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Helicobacter pylori FlgR Is an Enhancer-Independent Activator of σ^{54}-RNA Polymerase Holoenzyme

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

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

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

Flagellar gene regulation in *H. pylori* is complex, involving all three sigma factors found in the bacterium. Genes encoding flagellar components required early in flagellar biogenesis are transcribed by σ^{80}-RNA polymerase holoenzyme, the primary form of RNA polymerase holoenzyme in *H. pylori*, and are equivalent to the class II flagellar genes in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (31). Class III flagellar genes in *H. pylori* are transcribed by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) and encode basal body rod proteins (*flgBC*), the hook protein (*flgE*), and a minor flagellin (*flaB*) (54, 56). Expression of the σ^{54}-dependent flagellar genes requires a two-component system consisting of the sensor kinase HP0244 (3), which we refer to as FlgS, and the response regulator FlgR (54). The gene encoding FlgS appears to be within a class II operon (1, 58), which may provide a mechanism for controlling the hierarchical expression of the class III operons. The class IV flagellar genes in *H. pylori*, which include the major flagellin gene *flaA*, are transcribed by σ^{28}-RNA polymerase holoenzyme (22, 29). Temporal control of class IV genes appears to be coordinated through the regulation of both the expression and activity of σ^{28}. The gene encoding σ^{28}, *fliA*, is part of a class II operon (1, 58), and the activity of σ^{28} is negatively regulated through interactions with the anti-σ^{28} factor FlgM (9, 19).

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

Activators of σ^{54}-holoenzyme are modular in structure, generally consisting of an amino-terminal regulatory domain, a central domain responsible for transcriptional activation and ATP hydrolysis, and a carboxy-terminal DNA-binding domain (41, 64). The central activation domain belongs to the AAA+

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

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

superfamily of ATPases (ATPases associated with various cellular activities), the members of which are involved in diverse functions including transcription, DNA replication, protein folding and unfolding, proteolysis, and membrane fusion (39, 43, 65).

FlgR is unusual in that it lacks the DNA-binding domain found in other activators (Fig. 1); it consists of only an amino-terminal response regulator domain and an AAA+ domain. *Chlamydia trachomatis* CtcC was reported recently to lack the carboxy-terminal DNA-binding domain (23), indicating that FlgR is not unique in its unusual structural arrangement. Previous study on CtcC, however, did not address the issue of whether an enhancer is required for efficient CtcC-mediated transcriptional activation in *C. trachomatis*.

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

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strain DH5α [ϕ80d *lacZ* ΔM15 *recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*] was used for cloning and was cultured in Luria-Bertani (LB) medium at 37°C. *H. pylori* strains ATCC 43504 and 26695 were grown on tryptic soy agar (TSA) supplemented with 5% horse serum at 37°C under microaerobic conditions, which consisted of an atmosphere of 4% O_2, 5% CO_2, and 91% N_2. When included in the medium, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; bacitracin, 200 μg/ml; colistin, 15 μg/ml.

PCRs. Genomic DNA used for PCR was isolated from bacterial strains with the Wizard genomic DNA purification kit (Promega). PCR primers were from Integrated DNA Technologies. PCR amplifications were done with either *Taq* (Promega) or *Pfu* (Stratagene) DNA polymerase. DNA was amplified in 30 cycles with the following temperature regimen: 94°C for 2 min, 49°C for 1.5 min, and 72°C for 3 min. PCR products were cloned into the cloning vector pGEM-T (Promega), and sequencing of the cloned PCR products was performed at the Molecular Genetics Instrumentation Facility at the University of Georgia.

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

Construction of *H. pylori* mutant strains with insertions in *flgR* or *flgS*. Plasmid pGHPAY91 carries the *flgR* gene from *H. pylori* strain 26695 and was obtained from the American Type Culture Collection. A 1.4-kb EcoRI fragment from plasmid pHP1 (34) that carries the *Campylobacter coli aphA3* cassette was cloned into a unique StuI site within *flgR* in plasmid pGHPAY91 to create a suicide vector that was introduced into *H. pylori* strain ATCC 43504 by electroporation. Transformants were selected on TSA containing kanamycin, and genomic DNA from some of these colonies was analyzed by PCR to confirm that the chromosomal copy of *flgR* had been inactivated with the *aphA3* cassette. One of these strains was saved and named MGD1. An *H. pylori flgR* insertion mutant was similarly generated with a cassette bearing a chloramphenicol transacetylase (*cat*) gene from *C. coli*. The entire *flgR* from *H. pylori* strain 26695 was amplified by PCR and cloned in pGEM-T. A 1.3-kb EcoRI fragment from plasmid pSKAT4 (59) that carried the *C. coli cat* cassette was introduced into a unique Eco47III site within *flgR* in this plasmid, which then was transformed into *H. pylori* strain ATCC 43504 by electroporation. Transformants were selected on TSA containing chloramphenicol, and inactivation of the chromosomal copy of *flgR* by the *cat* cassette in these strains was confirmed by PCR. One *flgR*:*cat* mutant strain was saved and designated HP31.

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

Construction of *xylE* reporter genes. For construction of all of the reporter genes described below, genomic DNA from *H. pylori* strain 26695 was used as a

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

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

Plasmid constructions for expression of FlgR proteins. To produce a full-length histidine-tagged version of FlgR, *flgR* was amplified from pGHPAY91 and cloned into the expression vector pTrcHis-C (Invitrogen). The resulting plasmid, pMD20, introduced a sequence coding for a histidine tag to the 5′ end of *flgR*. To express a maltose-binding protein–FlgR fusion protein (MBP-FlgR), a DNA fragment bearing *flgR* was moved from pMD20 into a derivative of pMAL-c (New England Biolabs), resulting in plasmid pPBHP12. A plasmid that expressed the FlgR AAA+ domain (residues His-131 to Arg-381) with a histidine tag at the amino terminus was constructed by amplifying a 750-bp DNA fragment corresponding to this region of *flgR* with *H. pylori* 26695 genomic DNA as a template and cloning the PCR product into the expression vector pTrcHis-C to create plasmid pPBHP80. Plasmid pHX182, which expresses the *Sinorhizobium meliloti* DctD AAA+ domain (residues Leu-141 to Ser-390) linked to a histidine tag at the amino terminus, is a derivative of pTrcHis-C and was provided by Hao Xu (63).

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

Purification of FlgR proteins. Cultures of *E. coli* DH5α bearing plasmid pPBHP12 were grown in LB medium at 37°C to an OD_{650} of 0.5, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 1 mM to induce the expression of MBP-FlgR. After an additional 3 to 4 h of incubation, cells were harvested by centrifugation and the resulting cell pellet was resuspended in a mixture containing 20 mM Tris-HCl (pH 7.4), 5% (vol/vol) glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA, 200 mM KCl, and 0.5 mM phenylmethylsulfonyl fluoride (buffer C). Cells were lysed in a French pressure cell at 9,000 lb/in^2, and the resulting cell extract was clarified by centrifugation at 1,000 × *g* for 50 min. The supernatant was loaded onto an amylose-agarose (New England Biolabs) affinity column, and MBP-FlgR was eluted with buffer C plus 10 mM maltose. Protein fractions were pooled and dialyzed against 20 mM HEPES (pH 7.4)–5% (vol/vol) glycerol–1 mM DTT–100 mM potassium thiocyanate. Protein concentrations were determined by a bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard.

The histidine-tagged FlgR AAA+ domain was expressed in *E. coli* DH5α from plasmid pPBHP80. Cells were grown at 37°C to an OD_{650} of 0.8, at which point IPTG was added to the medium to a final concentration of 1 mM. Cultures were incubated for an additional 3 to 4 h. Cells were harvested by centrifugation, resuspended in 50 mM Tris-acetate (pH 8.2)–200 mM KCl–1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride, and lysed in a French pressure cell at 9,000 lb/in^2. The cell extract was clarified by centrifugation at 1,000 × *g* for 50 min, and the resulting supernatant was loaded onto a nickel-nitrilotriacetic acid resin column (QIAGEN). The histidine-tagged FlgR AAA+ domain was eluted in a buffer containing 300 mM NaCl, 50 mM sodium phosphate (pH 7.8), 250 mM imidazole, and 5% (vol/vol) glycerol. Fractions containing the FlgR AAA+ domain were pooled and dialyzed against 20 mM Tris-HCl (pH 8.8)–5% (vol/vol) glycerol–100 mM potassium thiocyanate–0.5 mM DTT (buffer A). The protein was then applied to a 5-ml HiTrapQ anion-exchange column (Pharmacia) and eluted in a linear gradient to 1 mM KCl in buffer A.

Assaying activity of FlgR proteins in *E. coli*. Plasmids pMD20, pPBHP80, and pHX182 were introduced into an *E. coli* DH5α strain that contained plasmid pRKMAZ:+UAS, which bears an *S. meliloti dctA′-′lacZ* reporter gene (25). Cultures were grown in LB medium at 37°C to an OD_{650} of 0.8, at which time IPTG was added to a final concentration of 1 mM. Cultures were incubated an addition 7 h, at which point six independent sets of whole cell β-galactosidase assays were done in duplicate as described previously (2), with activities expressed as Miller units (36).

Western blot analysis. *H. pylori* cells were lysed in sodium dodecyl sulfate loading buffer and then applied to a 10% polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes, which were probed with antiserum prepared in New Zealand White rabbits and directed against either full-length histidine-tagged FlgR (this study) or the *H. pylori* flagellum (kindly provided by Paul O'Toole, Massey University). A peroxidase-conjugated goat affinity-purified antibody against rabbit immunoglobulin G was used as the secondary antibody (ICN/Cappel). Cross-reactive protein bands were visualized by luminescence with an ECL kit (Amersham).

In vitro transcription assay. Single-round transcription assays were performed as described previously (21). Plasmid pJES534 (48) yields an ~155-nucleotide uracilless transcript from the *S. enterica* serovar Typhimurium *glnA* promoter and was used as a DNA template. Reaction mixtures contained 0.1 to 10 μM FlgR AAA+ domain protein, 1 U of *E. coli* RNA polymerase (Epicentre), 200 nM *S. enterica* serovar Typhimurium σ^{54}, 10 nM plasmid DNA, 4 mM ATP, 400 μM GTP, 5 μM CTP, and 7.5 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; Amersham). Proteins were incubated with the DNA template at 37°C for 10 min, after which time ATP was added to stimulate open-complex formation. After 10 min, the remaining nucleotides were added to allow synthesis of the transcripts along with 0.1 mg of heparin/ml to prevent further open-complex formation. Reactions were stopped after 10 min, and transcripts were visualized on a denaturing polyacrylamide gel, followed by exposure to X-ray film.

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

RESULTS

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

The *flgR* mutant strain expressed the major flagellin, FlaA, which is dependent on σ^{28} for its expression (29). This observation was consistent with the previous report for *H. pylori* strain G27 that *flgR* was not required for expression of *flaA* (54). This finding also illustrates a difference in the mechanisms for controlling the expression of σ^{28}-dependent flagellar genes in *S. enterica* serovar Typhimurium and *H. pylori*. In *S. enterica* serovar Typhimurium, σ^{28} function is inhibited by the anti-σ^{28} factor FlgM until the hook-basal body complex is completed, at which point FlgM is translocated out of the cell

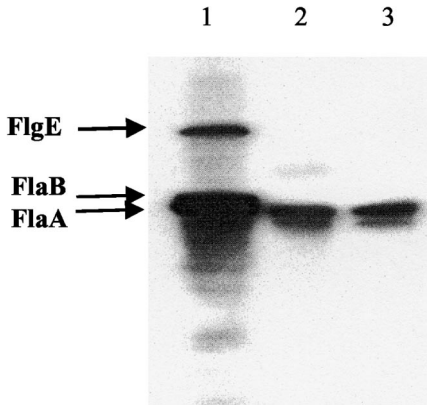

FIG. 2. Immunoblot of cell extracts of wild-type *H. pylori* and *flgR* and *flgS* mutant strains. Each lane contained cell extracts (50 μg of protein) from one of the *H. pylori* strains. The blot was probed with antiserum directed against the *H. pylori* flagellum. Lane 1, wild-type *H. pylori* ATCC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

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

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

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

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

Expression from a *flaA′-′xylE* reporter gene, which is dependent on σ^{28}-holoenzyme, and a *flgI′-′xylE* reporter gene, which is dependent on σ^{80}-holoenzyme, was compared with that from the *flaB′-′xylE* reporter genes (Table 1). Expression from the *flaA′-′xylE* reporter gene was independent of FlgR and FlgS, while expression from the *flgI′-′xylE* reporter gene was approximately twofold lower in the *flgR* mutant strain but not in the *flgS* mutant strain (Table 1). We do not understand why disruption of *flgR* resulted in lower expression from the *flgI′-′xylE* reporter gene, but it does not seem likely that it was due to the failure of the mutant strain to express the σ^{54}-dependent flagellar genes, since this would also require FlgS. The XylE activities observed for the flagellar reporter genes were consistent with the expected relative amounts of the products of these genes associated with the *H. pylori* flagellum (i.e., FlaA > FlaB > FlgI). Taken together, these data indicate that FlgR does not use an upstream activation sequence or enhancer to elicit its function on the *flaB* promoter.

FlgR levels in *H. pylori* are slightly higher than levels of σ^{54}-dependent activators in other bacteria. Transcriptional activation in the absence of DNA binding has been reported previously for other σ^{54}-dependent activators, including NtrC (42), DctD (18, 60), NifA (17), and PspF (20). In these previ-

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

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

[a] Values represent the averages of at least six replicates ± standard deviations.

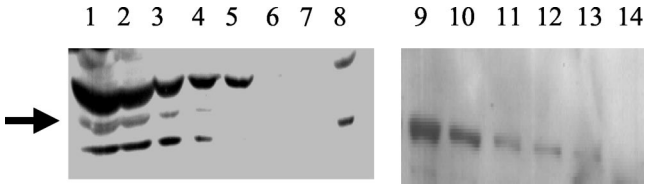

FIG. 3. Immunoblot of FlgR in *H. pylori* cell extracts. The immunoblot was probed with an antibody directed against the histidine-tagged FlgR protein. Arrow, band corresponding to FlgR. Different amounts of cell extract of wild-type *H. pylori* were loaded onto lanes 1 to 7 (lane 1, 1×10^9 cells; lane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

ous studies, the DNA-binding motifs of the activators were either deleted or mutated to eliminate DNA-binding activity or the upstream activation sequence of the target gene was removed. Transcriptional activation under these conditions required that the activator be present at higher-than-normal levels. We wished to estimate the levels of FlgR in *H. pylori* to determine if it was expressed at levels that were higher than those for σ^{54}-dependent activators that function by binding to an upstream activation sequence or enhancer.

Levels of NtrC in *E. coli* range from about 10 molecules of monomeric protein per cell when cells are grown in a medium with excess nitrogen to approximately 140 molecules per cell following starvation of cells for nitrogen (32, 49). We estimated FlgR levels in *H. pylori* strain ATCC 43504 by Western blotting using antiserum directed against the full-length histidine-tagged FlgR. For the Western blot, purified MBP-FlgR was used as a standard to avoid underestimating the FlgR concentration due to antibodies that may have recognized the histidine tag. Various amounts of *H. pylori* cells were lysed and loaded directly onto the sodium dodecyl sulfate-polyacrylamide gel for the Western blot assay. Under the assay conditions the detection limit for MBP-FlgR was ~12.5 ng, or approximately 0.14 pmol of MBP-FlgR monomer (Fig. 3). FlgR could be detected by the Western blot assay when as few as 10^8 cells were lysed and loaded on the gel, which corresponded to ~800 FlgR monomers per cell. Thus, assuming that the intracellular volumes of *H. pylori* and *E. coli* are similar, the level of FlgR in *H. pylori* appears to be about sixfold higher than that of NtrC in *E. coli* under conditions where these proteins are activating transcription.

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

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

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

In contrast to the results with the full-length protein, the FlgR AAA+ domain activated transcription from the *dctA′-′lacZ* reporter gene >120-fold above background levels when its expression was not induced with IPTG. The activity of the FlgR AAA+ domain in *E. coli* compared favorably with that of the DctD AAA+ domain, indicating that the FlgR AAA+ domain was able to function effectively with *E. coli* σ^{54}-holoenzyme. The activities of both the FlgR AAA+ domain and the DctD AAA+ domain decreased upon induction of these proteins with IPTG. This decrease in activity was not likely due to

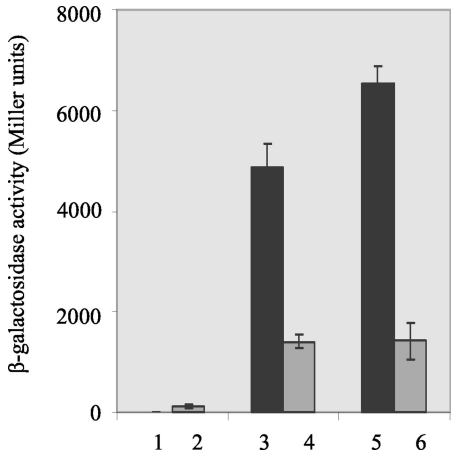

FIG. 4. In vivo activity of AAA+ domains of FlgR and DctD. Activity of full-length FlgR, the FlgR AAA+ domain, and the DctD AAA+ domain on a *dctA′-′lacZ* reporter gene in *E. coli*. Gray bars, activity for culture in which the expression of the FlgR or DctD proteins was induced with IPTG; black bars, activity for cultures in which the expression of the proteins was not induced with IPTG. Lanes 1 and 2, activity for full-length FlgR; lanes 3 and 4, activity for the FlgR AAA+ domain; lanes 5 and 6, activity for the DctD AAA+ domain.

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

Plasmid	Protein expressed	Glutamine synthetase activity (nmol of γ-glutamyl hydroxymate/min/A_{650} unit)[a]	
		−IPTG	+IPTG
None	None	5.46 ± 1.22	6.38 ± 2.00
pPBHP80	FlgR AAA+ domain	17.4 ± 5.42	15.4 ± 3.14
pHX182	DctD AAA+ domain	16.5 ± 5.07	9.62 ± 3.40

[a] Values represent the averages of at least six replicates ± standard deviations. −IPTG, without IPTG induction; +IPTG, with IPTG induction.

aggregation since these proteins were in the soluble fraction when we overexpressed them for purification. Since σ^{54}-dependent activators can bind σ^{54} (8), the FlgR and DctD AAA+ domains may have sequestered σ^{54} and prevented it from binding core RNA polymerase to form the holoenzyme.

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

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

DISCUSSION

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

Activators of σ^{54}-holoenzyme generally bind to sites that are located relatively far from the promoter and contact the closed complex through DNA looping to activate transcription (64). *H. pylori* FlgR lacks the DNA-binding domain associated with most other σ^{54}-dependent activators, and the results presented here demonstrate that FlgR does not require an enhancer or upstream activation sequence to activate transcription. We infer that FlgR binds σ^{54}-holoenzyme directly, either before or after formation of the closed promoter complex, to activate transcription. While mutant forms of other σ^{54}-dependent activators have been reported to activate transcription in the absence of DNA binding (17, 18, 20, 42), FlgR is unusual in that it represents a naturally occurring enhancer-independent activator of σ^{54}-holoenzyme. FlgR appears to be present at concentrations in the cell that are higher than those of NtrC in *E. coli*. The *glnA* enhancer facilitates oligomerization of NtrC, which is required for transcriptional activation from *glnA*p2 (48). Thus, higher concentrations of FlgR may be needed to compensate for the absence of enhancer binding and to allow oligomerization of the protein.

C. trachomatis CtcC was described recently as an activator of σ^{54}-holoenzyme that lacks a DNA-binding domain (23), and we infer that, like FlgR, CtcC does not require sequences upstream of the promoter to activate transcription. Search of the databases for additional σ^{54}-dependent activators that lack the DNA-binding domain identified activators from *Chlamydia pneumoniae*, *Chlamydia muridarum*, and *Chlamydophila caviae* with this unusual structural property (Fig. 1). Two other po-

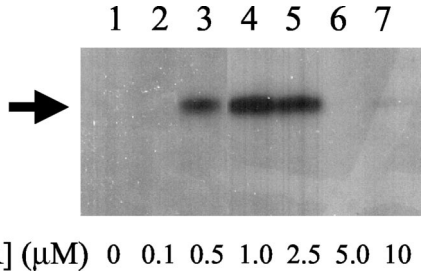

FIG. 5. In vitro transcriptional activation with the FlgR AAA+ domain. Reaction mixtures contained FlgR AAA+ domain (monomer) at the indicated concentrations. Arrow, transcript of the expected size (~155 nucleotides) from the *glnA* promoter.

tential σ^{54}-dependent activators that appear to lack the carboxy-terminal DNA-binding domain were found in database searches, one in *Xanthomonas campestris* pv. *campestris* ATCC 33913 (gene designated *pilR* in the database) and another in *P. putida* KT2440 (designated PP5166 in the database). There is some doubt, however, as to whether the sequences of these activators are correct or if they encode σ^{54}-dependent activators. The carboxy-terminal end of the deduced amino acid sequence of *X. campestris* PilR corresponds to a sequence located 28 amino acids before that of *H. pylori* FlgR, placing it within the conserved sensor II motif of the AAA+ domain. Since this motif is important for function of other σ^{54}-dependent activators, it is unlikely that such a truncation would result in an active protein. The carboxy-terminal end of the deduced amino acid sequence of *P. putida* PP5166 extends 3 residues beyond that of *H. pylori* FlgR. *P. putida* PP5166, however, has a poor match for the highly conserved GAFTGA motif in the C3 region of the protein (deduced amino acid sequence of this motif in PP5166 is GSHGGT). The GAFTGA motif, which is diagnostic of σ^{54}-dependent activators (45) functions in contacting σ^{54} and coupling ATP hydrolysis to open-complex formation, and substitutions within this motif often result in loss of activity (6, 8, 14, 28, 60, 61). Thus, PP5166 may not be an activator of σ^{54}-holoenzyme but may function with another form of RNA polymerase holoenzyme.

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

All of the chlamydial genomes that have been sequenced to date include an open reading frame that encodes a potential σ^{54}-dependent activator that lacks the DNA-binding domain. In contrast, *Helicobacter hepaticus* and *Campylobacter jejuni*, which are closely related to *H. pylori*, each have a single σ^{54}-dependent activator but these activators each have a potential DNA-binding domain (Fig. 1). This is somewhat surprising given that these activators appear to have the same role in flagellar biogenesis as *H. pylori* FlgR (15, 54, 57). We infer from this observation that loss of the DNA-binding domain in the chlamydial σ^{54}-dependent activators and its loss in *H. pylori* FlgR occurred independently of each other.

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

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