switch Proteins

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Helicobacter pylori uses flagellum-mediated chemotaxis to promote infection. Bacterial flagella change rotational direction by changing the state of the flagellar motor via a subcomplex referred to as the switch. Intriguingly, the H. pylori genome encodes four switch complex proteins, FliM, FliN, FliY, and FliG, instead of the more typical three of Escherichia coli or Bacillus subtilis. Our goal was to examine whether and how all four switch proteins participate in flagellation. Previous work determined that FliG was required for flagellation, and we extend those findings to show that all four switch proteins are necessary for normal numbers of flagellated cells. Furthermore, while fliY and fliN are partially redundant with each other, both are needed for wild-type levels of flagellation. We also report the isolation of an H. pylori strain containing an R54C substitution in fliM, resulting in bacteria that swim constantly and do not change direction. Along with data demonstrating that CheY-phosphate interacts with FliM, these findings suggest that FliM functions in H. pylori much as it does in other organisms.

Flagellar motility is important for gastric colonization by the ulcer-causing bacterium Helicobacter pylori and also for suborgan localization within the stomach (16–18, 33, 45). Flagellar motility is regulated by a set of signal transduction proteins, collectively referred to as the chemotaxis pathway, that control the migration of microbes in response to environmental cues. This pathway is well elucidated in organisms such as Escherichia coli, Salmonella enterica serovar Typhimurium (referred to hereinafter as S. Typhimurium), and Bacillus subtilis. Sequence analysis of the genomes of other flagellated bacteria, including H. pylori, has suggested that there is diversity in the set of chemotaxis proteins that a particular microbe contains. Here we analyze the diversity of H. pylori’s flagellar switch proteins, which control flagellar rotational direction.

The molecular mechanisms underlying chemotactic signal transduction in E. coli and S. Typhimurium have been extensively studied (7, 50). The overall function of this pathway is to convert the perception of local environmental conditions into a swimming response that drives bacteria toward beneficial conditions and away from harmful ones. Such migration is accomplished by interspersing straight, or smooth, swimming with periods of random reorientations or tumbles. Smooth swimming occurs when the flagella rotate counterclockwise (CCW), while reorienting occurs when the flagella rotate clockwise (CW). The chemotaxis signal transduction system acts to appropriately alter flagellar rotation. The canonical chemotaxis pathway consists of a chemoreceptor bound to the coupling protein CheW, which is in turn bound to the histidine kinase CheA. If a beneficial/attractant ligand is not bound (or a repellant is bound) to the chemoreceptor, CheA autophosphorylates and passes a phosphate to the response regulator CheY. Phosphorylated CheY (CheY-P) interacts with a protein complex called the flagellar switch (discussed at more length below). This interaction causes a switch in the direction of flagellar rotation from CCW to CW, thus reorienting the cells, via an as-yet-unknown mechanism (reviewed in references 23 and 29).

Bacterial flagella are complex, multiprotein organelles (reviewed in references 23, 25, and 29). Each flagellum is composed of several parts, including the filament, the hook, and the basal body (listed from outside the cell to inside the cytoplasm). The flagellar basal body spans from the outer membrane to the cytoplasm and is responsible for rotating the flagellum. This part of the flagellum is further made up of several subassemblies that are named for their locations. The innermost is called the switch or C ring, based on its location in the cytoplasm. The switch is comprised of three proteins in E. coli, FliM, FliN, and FliG (reviewed in references 23 and 29). Experimental evidence strongly suggests that these proteins, along with the stator proteins MotA and MotB, drive motor rotation, because one can obtain point mutations in these proteins that disrupt rotation but not flagellation. Null mutations, however, in fliM, fliN, or fliG also result in aflagellated cells, a phenotype that has been proposed to arise because these proteins are needed to complete the flagellar export apparatus (23).

There is extensive structural information about each of the switch proteins and their arrangement in the flagellum (reviewed in references 23 and 29, with additional key references

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§ Supplemental material for this article may be found at http://jb.asm.org.
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added below). There are 26 copies of FliG, 34 copies of FliM, and \( \sim 136 \) copies of FliN, arranged in a circular structure at the base of each flagellum. FliM is positioned between FliG and FliN and interacts with both. FliM also binds CheY-P via sequences in the first 16 amino acids, and elsewhere (15), to play a key role in switching flagellar rotation direction. FliG, the switch protein closest to the cytoplasmic membrane, interacts with the stator protein MotA, the FliF membrane protein that forms the flagellar basal-body MS ring, and the membrane-bound respiratory protein fumarate reductase (11). FliG has the most direct role in creating flagellar rotation. FliN is the most cytoplasmic component of the switch, and its role is not fully understood. FliN may play a role in switching by possibly binding CheY-P directly (36) and an additional role in flagellar assembly, because it binds to the flagellar export protein FliI and localizes it, along with its interaction partners FliG and FliJ, to the flagellum (20, 28, 36). FliN contains significant sequence similarity to secretion proteins of type III secretion systems of *Yersinia pestis* and *Shigella flexneri*. The conserved domain comprises most of FliN and is called a SpoA domain (37). Other FliN homologs include YscL and Spa33 (25).

The flagellar switch of another well-studied chemotactic microbe, *B. subtilis*, differs slightly in its protein makeup from that of *E. coli*. *B. subtilis* contains FliM and FliG, which function similarly to their *E. coli* counterparts, but instead of FliN it has a protein called FliY (6, 42). FliY of *B. subtilis* has two functional domains, one of which is homologous to *E. coli* FliN, while the other shares similarity with the *B. subtilis* chemotaxis protein CheC, which functions to dephosphorylate CheY-P. FliY is the most active known phosphatase of CheY-P in *B. subtilis* (40, 41).

*H. pylori* contains homologs of many of the chemotaxis and flagellar genes found in other organisms (32, 48). Curiously, its genome encodes four predicted flagellar switch proteins, FliG, FliM, and both FliY and FliN, although FliY was not annotated in the original genome analysis. Previous work had determined that *H. pylori* strain SS1 lacking fliG was aflagellated (1), but the other switch proteins had not been analyzed. As noted above, FliN and FliY share a FliN domain and so could have functional redundancy. FliY and fliM appear to reside in an operon, suggesting that the two encoded proteins function together (see Fig. 1 in the supplemental material).

Since having all four flagellar switch proteins in one microbe is unusual, we were curious as to whether all four serve “switch” functions. As noted above, fliM and fliG deletions typically result in an aflagellated phenotype in other organisms. Others had previously shown that fliG mutations have this phenotype in *H. pylori* (1), and we additionally show here that fliM null mutants are also almost completely aflagellate. In spite of a shared domain that might indicate functional redundancy, we show that fliN and fliY are each necessary for normal numbers of flagellated cells. Finally, we characterize a fliM point mutant that results in a lock-smooth swimming bias and demonstrate physical interaction between CheY-P and FliM, indicating that FliM responds to CheY signaling in *H. pylori* in a manner similar to that found in *E. coli*, *S. Typhimurium*, *B. subtilis*, and other studied organisms.

### Table 1. Strains and plasmids used in this study

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* Designations in parentheses are lab strain numbers.

**Materials and Methods**

**Bacterial strains.** The motile, human, wild-type *H. pylori* isolate G27 or its derivative NSH57 was used for all experiments. *E. coli* strain DH10B was used for cloning, and BL21 was used for protein expression. All strains and plasmids are listed in Table 1.

**Growth media and chemicals.** For solid-medium culture, *H. pylori* was grown on Columbia blood agar with 5% defibrinated horse blood and *H. pylori*-selective antibiotics (CHBA) as described previously (33). For liquid culture, *H. pylori* strains were grown in brucella broth (Becton Dickinson) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) (BB10) as described previously (33). For the selection of mutants, chloramphenicol was used at 5 to 10 \( \mu \text{g} / \text{ml} \) (*H. pylori*) or 20 \( \mu \text{g} / \text{ml} \) (*E. coli*), kanamycin was used at 15 \( \mu \text{g} / \text{ml} \), and sucrose was used at 6%.

For large-scale liquid growth of *H. pylori*, we adapted the methods of Deshpande et al. (13) for growth in a Bioflo 110 fermentor (New Brunswick Scientific) with a 7-liter vessel and 4 to 6 liters of BB10. A set point of 50% relative oxygen contents, respectively. CO 2 content was monitored via the effect on transcription (32). Amplification of DNA was carried out using Amplification of DNA was carried out using *Pfu* or *Pfu*-Turbo polymerase (Stratagene) or *Taq* polymerase (generous gift of D. Kellogg). DNA sequencing was performed by the UC Berkeley sequencing facility or the

**Plasmids.**

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<td>pBS-FliY::catmut</td>
<td>ΔfliY(28-854):cat</td>
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* Designations in parentheses are lab strain numbers. cat, cat mutant lacking its transcriptional terminator.

**References**

FHRC Genomics shared resource and analyzed using Sequencer (Gene Codes Corporation, Ann Arbor, MI).

**Purification of CheY and antibody production.** cheY was cloned from *H. pylori* G27 chromosomal DNA using primers H1-cheY-for and CheY-R1rev-r (see Table S1 in the supplemental material), cut with BamHI and EcoRI, and ligated into pGEX-6P2 cut with the same enzymes to create pGEX-HpCheY. GST-CheY was overexpressed in *E. coli* BL21 (DE3) at room temperature using 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were collected by centrifugation and ground to a fine powder with a mortar and pestle under liquid nitrogen. Cells were lysed by adding 5 volumes of buffer [phosphate-buffered saline plus 1.0 M NaCl, 0.5% Tween 20, 10 mM dithiothreitol (DTT)] and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Sigma-Aldrich or Calbiochem) and sonicated. Protein was purified using a GSTPrep FF 16/10 GST affinity column (GE Biosciences), using standard methods. GST was removed using PreScission protease (GE Biosciences) per the manufacturer’s instructions, followed by chromatography over the GST affinity column to remove GST and PreScission protease. Peak fractions containing *H. pylori* CheY (CheYHP) were concentrated by centrifugation with Centrifloc (Millipore) filters with a 3,000 molecular-weight cutoff. A 1.5 mg sample was collected to Animal Pharm for rabbit inoculation. This rabbit serum is hereinafter referred to as anti-CheY, with the fourth and final bleedings being used in this study. We verified the specificity of this antibody using wild-type *H. pylori* and several cheY mutants (data not shown).

**Purification of *H. pylori* GST-FliM.** *H. pylori* fliM was cloned from strain J99 genomic DNA using primers FliM-For-BamHI and FliM-REV-XHO1 (see Table S1 in the supplemental material) into the BamHI and XhoI sites of pGEX-4T1 to create pGEX-HpFliM. Purification was carried out as described for CheY above, except with slight modifications. To avoid the creation of insoluble inclusion bodies, cells were grown at room temperature and then transferred to 15°C for 18 h on CHBA. Grids with associated electron micrographs were visualized and modified using UCSF Chimera (http://www.cbl.ucsf.edu/chimera). As query sequences, we used CheC domain sequences containing which of these also bore flin sequences, using *H. pylori* Flin as the query protein.

**Cloning and mutating flin, flinA, and flinY.** Each flin gene and approximately 300 bp of the flanking region were amplified using PCR with genomic DNA from strain J99 and cloned into pBluescript (pBS), using the following primers: for flin, FlinMlocusfor and FlinMlocusrev; for flinA, FlinNlocusfor and FlinNlocusrev; and for flinY, FlinFlocusfor and FlinYlocusrev. Each PCR product was gel purified and treated with T4 kinase (New England Biolabs). These products were then cloned into EcoRV-cut pBS (Stratagene), creating the vectors pBS-Flin, pBS-FlinA, and pBS-FlinY. These plasmids were then used as templates in inverse PCR to create flinY (flin double mutants, *H. pylori* G27 ΔflinY::cat was transformed with ΔflinY::aphA3 cassette.

**Mapping and restoration of NSH57/ flin mutation.** Sequence polymorphisms between NSH57 and G27 were queried by amplifying and sequencing candidate genes HPG27_1004 (cheY3), HPG27_1005 (cheZ/cheY fusion), HPG27_1006 (cheW), HPG27_397 (fliM) from NSH57 genomic DNA and comparing them to their published BLASTn sequences. The NSH57 genomic DNA was amplified using primers flankfliM-up and kanupComp-fliMupdo; and flankfliM-down and kandownComp-fliMdownup. To create flinY/ΔflinY double mutants, *H. pylori* G27 ΔflinY::cat was transformed with ΔflinY::aphA3 cassette.

**Motility phenotypic analysis.** Soft-agar plates were made with brucella broth, 2% agar, as described for cheC. Plates were incubated at room temperature for 2 to 3 days before use. Strains were inoculated from CHBA plates using a pipette tip or sterilized aluminum sewing pin. Migration was monitored by measuring the colony diameter each day.

**For analysis of motility, overnight cultures of *H. pylori* grown in BB10 were viewed with a Nikon Eclipse TiE microscope under phase contrast to assess motility and spiral morphology. To visualize flagella, 1 ml of BB10 *H. pylori* was treated with 50 µl of 1-mg/ml FM 4-64 (Invitrogen/Molecular probes) for 20 to 30 min. Bacteria were observed under fluorescence with excitation using a 485-nm-band-pass filter (Omega 485DF22) and a 560-nm dichroic filter (506DCPL Dicrho), and emission was measured through a 550-nm-long-pass filter (Omega 550 alpha LP03 0037). For each strain, we examined 200 to 300 individual bacteria. For transmission electron microscopy of G27 and its flin, flinA, and flinY mutants, samples were prepared by gently placing carbon type B 300-mesh copper grids (Ted Pella, Inc.) onto *H. pylori* cells that had been grown for 15 to 18 h on CHBA. Grids with associated *H. pylori* were fixed by the addition of 1% glutaraldehyde and washed three to four times with deionized water to remove fixative. Cells were then stained with 1% phosphotungstic acid at neutral pH. Samples were viewed with a JEOL 1200EX electron microscope at various magnifications, and appropriate images were selected and photographed with a Gatan 982 II-1200 camera.
var/carbon-coated copper grids (Ted Pella, Inc.), rinsed with 0.1 M cacodylate buffer, rinsed again with water, and negatively stained with 1% uranyl acetate (Electron Microscopy Sciences). Excess uranyl acetate was rinsed from grids by sliding the grids over water-soaked filter paper. Grids were then dried overnight in a desiccator. Cells were imaged using a JEOL 1230 electron microscope and bottom-mounted Ultrascan 1000 2,000- by 2,000-pixel Gatan charge-coupled-device camera. Image brightness and contrast were adjusted as needed for printing with Adobe Photoshop Elements 3.0.

**RESULTS**

*H. pylori* genomes predict four switch proteins. The sequenced *H. pylori* genomes are all predicted to encode four flagellar switch proteins. The gene numbers for each *fli* gene in the *H. pylori* 26695 and G27 genomes, respectively, are as follows: *fliG*, HP0352 and HPG27_329; *fliM*, HP1031 and HPG27_397; *fliN*, HP0584 and HPG27_543; and *fliY*, HP1030 and HPG27_398 (4, 48). Our first goal was to assess whether all four of these switch proteins were involved in flagellar switch functions. *fliM* and *fliY* exist in a predicted operon downstream of the *fliA*-encoded sigma factor that controls portions of flagellar biosynthesis (see Fig. S1 in the supplemental material); *fliY* is followed by two non-flagellum-encoding genes. *fliG* is the third gene in a predicted seven-gene operon. The operon additionally contains the flagellar genes *fliF* and *fliH*, as well as several genes that are not obviously related to flagellar motility. *fliN* is the first gene in a predicted seven-gene operon with no annotated genes related to flagella or motility.

**A few other bacteria similarly contain both FliN and FliY.** As described above, *H. pylori* is unusual in that it contains two FliN domain proteins, FliN and FliY. FliY in both *B. subtilis* and *H. pylori* contains a carboxy-terminal FliN domain fused with a phosphatase/CheC-like domain. FliN domains of other bacteria are involved both in switch function and in facilitating export of flagellar proteins via interactions with FliH (20, 23, 28, 36). Because FliY contains a FliN domain, it should be sufficient to carry out FliN-related functions. It is thus not clear why a microbe would have both FliY and FliN. As a first step, we analyzed the ubiquity of having both FliN and FliY by analyzing 768 genomes available in the GenBank database as of June 2007. We searched for genomes that had a FliY, defined as one polypeptide homologous to both CheC and FliN, and then determined whether these genomes contain a separate protein homologous to FliN. We used CheC-based sequences from several organisms, as this protein is not well conserved between distantly related organisms (31). We found that of 86 predicted genomes encoding a FliY, those of all of the epsilonproteobacteria encoded a FliN as well (Table 2). A few other bacteria also appear to have genes that encode both FliY and FliN; however, the CheC domain of the FliY of these microbes was not detected using BLASTP with the *H. pylori* CheC domain of FliY but instead with another CheC, suggesting that the sequences have diverged. Bacteria with genes that encode both FliY and FliN include two species of *Clostridia*, *Moorella thermoacetica* and *Carboxydothermus hydrogenoformans*, and two members of the *Spirochaetales*, *Leptospira borgpetersenii* serovar Hardjo-bovis and *Leptospira interrogans* serovar Lai. Additionally, the genomes of several firmicutes also encoded both FliY and FliN, including *Bacillus anthracis*, *Bacillus cereus*, *Bacillus weihenstephanensis*, and all subtypes of *Bacillus thuringiensis*. Interestingly, a *fliY* mutant of *B. cereus* has been reported to retain flagella that support swimming motility but do not function for surface-associated swarming motility (38). All of the *Listeriaceae* contained very short sequences (<80 amino acids) that were only poorly homologous to the *H. pylori* FliY C-terminal domain and are included for completeness. This analysis suggests that the presence of both FliN and FliY is universal within the epsilonproteobacteria and is also found sporadically in phylogenetically diverse taxa.

Next we evaluated the likely role of these domains in the

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<th>Straina</th>
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### TABLE 2. Bacterial species predicted to contain both FliY and FliN

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{| Epsilonproteobacteria | Accession no. |
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<td><em>Leptospira interrogans</em> serovar Lai strain 56601</td>
<td>YP_789070.1</td>
</tr>
</tbody>
</table>

* a Bacteria that contain both FliY and FliN. Only one strain is listed if multiple strains of the same species all had similar FliY/FliN distributions, except in the case of the epsilonproteobacteria.

flagellar switch by examining the conservation of the FliN domains. As stated above, FliN proteins function both to promote flagellar rotation and to export flagellar proteins. It was thus possible that these two roles could have diverged to some extent in the H. pylori FliN domains, resulting in FliY playing one role and FliN another. We performed an alignment with the FliN domains from organisms that have both FliY and FliN, as well as with well-characterized FliN domains (Fig. 1).

There was significant residue and charge conservation in both FliN and the FliN domain of FliY (FliYN) in regions corresponding to those having roles in export and homodimerization (Fig. 1). There were, however, many positions that differed specifically between FliYN and FliN protein sequences. At most positions, the class of amino acid (e.g., small, hydrophobic, and positively charged, etc.) was generally well conserved between the two types of FliN domains. We did note, however, that although the class of amino acid was generally conserved, often the exact amino acid was not. For example, at the hydrophobic patch that mediates FliN-FliH interactions (36), FliN proteins had valines while FliY proteins typically had isoleucines (Fig. 1). Such differences could reflect slight differences in function between the two domains. Taken together, the alignment data suggest that both FliY and FliN largely retain regions involved in export, protein-protein interaction, and motility functions, although there are some differences.

The differences between the two domains generally reflect a different member of a specific type of amino acid. It thus seems likely that both FliN and FliYN retain the ability to carry out all FliN-related functions.

**Mutants lacking each switch gene are nonmotile.** Our bioinformatic analysis described above suggested that both FliY and FliN would function in flagellar switching. We thus determined the phenotypes of H. pylori strains with null mutations in their switch genes. Toward this end, we created chromosomal gene replacements of H. pylori strain G27 fliM, fliN, or fliY. In each case, the majority of the open reading frame was deleted in frame and replaced with a cat gene (that lacked its transcriptional terminator), as detailed in Table 1, to create null mutants. Previous work from our lab has shown that this cat allele is not polar (8, 24, 45), and we verified that genes downstream of fliN, fliM, and fliY were unaffected using reverse transcription-PCR (see Fig. S2 in the supplemental material), suggesting that these mutations are nonpolar. After creating these mutants, we analyzed them for motility using both phase-contrast microscopy to visualize swimming bacteria and a soft-agar motility assay. All switch mutants were nonmotile in both assays (data not shown), supporting the idea that each of these gene products plays a function in motility.

fliM mutants are almost completely aflagellated, while fliN and fliY mutants retain partial flagellation. To determine if any of the fli deletions resulted in flagellation but not motility (e.g., paralyzed flagella), we determined to what extent these mutants formed flagella. We used two approaches for visualizing flagella. First, we used a fluorescent dye, FM 4-64. Traditional methods of staining flagella depend on protein binding dyes such as Alexa Fluor 488, 532, 546, or 594 carboxylic acid succinimidyl ester (see, for example, Turner et al. [49]), but we found that this stain did not work well for H. pylori, likely because this microbe has a membranous sheath covering the flagella that blocks dye binding and also because the buffers and conditions necessary for dye-to-protein linkage resulted in a loss of viability of H. pylori (data not shown). The lipid-specific dye FM 4-64 stains the flagellar sheath but does not affect viability or motility in wild-type organisms (data not
shown). Using this dye, we found the parental *H. pylori* G27 strain to be 80% flagellated across 250 cells counted (Table 3). We found that the *fliM* and *fliY* mutants appeared aflagellated, as expected for switch gene mutants. Previous work had found the same phenotype for null mutation of *fliG* in *H. pylori* strain SS1 (1); strain G27 *fliG* mutants are also aflagellated (data not shown). Surprisingly, the *fliN* mutant displayed some flagellation, with 17% of cells displaying flagellum-like structures, with a high degree of variability between samples. This and the published analysis suggest that *fliM*, *fliG*, and *fliY* are essential for any flagellation, while *fliN* is required for full flagellation. Of note, both *fliN* and *fliY* contribute to flagellation in *H. pylori*.

To look more closely at the *fliN* mutant flagellar structure, as well as to verify the FM 4-64 findings, we analyzed the various switch mutants by electron microscopy. Electron microscopy of wild-type cells demonstrated that 64% were flagellated (Table 3 and Fig. 2A and B), with most of the flagella having the previously described terminal bulb structure (19). As expected from the fluorescent microscopy, the *fliN* mutants demonstrated a lower frequency of flagellation than was found for the wild type (40%) (Table 3), with some abnormalities, such as truncation, observed (Fig. 2C and D). In contrast to the fluorescence data, by transmission electron microscopy a few of the *fliY* mutant cells were found to be flagellated (9%), and these cells demonstrated fewer abnormalities than the *fliN* mutant (Fig. 2E and F). The *fliM* mutant was 99% aflagellated, al-

### Table 3. Flagellation state of fli mutants

<table>
<thead>
<tr>
<th>G27 strain</th>
<th>% Flagellated (no. of cells)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FM 4-64^a</td>
</tr>
<tr>
<td>Wild type</td>
<td>80</td>
</tr>
<tr>
<td><em>fliG</em> mutant</td>
<td>0</td>
</tr>
<tr>
<td><em>fliM</em> mutant</td>
<td>0</td>
</tr>
<tr>
<td><em>fliN</em> mutant</td>
<td>17</td>
</tr>
<tr>
<td><em>fliY</em> mutant</td>
<td>0</td>
</tr>
<tr>
<td><em>fliN</em> <em>fliY</em> mutant</td>
<td>0</td>
</tr>
</tbody>
</table>

^a For FM 4-64 analysis, at least 200 cells were analyzed for each mutant.
^b Some of the flagella were abnormal.

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**FIG. 2.** Flagellation of *H. pylori* fli mutants. Electron micrographs of *H. pylori* cells stained with phosphotungstate. In each panel, arrows mark flagella and arrowheads mark terminal bulb structures. (A) Wild-type (WT) G27; (B) detail of G27 wild-type flagella; (C) G27 Δ*fliN::cat* flagellated cells; (D) detail of G27 Δ*fliN::cat* flagella; (E) G27 Δ*fliY::cat*; (F) detail of G27 Δ*fliY::cat* mutant flagella; (G) G27 Δ*fliM::cat*; (H) rare G27 Δ*fliM::cat* flagellated cells; (I) G27 Δ*fliN::cat* Δ*fliY::aphA3* double mutants. Bar lengths are in micrometers. The squares in panel C are staining artifacts.
Identification of a FliM point mutation that affects switch bias but not flagellation. Studies of Salmonella have shown that FliM point mutations often confer switching phenotypes (39). We first observed that a G27 derivative selected for enhanced colonization of the murine stomach, NSH57, was unable to form large colonies typical of the wild type on soft agar (Fig. 3C). Examination of negatively stained NSH57 cells by transmission electron microscopy showed that 93 of 100 cells appeared normally flagellated (Fig. 3A). Observation of NSH57 by phase-contrast microscopy revealed that this strain does not exhibit switching behavior and appears locked in swim behavior (data not shown). Suspecting that the strain had acquired a mutation in a chemosensory signaling or switch complex protein during the mouse colonization process, we sequenced candidate genes (see Materials and Methods) and identified a single nucleotide change, fliM(C160T), which resulted in an R54C substitution in the FliM protein.

Using amino acid alignment and structural prediction based on the T. maritima FliM crystal structure (34), we pinpointed Arg54 as a solvent-exposed residue on the α1 helix domain (Fig. 3B). Arg54 aligns with the fifth residue in the crystallized N-terminal domain, corresponding to Arg53 in E. coli and Gln52 in B. subtilis, flagella. These findings thus further support the idea that both FliN and FliY are important for the construction of flagella, with FliY playing a more critical role.

Because both fliN and fliY single mutants were flagellated, we next determined whether the loss of both FliN and FliY proteins would result in a complete loss of flagellation. Following targeted deletion of both the fliY and fliN genes, we found that flagella were completely absent by FM 4-64 staining and electron microscopy as described above (Table 3 and Fig. 2I). One caveat is that we did not determine whether the ΔfliY::apha3 mutation is polar, although apha3 does not have a transcriptional terminator, and there are no genes for flagellar proteins downstream of fliY (see Fig. S1 in the supplemental material). This phenotype is reminiscent of the ΔfliM and ΔfliG deletions and demonstrates that fliY and fliN are partially redundant in terms of the extent of flagellation.

Identification of a FliM point mutation that affects switch direction. CheY-P interacts with FliM. In E. coli and B. subtilis, flagella switch direction when the response regulator CheY-P binds directly to FliM. This interaction, however, has not been demonstrated in most bacterial species, including H. pylori. Thus, to further confirm that FliM directly integrates the CheY chemotaxis signal, we cloned full-length FliMHP downstream of GST, placing GST at the amino-terminal end and full-length FliM at the C-terminal end, identical to the design used for E. coli FliM interaction studies (43). GST-FliMHP, as well as GST alone, was bound to
are particularly interesting, as they contain two genes contain-
uncharacterized. H. pylori

FIG. 4. Western blot of proteins that interact with GST-FliM, using anti-CheY antisera. Specific bands consistent with CheYHP are marked with arrows. On the left are molecular mass markers in kilodaltons. Lanes 1 and 2, presence of CheY in the starting materials (1, whole-
cell extract from wild-type H. pylori 2, whole-cell extract from H. pylori
CheA); 3, GST beads plus CheY-P (wild type); 4, GST-FliM beads
plus CheY-P (wild type); 5, GST beads plus unphosphorylated CheY
(extract from CheA); 6, GST-FliM beads plus unphosphorylated CheY
(extract from CheA). Lanes 3a to 6a were washed with 0.3 M KCl.
Lanes 3b to 6b were washed with 0.4 M KCl. Higher-KCl washes
resulted in the complete removal of CheY.

The extract was treated with the known two-component regu-
ulatory phosphate donor acetyl phosphate. In other systems,
treatment with acetyl phosphate phosphorylates CheY, en-
hancing its affinity for FliM by 20-fold (26, 47, 52). For com-
pletely nonphosphorylated CheY, we used extract from an H. pylori
cheA null strain. We thus had the following starting condi-
tions: (i) GST-FliMHP or GST bound to beads and (ii) extracts bearing either phosphorylated or nonphosphorylated
CheY. After being mixed, the beads were washed with increas-
ing amounts of KCl to remove bound proteins. Samples were
then analyzed by Western blotting using an anti-H. pylori CheY
antibody. A band consistent in size with CheY was signifi-
cantly enriched in the GST-FliMHP sample when the extract was
acetyl phosphate treated (Fig. 4). This band decreased in in-
tensity after the higher-KCl washes (data not shown). No pro-
tein consistent in size with CheY was found in samples in which
GST alone served as the bait or in which lysate derived from
the cheA deletion strain was not treated with acetyl phosphate.
There were additional strongly CheY antibody-reacting bands
that we were unable to identify using either Western analysis of
H. pylori lacking the CheV proteins that are homologous to
CheY or mass spectrometry of interacting proteins (data not
shown). These proteins appear in both FliM and GST samples,
suggesting that they are not specific to FliM and instead seem
to depend more on acetyl phosphate. Thus, it appears that H. pylori
CheY associates with FliM and that this association is
stronger in the presence of a phosphorylating agent.

DISCUSSION

Putative flagellar switch genes outside of the model organ-
isms E. coli, Salmonella species, and B. subtilis remain relatively
uncharacterized. H. pylori and related epsilonproteobacteria are
particularly interesting, as they contain two genes contain-
ing a fliN domain, fliN and fliY. We show here that having two
FliN domains is relatively uncommon outside the epsilonpro-
teobacteria, although not unique to them. Other work has
shown that proteins with FliN domains are key for flagellar
rotation and also appear to play a role in the export of other
flagellar proteins (20, 23, 30, 36).

Our sequence analysis demonstrates that several residues of
the two H. pylori FliN domains carried in FliN and FliY differ
by residue but not by residue type. These two domains bear
27% identity and 60% similarity to each other. Within our set
of sequences, several key residues thought to be involved in
export and protein-protein interaction are diagnostic either for
a FliN protein or for the FliN domain of a FliY protein within
this class; these residues, however, are of the same general
type. Our mutant analysis supports that both of these proteins
function in creating wild-type levels of flagellation in H. pylori.
We furthermore observed that the H. pylori FliY lacks a
CheY-P binding region at its N terminus that is found in B.
subtilis FliY (data not shown and reference 40). This CheY-P
binding sequence is typically also found in FliM and is a critical
CheY-P binding determinant in these proteins. H. pylori FliM
retains this sequence, and we show here that it binds CheY-P.
Whether the H. pylori FliY is able to bind CheY-P remains to
be determined, as does whether it can function as a phos-
phatase.

Our analysis of flagellar switch null mutants gave some ex-
pected and some unexpected results. The fliM null mutant was
mostly aflagellated, although we did observe, interestingly, very
rare fliM mutant cells that appeared to retain full flagella. We
never saw motile cells, however, suggesting that these flagella
were not functional. fliM null mutants in other bacteria are
usually described as completely aflagellated. The finding of
these very infrequent flagella suggests that H. pylori represents
an unusual case of sometimes being able to build flagella with-
out fliM. In contrast, mutants bearing null mutations in either
fliN or fliY were partially flagellated. This phenotype mostly
manifested in terms of the percentage of the population that
was flagellated. fliY mutants had about 9% of the cells flagel-
lated, while fliN mutants had about 40% of the population
flagellated, compared to wild-type G27 cells, with 64% of
the cells flagellated. Thus, it seems that either FliN or FliY allows
for partial flagellation. Neither mutant, however, was motile,
suggesting that both FliN and FliY are needed for functional
flagella. This phenotype is different from that reported for B.
cereus (38), another bacterium whose genome contains both
fliY and fliN. In this case, loss of fliY did not appreciably affect
flagellation or motility, consistent with fliN being able to sub-
stitute for fliY in this microbe. Loss of fliY did, however, cause
cells to exhibit more tumble bias and to lose chemotactic and
solid-surface swarming abilities, perhaps due to the loss of the
CheC-like phosphatase portion of fliY. In E. coli, FliN is found
at about 100 copies/flagellum in tetramers that associate with
one FliM (35). Thus, one could imagine that an individual H. pylori
flagellum might contain both FliN and FliY. Loss of either the fliY or fliN gene might generate mutants that simply
do not have enough FliN or FliY to create functional flagella.
In support of this idea, Tang et al. found that E. coli mutants
that expressed less fliN had more dramatic effects on flagellar
function than on flagellation per se (44). They found that small
amounts of fliN could result in flagellated cells that were not
fully motile. In this scenario, the FliN domains of both FliY
and FliN are equivalent, which, although plausible, does not
explain why a bacterium would have both. Another possible
scenario is that the two FliN domains have somewhat unique
functions, with perhaps one being more involved in a function such as export. Although we did not observe any gross differences in the infrequent flagella of either such as export. Although we did not observe any gross differences in, for example, C-ring structure. Additionally, because a loss of flagellar switch proteins can feed back onto the transcription of other flagellar genes, it is hard to separate transcriptional from posttranscriptional effects. Another possibility is that either FlIM domain could be acting as a molecular “spare part” reminiscent of the flbB homolog HP1575, which can compensate for the loss of only the C-terminal domain of flbB (51).

Apart from its important role in flagellar assembly, we have confirmed that *H. pylori* FlIM is involved in switching the direction of flagellar rotation, presumably in response to signals from the phosphorylated chemosensory signaling protein CheY, to which it directly binds. We identified Arg54 as an important residue for FlIM’s switching function. This residue lies in the α1 helix domain, which is involved in FlIM oligomerization, but also just downstream of a 35-residue region that links the canonical CheY binding site to the rest of the protein (34). This linker is believed to be unstructured in the absence of CheY but important for structurally transmitting the switch signal from the binding site to the rest of the protein upon CheY binding (34). Other work has shown that point mutations in FlIM can confer either CW or CCW switch bias (39). Söckett and colleagues isolated FlIM point mutations that were either CW biased to suppress loss of CheY or CCW biased to suppress loss of CheZ. The authors found that CCW-biased mutations predominated in the first 50 residues comprising the CheY binding and linker regions, whereas CW-biased mutations predominated in residues 51 to 74 (α1) (39). The observations that the R54C substitution confers a CCW bias and that it is positioned at the end of a highly conserved region that extends from the linker suggest that the functional importance of this residue in *H. pylori* may be related to that of the linker, although we cannot exclude the possibility of Arg54 contributing to FlIM-FlIM interactions. Further experiments that look at other Arg54 substitutions, as well as experiments that genetically target the highly conserved residues adjacent to Arg54 and the canonical CheY binding site, are needed to fully assess how these N-terminal motifs affect CheY binding and/or switch function in *H. pylori*.

In this work we have characterized the flagellar switch proteins of *H. pylori*. We and others have demonstrated that all four, flfM, flgG, flIN, and flfY, are required for wild-type numbers of flagellated cells, supporting the idea that they are all part of the flagellar apparatus. We further demonstrated evidence that FlIM functions in flagellar rotation switching and that this protein associates with CheY-P.

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REFERENCES


