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Helicobacter pylori FlhA Binds the Sensor Kinase and Flagellar Gene Regulatory Protein FlgS with High Affinity

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ABSTRACT

Flagellar biogenesis is a complex process that involves multiple checkpoints to coordinate transcription of flagellar genes with the assembly of the flagellum. In Helicobacter pylori, transcription of the genes needed in the middle stage of flagellar biogenesis is governed by RpoN and the two-component system consisting of the histidine kinase FlgS and response regulator FlgR. In response to an unknown signal, FlgS autophosphorylates and transfers the phosphate to FlgR, initiating transcription from RpoN-dependent promoters. In the present study, export apparatus protein FlhA was examined as a potential signal protein. Deletion of its N-terminal cytoplasmic sequence dramatically decreased expression of two RpoN-dependent genes, flaB and flaE. Optical biosensing demonstrated a high-affinity interaction between FlgS and a peptide consisting of residues 1 to 25 of FlhA (FlhA NT). The K P (equilibrium dissociation constant) was 21 nM and was characterized by fast-on (k on = 2.9 × 104 M−1s−1) and slow-off (k off = 6.2 × 10−3 s−1) kinetics. FlgS did not bind peptides consisting of smaller fragments of the FlhA NT sequence. Analysis of binding to purified fragments of FlgS demonstrated that the C-terminal portion of the protein containing the kinase domain binds FlhA NT. FlhA NT binding did not stimulate FlgS autophosphorylation in vitro, suggesting that FlhA facilitates interactions between FlgS and other structures required to stimulate autophosphorylation.

IMPORTANCE

The high-affinity binding of FlgS to FlhA characterized in this study points to an additional role for FlhA in flagellar assembly. Beyond its necessity for type III secretion, the N-terminal cytoplasmic sequence of FlhA is required for RpoN-dependent gene expression via interaction with the C-terminal kinase domain of FlgS.

Helicobacter pylori is an epsilonproteobacterium that can cause significant pathologies in the stomach (1–3). Approximately 50% of the world population is infected with H. pylori, although only a small fraction of infected individuals have symptoms. H. pylori possesses 2 to 6 polar flagella that are used to burrow through the mucus layer lining the stomach epithelium. Colonization of the gastric mucosa is dependent on motility, as nonflagellated mutants are unable to colonize (4).

The flagellum itself is a complex structure comprised of the basal body, hook, and filament (5). The basal body is located within the cell envelope and contains the flagellar protein export apparatus, the various flagellar rings, the rod, and motor components. The export apparatus is responsible for the secretion of axial components of the flagellum and consists of six proteins located within the inner membrane (FlfO, FlfP, FlfQ, FlfR, FlfA, and FlfB) plus three cytoplasmic proteins that bring flagellar substrates to the integral membrane component of the export apparatus (FlfH, Flfi, and Flfi). The C ring (or switch complex) is located at the cytoplasmic side of the inner membrane. In H. pylori it consists of four proteins, FlfG, FlfM, FlfN, and FlfY. In addition to controlling the rotational direction of the flagellum, the C ring works with the soluble components of the export apparatus to bring flagellar substrates to the export apparatus for secretion (6, 7). The rod proteins are the first proteins exported and are followed by hook proteins. The hook serves as a universal joint between the rod and the filament, transmitting torque from the motor to the filament. The filament is assembled after the completion of the hook and involves the minor flagellin FlaB and the major flagellin FlaA.

Flagellar biogenesis is a complex process that involves the coordinated expression of over 50 structural and regulatory genes with assembly of the nascent flagellum. In H. pylori, temporal expression of flagellar genes is controlled by the three sigma factors found in the bacterium: RpoD (σ 28), RpoN (σ 24), and FlfA (σ 28). Transcription of the early flagellar genes, which encode components of the basal body, is regulated by RpoD. Genes whose products are needed for flagellar biogenesis following assembly of the basal body include components of the hook and a minor flagellin, and transcription of these genes is regulated by RpoN. Transcription of the RpoN-dependent genes is regulated by the two-component system consisting of the sensor kinase FlgS and the response regulator FlgR (8, 9). FlgS undergoes autophosphorylation in response to an unknown signal and subsequently transfers the phosphate to FlgR. Based on characterization of other RpoN-dependent genes (10), phosphorylation of FlgR likely pro-
motates multimerization of the protein, which allows it to interact productively with RpoN-RNA polymerase holoenzyme to stimulate transcription. Components of the export apparatus are required for transcription of the RpoN-dependent genes, as deletions of flhB, flfO, and flhA result in decreased expression of these genes in *H. pylori* (11–13). The transcription of genes whose products are required later in flagellar biogenesis, which include the major flagellin, is regulated by FlIA. The activity of FlIA is regulated by the anti-sigma factor FlgM which, when bound to FlIA, represses its activity (14). In *Salmonella*, inhibition of FlIA activity is relieved when FlgM is secreted through the nascent flagellum as a filament-type substrate (15). FlgM has not been shown to be exported in *H. pylori*, but it may bind FlhA to alleviate the repression of FlIA (16).

In this study, we sought to identify the activating signal that is sensed by FlgS to initiate signal transduction, resulting in expression of the RpoN regulon. In a previous study, we showed that a truncated form of the export apparatus component FlhA, consisting of only the first 77 residues of the protein, is sufficient to support transcription of RpoN-dependent genes (13). The 25 amino acid residues at the N terminus of FlhA (FlhA<sub>NT</sub>) are predicted to be exposed on the cytoplasmic side of the membrane. They are the only significant components of the 77-amino-acid sequence likely to be exposed to cytoplasmic FlgS, since putative transmembrane domains span residues 26 to 48 and 50 to 69. We show here that an FlhA variant lacking FlhA<sub>NT</sub> is unable to support transcription of RpoN-dependent genes, suggesting a role for FlhA<sub>NT</sub> in regulating expression of the RpoN regulon.

Using optical biosensing, we show that a peptide corresponding to FlhA<sub>NT</sub> binds FlgS with nanomolar affinity via the C-terminal half of FlgS. In *vitro* phosphorylation studies suggest that binding of the FlhA<sub>NT</sub> peptide does not stimulate FlgS autophosphorylation. Our results suggest a mechanism by which transcription of RpoN-dependent genes is initiated via an early flagellar assembly checkpoint that involves the amino-terminal segment of FlhA.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Escherichia coli* strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. Kanamycin (30 μg/ml) or ampicillin (100 μg/ml) was added to the medium when appropriate. *H. pylori* strains were grown on tryptic soy agar (TSA) supplemented with 10% horse serum at 37°C under an atmosphere consisting of 2% O<sub>2</sub>, 5% CO<sub>2</sub>, and 93% N<sub>2</sub>. Kanamycin (30 μg/ml) or ampicillin (100 μg/ml) was added to the medium for culturing *H. pylori*. *H. pylori* strains B128 and ATCC 43504 as previously described (13). Briefly, the region corresponding to 90 nucleotides upstream of the start codon through codon 77 of *flhA* was replaced with a chloramphenicol cassette. Genomic DNA from *H. pylori* was extracted using the Wizard genomic DNA purification kit (Promega) and used as the template for creating *flhA* mutants. This region was used to perform overlapping PCR (qRT-PCR) as described in reference 12. Primers used for this study are listed in Table 1.

### Strain construction. **ΔflhA** mutants were constructed in *H. pylori* strains B128 and ATCC 43504 as previously described (13). Briefly, the region corresponding to 90 nucleotides upstream of the start codon through codon 77 of *flhA* was replaced with a chloramphenicol transacylase (*cat*) cassette. Genomic DNA from *H. pylori* was extracted using the Wizard genomic DNA purification kit (Promega) and used as the template for creating *flhA* alleles. The **ΔflhA** mutant was complemented with *flhA* alleles expressed from the native *flhA* promoter and carried on the shuttle vector pHE3 (17). Primers SphI flhA F2 and KpnI flhA R2 were used to amplify *flhA* and its native promoter. This region was cloned into pCR2.1 and subcloned into pHel3 via the SphI and KpnI sites. The resulting plasmid containing the *flhA* region was called *pf1flhA*. Another plasmid was generated to create an in-frame deletion of codons 2 to 24 (pf1flhA<sub>25NT</sub>). Primers SphI Pf1flhA F2 and flhA24 R were used to amplify the *flhA* promoter and the start codon of *flhA*. Primers flhA24 F and KpnI flhA R2 were used to amplify codons 25 to 56 nucleotides downstream of the *flhA* stop codon. Primer flhA24 F contains sequences reverse and complementary to *flhA* R2. This region was used to perform overlapping PCR to create an in-frame deletion of codons 2 through 24. The resulting amplicon was cloned into pCR2.1 and subcloned into pHel3, and the resulting plasmid was named pf1flhA<sub>25NT</sub>. Plasmids pf1flhA and pf1flhA<sub>25NT</sub> were introduced into the ΔflhA mutant by natural transformation. All constructs were confirmed by PCR and sequencing of the resulting amplicon. Primers used for strain construction are listed in Table 1.

Plasmids encoding fragments of *flgS* were synthesized by DNA 2.0 (Menlo Park, CA). The parent vector pH414 (DNA 2.0) contains a T7 promoter and a terminator sequence flanking a multiple cloning site. DNA sequences encoding residues 1 to 169 and residues 170 to 230 along with an N-terminal His tag were cloned into the NdeI and BamHI site of this vector to create the plasmids pf2flgS<sub>NT</sub> and pf2flgS<sub>C</sub>, respectively. For expression and purification, pf2flgS<sub>NT</sub> and pf2flgS<sub>C</sub> were transformed into *E. coli* BL21(DE3) pLysS.

**Motility assay.** Motility of *H. pylori* cells was assayed on soft agar plates consisting of Mueller–Hinton broth and 0.4% Noble agar. After autoclaving, sterile heat-inactivated horse serum (10% final concentration), FeSO<sub>4</sub> (10 μM final concentration), and MES (20 mM final concentration) were added to the medium. A sterile toothpick was used to inoculate cells into the center of the agar. Three inocula were used per strain. Plates were incubated at 37°C under an atmosphere of 2% O<sub>2</sub>, 5% CO<sub>2</sub>, and 93% N<sub>2</sub>. Diameters of the halos formed by cells migrating from the point of inoculation were measured after 1 week. Diameter averages were tested for significance with Student’s t-test.

**qRT-PCR.** RNA was extracted as previously described (13). Briefly, *H. pylori* cells were grown on TSA supplemented with 10% horse serum for 18 h. Cells were harvested and resuspended in 100 μl of distilled water. RNA was extracted using the Aurum total RNA minikit (Bio-Rad), and contaminating DNA was removed using the Turbo DNA-free kit (Ambion). Single-stranded cDNA was synthesized from 200 ng RNA using the iScript cDNA synthesis kit (Bio-Rad). Relative transcript levels of *flhB*, *flgE*, and *flaB* were determined using quantitative reverse-transcription PCR (qRT-PCR) as described in reference 12. Primers used for this study are listed in Table 1. Significance was determined using the Student’s t-test.

**FlgS purification.** *flgS* was amplified from *H. pylori* ATCC 43504 using primers FlgS F and FlgS R with Pfu polymerase. The amplicon was cloned into pCR2.1 and subcloned into pHel3 via the SphI and KpnI sites. *flgS* was amplified from *E. coli* strain BL21(DE3) pLysS using primers FlgS F and FlgS R with Pfu polymerase. The amplicon was cloned into pCR2.1 and subcloned into pHel3 via the SphI and KpnI sites.

### TABLE 1 Primers and peptides used in this study

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlhA&lt;sub&gt;NT&lt;/sub&gt;</td>
<td>Biotin-MANERSKLFKKTFPVFKRFLQSKD</td>
</tr>
<tr>
<td>FlhA&lt;sub&gt;NT&lt;/sub&gt;</td>
<td>Biotin-MANERSKLFKKTF</td>
</tr>
<tr>
<td>FlhA&lt;sub&gt;25NT&lt;/sub&gt;</td>
<td>Biotin-PFYKRFQSKD</td>
</tr>
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into the Ndel and the BamHI sites of pET19b. The resulting construct (pflgS) was verified by PCR and sequencing. For expression and purification of FlgS, pflgS was transformed into E. coli KRX cells. Cultures were grown at 37°C in Terrific broth to an optical density at 600 nm (OD600) of 1. Rhamnose (0.1% final concentration) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) were added to the culture to induce expression of His-FlgS. After overnight growth at room temperature, cells were harvested by centrifugation and resuspended into buffer A (50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 0.05% Tween 20). All purification steps were carried out at 4°C. Cells were lysed by three passages through a French pressure cell at 10,000 kPa. Unlysed cells were removed by centrifugation for 15 min at 6,000 × g. His-FlgS was purified using His-Pur nickel-nitrilotriacetic acid (Ni-NTA) resin (Thermo Scientific). The His-Pur Ni-NTA resin was equilibrated in buffer A and then incubated with the lysate for 1 h on an end-over-end tube rotator. The resin containing any bound proteins was separated from unbound proteins by centrifugation and washed with buffer B (50 mM phosphate buffer, pH 8.0, 500 mM NaCl, 25 mM imidazole, 10% glycerol, 0.05% Tween 20). The resin mixture then was transferred to a column for further washing with buffer B. His-FlgS was eluted from the resin using buffer C (50 mM phosphate buffer, pH 8.0, 500 mM NaCl, 250 mM imidazole, 10% glycerol, 0.05% Tween 20). For phosphorylation studies, eluted proteins were dialyzed in 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.05% Tween 20, 100 mM potassium thiocyanate, and 0.1 mM EDTA, pH 7.4. For biosensing experiments, FlgS was exchanged into binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 10% glycerol, 1 mM dithiothreitol). Protein concentration was determined by a bicinchoninic acid protein assay (Pierce) or Bradford assay. FlgSN and FlgSC were purified as described for FlgS, with minor modifications.

Optical biosensing. All biolayer interferometry (BLI) experiments were performed on a ForteBio (Menlo Park, CA) Octet QK biosensor using streptavidin (SA) sensors. Assays were performed in 96-well microplates at 25°C. All volumes were 200 μl. Ligand peptides used in this study were synthesized with an N-terminal biotin group (Biomatik, Cambridge, Canada). Their sequences are shown in Table 1. After loading ligands onto SA sensors, a baseline was established in binding buffer prior to monitoring association at various analyte concentrations. Dissociation subsequently was measured in buffer only. Nonspecific binding was measured by screening sensors not exposed to ligand versus analytes under conditions identical to those of the binding assays and with the same analyte samples. Raw data were analyzed with GraphPad Prism, and constants were determined by fits to appropriate models.

In vitro phosphorylation assay. The phosphorylation state of FlgS was monitored using a phosphate affinity polyacrylamide (Phos-tag) gel electrophoresis system (Wako Chemistry USA, Inc.) as previously described (18). A buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM KCl, and 20 mM MgCl2 was used for all dilutions. Reaction mixtures contained 2 μg FlgS and 200 nM FlgSN and were initiated with 1 mM ATP in a total volume of 25 μl. Reaction mixtures were incubated at room temperature for 2 min, 5 min, and 10 min before stopping the reaction with the addition of 6× SDS-PAGE loading dye (168 mM Tris base, pH 6.8, 7% SDS, 0.3% bromophenol blue, 34% glycerol, 4% 2-mercaptoethanol). Phos-tag gels were made according to the supplier’s protocols. Fifteen microfilters of the reaction mixture was loaded onto the gel. Gels were stained with Coomassie brilliant blue to visualize both unphosphorylated and phosphorylated forms of FlgS.

RESULTS

The amino terminus of FlhA is required for motility in H. pylori ATCC 43504. FlhA is required for transcription of the RpoN regulon (13). It is a large protein of 733 amino acid residues in length and is a member of the membrane-integrated portion of the flagellar protein export apparatus. Like its homolog in Salmonella, hydrophathy analysis of FlhA predicts a small N-terminal cytoplasmic region, followed by eight membrane-spanning helices and a large cytoplasmic region (Fig. 1A). A strain carrying a complete deletion of flhA (the ΔflhA strain) does not support transcription of the RpoN regulon. However, a strain capable of expressing only the first 77 residues of FlhA (the ΔflhA77 strain) containing the N-terminal cytoplasmic region and the first two transmembrane segments is able to support wild-type levels of RpoN-dependent gene transcription (13). Therefore, we hypothesized that the N-terminal cytoplasmic region of FlhA (FlhATN) plays a role in regulation of the RpoN regulon, perhaps via interaction with a regulatory protein, such as FlgS.

To test this hypothesis, we monitored the ability of a plasmid bearing the full-length flhA allele (pflhA) and a plasmid bearing an flhA allele which lacked codons 2 through 24 (pflhAΔNT) to complement motility defects of the ΔflhA mutant. The plasmid-borne flhA alleles possessed the native flhA promoter. Plasmids were

FIG 1 (A) Transmembrane topography as inferred by hydrophathy analysis. FlhA_TM, the transmembrane domain; FlhA_N, cytoplasmic domain. The sequence deleted (residues 2 to 24) for construct pflhAΔNT is indicated at the left. (B) Motility assays. Top row, the ΔflhA mutant from parent strain 43504 transformed with empty vector, pflhA, and pflhAΔNT. Bottom row, 43504/ΔflhA deletion strain transformed with the same plasmids as the wild type. (C) Strain-specific motility differences. H. pylori strains 43504 and BI28 were used as the parental strains. The parent strain for ΔflhA deletion is indicated by color, with motility results quantified for each transformation. Error bars indicate standard errors from three separate trials.
transformed into wild-type and ΔflhA mutant cells. Transformation with pflhA partially restored motility in the ΔflhA background (the resulting strain was called the ΔflhA/pflhA NT strain) (Fig. 1B). Introduction of pflhA in the wild-type background (resulting in the WT/pflhA NT strain) reduced motility, possibly due to elevated levels of FlhA from the plasmid-based expression method. We have obtained similar results in previous experiments where overexpression of an export apparatus protein in otherwise wild-type cells resulted in decreased motility in soft agar medium (12, 19). In contrast to pflhA, the introduction of pflhA NT in the ΔflhA mutant (the ΔflhA/pflhA NT strain) did not enhance the motility of the strain (Fig. 1B), suggesting that the N-terminal portion of FlhA is required for flagellar biogenesis. Introduction of pflhA NT in the wild-type background (the WT/pflhA NT strain) inhibited motility. We infer from these results that the truncated FlhA NT variant is stably expressed and inserted into the export apparatus, where it interferes with the function of full-length FlhA proteins in H. pylori ATCC 43504.

Complementation differences were observed between the two background strains, B128 and ATCC 43504. In both ΔflhA strains, transformation with pflhA restored motility, whereas pflhA NT did not. However, significant differences in inhibition of motility upon transformation of the wild type were observed, particularly by pflhA NT (10-mm halo in ATCC 43504, 22-mm halo in B128). Differences in membrane assembly consistent with earlier reports may account for the observation that truncated forms of FlhA were able to associate with the membrane in ATCC 43504 but not in B128 (13).

The N terminus of FlhA is required for RpoN-dependent flagellar gene expression. To determine whether the FlhA NT allele is required for transcription of the RpoN regulon, quantitative reverse transcription-PCR (qRT-PCR) was used to monitor expression of two RpoN-dependent genes, flaB and flaF. Transcript levels of flaB and flaF in the ΔflhA mutant were consistent with our previous results (13), where there was a very low basal level of transcription of flaB and transcription of flaF was about 6-fold lower than wild-type levels (Fig. 2). Introduction of pflhA into the

ΔflhA mutant partially restored transcript levels, while the introduction of pflhA NT did not restore transcript levels in the ΔflhA mutant. These findings suggest FlhA NT is required to initiate signal transduction resulting in transcriptional activation of the RpoN regulon. Since FlhA NT is predicted to be exposed on the cytoplasmic side of the membrane, we postulated that FlgS recognizes FlhA NT as part of an assembly checkpoint to initiate signal transduction.

The N terminus of FlhA binds FlgS via its C-terminal kinase domain. To test the hypothesis that FlgS recognizes FlhA NT, a synthetic peptide consisting of the first 25 residues of FlhA was used as a ligand to measure interactions with FlgS using biolayer interferometry (BLI). BLI is an optical biosensing technique similar to the better-known surface plasmon resonance (SPR) (20). It allows for measurement of intermolecular interactions in real time (21–23). Ligand molecules are tethered to fiber-optic sensors and then exposed to various concentrations of an analyte. Binding is measured by the shift of the interference pattern of white light reflected from the end of the sensor. After an association phase, sensors are moved to a buffer-only solution and dissociation is monitored. By examining several different analyte concentrations and fitting raw data to global binding models, rate and affinity constants can be determined for ligand-analyte interactions.

As shown in Fig. 3, binding at five different FlgS concentrations ranging from 31 to 500 nM readily fit a one-state global model. K_d (equilibrium dissociation constant) was 21 nM, with subsidiary fast-on kinetics (k_{on}) of 2.9 × 10^2 M^{-1} s^{-1} and slow-off kinetics (k_{off}) of 6.2 × 10^{-4} s^{-1}. Goodness-of-fit parameters indicate excellent fits, e.g., the standard error of the K_d is 440 pM. Controls of analyte screened against sensors without ligands resulted in negligible nonspecific binding. Microscale thermophoresis (24), a solution method not requiring the tethering of one of the binding partners to a surface, yielded a K_d of 62 nM, in reasonable agreement with the BLI results (see Fig. S1 in the supplemental material).

To more specifically determine which region of FlgS is involved in binding, interactions of FlhA NT were analyzed against fragments of FlgS. Residues 1 to 169 (termed FlgS NT) did not bind FlhA NT. However, residues 170 to 315 (termed FlgS) bound with high affinity (Fig. 4). A one-state global fit of five FlgS concentrations gave a k_{on} of 2.1 × 10^3 M^{-1} s^{-1} and k_{off} of 7.1 × 10^{-4} s^{-1} for an overall K_d of 33 nM. Fits to the raw data were excellent, but
not as good as those for full-length FlgS, e.g., the standard error for $K_{D}$ was 1 nM. However, fits to a parallel two-state model yielded $K_{D}$ of 24 nM and 17 μM, with the majority of the amplitudes accounted for in the high-affinity $K_{D}$ (see Fig. S2 in the supplemental material), suggesting that the secondary event is an artifact and that the affinity of FlgSC is nearly identical to that of full-length FlgS (see Discussion).

An initial attempt to discern critical residues for binding was performed with synthetic peptides representing residues 1 to 14 (FlhA<sub>NT1</sub>) and 15 to 25 (FlhA<sub>NT2</sub>). Neither evinced binding to FlgS, as a response was indistinguishable from background noise (Fig. 5), suggesting that the binding site is disrupted in the smaller peptides.

**FlhA<sub>NT</sub> does not stimulate autophosphorylation of FlgS.** To test the hypothesis that interaction with FlhA<sub>NT</sub> stimulates autophosphorylation of FlgS, 200 nM FlhA<sub>NT</sub> was incubated with 1.78 μM FlgS. Phosphorylated proteins were separated from unphosphorylated proteins using the Phos-tag gel system where phosphorylated proteins migrate slower through the gel than unphosphorylated proteins. Under our assay conditions, we observed a low level of phosphorylation of FlgS in the presence of ATP (Fig. 6, lane 2). The addition of FlhA<sub>NT</sub> did not increase the amount of phosphorylated protein, indicating that FlhA<sub>NT</sub> does not act by itself to stimulate the autokinase activity of FlgS (Fig. 6, lane 3).

**DISCUSSION**

Like its homologs in *Salmonella* and other species, *H. pylori* flhA is required for motility, presumably to effect flagellar type III secretion (19,25). The N-terminal soluble cytoplasmic sequence also is required in both *Salmonella* and *H. pylori*, as its deletion eliminated the ability of flhA to complement ΔflhA mutations in these bacteria (Fig. 1B)(19). In the study by McMurry et al., an FlhA variant lacking residues 18 to 22 was able to complement an flhA mutant in *Salmonella*, while another variant that lacked residues 2 to 22 was unable to complement the same flhA mutant (19). We observed similarities in which expression of *H. pylori* FlhA in an flhA mutant was able to complement motility, whereas the expression of a truncated form of FlhA lacking the N-terminal segment (FlhA<sub>NT</sub>) was unable to complement motility (Fig. 1B). While for technical reasons (e.g., lack of antiserum directed against *H. pylori* FlhA) fractionation and protease protection assays could not be performed, the observation that FlhA<sub>NT</sub> inhibits wild-type mo-
tility strongly suggests proper assembly of \textit{H. pylori} FlhA\textsubscript{ANT} into the export apparatus. FlhA\textsubscript{ANT} has been proposed to interact with FlII, as either overproduction of FlII or bypass mutations in FlhA\textsubscript{ANT} improved motility in a \(\Delta fi{I}{I}H\) mutant background, suggesting that FlhA\textsubscript{ANT} has a role in substrate export as well (26).

\textit{H. pylori} FlhA also is involved in regulating the transcription of flagellar genes, as a complete deletion of flhA abolished transcription of the RpoN-dependent genes examined (13). The same study also showed that the first 77 residues were sufficient to support RpoN-dependent expression at about 60% of the level for the wild type. In the present study, we focused on the sequence N terminal to the first transmembrane segment. qRT-PCR results indicated that the first 25 residues are necessary for transcription of the RpoN-dependent genes flaB and fliE (Fig. 2).

Transcription of the RpoN-dependent genes is regulated by the FlgS/FlgR two-component system in which FlgS must interact with a signal to initiate signal transduction, which culminates in FlgS/FlgR two-component system in which FlgS must interact with a signal to initiate signal transduction, which culminates in FlgS autokinase activity, or it may be unable to stimulate FlgS activity outside its native context within the export apparatus. FlhA\textsubscript{ANT} improved motility in a FliI, as either overproduction of FliI or bypass mutations in the export apparatus. FlhA\textsubscript{ANT} has been proposed to interact with the export apparatus component FliO for optimal expression of flagellar genes in \textit{H. pylori} (30, 31). We plan to investigate these possibilities in subsequent studies.

ACKNOWLEDGMENTS

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