Flagellar Localization of a *Helicobacter pylori* Autotransporter Protein

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**ABSTRACT** *Helicobacter pylori* contains four genes that are predicted to encode proteins secreted by the autotransporter (type V) pathway. One of these, the pore-forming toxin VacA, has been studied in great detail, but thus far there has been very little investigation of three VacA-like proteins. We show here that all three VacA-like proteins are >250 kDa in mass and localized on the surface of *H. pylori*. The expression of the two vacA-like genes is upregulated during *H. pylori* colonization of the mouse stomach compared to *H. pylori* growth in vitro, and a wild-type *H. pylori* strain out competed each of the three corresponding isogenic mutant strains in its ability to colonize the mouse stomach. One of the VacA-like proteins localizes to a sheath that overlies the flagellar filament and bulb, and therefore, we designate it FaaA (flagella-associated autotransporter A). In comparison to a wild-type *H. pylori* strain, an isogenic faaA mutant strain exhibits decreased motility, decreased flagellar stability, and an increased proportion of flagella in a nonpolar site. The flagellar localization of FaaA differs markedly from the localization of other known autotransporters, and the current results reveal an important role of FaaA in flagellar localization and motility.

**IMPORTANCE** The pathogenesis of most bacterial infections is dependent on the actions of secreted proteins, and proteins secreted by the autotransporter pathway constitute the largest family of secreted proteins in pathogenic Gram-negative bacteria. In this study, we analyzed three autotransporter proteins (VacA-like proteins) produced by *Helicobacter pylori*, a Gram-negative bacterium that colonizes the human stomach and contributes to the pathogenesis of gastric cancer and peptic ulcer disease. We demonstrate that these three proteins each enhance the capacity of *H. pylori* to colonize the stomach. Unexpectedly, one of these proteins (FaaA) is localized to a sheath that overlies *H. pylori* flagella. The absence of FaaA results in decreased *H. pylori* motility as well as a reduction in flagellar stability and a change in flagellar localization. The atypical localization of FaaA reflects a specialized function of this autotransporter designed to optimize *H. pylori* colonization of the gastric niche.

*Helicobacter pylori* is a Gram-negative bacterium that colonizes the stomach in about 50% of humans worldwide (1–4). *H. pylori* colonization of the stomach results in gastric mucosal inflammation and is a significant risk factor for the development of distal gastric adenocarcinoma and peptic ulcer disease (3–5). One of the major virulence factors of *H. pylori* is a secreted protein known as vacuolating toxin (VacA) (7–9, 12). VacA is produced as a 140-kDa VacA protoxin that undergoes proteolytic cleavage to yield an 88-kDa protein that exhibits toxin activity (10, 11). The 88-kDa protein is secreted as a soluble protein into the extracellular space, or alternatively, it can remain attached to the bacterial cell surface (12, 13). VacA inserts into membranes to form anion-selective channels and can cause a wide array of alterations in host cells (7–9, 12).

Analysis of *H. pylori* genomes has revealed the existence of three vacA-like genes (14, 15). In strains 26695 and J99 (the first two *H. pylori* strains for which complete genome sequences were determined), these are designated HP0289/JHP0274, HP0609-JHP0610, and HP0922/JHP0856 (14, 15). Several large-scale transposon mutagenesis studies provided evidence that two of the vacA-like genes are important for colonization of the rodent stomach by *H. pylori*. Specifically, a signature-tagged mutagenesis screen identified HP0289/JHP0274 as a gene required for *H. pylori* colonization of the gerbil stomach (16). HP0289/JHP0274 was required for colonization of the mouse stomach by *H. pylori* strain LSH100 (a derivative of G27) but not by *H. pylori* strain SS1 (17). Another transposon mutagenesis screen identified HP0609/JHP0556 as a gene required for *H. pylori* colonization of the mouse stomach (18). The HP0289/JHP0274 promoter is upregulated upon *H. pylori* colonization of the mouse stomach compared to *H. pylori* growth in vitro (6), and a recent study showed that, in comparison to a wild-type *H. pylori* strain, an HP0289/JHP0274 mutant stimulated greater expression of interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) by gastric epithelial cells; therefore, it was proposed that the protein encoded by HP0289/JHP0274 (also known as ImaA) has immunomodulatory properties (17).

The genes encoding the VacA-like proteins are among the most conserved in the *H. pylori* genome and encode proteins with predicted molecular masses of 313 kDa, 348 kDa, and 260 kDa (correspond-
ing to the genes JHP0274, JHP0556, and JHP0856, respectively, in strain J99) (14, 15). Comparison of the sequences of the three VacA-like proteins with that of VacA shows that the highest level of similarity is within the C-terminal domains; other regions of the proteins exhibit very low levels of sequence similarity (15). The C-terminal region of VacA is a β-barrel domain that is required for secretion of VacA through an autotransporter (type V) pathway (19–21). Based on the sequence relatedness of the three VacA-like proteins to VacA within the β-barrel domain, it is presumed that the VacA-like proteins are also secreted by this route. Proteins secreted by the autotransporter pathway constitute the largest family of secreted proteins in Gram-negative bacteria (22–24). These proteins typically consist of three domains: (i) an N-terminal signal peptide, which is required for secretion across the inner membrane, (ii) a passenger domain, and (iii) a C-terminal β-domain, which facilitates translocation of the passenger domain across the outer membrane (15, 22–24). The passenger domains can have a wide variety of functions related to pathogenesis, including adhesion, autoaggregation, invasion, biofilm formation, and cytotoxicity. Structural analyses of several autotransporter passenger domains have revealed a conserved right-handed parallel β-helical fold (25–27). However, the primary amino acid sequences and specific functions of individual passenger domains are quite variable (22–24).

In the present study, we sought to learn more about the expression, subcellular localization, and in vivo roles of the VacA-like proteins. We show that all three vacA-like genes are expressed at increased levels when *H. pylori* colonizes the mouse stomach compared to *H. pylori* growth in vitro, and all three VacA-like proteins enhance the capacity of *H. pylori* to colonize the stomach. Studies of the localization of these proteins indicate that two VacA-like proteins localize in association with the bacterial body, whereas the third protein (which we designate FaaA, for flagella-associated autotransporter A) is detected as a component of the sheath overlying the flagellar filament and bulb. A faaA isogenic mutant exhibits decreased motility, decreased flagellar stability, and mislocalized flagella. Collectively, these results reveal that the localization of FaaA differs markedly from the localization of other known autotransporters and that FaaA has an important role in flagellar functions.

**RESULTS**

**Detection of VacA-like proteins.** *H. pylori* genomes contain three vacA-like genes, which are designated imaA (17), faaA, and vlpC (defined in Materials and Methods). These three genes, each >7 kb in length, are among the largest in the *H. pylori* genome. As a first step in analyzing the three VacA-like proteins, we genetically modified *H. pylori* strain 60190 to yield strains encoding c-Myc-tagged versions of each VacA-like protein. A previous study identified the imaA promoter as one of several promoters that are induced during *H. pylori* colonization of the mouse stomach compared to growth in vitro (6). Therefore, we hypothesized that the other vacA-like genes might be subject to similar regulation. C57BL/6 mice were orogastrically infected with *H. pylori* strain G27, and the animals were euthanized 2, 6, and 16 weeks thereafter. Transcription of imaA, faaA, and vlpC by *H. pylori* in the mouse stomach was analyzed by real-time reverse transcription-PCR (RT-PCR), as described in Materials and Methods. The transcription of all three genes was increased upon *H. pylori* colonization of the mouse stomach compared to *H. pylori* growth in vitro (Fig. 2A). The relative increase in transcription (in vivo compared to in vitro) was greatest for imaA at all three time points. These data confirm that imaA expression is increased during bacterial growth in vivo compared to bacterial growth in vitro and indicate that the other vacA-like genes also exhibit increased transcription in vivo.

A vacA mutant strain was previously reported to have a colonization defect compared to a wild-type strain in mouse infection experiments (29). To assess a potential role of VacA-like proteins in vivo, we performed competition experiments. Since *H. pylori* strain G27 colonized a relatively low proportion of challenged mice, we used *H. pylori* strain X47 for these experiments. Mice were coinfected with 1:1 mixtures of the wild-type (WT) strain plus individual isogenic imaA, faaA, or vlpC mutants. After 2 weeks of infection, mice were euthanized, and bacterial colonization was assessed by analyzing bacterial growth on antibiotic-containing media that were selective for either the WT strain (metronidazole resistant) or the mutant strains (chloramphenicol resistant). The total bacterial densities in these animal stomachs ranged from 10^5 to 10^6 CFU/g for each animal; the bacterial densities did not differ significantly between animals challenged with...
different strains. A competitive index was determined as described in Materials and Methods. The WT bacteria outcompeted all three mutant strains (Fig. 2B) \((P < 0.04\) for each mutant). Collectively, these experiments provide evidence that each of the VacA-like proteins has an important role in vivo.

Localization of FaaA to flagella. To further investigate the localization of the VacA-like proteins, we first utilized immunofluorescence microscopy to analyze \(H. pylori\) strains producing c-Myc-tagged versions of ImaA, FaaA, and VlpC. In these immunofluorescence studies, ImaA and VlpC were localized on the surfaces of the bacteria, as expected based on their sensitivity to proteinase K, and were localized to a bacterial pole (Fig. 3A to D). In contrast, FaaA was localized to a site external to the bacterial body (Fig. 3E and F). Based on the immunofluorescence results, we hypothesized that FaaA might be localized to the flagella. To test this hypothesis, we generated preparations enriched in flagella from an \(H. pylori\) strain that produced a c-Myc tagged form of FaaA and from a control strain that produced a c-Myc-tagged form of ImaA. The presence of FaaA and ImaA in cell lysates and in the flagellar preparations was then examined by Western blotting using an anti-c-Myc antibody. As a control, we analyzed the presence of FlaA, the major flagellin subunit. As expected, FaaA was enriched in the flagellar preparations compared to total cell lysates (Fig. 3G), indicating an enrichment of flagellar components in the flagellar preparations. FaaA was also enriched in the flagellar preparations, whereas ImaA was not. These results support the hypothesis that FaaA is associated with flagella.

We then used immunoelectron microscopy to investigate potential flagellar localization of FaaA. \(H. pylori\) is characterized by the presence of multiple unipolar flagella (30, 31), and distinctive features of \(H. pylori\) flagella include the presence of a terminal bulb and a flagellar sheath (30–34). Analysis of FaaA localization by EM and immunogold staining revealed that FaaA localized to the flagellar sheath that covers the flagellar filament and the flagellar bulb (Fig. 4A to D). In contrast, ImaA, VlpC, and VacA were not detected in association with flagella (Fig. 4E to G) but were detected at the nonflagellar bacterial pole (see Fig. S2 in the supplemental material). Gold labeling was not detected in any of the negative-control samples; these included \(H. pylori\) lacking the c-Myc tag and processed in parallel with the other strains (Fig. 4H) and strains producing c-Myc-tagged versions of ImaA, FaaA, or VlpC that were unlabeled with secondary antibody conjugated to gold particles alone (primary antibody omitted) (data not shown). Quantitative analyses confirmed that FaaA localized mainly to the flagella (Table 1) \((P < 0.0001)\). We also used immunofluorescence and electron microscopy to analyze two other \(H. pylori\) strains (99 and X47) that produced c-Myc-tagged versions of ImaA, FaaA, and VlpC. Results in these strains were similar to what was observed in studies of strain 60190 and confirmed that FaaA localized to the flagella (Fig. 4D and data not shown).

Flagellar alterations in a \(faaA\) mutant strain. Since FaaA was localized to the flagella, we hypothesized that a \(faaA\) mutant strain might have a defect in flagellar morphology. To test this hypoth-
alleles. Isogenic mutants were rarely detected in preparations of the WT strain or mutants with restored faaA allele. In comparison to WT bacteria, faaA mutant bacteria produced reduced levels of FlaA, based on Western blot analysis (Fig. 6A). In a similar analysis of imaA and vlpC mutants compared to WT bacteria, there was no detectable difference in FlaA production (Fig. 6A). To investigate whether the observed reduction in FlaA protein production in the faaA mutant strain was due to a reduction in flaA transcription, we performed real-time RT-PCR analysis. We did not detect any decreased flaA transcription in the faaA mutant strain, but in fact, we detected increased flaA transcription in the faaA mutant compared to the WT strain (Fig. 6C) ($P < 0.03$). These results suggest that the reduced level of FlaA in a faaA mutant strain is due to reduced FlaA stability.

**Decreased motility of faaA mutant strains.** Since FaaA is localized to flagella and various flagellar alterations were detected in

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**FaaA plays a role in flagellar stability.** To further examine the role of FaaA in flagellar stability, we examined the production of FlaA (the major flagellin) in a WT strain, in isogenic faaA, imaA and vlpC mutants, and in a mutant with a restored faaA allele. In comparison to WT bacteria, faaA mutant bacteria produced reduced levels of FlaA, based on Western blot analysis (Fig. 6A). In a similar analysis of imaA and vlpC mutants compared to WT bacteria, there was no detectable difference in FlaA production (Fig. 6A). To investigate whether the observed reduction in FlaA protein production in the faaA mutant strain was due to a reduction in flaA transcription, we performed real-time RT-PCR analysis. We did not detect any decreased flaA transcription in the faaA mutant strain, but in fact, we detected increased flaA transcription in the faaA mutant compared to the WT strain (Fig. 6C) ($P < 0.03$). These results suggest that the reduced level of FlaA in a faaA mutant strain is due to reduced FlaA stability.

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The motility of a WT strain, a mutant strain exhibited decreased motility (Fig. 7A). There were no significant differences in the motility (Fig. 7A). Restoration of an intact copy of \textit{faaA} in the \textit{faaA} mutant strain demonstrated attenuated colonization compared to the WT strain (Fig. 8A) ($P = 0.0172$). In contrast, there was no significant difference at 1 month postinfection (Fig. 8B). These data, along with the competition experiments (Fig. 2), indicate that \textit{faaA} is required for optimal \textit{H. pylori} colonization during early stages of infection, when flagella are required for bacterial entry into the gastric mucus layer (35, 36).

**DISCUSSION**

The \textit{H. pylori} genome contains three \textit{vacA}-like genes that have C-terminal regions related to that of \textit{vacA}, which encodes a secreted toxin. The C-terminal regions shared by \textit{VacA} and the \textit{vacA}-like proteins correspond to a predicted $\beta$-barrel domain that is required for protein secretion via the autotransporter pathway. The results of the present study show that these three \textit{VacA}-like proteins share several common features. ImaA, \textit{faaA}, and VlpC are among the largest proteins produced by \textit{H. pylori} (each $>250$ kDa in mass), and they are each localized on the bacterial surface. The three \textit{vacA}-like genes are all upregulated during \textit{H. pylori} colonization of the mouse stomach compared to \textit{H. pylori} growth in vitro. Finally, \textit{imaA}, \textit{faaA}, and VlpC mutants each have a competitive disadvantage compared to the WT strain in mouse colonization experiments.

Unlike ImaA and VlpC, which are localized to a bacterial pole, \textit{faaA} is localized to the flagella. Correspondingly, \textit{faaA} mutants exhibit multiple flagellar abnormalities, including absence of flagella, decreased numbers of flagella, increased flagellar fragility, and mislocalization of flagella to the lateral side of the bacteria instead of the pole. In addition, \textit{faaA} mutants exhibit decreased motility compared to the WT strain. Thus, \textit{faaA} is required not only for flagellar stability and proper flagellar localization but also for optimal flagellar function. In an analysis of gastric colonization, a \textit{faaA} mutant strain colonized the mouse stomach less efficiently than WT bacteria at an early time point postinfection, which is consistent with a known essential role of motility at early stages of \textit{H. pylori} infection (36). \textit{FaA} might also have a role at later stages of infection, since flagella are likely to be required for continuous \textit{H. pylori} colonization of the gastric mucus layer during the natural turnover of gastric mucus and exfoliation of gastric epithelial cells.

To the best of our knowledge, flagellar localization of autotransporter proteins has not previously been reported. The atypical localization of \textit{FaA} is probably attributable at least in part to unusual features of \textit{H. pylori} flagella. \textit{H. pylori} contains 2 to 6 polar flagella that are characterized by the presence of a sheath and a terminal bulb (30–34). Thus far, there has been relatively little analysis of the \textit{H. pylori} flagellar sheath, and only one protein, HpaA, has been previously localized to this site (37–39). We speculate that the flagellar sheath contains multiple components that are derived from the \textit{H. pylori} outer membrane. Therefore, we propose that \textit{FaA} is exported to the outer membrane and subse-

![Immunogold EM analysis of FaA localization.](image-url)

**FIG 4** Immunogold EM analysis of FaA localization. \textit{H. pylori} strains were immunolabeled with primary antibodies to either VacA or c-Myc, followed by secondary antibodies conjugated to 10-nm immunogold particles. (A to C) 60190 \textit{FaA} c-Myc labeled with an anti-c-Myc antibody, demonstrating FaA localization to the flagellar filament and flagellar bulb. (D) X47 \textit{FaA} c-Myc labeled with an anti-c-Myc antibody; (E) 60190 \textit{imaA} c-Myc labeled with an anti-c-Myc antibody; (F) 60190 (no c-Myc tag) labeled with an anti-VacA antibody; (G) 60190 VlpC c-Myc labeled with an anti-c-Myc antibody; (H) 60190 (no c-Myc tag) labeled with an anti-c-Myc antibody. FaA localizes to flagella, whereas VacA, \textit{imaA}, and VlpC do not. The experiment was performed three times in multiple strains with similar results. Bars, 100 nm.

\textit{faaA} mutant strains, we hypothesized that \textit{FaA} might be required for optimal \textit{H. pylori} motility. To test this hypothesis, we analyzed the motility of a WT strain, a \textit{faaA} isogenic mutant, and a mutant with a restored intact copy of \textit{faaA}. In comparison to the WT strain, the \textit{faaA} mutant strain exhibited decreased motility (Fig. 7A). Restoration of an intact \textit{faaA} gene reversed the defect in motility (Fig. 7A). There were no significant differences in the motility of \textit{imaA} or VlpC mutants compared to the WT strain (Fig. 7B). These results indicate that \textit{faaA} is required not only for flagellar stability and proper flagellar localization but also for optimal motility.
TABLE 1 Analysis of FaaA localization a,b

| Strain          | Mean no. of gold particles per bacterium at site | Flagella | Flagellar pole | Nonflagellar pole | Side of bacterium |
|-----------------|-----------------------------------------------|----------|----------------|-------------------|------------------|
| 60190 FaaA c-Myc | 2.7 ± 0.5                                     | 0.87 ± 0.18 | 0              | 0.56 ± 0.13       |
| 60190 faaA mutant | 0                                              | 0        | 0              | 0                 |
| 60190           | 0                                              | 0        | 0              | 0.028 ± 0.17      |

a FaaA localization in the indicated strains was analyzed by immunogold EM, using an anti-c-Myc antibody followed by a secondary antibody conjugated to immunogold particles. Mean ± SEM values are reported.

b The numbers of bacteria that were analyzed to generate the data were 45 for 60190 FaaA c-Myc, 123 for the 60190 faaA mutant, and 37 for 60190.

quently becomes a component of the flagellar sheath. Interestingly, we noted that levels of FlaA (the major component of flagella) were diminished in a faaA mutant, but there was no detectable reduction in flaA transcription in this mutant. We hypothesize that FaaA interacts directly or indirectly with multiple flagellar proteins; thus, the absence of FaaA may result in decreased protein stability of FlaA and possibly decreased stability of other flagellar components as well.

In summary, this study highlights important features of the H. pylori VacA-like proteins, including upregulation of the corresponding genes in vivo and a role for these proteins in colonization of the mammalian stomach. Unexpectedly, we show that one of these proteins, FaaA, localizes to flagella and that FaaA is required for proper flagellar localization and optimal flagellar function. This unusual localization and function of an autotransporter protein presumably reflects an adaptation designed to optimize H. pylori colonization of the gastric mucosal niche.

MATERIALS AND METHODS

Bacterial strains and culture conditions. H. pylori strains 60190 (ATCC 49503), 60190 vacA:cat-rdxA (40), and J99 (ATCC 700824) and mouse-adapted versions of strain G27 and X47 were selected for use in this study. H. pylori strains were routinely grown at 37°C on Trypticase soy agar plates containing 5% sheep blood in ambient air containing 5% CO2. H. pylori mutant strains were selected based on resistance to chloramphenicol (2.5 μg ml−1) or resistance to metronidazole (7.5 μg ml−1) on brucella agar plates (brucella broth containing 1.35% agar and 10% fetal bovine serum [FBS]). H. pylori liquid cultures were grown in brucella broth (Sigma) supplemented with 5 to 10% fetal bovine serum (Atlanta Biologicals) or cholesterol (Gibco) (41). Prior to mouse infections, H. pylori strains were grown in brucella broth containing 10% FBS and 10 μg ml−1 vancomycin at 37°C under microaerobic conditions generated by a GasPakEZ Campy Container System (BD Biosciences).

Mutagenesis of imaA, faaA, and vlpC and production of c-Myc-tagged proteins. In this present study, we designate HP0289/HP0274 as imaA (for immunomodulatory autotransporter protein A [17]), HP0609/HP0051 as faaA (for flagella-associated autotransporter A), and HP0922/HP0856 as vlpC (for VacA-like protein C). To introduce mutations into these genes, we used a previously described mutagenesis method (42, 43). As a first step, metronidazole-resistant forms of strains 60190, X47, and J99 (60190ΔrdxA, X47ΔrdxA, and J99ΔrdxA) were generated by deleting the rdxA gene (43). Next, fragments of each gene (nucleotides 1,261 to 3,530 for imaA, 2,041 to 4,284 for faaA, and 1,390 to 3,639 for vlpC, with numbers based on the DNA sequences from H. pylori strain J99) were PCR amplified from H. pylori 60190, X47, and J99 genomic DNA and cloned into pGEM-T Easy (Promega). These plasmids were then used as templates for inverse PCR to generate modified plasmids containing a BamHI site after nucleotide 2199, 2997, and 2067 (numbers based on the sequences of genes in H. pylori strain J99) in imaA, faaA, and vlpC, respectively. The locations of the BamHI sites were selected based on the identification of regions that are predicted to be surface-exposed in a Hopp-Woods hydrophobicity analysis (http://www.liv.virginia.edu/molkit/hydrophy.html). A cat-rdxA cassette was cloned into the BamHI site, and cat-rdxA-containing plasmids, which are unable to replicate in H. pylori, were then transformed into H. pylori 60190ΔrdxA, X47ΔrdxA, and J99ΔrdxA, thereby allowing insertion of the cat-rdxA cassette into imaA, faaA, or vlpC. Single colonies were selected based on chloramphenicol resistance and metronidazole sensitivity. In each case, correct insertion of the cat-rdxA cassette was confirmed by PCR analysis.

To restore an intact copy of the relevant gene in strains that had been mutated and simultaneously insert a sequence encoding a c-Myc epitope tag into the gene of interest, we used a counterselection method, as described previously (42, 43). A DNA sequence encoding a c-Myc tag (5’ GAA CAA AAA CTT ATT AGT GAA GAA GAT CTT 3’) was inserted into the BamHI site in the plasmids described above using a QuickChange II XL site-directed mutagenesis kit (Agilent). Correct insertion of the c-Myc tag was confirmed by DNA sequencing. Plasmids containing the c-Myc-tagged versions of each VacA-like protein were then transformed into the appropriate H. pylori strains containing the cat-rdxA cassette, and metronidazole-resistant transformants were selected. This resulted in replacement of the cat-rdxA cassette with a sequence that contained the c-Myc tag.

Experiments in this study analyzed properties of metronidazole-resistant strains that contain WT copies of imaA, faaA, and vlpC, compared to isogenic mutant strains with disruptions of imaA, faaA, and vlpC and derivatives that contain restored intact forms of these genes with a c-Myc epitope tag. For convenience, the parental strains are designated WT here (despite the presence of the rdxA mutation) because they contain WT copies of all three vacA-like genes.

Detection of c-Myc-tagged proteins. The presence of c-Myc-tagged VacA-like proteins was assessed by Western blot analysis using an anti-c-Myc antibody (4F6, 1:1,000; Vanderbilt Monoclonal Antibody Core) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Promega). Proteins were visualized by incubation with chemiluminescent substrate solution (Pierce) and exposure to X-ray film.

Proteinase K susceptibility. The susceptibility of the VacA-like proteins to proteinase K digestion was assessed using a modified version of previous protocols (43, 44). H. pylori strains were grown in liquid medium for 18 h and then harvested and washed with PBS. Bacteria were then resuspended in RPMI medium only or RPMI medium containing 50 μg/ml of proteinase K and incubated at 37°C for 30 min. Proteinase K activity was abrogated by the addition of phenylmethylsulfonyl fluoride (PMSF; 2 mM final concentration). The bacteria were washed in RPMI containing 2 mM PMSF, resuspended in SDS sample buffer, and analyzed by immunoblotting using an anti-c-Myc antibody. As controls, we monitored proteolysis of VacA, which is known to be localized to the bacterial surface (13), and heat shock protein B (HspB), which is localized within the cytoplasm, using antisera directed toward these proteins. Antibody concentrations used were 1:1,000 (anti-c-Myc), 1:10,000 (anti-VacA), and 1:20,000 (anti-HspB).

Immunofluorescence microscopy. H. pylori strains were washed in PBS (pH 7.4), fixed in PBS containing 2.5% glutaraldehyde and 2.0% paraformaldehyde for 1 h at room temperature, washed twice with PBS, and blocked for 1 h in PBS containing 0.1% bovine serum albumin. Cells were incubated with
anti-c-Myc antibody for 4 h at 4°C. Afterward, cells were washed three times with PBS and then incubated overnight at 4°C with an Alexa Fluor 488-conjugated secondary antibody (goat anti-mouse IgG; Invitrogen). Bacterial cells were washed three times with PBS before being counterstained with propidium iodide. As negative controls, replicate samples were processed by applying secondary antibodies alone. Samples were mounted using ProLong Gold antifade reagent (Invitrogen) and viewed using a Zeiss Axioptot wide-field microscope or a Zeiss LSM710 confocal laser scanning microscope.

**Immunoelectron microscopy.** Immunoelectron microscopy was performed as described previously (43). Briefly, *H. pylori* strains were washed in 0.05 M sodium cacodylate buffer (pH 7.4) and then fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.05 M sodium cacodylate buffer for 1 h at room temperature. Cells were washed twice with sodium cacodylate buffer and blocked for 1 h in sodium cacodylate buffer containing 0.1% gelatin. Cells were incubated with primary antibody (mouse monoclonal anti-c-Myc) for 4 h at 4°C. Afterward, cells were washed three times with sodium cacodylate buffer before incubation overnight at 4°C with goat anti-mouse IgG conjugated to 10 nm gold particles (Ted Pella) or 25 nm gold particles (Electron Microscopy Sciences). The following day, bacterial cells were spotted onto Formvar-coated grids (Electron Microscopy Sciences) and negatively stained with 1% ammonium molybdate. As negative controls, replicate samples were processed by applying secondary antibodies alone. Samples were viewed with a FEI T-12 or a Philips C-12 transmission electron microscope.

**FaaA labeling and localization were quantified by counting the number of gold particles. Quantification was done by one person (J.N.R.), who was blinded to the identity of the samples.**

**Electron microscopy analysis of flagella.** *H. pylori* strains were cultured on blood agar plates or in brucella broth (BB)-5% FBS overnight at 37°C in 5% CO₂. Cells were harvested from the plate in 0.05 M sodium cacodylate buffer (pH 7.4) for an initial wash. Bacterial cells were spotted onto Formvar-coated grids and negatively stained with 1% ammonium molybdate. Samples were viewed with a FEI T-12 or a Philips C-12 transmission electron microscope. The number and localization of flagella were quantified by counting the number of flagella per bacterium. Quantification was done by one person (J.N.R.), who was blinded to the identity of the samples.

**Motility assay.** *H. pylori* motility was analyzed as described previously (36, 45). Briefly, 1-µl aliquots of bacterial suspensions (overnight broth cultures back-diluted to an optical density at 600 nm [OD₆₀₀] of 0.1) were stabbed into soft agar plates composed of BB–10% FBS and 0.35% agar. The plates were then incubated for 5 days at 37°C, and the diameters of the bacterial halos were measured each day.

**Enrichment of flagella.** A preparation enriched in flagella was prepared using a protocol adapted from previously described methods (46–48). Briefly, *H. pylori* strains were grown in liquid culture at 37°C for 18 h under microaerobic conditions. Bacteria were centrifuged at 6,000 rpm for 10 min, pellets were resuspended in sucrose solution (0.5 M sucrose, 10 mM Tris-HCl [pH 8.0]) containing lysozyme (0.02 mg/ml final concentration), EDTA (10 mM final concentration), and Zwitter-
FIG 6 FaaA has a role in flagellar stability. (A) WT H. pylori strain J99, isogenic faaA, vlpC, and imaA mutant strains, and a faaA mutant with a restored intact faaA allele were cultured overnight in BB-FBS, and the presence of FlaA was assessed by Western blotting. The presence of HspB was monitored as a control. The level of FlaA was reduced in the faaA mutant compared to the other strains. The experiment was performed multiple times with similar results. (B) Transcription of flaA was analyzed by real-time RT-PCR, as described in Materials and Methods. RNA from WT bacteria served as the calibrator, and relative units are shown in comparison to the WT strain. Transcription of flaA was increased in the faaA mutant strain compared to the WT strain. Error bars represent mean ± standard error from combined results of three independent experiments performed in triplicate.

Infection of mice with H. pylori. Eight-week-old Helicobacter-free male C57BL/6 mice (Jackson Laboratory) were used in all experiments, with a minimum of 5 to 10 mice per group. Prior to infection of mice, H. pylori was inoculated into liquid medium and cultured for 18 h under microaerobic conditions. Mice were orogastrically inoculated with a suspension of 5 × 10⁸ CFU of H. pylori in 0.5 ml of brucella broth without supplemental FBS (49). For competition experiments, mice were coinfected with a 1:1 ratio of the WT strain plus imaA, faaA, or vlpC mutant H. pylori strains, using a total input of approximately 5 × 10⁸ CFU in 0.5 ml of brucella broth. The inocula of the WT and mutant strains used for coinfection experiments were verified to contain equivalent CFU/ml, based on colony counting. Mice were orogastrically infected with two doses, administered two days apart. For in vivo gene expression studies, mice were euthanized at 2, 6, and 16 weeks postinfection. For competition experiments, mice were euthanized at 2 weeks postinfection. For colonization studies in which animals were infected with a single strain, mice were euthanized at 4 days or 1 month postinfection.

FIG 7 Mutation of faaA results in decreased motility. Motility of WT H. pylori strain J99, isogenic imaA, faaA, and vlpC mutant derivatives, and mutants with restored intact forms of imaA, faaA, and vlpC was assessed. Bacterial suspensions were inoculated into semisolid brucella medium, and the outward migration was measured over a period of 5 days. (A) Analysis of the motility of a faaA mutant compared to the WT strain and a mutant with a restored intact faaA. (B) Quantification of H. pylori motility. The motility of the faaA mutant was significantly decreased compared to that of the WT strain and the other strains tested. Data are means ± standard error from combined results of three independent experiments, each performed in triplicate. The asterisk indicates a P value of <0.05 compared to all other strains (Student’s t test).
FIG 8 Role of FaaA in *H. pylori* colonization of the mouse stomach. C57BL/6 mice were infected with WT *H. pylori* strain X47 or an isogenic mutant for 4 days or 1 month. Mice were then euthanized and *H. pylori* colonization of the stomach was analyzed as described in Materials and Methods. (A) C57BL/6 mice were orogastrically infected with either the WT strain or an isogenic *faaA* mutant strain (WT, *n* = 5; *faaA* mutant, *n* = 14) for 4 days. The asterisk indicates a *P* value of 0.019 (Mann-Whitney U test). (B) Mice were infected with the WT strain or a *faaA* mutant strain (WT, *n* = 5; *faaA* mutant, *n* = 10) for 1 month. CFUs for individual mice are shown. Medians with interquartile ranges are shown.

Processing of mouse stomachs and culturing of *H. pylori* from mouse stomachs. Mouse stomachs were processed as described previously, with minor modifications (49). The stomach was removed from each mouse by excising between the esophagus and the duodenum. The forestomach (nonglandular stomach) was removed from the glandular stomach and discarded. The glandular stomach was opened and rinsed gently in PBS. For colonization studies, the glandular stomach was cut in half and placed into brucella broth for immediate processing. Gastric tissue was then homogenized using a Tissue Tearor (BioSpec Products), and serial dilutions of the homogenate were plated on Trypticase soy agar plates containing 5% sheep blood, 10 μg ml⁻¹ nalidixic acid, 100 μg ml⁻¹ vancomycin, 2 μg ml⁻¹ amphotericin, and 200 μg ml⁻¹ bacitracin. For coinfection experiments, plates also contained either chloramphenicol (2.5 μg ml⁻¹) or metronidazole (7.5 μg ml⁻¹), in order to permit selective isolation of mutant and WT strains, respectively (the WT strain X47 used in these experiments is metronidazole resistant, and the *imaA*, *faaA*, and vlpC mutants are chloramphenicol resistant). Plates were cultured under microaerobic conditions for 5 days before colonies were counted.

For experiments designed to analyze bacterial transcription *in vivo*, stomachs were incised and washed as described above, the gastric mucosa was scraped with cell scrapers (Fisher), and the scraped mucosa was placed in RNAProtect (Qiagen). Scrapings from 3 or 4 mouse stomachs were combined and analyzed as a single pooled sample. RNA was isolated and RT-PCR was performed as described above. Competitive index was determined by dividing the number of cultured mutant bacteria by the number of cultured WT bacteria, followed by corrections for any deviations from an input ratio of 1:1 (17).

Statistical analysis. Gene transcription data, motility data, mouse colonization data, competition data, and flagellar localization data were analyzed using Student's *t* test. Bacterial colonization densities were analyzed using the Mann-Whitney *U* test. Quantitative data pertaining to FaaA labeling, flagellar numbers, and flagellar localization were analyzed using the Kruskal-Wallis test. All statistical analyses were performed using the GraphPad Prism 5 program.

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine and the VA Institutional Animal Care and Use Committee (IACUC protocol V/10/157 and M/06/333).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00613-12/-/DCSupplemental.

Figure S1, TIF file, 0.7 MB.
Figure S2, TIF file, 7.3 MB.

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