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Stable Accumulation of σ^{54} in *Helicobacter pylori* Requires the Novel Protein HP0958

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Several flagellar genes in *Helicobacter pylori* are dependent on σ^{54} (RpoN) for their expression. These genes encode components of the basal body, the hook protein, and a minor flagellin, FlaB. A protein-protein interaction map for *H. pylori* constructed from a high-throughput screen of a yeast two-hybrid assay (http://pim.hybrigenics.com/pimriderext/common/) revealed interactions between σ^{54} and the conserved hypothetical protein HP0958. To see if HP0958 influences σ^{54} function, the corresponding gene was disrupted with a kanamycin resistance gene (*aphA3*) in *H. pylori* ATCC 43504 and the resulting mutant was analyzed. The *hp0958:aphA3* mutant was nonmotile and failed to produce flagella. Introduction of a functional copy of *hp0958* into the genome of the *hp0958:aphA3* mutant restored flagellar biogenesis and motility. The *hp0958:aphA3* mutant was deficient in expressing two σ^{54}-dependent reporter genes, *flaB′-′xylE* and *hp1120′-′xylE*. Levels of σ^{54} in the *hp0958* mutant were substantially lower than those in the parental strain, suggesting that the failure of the mutant to express the genes in the RpoN regulon and produce flagella was due to reduced σ^{54} levels. Expressing σ^{54} at high levels by putting *rpoN* under the control of the *ureA* promoter restored flagellar biogenesis and motility in the *hp0958:aphA3* mutant. Turnover of σ^{54} was more rapid in the *hp0958:aphA3* mutant than it was in the wild-type strain, suggesting that HP0958 supports wild-type σ^{54} levels in *H. pylori* by protecting it from proteolysis.

Helicobacter pylori, a member of the ε subdivision of the proteobacteria, colonizes the human gastric epithelium, which leads to a gastric inflammation that can progress to chronic gastritis, peptic ulcer, gastric cancer, or mucosa-associated lymphoma (6, 10, 12). *H. pylori* must be motile to colonize the gastric epithelium (13, 14), and motility by the bacterium occurs through the action of two to six polar flagella.

Flagellar biogenesis in *H. pylori* involves the coordinated expression of over 40 flagellar genes scattered throughout the genome and organized into 25 or more transcriptional units (1, 33). Transcriptional regulation of these flagellar operons in *H. pylori* is complex, involving all three σ factors found in the bacterium, σ^{80} (the primary σ factor in *H. pylori*), σ^{54} (RpoN), and σ^{28} (FliA) (7, 15, 19, 29, 30, 32). In this regard regulation of flagellar biogenesis in *H. pylori* is similar to that of *Vibrio cholerae* and *Pseudomonas aeruginosa*, which also require both σ^{54} and σ^{28} for expression of different classes of flagellar genes (11, 23, 34).

In *H. pylori*, σ^{80} is required for transcription of flagellar genes whose products are needed early in flagellar biogenesis and include basal body proteins and components of the flagellar protein export apparatus (30). *H. pylori* flagellar genes whose products are required midway through flagellar assembly are dependent on σ^{54} for their expression and include genes that encode the proximal rod proteins of the basal body (*flgBC*), the hook protein (*flgE*), hook-associated proteins (*flgK* and *flgL*), and a minor flagellin (*flaB*) (19, 29, 32). Finally, genes whose products are needed at the end of flagellar biogenesis are transcribed by σ^{28}-RNA polymerase holoenzyme and include genes encoding the major flagellin (*flaA*), the filament cap protein (*fliD*), and flagellar protein chaperones (*fliS* and *fliT*) (15, 16, 19).

Transcription of σ^{54}-dependent genes in *H. pylori* requires the activator FlgR, which belongs to the NtrC family of transcriptional activators (7, 29). FlgR is a response regulator of a two-component signal transduction system and must be phosphorylated by its cognate sensor kinase, FlgS, to activate transcription (2, 7, 19). Sensor kinases are often responsive to environmental or cellular signals, but it is not known if FlgS responds to such cues. Activators of σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) typically bind enhancers located upstream of the promoters of the genes which they activate (8, 25). After binding to the enhancer, the activator contacts σ^{54}-holoenzyme bound at the promoter in a closed complex via looping of the DNA between the enhancer and promoter (26, 31). The activator hydrolyzes ATP and couples energy released from hydrolysis to stimulate the isomerization of the closed promoter complex to an open complex that is competent to initiate transcription (22, 35). Unlike most σ^{54}-dependent activators, *H. pylori* FlgR lacks a DNA-binding domain and apparently contacts σ^{54}-holoenzyme directly rather than through DNA looping to activate transcription (7).

In addition to the involvement of the FlgS/FlgR system in the regulation of σ^{54}-dependent genes, other factors may regulate expression of genes of the RpoN regulon. A protein-protein interaction map for *H. pylori* was constructed using a high-throughput screen of a yeast two-hybrid system (24). The protein-protein interaction map indicated interactions between σ^{54} and the conserved hypothetical protein HP0958 (Hybrigenics PimRider database; http://pim.hybrigenics.com/pimriderext/common/). To determine if HP0958 influences

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σ^{54} function in *H. pylori*, we disrupted its corresponding gene with a cassette bearing a kanamycin resistance gene (*aphA3*) in *H. pylori* ATCC 43504 and analyzed the phenotype of the resulting mutant. The *hp0958:aphA3* mutant was nonmotile and aflagellated, which appeared to result from reduced levels of σ^{54}. The turnover rate of σ^{54} in the *hp0958:aphA3* mutant was significantly higher than that in the parental strain, suggesting that HP0958 protects σ^{54} from proteolysis.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* DH5α [ϕ80 *lacZ*Δ M15 *recA1* e*ndA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR*Δ (*lacZYA-argF*)U169] was grown in Luria-Bertani broth at 37°C. *H. pylori* ATCC 43504 was grown on blood agar supplemented with 10% sheep blood or tryptic soy agar supplemented with 5% horse serum (TSA serum) and grown at 37°C under an atmosphere of 4% oxygen, 5% carbon dioxide, and 91% nitrogen. Serum-free medium was used to grow liquid cultures of *H. pylori* in brain heart infusion broth supplemented with 0.1% β-cyclodextrin (Sigma) as described previously (9). Motility agar plates consisted of Mueller-Hinton broth supplemented with 5% horse serum and contained 0.35% agar. Sterile toothpicks were used to inoculate the motility agar with *H. pylori* strains, and motility was scored after incubating the plates at 37°C under an atmosphere of 4% oxygen, 5% carbon dioxide, and 91% nitrogen for 4 to 5 days. When required, medium was supplemented with 30 μg/ml chloramphenicol, kanamycin, or tetracycline.

Protein purification. Details for construction of plasmids used for overproduction of *H. pylori* proteins are available upon request. PCR products used to construct the expression vectors were sequenced at the Integrated Biotechnology Laboratories at the University of Georgia to verify that no errors had been introduced during amplification.

A DNA fragment bearing *hp0958* was amplified by PCR from *H. pylori* 26695 genomic DNA and cloned into plasmid pJES489, which is a derivative of pMAL-c (New England Biolabs), to create a fusion of *hp0958* and *E. coli malE* (encoding the maltose-binding protein [MBP]). MBP-HP0958 was expressed from this plasmid in *E. coli* DH5α by growing a 1-liter culture to an optical density of 0.3 and then adding isopropyl-β-D-thiogalactoside (1 mM final concentration). After 3.5 h, cells were harvested; resuspended in 50 mM Tris-acetate, pH 8.2, 200 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT); and lysed with a French pressure cell at 7,000 lb/in^2. The crude cell extract was centrifuged at 17,000 × *g* for 45 min, and the resulting supernatant liquid was applied to an amylose-agarose (New England Biolabs) affinity column that had been equilibrated previously with 20 mM Tris-HCl, pH 7.4, 5% (wt/vol) glycerol, 1 mM EDTA, 1 mM DTT, and 200 mM KCl (buffer A). MBP-HP0958 was eluted from the column with buffer A plus 10 mM maltose; dialyzed against 20 mM Tris-HCl, pH 8.8, 0.5 mM DTT, and 5% (wt/vol) glycerol (buffer C); and then applied to a High Trap Q anion-exchange column (5 ml; Amersham Biosciences) that had been equilibrated previously with buffer C. MBP-HP0958 was eluted from the column with a gradient to buffer C plus 1 M KCl at a salt concentration of ~0.2 M.

H. pylori rpoN was amplified by PCR from *H. pylori* strain 26695 and cloned into pJES489 to create a chimeric *malE-rpoN* gene. MBP-σ^{54} protein was overproduced in *E. coli* DH5α and purified through the amylose-agarose affinity chromatography step as described for MBP-HP0958. Fractions containing the protein were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mM maltose, and 5% (wt/vol) glycerol (buffer B). The dialyzed fractions were applied to a heparin-agarose column which was equilibrated with buffer B. After being washed with buffer B, MBP-σ^{54} was eluted from the column at ~0.2 M KCl with a linear gradient to buffer plus 1 M KCl.

H. pylori flaB was amplified by PCR from *H. pylori* strain 26695 and cloned into pJES489 to create a chimeric *malE-flaB* gene. MBP-FlaB protein was overproduced in *E. coli* DH5α and purified as described for MBP-HP0958. Antisera directed against MBP-HP0958, MBP-σ^{54}, or MBP-FlaB were raised in New Zealand White rabbits by Cocalico Labs, Reamstown, PA.

Construction of *H. pylori* mutant strains. Mutant strains were constructed using suicide vectors derived from pGEM-T (Promega) and contained fragments of the genes targeted for mutagenesis. Targeted genes were amplified from *H. pylori* 26695 genomic DNA by PCR, cloned into pGEM-T, and disrupted with a 1.4-kb EcoRI fragment bearing a *Campylobacter coli aphA3* (conferring resistance to kanamycin) cassette from plasmid pHP1 (17). Suicide vectors were introduced into *H. pylori* ATCC 43504 by natural transformation, and recombinants in which the chromosomal copy of the targeted gene had been replaced by the disrupted copy of the gene were selected on TSA serum supplemented with kanamycin. Insertion of the *aphA3* cassette in the targeted gene was verified by PCR using a set of primers that flanked the site of insertion. An *H. pylori rpoN* mutant was constructed by introducing an EcoRI site ~370 bp from the start codon of *H. pylori rpoN* using the QuickChange II site-directed mutagenesis kit (Stratagene) and cloning the *aphA3* cassette into this site. An *H. pylori hp0958* mutant was constructed following introduction of the *aphA3* cassette within an NheI site, which resulted in disruption of the gene at codon 76. An *H. pylori hp0959* mutant was generated by inserting the *aphA3* cassette into a BamHI site located ~450 bp downstream of the start codon of *hp0959*.

Complementation of *hp0958* mutant. A ~1.5-kb PCR product that carried most of *hp0959* and all of *hp0958* was cloned into pGEM-T and sequenced. The cloned DNA fragment was introduced into the EcoRV site of plasmid pEU39Cm (21), which carries a copy of *H. pylori hp0405* disrupted with a cassette containing *C. coli* chloramphenicol transacetylase (*cat*). The EcoRV site is present in *hp0405* and is adjacent to the *cat* cassette. The resulting suicide vector was transformed into the *hp0958:aphA3* mutant, and recombinants in which the chromosomal copy of *hp0405* had been replaced with the disrupted gene carrying *hp0959-hp0958* along with the *cat* cassette were selected on TSA serum supplemented with chloramphenicol. Introduction of *hp0959-hp0958* into the *hp0405* locus was verified by PCR using a set of primers in which one was internal to *cat* and the other was within *hp0405*. PCR was also used to confirm that this strain retained the *aphA3* cassette in the *hp0958* locus.

Overproduction of σ^{54} and HP0958 in *H. pylori*. *H. pylori rpoN* was introduced into plasmid pPA (5) to place it under the control of the *H. pylori ureA* promoter. A ~1.4-kb DNA fragment bearing the *ureA* promoter plus *rpoN* from the resulting plasmid was cloned into the EcoRV site of plasmid pEU39Cm. This plasmid was transformed into the *hp0958:aphA3* mutant and into wild-type *H. pylori* to introduce the *rpoN* allele under the control of the *ureA* promoter into the *hp0405* locus as described above.

Similarly, *H. pylori hp0958* was placed under the control of the *ureA* promoter by introducing it into plasmid pPA. A ~975-bp DNA fragment bearing the P_{ureA}-*hp0958* allele was cloned into pEU39Cm, and the resulting plasmid was transformed into the wild-type or *hp0958:aphA3* mutant strains to introduce the P_{ureA}-*hp0958* allele into the *hp0405* locus.

Construction of *H. pylori* reporter strains. Reporter genes in which the *H. pylori flaB* promoter region (positions −67 to +26 relative to the transcriptional start site) or the *H. pylori flaA* promoter region (positions −126 to +47 relative to the transcriptional start site) was joined to promoterless *Pseudomonas putida xylE* (encoding catechol 2,3-dioxygenase) have been described previously (7). An *orf1120′-′xylE* reporter gene was constructed by cloning a DNA fragment corresponding to positions −70 to +44 relative to the transcriptional start site of the *hp1120-flgK* operon upstream of *xylE*. The *flaB′-′xylE*, *hp1120′-′xylE*, and *flaA′-′xylE* reporter genes were cloned into the EcoRV site of plasmid pEU39Cm to create suicide vectors that were used to introduce the reporter genes into the *hp0405* locus of various *H. pylori* strains as described above.

To construct a strain with an *rpoN′-′xylE* reporter gene, the start codon of the *P. putida xylE* gene was joined in-frame to codon 61 of *H. pylori rpoN* that had been cloned previously in pGEM-T. A 1.3-kb fragment bearing *C. coli cat* was cloned immediately downstream of *xylE* in the EcoRI site that had been introduced previously in *rpoN*. The resulting suicide vector was transformed into wild-type *H. pylori* and the *hp0958:aphA3* mutant. Recombinants in which the chromosomal copy of *rpoN* was replaced with the *rpoN′-′xylE* reporter and the *cat* cassette were selected on TSA serum supplemented with chloramphenicol and verified by PCR.

XylE assays. Whole-cell XylE assays were carried out essentially as described previously (7). The rate of product (2-hydroxymuconic semialdehyde) formation was determined, and activities were expressed as μmoles product formed/min/10^8 *H. pylori* cells from at least 10 independent assays for each sample.

Electron microscopy. *H. pylori* cells were grown on blood agar for 48 h at 37°C and then gently resuspended in phosphate-buffered saline, pH 7.4. Samples were negatively stained with 2% phosphotungstic acid (pH 7.0) on Formvar-coated copper grids and observed with a JEOL 100CK electron microscope (JEOL USA, Peabody, MA) at the Center for Advanced Ultrastructural Research at the University of Georgia. Micrographs were taken at an accelerating voltage of 80 kV.

Western blot analysis. Immunoblotting with primary antibodies directed against MBP-HP0958, MBP-σ^{54}, MBP-FlaB, or VacA (Austral Biologicals, San Ramon, CA) was done as described previously (7). The bound antibody was detected by enhanced chemiluminescence using peroxidase-coupled goat anti-rabbit antibody as the secondary antibody (MP Biomedicals Inc., Aurora, ID). Antibody directed against MBP-σ^{54} was affinity purified prior to use as follows.

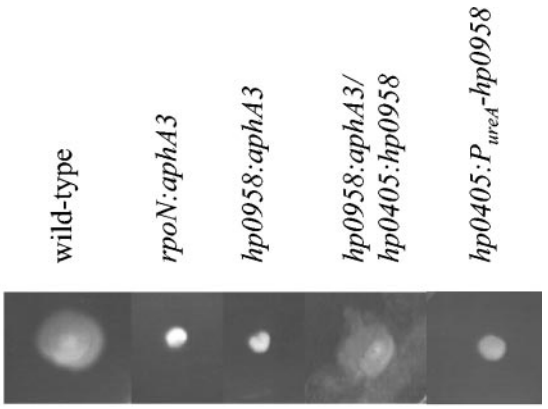

FIG. 1. Motilities of *H. pylori* 43504 and various mutant derivatives. Cells were inoculated on semisolid motility agar plates with a sterile toothpick and incubated for 4 to 5 days at 37°C under microaerophilic conditions. Strains that were tested for motility were *H. pylori* 43504 (wild type), an *rpoN:aphA3* mutant, an *hp0958:aphA3* mutant, an *hp0958:aphA3* mutant in which a functional copy of *hp0958* was introduced into the *hp0405* locus (*hp0958:aphA3*/*hp0405:hp0958*), and *H. pylori* 43504 bearing the P_{ureA}-*hp0958* allele in the *hp0405* locus (*hp0405:*P_{ureA}-*hp0958*).

Purified histidine-tagged σ54 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and visualized by staining with Ponceau red. The band was excised, and the membrane strip was rinsed in distilled water, blocked with a 2% nonfat dry milk solution, washed with Tris-buffered saline containing 0.05% Tween 20 (TBST), and incubated overnight with 20 ml of a 1:5 dilution of antiserum directed against MBP-σ54. The membrane strip was washed five times with TBST and incubated with an acidic buffer (0.2 M glycine, 0.2 M sodium chloride, 1% gelatin, pH 2.8) for 15 min to elute the antibody. The eluate was dialyzed overnight against a buffer containing 50 mM citric acid and 50 mM Na_2HPO_4, pH 5.5, followed by overnight dialysis against Tris-buffered saline.

σ54 protein stability assay. *H. pylori* cultures were grown to mid-log phase in serum-free medium as described previously (9), at which point tetracycline was added to stop protein synthesis. Aliquots were removed at various times following tetracycline addition. Cells were recovered by centrifugation and resuspended in phosphate-buffered saline. Approximately 10^8 cells from each sample were lysed and analyzed by Western blotting.

To assay for secreted proteins, *H. pylori* cultures were grown to mid-log phase in serum-free medium as described previously (9). Cells were removed by centrifugation for 15 min at 20,000 × *g* at 4°C. The resulting supernatant liquids were filtered through a 0.22-μm-pore-size membrane filter to remove any residual bacteria. Extracellular proteins were precipitated using a modified trichloroacetic acid method as described previously (9), and approximately 20 μg of precipitated protein was analyzed by Western blotting.

RESULTS

Inactivation of *hp0958* interferes with flagellar biogenesis. *H. pylori hp0958* encodes a protein of unknown function that is predicted to be 254 amino acid residues in length. To determine if the interactions between HP0958 and σ54 observed in the yeast two-hybrid assay were of physiological relevance, *hp0958* was disrupted by introducing a cassette bearing *aphA3* into codon 76 of the gene in *H. pylori* ATCC 43504. The *hp0958:aphA3* mutant was nonmotile when tested on motility agar (Fig. 1) as well as when examined microscopically. Since loss of motility could have resulted from defects of either flagellar assembly or function, the *hp0958* mutant was examined by transmission electron microscopy for the presence of flagella. Sheathed polar flagella were readily apparent in the wild-type *H. pylori* strain but were absent in the *hp0958* mutant (data not shown).

The gene immediately downstream of *hp0958* is *kdtA*, which encodes 3-deoxy-D-manno-octulosonic acid transferase, an enzyme that is essential for lipopolysaccharide (LPS) biosynthesis (3, 4). Mutations in *H. pylori* that interfere with both LPS biosynthesis and flagellar biogenesis have been described previously (18), and so we wished to verify that the effect on flagellar biogenesis that we observed in the *hp0958:aphA3* mutant was not due to polar effects on *kdtA*. Mass spectrometry analysis of purified LPS from the *hp0958* mutant and wild-type strains revealed no differences between the two strains (data not shown), which argued strongly against the *aphA3* cassette in *hp0958* interfering with expression of *kdtA* or other genes downstream of *hp0958*. To confirm that disruption of *hp0958* was responsible for the phenotype of the mutant strain, we complemented the *hp0958:aphA3* mutant by introducing a functional copy of *hp0958* in the mutant strain. The *hp0958* gene along with most of the gene immediately upstream of *hp0958* (*hp0959*) was introduced into the *hp0958:aphA3* mutant at the *hp0405* locus. *H. pylori hp0405* encodes a predicted NifS-like protein of unknown function, and this locus was used previously to construct *H. pylori* merodiploid strains (20, 21). Disruption of *hp0405* does not affect motility. Introduction of a functional copy of *hp0958* into the *hp0958:aphA3* mutant allowed expression of HP0958 at wild-type level (Fig. 2A) and restored flagellar synthesis and motility (Fig. 1). These results demonstrated that disruption of *hp0958* was responsible for the lesion in flagellar biogenesis in the original mutant. Since *hp0958* and *hp0959* are separated by only 10 bp of DNA, the restoration of HP0958 expression to wild-type level in the complemented strain suggests that the promoter for *hp0958* is in *hp0959*.

Homologs of HP0958 are found in several bacteria, including all of the ε-proteobacteria whose genomes have been sequenced to date (*Helicobacter hepaticus*, *Campylobacter jejuni*, and *Wolinella succinogenes*). In all of these representatives of ε-proteobacteria, the gene located immediately upstream of the *hp0958* homolog shares homology with *hp0959*, and in *W. succinogenes* the two genes appear to be fused. This prompted us to investigate whether HP0959 also had a role in flagellar biogenesis. The *aphA3* cassette was used to disrupt *hp0959* in *H. pylori* ATCC 43504, and the resulting mutant expressed wild-type levels of HP0958 and was flagellated and motile (data not shown). These data indicate that, despite the apparent fusion of HP0958 and HP0959 homologs in *W. succinogenes*, HP0959 is not required for HP0958 function in *H. pylori* flagellar biogenesis.

Inactivation of HP0958 interferes with expression of flagellar genes in the RpoN regulon. Given the interaction between σ54 and HP0958 in the yeast two-hybrid assay and our observation that HP0958 is required for flagellar biogenesis, we reasoned that HP0958 may influence σ54 function in *H. pylori*. To test this hypothesis, FlaB levels were assessed by Western blotting in *H. pylori* ATCC 43504 and the mutant strains. The *flaB* gene is part of the RpoN flagellar regulon (19, 29), and we observed that an *rpoN* mutant failed to accumulate FlaB (Fig. 2B). A faint cross-reacting protein band was seen just below where FlaB was expected to migrate in the lane with cell lysate from the *rpoN* mutant. FlaA shares 58.4% amino acid identity

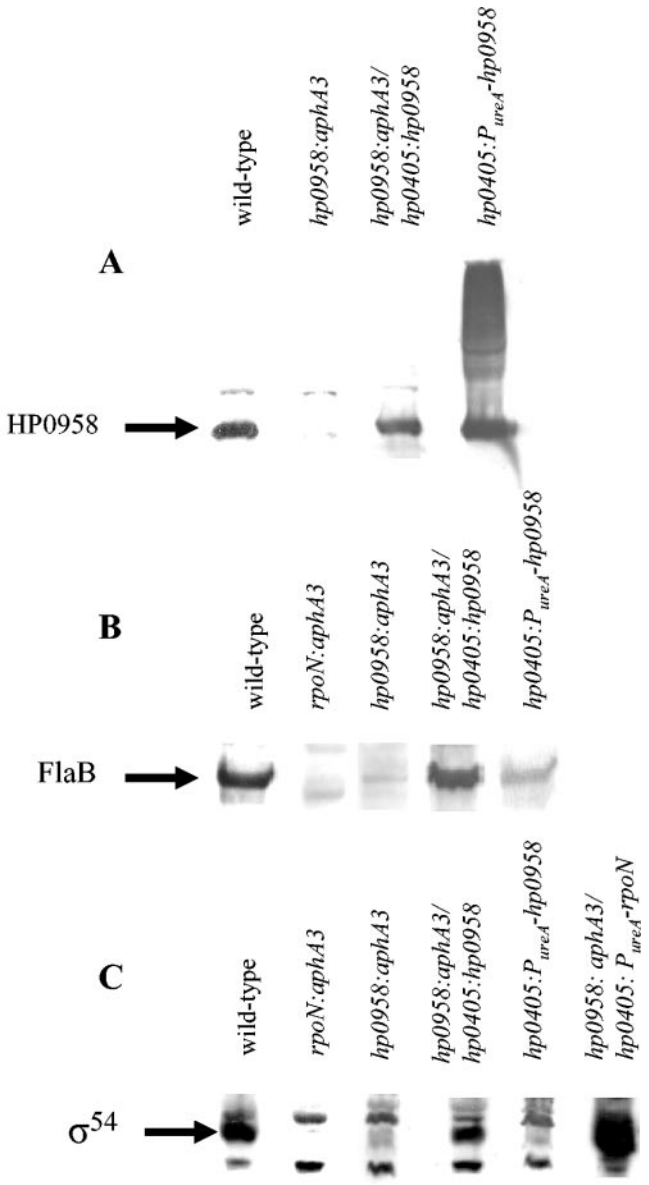

FIG. 2. Western blot analysis of HP0958, FlaB, and σ^{54} in various *H. pylori* strains. For panels A and B, approximately 10^8 cells were lysed and loaded in each lane, while $\sim 4 \times 10^8$ cells were lysed and loaded in each lane in panel C. Membranes were probed with antiserum directed against MBP-HP0958 (A), MBP-FlaB (B), or MBP-σ^{54} (C). Relevant genotypes of the strains that were analyzed are indicated above the lanes and include *H. pylori* 43504 (wild type), an *rpoN:aphA3* mutant, an *hp0958:aphA3* mutant, an *hp0958:aphA3* mutant complemented with a copy of *hp0958* in the *hp0405* locus (*hp0958:aphA3*/*hp0405:hp0958*), an *hp0958:aphA3* mutant in which σ^{54} was overproduced (*hp0958*:*aphA3*/*hp0405*:P_{ureA}-*rpoN*), and *H. pylori* 43504 bearing a P_{ureA}-*hp0958* allele in the *hp0405* locus (*hp0405*:P_{ureA}-*hp0958*). Arrows indicate the positions of the proteins analyzed in each Western blot.

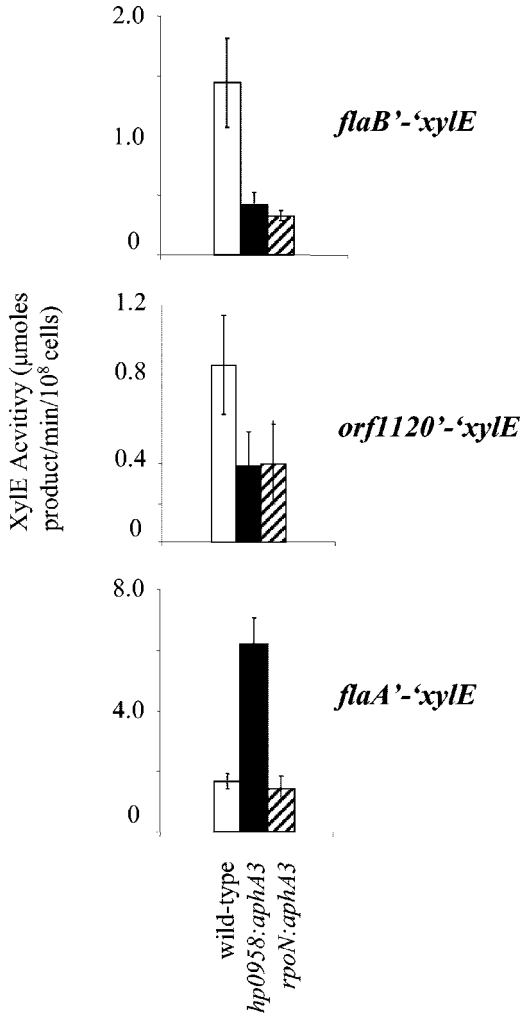

FIG. 3. Expression of *flaB'-'xylE*, *hp1120'-'xylE*, and *flaA'-'xylE* reporter genes in various *H. pylori* strains. XylE activities were measured for the reporter genes indicated to the right of the graphs in *H. pylori* 43504 (wild type; open bars), an *hp0958:aphA3* mutant (filled bars), and an *rpoN:aphA3* mutant (striped bars). Values represent the averages of at least 10 assays, and standard deviations for these values are indicated by the error bars.

with FlaB and is slightly smaller than FlaB (53.3 kDa versus 53.9 kDa), and we infer that this cross-reacting band is FlaA. Consistent with this hypothesis, the FlaB antiserum cross-reacted weakly with purified histidine-tagged FlaA (data not shown). The level of FlaB in the *hp0958:aphA3* mutant was dramatically reduced but was restored to wild-type level in the *hp0958*:*aphA3* mutant carrying a functional copy of *hp0958* in the *hp0405* locus, as expected from the restoration of flagellar synthesis and motility (Fig. 2B).

To determine if the reduced levels of FlaB in the *hp0958:aphA3* mutant were due to decreased transcription of *flaB*, we introduced a *flaB'-'xylE* reporter gene into the *hp0405* locus of the mutant. The *flaB'-'xylE* reporter gene was also introduced into the *hp0405* locus of the *rpoN:aphA3* and wild-type strains. Expression of the *flaB'-'xylE* reporter gene was reduced in the *hp0958*:*aphA3* mutant to a level that was comparable to that in the *rpoN:aphA3* mutant (Fig. 3), indicating that the reduced level of FlaB in the *hp0958:aphA3* mutant was due to decreased transcription of *flaB*. Expression of another RpoN-dependent reporter gene, *hp1120'-'xylE*, was reduced in the *hp0958* mutant (Fig. 3), suggesting that HP0958 is required for transcription of genes of the RpoN flagellar regulon. A σ^{28}-

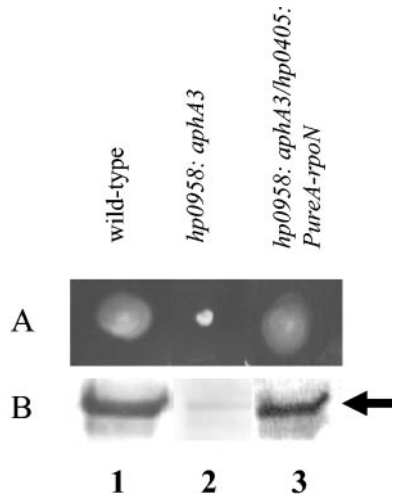

FIG. 4. Overproduction of σ^{54} in the *hp0958:aphA3* mutant restores motility and FlaB synthesis. (A) The motilities of *H. pylori* 43504 (wild type), an *hp0958:aphA3* mutant, and an *hp0958:aphA3* mutant in which σ^{54} was overproduced from the *ureA* promoter were assessed in semisolid agar. (B) *H. pylori* strains ($\sim 10^8$ cells) were analyzed by Western blotting for FlaB (indicated by arrow). Lane 1, *H. pylori* 43504; lane 2, *hp0958:aphA3* mutant; lane 3, *hp0958:aphA3* mutant bearing a P_{ureA}-*rpoN* allele in the *hp0405* locus (*hp0958:aphA3/hp0405:* P_{ureA}-*rpoN*).

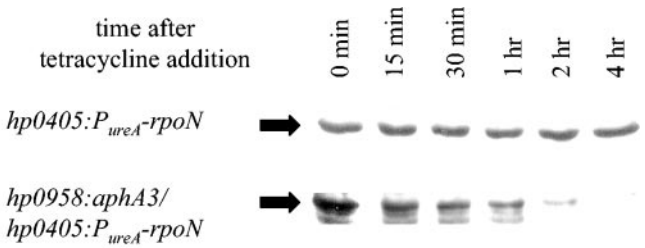

FIG. 5. Comparison of σ^{54} stability in an *hp0958:aphA3* mutant and its parental strain. Levels of σ^{54} in an *hp0958:aphA3* mutant that overproduced σ^{54} from a P_{ureA}-*rpoN* allele (*hp0958:aphA3/hp0405:* P_{ureA}-*rpoN*) and its parental strain (*hp0405:*P_{ureA}-*rpoN*) were analyzed by Western blotting at various times following the addition of tetracycline. Approximately 10^8 cells were lysed and loaded in each lane. σ^{54} is indicated by the arrows.

dependent *flaA′-′xylE* reporter gene was introduced into the *H. pylori* strain to determine if HP0958 was required for expression of flagellar genes outside the RpoN regulon. Expression of the *flaA′-′xylE* reporter gene in the *hp0958:aphA3* mutant was about threefold higher than that in the wild-type and *rpoN:aphA3* mutant strains (Fig. 3), indicating that HP0958 is not required for expression of flagellar genes in the FliA regulon.

Inactivation of HP0958 results in a decreased level of σ^{54}. To determine if the decreased expression of *flaB* in the *hp0958:aphA3* mutant was due to decreased activity or levels of σ^{54}, σ^{54} levels in the *hp0958:aphA3* mutant and its parental strain were compared. As shown in Fig. 3, the level of σ^{54} in the *hp0958:aphA3* mutant was significantly reduced compared to the wild-type strain. Introduction of a functional copy of *hp0958* in the *hp0405* locus of the *hp0958:aphA3* mutant restored the level of σ^{54} to close to wild-type level, demonstrating that HP0958 is required for the accumulation of a wild-type level of σ^{54}.

To test if overproduction of σ^{54} in the *hp0958:aphA3* mutant would restore flagellar synthesis and motility, *rpoN* was placed under the control of the *ureA* promoter and introduced into the *hp0405* locus of the *hp0958:aphA3* mutant. The level of σ^{54} in this strain exceeded that of the wild-type strain (Fig. 2C) and restored motility and expression of *flaB* (Fig. 4). Taken together, these data suggest that HP0958 is required for accumulation, but not function, of σ^{54}.

Overproduction of HP0958 interferes with its function. In the initial attempt to complement the *hp0958:aphA3* mutation, an *hp0958* allele under the control of the *ureA* promoter (P_{ureA}-*hp0958*) was introduced into the *hp0405* locus of the mutant, which failed to restore motility (data not shown). Introduction of the P_{ureA}-*hp0958* allele into wild-type *H. pylori* interfered with motility (Fig. 1), and as with inactivation of *hp0958*, it resulted in decreased levels of FlaB and σ^{54} (Fig. 2B and C). The level of HP0958 in the strain with the P_{ureA}-*hp0958* allele was ~10-fold higher than the wild-type level, which was estimated by determining the lowest number of cells needed to visualize the protein by Western blotting (data not shown). We do not know why overproduction of HP0958 interfered with its function. Behavior of HP0958 overproduced in *H. pylori* in the sodium dodecyl sulfate-polyacrylamide gel was unusual in that much of the protein migrated with a reduced mobility (Fig. 2A). This may have resulted from cross-linking or other stable interactions between HP0958 monomers which could have interfered with activity of the protein.

HP0958 prevents the rapid turnover of σ^{54}. To address whether HP0958 exerts its control over σ^{54} accumulation posttranslationally, we introduced an *rpoN′-′xylE* reporter gene into the *hp0958:aphA3* mutant and its parental strain. This *rpoN′-′xylE* reporter gene encoded a translational fusion in which *xylE* was joined in-frame with codon 61 of *H. pylori rpoN*. Expression of the *rpoN′-′xylE* reporter gene was indistinguishable in the two strains (0.47 ± 0.10 units XylE activity/10^8 cells for the parental strain versus 0.51 ± 0.07 units XylE activity/10^8 cells for the *hp0958:aphA3* mutant), which argued that HP0958 affects σ^{54} levels in *H. pylori* at a posttranslational step.

To determine if HP0958 influences σ^{54} stability, σ^{54} levels were monitored in wild-type and *hp0958:aphA3* mutant strains where *rpoN* was under the control of the *ureA* promoter. Strains were grown in serum-free medium to mid-log phase, at which time tetracycline was added to block translation. Samples were analyzed for σ^{54} at various times following the addition of tetracycline. Levels of σ^{54} in the *hp0958:aphA3* mutant decreased with an apparent half-life of ~33 min, whereas σ^{54} levels remained constant for at least 4 h in the wild-type strain (Fig. 5). In the absence of tetracycline, σ^{54} levels remained constant in the wild-type and the *hp0958:aphA3* mutant strains over the entire course of the assay (data not shown). We were unable to detect σ^{54} in the extracellular proteins of the *hp0958:aphA3* mutant or its parental strain, but in a positive control, the vacuolating cytotoxin VacA was detected in the extracellular proteins of both strains (data not shown). Taken together, these data suggest that the rapid decrease in σ^{54} levels in the *hp0958:aphA3* mutant does not result from secretion but rather from protein degradation.

DISCUSSION

We demonstrate here that HP0958 is required for the normal accumulation of σ^{54} in *H. pylori*, indicating that it may play a regulatory role in modulating σ^{54} levels under different growth or environmental conditions. HP0958 appears to influence σ^{54} levels by protecting it from proteolysis. Regulated turnover is a mechanism by which the levels of other σ factors are sometimes controlled. *E. coli* σ^S, for example, is degraded by ClpXP protease but is protected from proteolysis under certain conditions by the chaperone DnaK (27, 28). Like DnaK, HP0958 may function as a chaperone to protect σ^{54} from proteolysis, which would explain the interactions between HP0958 and σ^{54} in the yeast two-hybrid assay. Suppression of the *hp0958:aphA3* mutation by overexpressing *rpoN* may be explained by high levels of σ^{54} overwhelming the proteolysis of the protein. Alternatively, HP0958 may modify σ^{54} to protect it from proteolysis. The suppression of the *hp0958* mutation by overproducing σ^{54}, however, suggests that any such potential modification of σ^{54} is not needed to convert it from an inactive to an active form. Thus, the only essential role that HP0958 appears to play in flagellar biogenesis is in maintaining σ^{54} levels capable of supporting efficient expression of the RpoN regulon. HP0958 may have an additional, nonessential role in regulating the FliA flagellar regulon, however, since expression of the *flaA′-′xylE* reporter gene was elevated in the *hp0958* mutant but not in the *rpoN* mutant. HP0958 may affect *flaA* expression by influencing the level or activity of the anti-σ^{28} factor FlgM or another factor that inhibits expression of *flaA*.

We have not observed any other phenotypes for the *hp0958:aphA3* mutant. HP0958 interacts with other *H. pylori* proteins in the yeast two-hybrid assay, including FliH (a regulator of the flagellar protein export apparatus), HP1462 (a putative secreted protein involved in motility), and TonB1 (a putative siderophore-mediated iron transport protein). Thus, HP0958 may have other roles in addition to maintaining a wild-type level of σ^{54}. Since σ^{54} is required for flagellar biogenesis, the interactions between HP0958 and FliH are intriguing. Inactivation of *fliH*, however, does not affect σ^{54} levels in *H. pylori* (L. Pereira, unpublished data). Despite several attempts, we were unable to construct an *hp0958*/*fliH* double mutant, suggesting that this combination of mutations is lethal.

Comparison of the genome sequences of *H. pylori*, *H. hepaticus*, *C. jejuni*, and *W. succinogenes* reveals similar organization of flagellar genes and potential regulatory mechanisms controlling their expression. All of these ε-proteobacteria possess HP0958 homologs, and we anticipate that these proteins have roles in maintaining wild-type σ^{54} levels in these bacteria. FlgR is the only σ^{54}-dependent activator present in *H. pylori*, *H. hepaticus*, and *C. jejuni*, suggesting that σ^{54} is dedicated for flagellar biogenesis in these bacteria. *W. succinogenes*, however, possesses two σ^{54}-dependent activators, FlgR and NifA, which appear to be required for the expression of flagellar and nitrogen fixation genes, respectively. Thus, the HP0958 homolog in *W. succinogenes* may be required for flagellar biogenesis and nitrogen fixation.

In addition to the ε-proteobacteria, homologs of HP0958 are present in a number of bacteria from diverse phylogenic groups, including *Aquifex aeolicus*, *Bacteroides thetaiotaomicron*, *Chlamydia trachomatis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Thermoanaerobacter tengcongensis*, *Chlorobium tepidum*, *Borrelia burgdorferi*, and *Treponema pallidum*. Many of these bacteria possess σ^{54}, and the HP0958 homologs in these bacteria may have roles in modulating σ^{54} levels. Elucidating the mechanism by which HP0958 influences σ^{54} levels in *H. pylori* is certain to lead to a better understanding of the function of its homologs in other bacteria.

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REFERENCES

1. **Alm, R. A., L.-S. L. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smithe, B. Noonon, B. D. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, J. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. R. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176–180.
2. **Beier, D., and R. Frank.** 2000. Molecular characterization of two-component systems of *Helicobacter pylori*. J. Bacteriol. **182:**2068–2076.
3. **Belunis, C. J., S. M. Clementz, S. M. Carty, and C. R. Raetz.** 1995. Inhibition of lipopolysaccharide biosynthesis and cell growth following inactivation of the *kdtA* gene in *Escherichia coli*. J. Biol. Chem. **270:**27646–27652.
4. **Belunis, C. J., and C. R. Raetz.** 1992. Biosynthesis of endotoxins. Purification and catalytic properties of 3-deoxy-D-octolusonic acid transferase from *Escherichia coli*. J. Biol. Chem. **267:**9988–9997.
5. **Benoit, S., and R. J. Maier.** 2003. Dependence of *Helicobacter pylori* urease activity on the nickel-sequestering ability of the UreE accessory protein. J. Bacteriol. **185:**4787–4795.
6. **Blaser, M. J.** 1993. *Helicobacter pylori*: microbiology of a 'slow' bacterial infection. Trends Microbiol. **1:**255–260.
7. **Brahmachary, P., M. G. Dashti, J. W. Olson, and T. R. Hoover.** 2004. *Helicobacter pylori* FlgR is an enhancer-independent activator of σ^{54}-RNA polymerase holoenzyme. J. Bacteriol. **186:**4535–4542.
8. **Buck, M., S. Miller, M. Drummond, and R. Dixon.** 1986. Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature (London) **320:**374–378.
9. **Bumann, D., S. Aksu, M. Wendland, K. Janek, U. Zimny-Arndt, N. Sabarth, T. F. Meyer, and P. R. Jungblut.** 2002. Proteome analysis of secreted proteins of the gastric pathogen *Helicobacter pylori*. Infect. Immun. **70:**3396–3403.
10. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135–145.
11. **Dasgupta, N., M. C. Wolfgang, A. L. Goodman, S. K. Arora, J. Jyot, S. Lory, and R. Ramphal.** 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**809–824.
12. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720–741.
13. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. **57:**1119–1125.
14. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. **37:**123–127.
15. **Kim, J. S., J. H. Chang, S. I. Chung, and J. S. Yum.** 1999. Molecular cloning and characterization of the *Helicobacter pylori fliD* gene, an essential factor in flagellar structure and motility. J. Bacteriol. **181:**6969–6976.
16. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol. Microbiol. **6:**2863–2874.
17. **McGee, D. J., F. J. Radcliff, R. L. Mendz, R. L. Ferrero, and H. L. Mobley.** 1999. *Helicobacter pylori rocF* is required for arginase activity and acid protection in vitro but is not essential for colonization of mice or for urease activity. J. Bacteriol. **181:**7314–7322.
18. **Merkx-Jacques, A., R. K. Obhi, G. Bethune, and C. Creuzenet.** 2004. The *Helicobacter pylori flaA1* and *wbpB* genes control lipopolysaccharide and flagellum synthesis and function. J. Bacteriol. **186:**2253–2265.
19. **Niehus, E., H. Gressmann, F. Ye, R. Schlapbach, M. Dehio, C. Dehio, A. Stack, T. F. Meyer, S. Suerbaum, and C. Josenhans.** 2004. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori*. Mol. Microbiol. **52:**947–961.
20. **Olson, J. W., M. K. Johnson, and R. J. Maier.** 2000. Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39:**16213–16219.

21. **Olson, J. W., N. S. Mehta, and R. J. Maier.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol. Microbiol. **39:**176–182.
22. **Popham, D., D. Szeto, J. Keener, and S. Kustu.** 1989. Function of a bacterial activator protein that binds to transcriptional enhancers. Science **243:**629–635.
23. **Prouty, M. G., N. E. Correa, and K. E. Klose.** 2001. The novel σ^{54}- and σ^{28}-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol. Microbiol. **39:**1595–1609.
24. **Rain, J.-C., L. Selig, H. De Reuse, V. Battaglia, C. Reverdy, S. Simon, G. Lenzen, F. Petel, J. Wojcik, V. Schachter, Y. Chemama, A. Labigne, and P. Legrain.** 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature **409:**211–215.
25. **Reitzer, L. J., and B. Magasanik.** 1986. Transcription at *glnA* of *E. coli* is stimulated by activator bound to sites far from the promoter. Cell **45:**785–792.
26. **Rippe, K., M. Guthold, P. H. von Hippel, and C. Bustamante.** 1997. Transcriptional activation *via* DNA-looping: visualization of intermediates in the activation pathway of *E. coli* RNA polymerase σ^{54} holoenzyme by scanning force microscopy. J. Mol. Biol. **270:**125–138.
27. **Rockabrand, D., K. Livers, T. Austin, R. Kaiser, D. Jensen, R. Burgess, and P. Blum.** 1998. Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli*. J. Bacteriol. **180:**846–854.
28. **Schweder, T., K. H. Lee, O. Lomovskaya, and A. Matin.** 1996. Regulation of *Escherichia coli* starvation factor (σ^s) by ClpXP protease. J. Bacteriol. **178:**470–476.
29. **Spohn, G., and V. Scarlato.** 1999. Motility of *Helicobacter pylori* is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. J. Bacteriol. **181:**593–599.
30. **Spohn, G., and V. Scarlato.** 2001. Motility, chemotaxis, and flagella, p. 239–257. *In* S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM Press, Washington, D.C.
31. **Su, W., S. Porter, S. Kustu, and H. Echols.** 1990. DNA-looping and enhancer activity: association between DNA-bound NTRC activator and RNA polymerase at the bacterial *glnA* promoter. Proc. Natl. Acad. Sci. USA **87:**5504–5508.
32. **Suerbaum, S., C. Josenhans, and A. Labigne.** 1993. Cloning and genetic characterization of the *Helicobacter pylori* and *Helicobacter mustelae flaB* flagellin genes and construction of *H. pylori flaA*- and *flaB*-negative mutants by electroporation-mediated allelic exchange. J. Bacteriol. **175:**3278–3288.
33. **Tomb, J.-F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539–547.
34. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172:**389–396.
35. **Weiss, D. S., J. Batut, K. E. Klose, J. Keener, and S. Kustu.** 1991. The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. Cell **67:**155–167.