JOURNAL OF BACTERIOLOGY, Dec. 2009, p. 7147–7156 Vol. 191, No. 23
0021-9193/09/$12.00 doi:10.1128/JB.00749-09

Functional Analysis of the *Helicobacter pylori* Flagellar Switch Proteins▽§

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Received 10 June 2009/Accepted 11 September 2009

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/.

▽ Published ahead of print on 18 September 2009.

added below). There are 26 copies of FliG, 34 copies of FliM, and ~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 FliH and localizes it, along with its interaction partners FliI 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 or PFAM PF01052 domain. 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. S1 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

Strain[a]	Genotype or description[b]	Reference or source
H. pylori		
G27	Wild type	9
NSH57	Mouse-adapted G27	3
J99	Wild type	2
G27 *cheA* (KO857)	Δ*cheA*::*cat*	This study
G27 *fliM* (KO1060)	Δ*fliM*(1-105)::cat_{mut}	This study
G27 *fliN* (KO1061)	Δ*fliN*(1-345)::cat_{mut}	This study
G27 *fliY* (KO1062)	Δ*fliY*(28-854)::cat_{mut}	This study
G27 *fliN fliY* (KO1065)	KO1061 Δ*fliY* (28-854)::*aphA3*	This study
NSH57 *fliM* (LSH99)	NSH57 Δ*fliM*::*cat-sacB*	This study
NSH57 *fliM* restored (LSH100)	NSH57 *fliM*(*160C*)	This study
E. coli		
DH10B	Cloning strain	Lab stock (21)
BL21(DE3)pLysS	*E. coli* B F^- *dcm hsdS*(r_B^- m_B^-) *gal* λ(DE3)pLysS	Promega
Plasmids		
pGEX-4T1	GST fusion plasmid	GE Biosciences
pGEX-6P2	GST fusion plasmid	GE Biosciences
pGEX-FliM	pGEX-4T1::$fliM_{HP}$ from strain J99	This study
pGEX-CheY	pGEX-6P2::$cheY_{HP}$ from strain G27	This study
pBS	Cloning plasmid	Stratagene
pBS-catmut	pBS with *C. coli cat* gene lacking transcription term	45
pBS-FliM	pBS::$fliM_{HP}$ from strain G27	This study
pBS-FliN	pBS::$fliN_{HP}$ from strain G27	This study
pBS-FliY	pBS::$fliY_{HP}$ from strain G27	This study
pBS-fliM::catmut	Δ*fliM*(1–105)::cat_{mut}	This study
pBS-fliN::catmut	Δ*fliN*(1–345):: cat_{mut}	This study
pBS-fliY::catmut	Δ*fliY*(28–854):: cat_{mut}	This study

[a] Designations in parentheses are lab strain numbers.
[b] cat_{mut}, *cat* mutant lacking its transcriptional terminator.

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 μg/ml (*H. pylori*) or 20 μg/ml (*E. coli*), kanamycin was used at 15 μg/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 was established, after sparging with N_2 gas and air to establish 0 and 100% relative oxygen contents, respectively. CO_2 content was monitored via the effect that injection had on pH. The pH was kept at 6.5 by injection of CO_2. Cells were collected by centrifugation, ground in liquid nitrogen as described for glutathione *S*-transferase (GST)–CheY below, and stored at −20°C.

For long-term storage at −70°C, *H. pylori* was stored in BB10–1% (wt/vol) β-cyclodextrin–25% glycerol–5% dimethyl sulfoxide.

Plasmid preparation was done using kits from Qiagen. For preparation of genomic DNA, DNeasy kits (Qiagen) or Wizard genomic kits (Promega) were used. All restriction and DNA modification enzymes were from New England Biolabs or Gibco. 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

FHCRC Genomics shared resource and analyzed using Sequencher (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 ($CheY_{HP}$) were concentrated by centrifugation with Centricon (Millipore) filters with a 3,000-molecular-weight cutoff. A 1.5-mg sample was delivered to Animal Pharm for rabbit inoculation. This rabbit serum is hereinafter referred to as anti-CheY, with the fourth and final bleeds 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 with slight modifications. To avoid the creation of insoluble inclusion bodies, cells were grown at room temperature and then transferred to 15°C for overexpression with 60 μM IPTG for 10 to 12 h. Purification was as described above for CheY, except that the steps involving PreScission protease were omitted. Peak fractions were dialyzed into 250 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.15% Tween 20. Glycerol was added to 10%, and samples were stored at −20°C. The yield was typically 4 to 8 mg for 8 liters of culture of GST-HpFliM. GST was also purified using this method.

FliM protein-protein interaction assay. GST-HpFliM (0.6 to 1 mg) or equimolar GST was bound to 0.8 to 1.2 ml of settled GST · Mag agarose beads (Novagen/EMD Biosciences) for 1 hour in 50 mM K^+ HEPES, pH 7.6, 250 mM KCl, 5 mM $MgCl_2$, 0.15% Tween 20, and 0.5% Brij 35, and then the beads were washed three times with the same buffer. *H. pylori* cell powder from G27 or its isogenic *cheA* mutant was resuspended in a 50 mM room temperature solution of K^+ HEPES, pH 7.6, 250 mM KCl, 5 mM $MgCl_2$, 0.15% Tween 20, 10 mM DTT, and 1 mM AEBSF, sonicated briefly, and clarified by centrifugation at 100,000 × *g* for 1.5 h. $CheY_{HP}$ would not bind to $FliM_{HP}$ below 250 mM KCl, independent of phosphorylation (data not shown). The *cheA* mutant lysate was included to eliminate all background phosphorylation of $CheY_{HP}$. To phosphorylate proteins in the *H. pylori* extract, Li^+ K^+-acetyl phosphate (Sigma-Aldrich) was added to 10 mM. Lysates with or without acetyl phosphate were mixed with magnetic beads bound to GST or GST-$FliM_{HP}$ for a total of four experimental conditions. Beads were then washed twice with 10 ml of the buffer 50 mM K^+ HEPES, pH 7.6, 5 mM $MgCl_2$, 0.15% Tween 20 with or without 10 mM acetyl phosphate and with increasing concentrations of salt at 300, 400, and 500 mM KCl. All samples were saved by the addition of glycerol to 10% and storage at −20°C. Due to the labile nature of acetyl phosphate and the short half-life of CheY-P in other organisms, all buffers were used within 15 min of the addition of Li^+ K^+ acetyl phosphate and all incubation steps were short (5 to 7 min in length).

Analysis of proteins interacting with GST-FliM and GST. Samples from the GST-FliM pull-down assay were analyzed by Western blotting on a sodium dodecyl sulfate–12% polyacrylamide gel followed by transfer to immunoblot polyvinylidene difluoride membranes (Bio-Rad). Rabbit polyclonal anti-CheY was used at a 1:2,500 dilution, and horseradish peroxidase-conjugated chicken anti-rabbit antibodies (Santa Cruz Biotech) were used at a 1:5,000 dilution. Results were visualized using luminol and Biomax light film (Kodak).

Protein sequence and analysis. Alignments of selected FliN and FliM domains were generated with ClustalW (46) and visualized using Boxshade (http://www.ch.embnet.org). Structural prediction was performed with PHYRE (5), and three-dimensional molecular structures were visualized and modified using PyMOL (http://www.pymol.org) (12).

To identify all microbial species carrying FliY proteins, a BLASTP search was done against all available microbial sequences accessible at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). As query sequences, we used CheC domain sequences contained in either FliY or CheC from *H. pylori* 26695, *Treponema pallidum* subsp. *pallidum* strain Nichols, *Thermotoga maritima* MSB8, *Pyrococcus horikoshii* OT3, *Bacillus subtilis* subsp. *subtilis* strain 168, and *Vibrio cholerae* O1 biovar eltor strain N16961. After identifying all CheC-homologous sequences, we then determined which of these also bore FliN sequences, using *H. pylori* FliN as the query protein.

Cloning and mutating *fliM*, *fliN*, and *fliY*. Each *fli* 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 *fliM*, FliMlocusfor and FliMlocusrev; for *fliN*, FliNlocusfor and FliNlocusrev; and for *fliY*, FliYlocusfor and FliYlocusrev. 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-FliM, pBS-FliN, and pBS-FliY. These plasmids were then used as templates in inverse PCR to delete most of the *fli* gene. Primers used were as follows: for *fliM*, fliMcircfor and fliMcircrev-2; for *fliN*, fliNcircfor and fliNcirevrev; and for *fliY*, fliYcircfor-2 and fliYcircrev-2. Each of these PCR products was gel purified and ligated with a chloramphenicol acetyltransferase gene lacking a terminator obtained from the vector pCat-mut (cat_{mut}) (45) using HincII. These ligations created the vectors pBS-FliM::catmut, pBS-FliN::catmut, and pBS-FliY::catmut.

A Δ*fliY*::*aphA3* cassette was created using the vector-free allelic replacement method described by Chalker et al. (10). The three pieces were *fliY* upstream, with primers flankfliY-up and kanupComp-fliYupdo; *aphA3*, with primers kanupComp-fliYupdo and kandownComp-fliYdownup; and *fliY* downstream, with primers flankfliY-down and kandownComp-fliYdownup.

Introduction of DNA in *H. pylori* was as described previously (37), with the addition of cell-free *H. pylori* G27 extract under conditions that methylated the plasmid DNA, allowing it to bypass the robust *H. pylori* restriction barrier (14). To create *fliN fliY* double mutants, *H. pylori* G27 Δ*fliN*::*cat* was transformed with Δ*fliY*::*aphA3*.

Mapping and restoration of NSH57 *fliM* mutation. Sequence polymorphisms between NSH57 and G27 were queried by amplifying and sequencing candidate genes HPG27_1004 (*cheV3*), HPG27_1005 (*cheA*/*cheY* fusion), HPG27_1006 (*cheW*), and HPG27_397 (*fliM*) from NSH57 genomic DNA and comparing them to the published G27 sequence (4). To reengineer the NSH57 *fliM*(*C160T*) mutation back to the wild-type allele, we first utilized SOEing (splicing by overlap extension) PCR to generate a *fliM* null allele with insertion of a *cat sacB* cassette (10, 22) and concomitant deletion of the *fliM* coding sequence from position 55 to position 1001. Transformation of the SOEing PCR product into NSH57 resulted in strain LSH99 (Δ*fliM*::*cat sacB*). Sucrose sensitivity conferred by the *sacB* marker enabled selection of the wild-type allele upon transformation of LSH99 with a PCR product containing the *fliM* gene that had been amplified from wild-type G27 genomic DNA. Restoration of *fliM*(*160C*) in the resulting strain, LSH100, was confirmed by PCR amplification and sequencing. All primers utilized for NSH57 candidate gene sequencing and *fliM* allelic replacement are listed in Table S1 in the supplemental material.

Motility phenotypic analysis. Soft-agar plates were made with brucella broth, 2.5 to 5% FBS, and 0.3 to 0.35% agar as described previously (27). Plates were incubated at room temperature for 2 to 3 days before use. Strains were inoculated from CHBA plates or overnight liquid cultures grown in BB10 into the soft-agar 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 E600 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 (560DCLP Dichroic), and emission was measured through a 550-nm-long-pass filter (Omega 550 alpha LP030 0037). For each strain, we examined 200 to 300 individual bacteria. For transmission electron microscopy of G27 and its *fliM*, *fliN*, and *fliY* 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% phosphotungstate 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 792 Bioscan camera.

For transmission electron microscopy of NSH57 and its mutants, the cells were grown in shaken BB10 liquid culture to an optical density at 600 nm of 0.5 to 1.0 and fixed in half-strength Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer). Cells were applied to 200-mesh Form-

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 contained 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

TABLE 2. Bacterial species predicted to contain both FliY and FliN

Strain[a]	Accession no.[b]	
	FliY	FliN
Firmicutes		
Bacillus anthracis strain Ames	NP_844108.1	NP_844145.1
Bacillus cereus ATCC 10987	NP_978070.1	NP_978104.1
Bacillus thuringiensis serovar *israelensis* ATCC 35646	ZP_00740677.1	ZP_00743099.1
Bacillus thuringiensis serovar kondukian strain 97-27	YP_035851.1	YP_035883.1
Bacillus thuringiensis strain Al Hakam	YP_894318.1	YP_894349.1
Bacillus weihenstephanensis KBAB4	ZP_01183416.1	HP_01186704.1
Listeria innocua Clip11262	NP_470051.1	NP_470049.1
Listeria monocytogenes EGD-e	NP_464227.1	ZP_00232875.1
Listeria welshimeri serovar 6b strain SLCC5334	YP_848870.1	YP_848868.1
Carboxydothermus hydrogenoformans Z-2901	YP_359867.1	YP_359850.1
Moorella thermoacetica ATCC 39073	YP_429666.1	YP_429645.1
Epsilonproteobacteria		
Campylobacter coli RM2228	ZP_00367872.1	ZP_00367623.1
Campylobacter concisus 13826	ZP_013733.76	ZP_01374235.1
Campylobacter curvus 525.92	ZP_01806896.1	ZP_01806986.1
Campylobacter fetus subsp. Fetus 82-40	YP_892647.1	YP_891558.1
Campylobacter jejuni RM1221	YP_178081.1	YP_178419.1
Campylobacter lari RM2100	ZP_00368719.1	ZP_00368652.1
Campylobacter upsaliensis RM3195	ZP_00369881.1	ZP_00370648.1
Helicobacter acinonychis strain Sheeba	YP_664894.1	YP_665155.1
Helicobacter hepaticus ATCC 51449	NP_860679.1	NP_861108.1
Helicobacter pylori 26695	NP_207820.1	NP_207379.1
Helicobacter pylori HPAG1	YP_627158.1	HP_627304.1
Helicobacter pylori J99	NP_223113.1	NP_223249.1
Helicobacter pylori G27	YP_002266029.1	YP_002266028.1
Thiomicrospira denitrificans ATCC 33889	YP_393222.1	YP_393679.1
Wolinella succinogenes DSM 1740	NP_907768.1	NP_907256.1
Spirochaetales		
Leptospira borgpetersenii serovar Hardjo-bovis JB197	YP_800948.1	YP_797771.1
Leptospira interrogans serovar Lai strain 56601	NP_712794.1	NP_712250.1

[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.

[b] NCBI reference sequence numbers (http://www.ncbi.nlm.nih.gov/protein/).

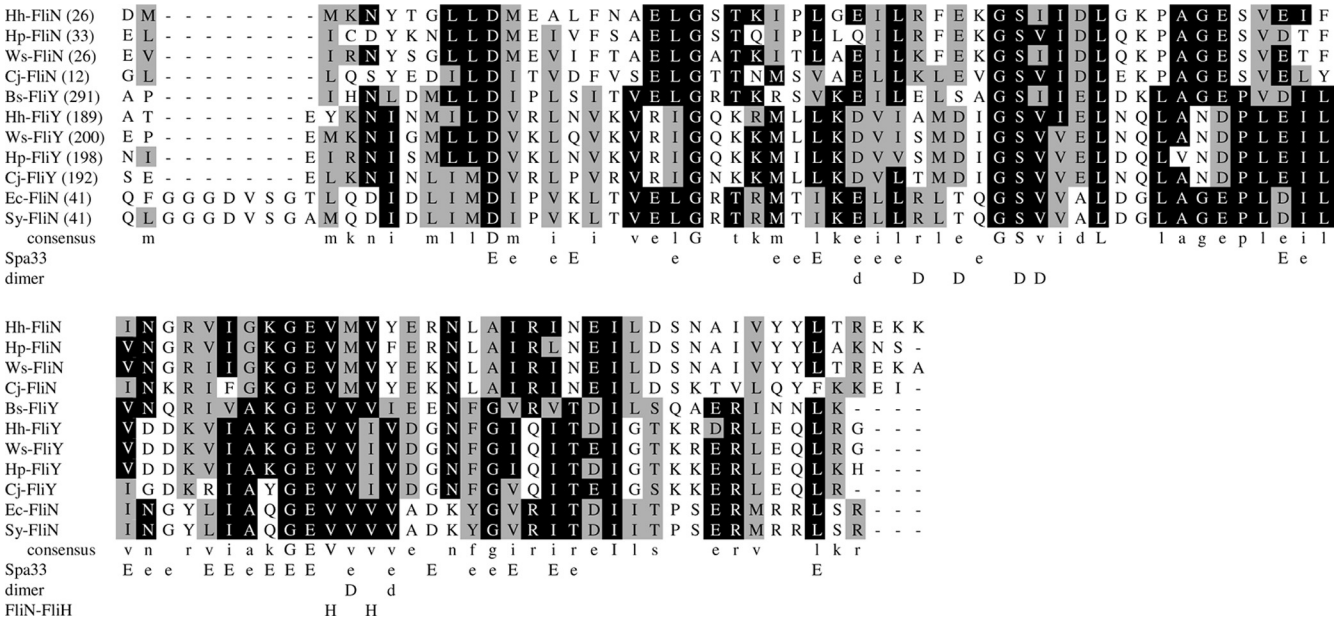

FIG. 1. Alignments of selected FliN domains. Ec, *Escherichia coli* K-12-MG1655; Sy, *Salmonella* Typhimurium LT2 SGSC1412; Hp, *Helicobacter pylori* 26695; Hh, *Helicobacter hepaticus* ATCC 51449; Cj, *Campylobacter jejuni*; Ws, *Wolinella succinogenes* DSMZ 1740; Bs, *Bacillus subtilis.* The first residue shown is indicated after each name. E denotes residues important for export in Spa33 domains (44), D denotes regions important for forming multimers, and H denotes residues critical for FliH interactions (36). Uppercase letters in these labels denote residues that do not vary significantly between epsilonproteobacterial FliY and FliN proteins, while lowercase letters denote those that may differentiate FliY and FliN.

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 ($FliY_N$) in regions corresponding to those having roles in export and homodimerization (Fig. 1). There were, however, many positions that differed specifically between $FliY_N$ 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 $FliY_N$ 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

TABLE 3. Flagellation state of *fli* mutants

G27 strain	% Flagellated (no. of cells)	
	FM 4-64[a]	Electron microscopy
Wild type	80	64 (554)
fliG mutant	0	0 (156)
fliM mutant	0	1 (200)
fliN mutant	17	40.4[b](252)
fliY mutant	0	9.4[b](191)
fliN fliY mutant	0	0 (200)

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

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-

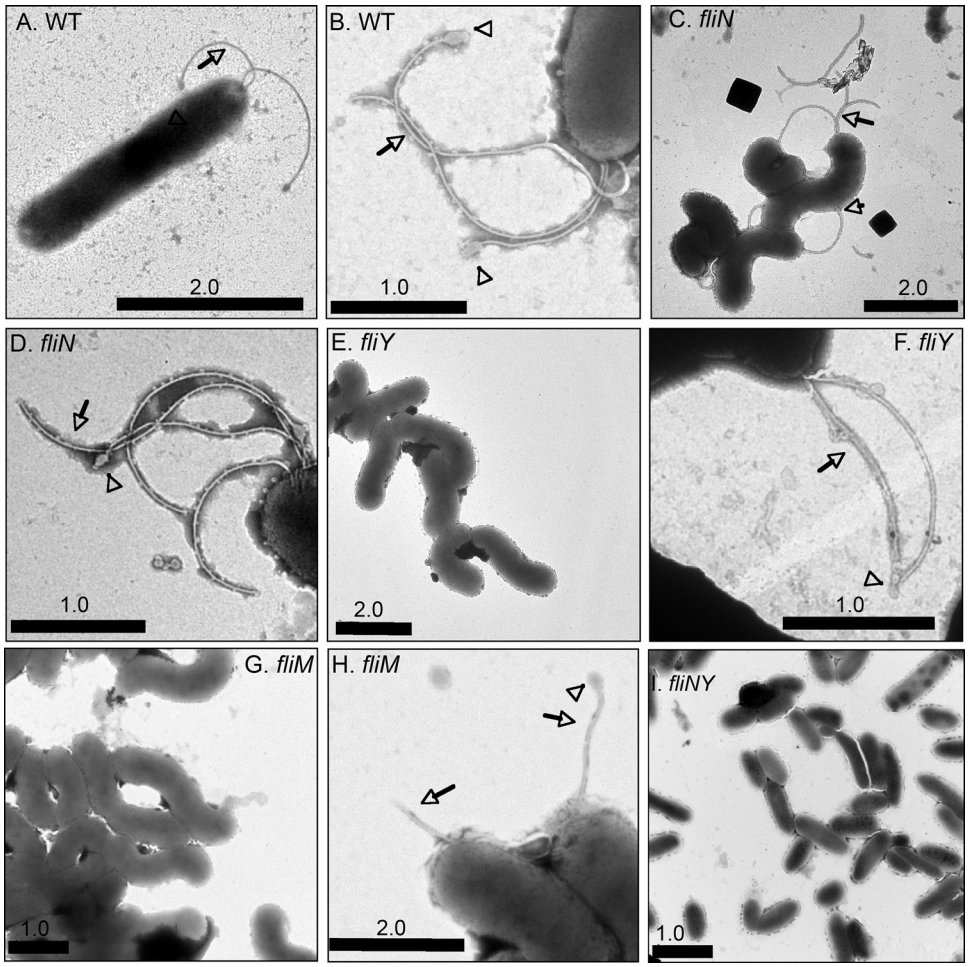

FIG. 2. Flagellation of *H. pylori fli* mutants. Electron micrographs of *H. pylori* cells stained with phosphotungstate. In each panel, arrows mark flagella and arrowheads mark terminal bulb structures. (A) Wild-type (WT) G27; (B) detail of G27 wild-type flagella; (C) G27 Δ*fliN*::*cat* flagellated 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.

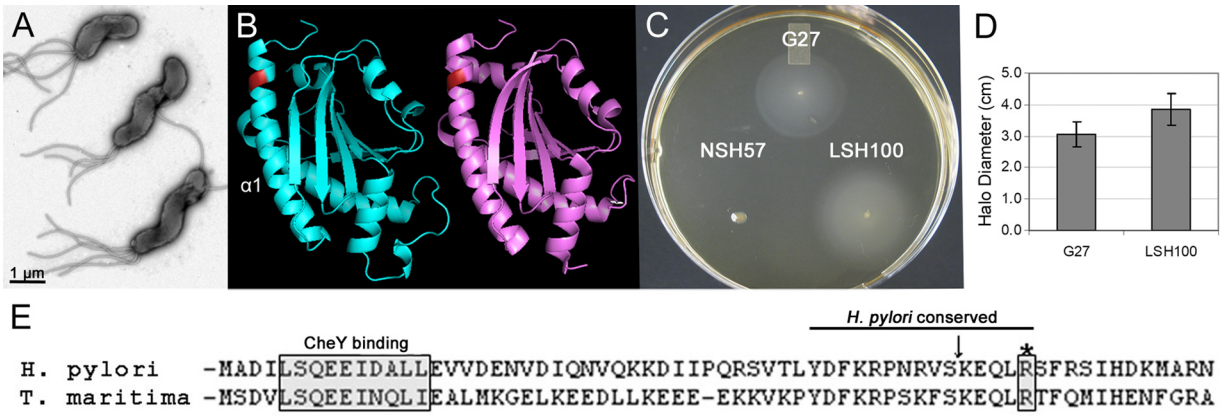

FIG. 3. Characterization of *H. pylori* strain NSH57 containing a FliM point mutation. (A) Electron micrograph of NSH57 cells with characteristically normal flagella. (B) Ribbon diagram of the predicted *H. pylori* FliM structure (left, teal) generated by threading the protein sequence on the *T. maritima* FliM crystal structure (right, pink). The position of Arg54 within the α1 helix is highlighted in red on the predicted *H. pylori* structure, as is the corresponding arginine (Arg53) on the *T. maritima* structure. (C) Halo formation after 5 days of growth in 0.3% soft agar. (D) Average halo diameters (± standard deviations) from two independent trials of 20 stabs each. (E) N-terminal sequence alignment of the *H. pylori* and *T. maritima* FliM proteins. Boxed are the predicted CheY binding domain and (with star) the site of the R54C mutation. The arrow indicates the start of the crystallized α1 domain.

though we were able to find very rare cells that had relatively normal-looking flagella (Table 3 and Fig. 2G and H). We additionally did not find free flagella in the surroundings, suggesting that none of the mutants had particularly fragile 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 Δ*fliY*::*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 *fliM* and *fliG* 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 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 α1 domain, corresponding to Arg53 in *T. maritima* and Gln52 in *S.* Typhimurium, and is preceded by a 35-residue linker region (Fig. 3E). At the amino-terminal end, the canonical CheY binding site appears conserved in *H. pylori*, spanning residues 5 to 14 (data not shown). Interestingly, a 15-residue sequence beginning with Tyr40 in the linker region and ending with Arg54 is conserved with 100% identity in all *H. pylori* FliM sequences and 33 of 35 sequences annotated as FliM in the epsilonproteobacteria (GenBank taxid 29547) (data not shown); *Nitratiruptor* sp. strain SB155-2 and *Arcobacter butzleri* FliM sequences each differed from the consensus at multiple positions but conserved a basic residue at position 54.

To determine whether this single nucleotide change in *fliM* was responsible for NSH57's altered swimming behavior, we replaced the NSH57 *fliM*(*C160T*) allele with the G27 wild-type *fliM* in a restored strain that we designated LSH100. LSH100 was observed to exhibit switching behavior and, as shown in Fig. 3D, slightly superseded G27 in its ability to form halos in soft agar. Together, these data suggest that residue 54 and likely other conserved N-terminal portions of *H. pylori* FliM are important for switching the rotational direction of the flagellar motor.

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 probed whether *H. pylori* proteins, and specifically CheY, would interact with $FliM_{HP}$. Toward this end, we cloned full-length $FliM_{HP}$ 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-$FliM_{HP}$, as well as GST alone, was bound to

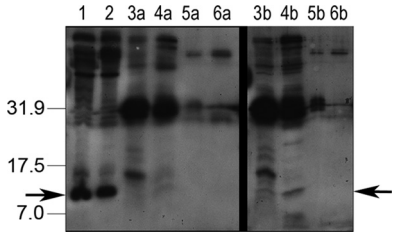

FIG. 4. Western blot of proteins that interact with GST-FliM, using anti-CheY antisera. Specific bands consistent with $CheY_{HP}$ are marked with arrows. On the left are molecular mass markers in kilodaltons. Lanes: 1 and 2, presence of CheY in the starting materials (1, whole-cell extract from wild-type *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.

glutathione beads and then mixed with total *H. pylori* extract. The extract was treated with the known two-component regulatory phosphate donor acetyl phosphate. In other systems, treatment with acetyl phosphate phosphorylates CheY, enhancing its affinity for FliM by 20-fold (26, 47, 52). For completely nonphosphorylated CheY, we used extract from an *H. pylori cheA* null strain. We thus had the following starting conditions: (i) GST-$FliM_{HP}$ or GST bound to beads and (ii) extracts bearing either phosphorylated or nonphosphorylated CheY. After being mixed, the beads were washed with increasing 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 significantly enriched in the GST-$FliM_{HP}$ sample when the extract was acetyl phosphate treated (Fig. 4). This band decreased in intensity after the higher-KCl washes (data not shown). No protein 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 organisms *E. coli*, *Salmonella* species, and *B. subtilis* remain relatively uncharacterized. *H. pylori* and related epsilonproteobacteria are particularly interesting, as they contain two genes containing a *fliN* domain, *fliN* and *fliY*. We show here that having two FliN domains is relatively uncommon outside the epsilonproteobacteria, 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 phosphatase.

Our analysis of flagellar switch null mutants gave some expected 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 without *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 flagellated, 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 substitute 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 *fliY* or *fliN* mutants that would support a particular role for either protein, there may be more subtle 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 FliN domain could be acting as a molecular "spare part" reminiscent of the *flhB* homolog HP1575, which can compensate for the loss of only the C-terminal domain of *flhB* (51).

Apart from its important role in flagellar assembly, we have also 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). Sockett 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, though 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, *fliM*, *fliG*, *fliN*, and *fliY*, 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.

ACKNOWLEDGMENTS

We are grateful to Noelani Villenueve for initiating the *fliY*::*aphA3* mutant construction, to Karianne Terry for creating G27 *cheA*, and to Will Sause for locating information needed for the manuscript.

The project described was supported by grant numbers AI050000 (to K.M.O.) and AI054423 (to N.R.S) from the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health. The contents of this paper are solely our responsibility and do not necessarily represent the official views of the NIH.

REFERENCES

1. **Allan, E., N. Dorrell, S. Foynes, M. Anyim, and B. W. Wren.** 2000. Mutational analysis of genes encoding the early flagellar components of *Helicobacter pylori*: evidence for transcriptional regulation of flagellin A biosynthesis. J. Bacteriol. **182:**5274–5277.
2. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176–180.
3. **Baldwin, D. N., B. Shepherd, P. Kraemer, M. K. Hall, L. K. Sycuro, D. M. Pinto-Santini, and N. R. Salama.** 2007. Identification of *Helicobacter pylori* genes that contribute to stomach colonization. Infect. Immun. **75:**1005–1016.
4. **Baltrus, D. A., M. R. Amieva, A. Covacci, T. M. Lowe, D. S. Merrell, K. M. Ottemann, M. Stein, N. R. Salama, and K. Guillemin.** 2009. The complete genome sequence of *Helicobacter pylori* strain G27. J. Bacteriol. **191:**447–448.
5. **Bennett-Lovsey, R. M., A. D. Herbert, M. J. Sternberg, and L. A. Kelley.** 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins **70:**611–625.
6. **Bischoff, D. S., and G. W. Ordal.** 1992. Identification and characterization of FliY, a novel component of the *Bacillus subtilis* flagellar switch complex. Mol. Microbiol. **6:**2715–2723.
7. **Blair, D. F.** 1995. How bacteria sense and swim. Annu. Rev. Microbiol. **49:**489–522.
8. **Castillo, A. R., S. S. Arevalo, A. J. Woodruff, and K. M. Ottemann.** 2008. Experimental analysis of *Helicobacter pylori* transcriptional terminators suggests this microbe uses both intrinsic and factor-dependent termination. Mol. Microbiol. **67:**155–170.
9. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. USA **93:**14648–14653.
10. **Chalker, A. F., H. W. Minehart, N. J. Hughes, K. K. Koretke, M. A. Lonetto, K. K. Brinkman, P. V. Warren, A. Lupas, M. J. Stanhope, J. R. Brown, and P. S. Hoffman.** 2001. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J. Bacteriol. **183:**1259–1268.
11. **Cohen-Ben-Lulu, G. N., N. R. Francis, E. Shimoni, D. Noy, Y. Davidov, K. Prasad, Y. Sagi, G. Cecchini, R. M. Johnstone, and M. Eisenbach.** 2008. The bacterial flagellar switch complex is getting more complex. EMBO J. **27:**1134–1144.
12. **DeLano, W. L.** 2002. Unraveling hot spots in binding interfaces: progress and challenges. Curr. Opin. Struct. Biol. **12:**14–20.
13. **Deshpande, M., E. Calenoff, and L. Daniels.** 1995. Rapid large-scale growth of *Helicobacter pylori* in flasks and fermentors. Appl. Environ. Microbiol. **61:**2431–2435.
14. **Donahue, J. P., D. A. Israel, R. M. Peek, M. J. Blaser, and G. G. Miller.** 2000. Overcoming the restriction barrier to plasmid transformation of *Helicobacter pylori*. Mol. Microbiol. **37:**1066–1074.
15. **Dyer, C. M., A. S. Vartanian, H. Zhou, and F. W. Dahlquist.** 2009. A molecular mechanism of bacterial flagellar motor switching. J. Mol. Biol. **388:**71–84.
16. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. **37:**123–127.
17. **Eaton, K. A., S. Suerbaum, C. Josenhans, and S. Krakowka.** 1996. Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. Infect. Immun. **64:**2445–2448.
18. **Foynes, S., N. Dorrell, S. J. Ward, R. A. Stabler, A. A. McColm, A. N. Rycroft, and B. W. Wren.** 2000. *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. Infect. Immun. **68:**2016–2023.
19. **Geis, G., H. Leying, S. Suerbaum, U. Mai, and W. Opferkuch.** 1989. Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. J. Clin. Microbiol. **27:**436–441.
20. **Gonzalez-Pedrajo, B., T. Minamino, M. Kihara, and K. Namba.** 2006. Interactions between C ring proteins and export apparatus components: a possible mechanism for facilitating type III protein export. Mol. Microbiol. **69:**984–988.
21. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
22. **Humbert, O., and N. R. Salama.** 2008. The *Helicobacter pylori* HpyAXII restriction-modification system limits exogenous DNA uptake by targeting GTAC sites but shows asymmetric conservation of the DNA methyltransferase and restriction endonuclease components. Nucleic Acids Res. **36:**6893–6906.
23. **Kojima, S., and D. F. Blair.** 2004. The bacterial flagellar motor: structure and function of a complex molecular machine. Int. Rev. Cytol. **233:**93–134.

24. **Lowenthal, A. C., C. Simon, A. S. Fair, K. Mehmood, K. Terry, S. Anastasia, and K. M. Ottemann.** 2009. A fixed-time diffusion analysis method determines that the three cheV genes of Helicobacter pylori differentially affect motility. Microbiology **155:**1181–1191.
25. **Macnab, R. M.** 2003. How bacteria assemble flagella. Annu. Rev. Microbiol. **57:**77–100.
26. **McCleary, W. R., and J. Stock.** 1994. Acetyl phosphate and the activation of two-component response regulators. J. Biol. Chem. **269:**31567–31572.
27. **McGee, D. J., M. L. Langford, E. L. Watson, J. E. Carter, Y.-T. Chen, and K. M. Ottemann.** 2005. Colonization and inflammation deficiencies in Mongolian gerbils infected by *Helicobacter pylori* chemotaxis mutants. Infect. Immun. **73:**1820–1827.
28. **McMurry, J. L., J. W. Murphy, and B. Gonzalez-Pedrajo.** 2006. The FliN-FliH interaction mediates localization of flagellar export ATPase FliI to the C ring complex. Biochemistry **45:**11790–11798.
29. **Minamino, T., K. Imada, and K. Namba.** 2008. Molecular motors of the bacterial flagella. Curr. Opin. Struct. Biol. **18:**693–701.
30. **Minamino, T., B. González-Pedrajo, M. Kihara, K. Namba, and R. M. Macnab.** 2003. The ATPase FliI can interact with the type III flagellar protein export apparatus in the absence of its regulator, FliH. J. Bacteriol. **185:**3983–3988.
31. **Muff, T. J., and G. W. Ordal.** 2008. The diverse CheC-type phosphatases: chemotaxis and beyond. Mol. Microbiol. **70:**1054–1061.
32. **O'Toole, P. W., M. C. Lane, and S. Porwollik.** 2000. *Helicobacter pylori* motility. Microbes Infect. **2:**1207–1214.
33. **Ottemann, K. M., and A. C. Lowenthal.** 2002. *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect. Immun. **70:**1984–1990.
34. **Park, S., B. Lowder, A. M. Bilwes, D. F. Blair, and B. R. Crane.** 2006. Structure of FliM provides insight into assembly of the switch complex in the bacterial flagella motor. Proc. Natl. Acad. Sci. USA **103:**11886–11891.
35. **Paul, K., and D. F. Blair.** 2006. Organization of FliN subunits in the flagellar motor of *Escherichia coli*. J. Bacteriol. **188:**2502–2511.
36. **Paul, K., J. G. Harmon, and D. F. Blair.** 2006. Mutational analysis of the flagellar rotor protein FliN: identification of surfaces important for flagellar assembly and switching. J. Bacteriol. **188:**5240–5248.
37. **Salama, N. R., G. Otto, L. Tompkins, and S. Falkow.** 2001. Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. Infect. Immun. **69:**730–736.
38. **Senesi, S., F. Celandroni, S. Salvetti, D. J. Beecher, A. C. L. Wong, and E. Ghelardi.** 2002. Swarming motility in *Bacillus cereus* and characterization of a *fliY* mutant impaired in swarm cell differentiation. Microbiology **148:**1785–1794.
39. **Sockett, H., S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab.** 1992. Molecular analysis of the flagellar switch protein FliM of *Salmonella typhimurium*. J. Bacteriol. **174:**793–806.
40. **Szurmant, H., H. W. Bunn, V. J. Cannistraro, and G. W. Ordal.** 2003. *Bacillus subtilis* hydrolyzes CheY-P at the location of its action, the flagellar switch. J. Biol. Chem. **278:**48611–48616.
41. **Szurmant, H., T. J. Muff, and G. W. Ordal.** 2004. *Bacillus subtilis* CheC and FliY are members of a novel class of CheY-P-hydrolizing proteins in the chemotactic signal transduction cascade. J. Biol. Chem. **279:**21787–21792.
42. **Szurmant, H., and G. W. Ordal.** 2004. Diversity in chemotaxis mechanisms among the bacteria and archaea. Microbiol. Mol. Biol. Rev. **68:**301–319.
43. **Tang, H., T. F. Braun, and D. F. Blair.** 1996. Motility protein complexes in the bacterial flagellar motor. J. Mol. Biol. **261:**209–221.
44. **Tang, H., S. Billings, X. Wang, L. Sharp, and D. F. Blair.** 1995. Regulated underexpression and overexpression of the FliN protein of *Escherichia coli* and evidence for an interaction between FliN and FliM in the flagellar motor. J. Bacteriol. **177:**3496–3503.
45. **Terry, K., S. M. Williams, L. Connolly, and K. M. Ottemann.** 2005. Chemotaxis plays multiple roles during *Helicobacter pylori* animal infection. Infect. Immun. **73:**803–811.
46. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
47. **Toker, A. S., and R. M. Macnab.** 1997. Distinct regions of bacterial flagellar switch protein FliM interact with FliG, FliN and CheY. J. Mol. Biol. **273:** 623–634.
48. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:** 539–547.
49. **Turner, L., W. S. Ryu, and H. S. Berg.** 2000. Real-time imaging of fluorescent flagellar filaments. J. Bacteriol. **182:**2793–2801.
50. **Wadhams, G. H., and J. P. Armitage.** 2004. Making sense of it all: bacterial chemotaxis. Nat. Rev. Mol. Cell Biol. **5:**1024–1037.
51. **Wand, M. E., R. E. Sockett, K. J. Evans, N. Doherty, P. M. Sharp, K. R. Hardie, and K. Winzer.** 2006. *Helicobacter pylori* FlhB function: the FlhB C-terminal homologue HP1575 acts as a "spare part" to permit flagellar export when the HP0770 $FlhB_{CC}$ domain is deleted. J. Bacteriol. **188:**7531–7541.
52. **Welch, M., K. Oosawa, S. Aizawa, and M. Eisenbach.** 1993. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. Proc. Natl. Acad. Sci. USA **90:**8787–8791.