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Posttranscriptional Regulation of Flagellin Synthesis in *Helicobacter pylori* by the RpoN Chaperone HP0958▽†

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The *Helicobacter pylori* protein HP0958 is essential for flagellum biogenesis. It has been shown that HP0958 stabilizes the σ^{54} factor RpoN. The aim of this study was to further investigate the role of HP0958 in flagellum production in *H. pylori*. Global transcript analysis identified a number of flagellar genes that were differentially expressed in an HP0958 mutant strain. Among these, the transcription of the major flagellin gene *flaA* was upregulated twofold, suggesting that HP0958 was a negative regulator of the *flaA* gene. However, the production of the FlaA protein was significantly reduced in the HP0958 mutant, and this was not due to the decreased stability of the FlaA protein. RNA stability analysis and binding assays indicated that HP0958 binds and destabilizes *flaA* mRNA. The HP0958 mutant was successfully complemented, confirming that the mutant phenotype described was due to the lack of HP0958. We conclude that HP0958 is a posttranscriptional regulator that modulates the amount of the *flaA* message available for translation in *H. pylori*.

Helicobacter pylori is a gram-negative microaerophilic epsilon-proteobacterium that colonizes the human gastric mucosa. *H. pylori* infection elicits gastric inflammation and may cause gastritis, peptic ulcers, duodenal ulcers, and gastric adenocarcinoma (5, 10, 20, 59). The motility of *H. pylori* is an essential requirement for colonization of the gastric niche (13, 14). The pathogenic bacterium is motile by means of multiple polar sheathed flagella (5, 17, 18). In addition to motility, flagella in some bacteria mediate adhesion to, and interaction with, host cells (26). The interaction between *H. pylori* and gastric cells induces a cytokine response in gastric epithelial cells via Toll-like receptors (TLR) (25, 53). However, the flagellar filament in *H. pylori* is not recognized by TLR5 (16).

In the *Enterobacteriaceae*, the flagellar apparatus is the result of the sequential assembly of more than 40 flagellar proteins (3, 30, 56). Flagellar biogenesis in *H. pylori* shares common features with those of model organisms like *Salmonella* and *Escherichia* spp. (40, 41), and most of the relevant genes for structural and regulatory proteins required for *H. pylori* flagellum biogenesis have been annotated (37, 56). The transcription of flagellar genes is under the control of three main RNA polymerase sigma factors, σ^{28}, σ^{54}, and σ^{80}, and is also modulated by an anti-σ^{28} factor, FlgM (8, 9, 27, 37, 42, 51). The master regulator $FlhC_4D_2$, present in most *Enterobacteriaceae*, has not been annotated in *H. pylori*, highlighting the distinct regulatory network in this organism (37). Three flagellar gene classes have been defined (37). Early flagellar proteins required in flagellar biogenesis (class I) are under the transcriptional regulation of σ^{80} (37). RpoN (σ^{54}) controls the transcription of the middle flagellar structural genes (class II), including the gene for the hook-length protein FliK (37). RpoN activity is regulated by its activator FlgR and its cognate histidine kinase FlgS (HP0244) (8, 54), similarly to the FleR/FleS system in *Pseudomonas aeruginosa* (46). The transcription of the late flagellar genes (class III), including the gene encoding the major flagellin FlaA, is under the control of FliA (σ^{28}) (37). FliA activity is tightly repressed by the anti-σ^{28} factor FlgM (9). This repression is relieved when FlgM is secreted from the cell upon completion of the hook structure (29), though FlgM secretion has not actually been demonstrated for *H. pylori*. The hook length is under the control of FliK in *Salmonella* (15), which we recently identified as the product of the gene HP0906 in *H. pylori* (49). Completion of the hook structure modulates the activity of the RpoN regulon in *H. pylori*.

Based on the data from a high-throughput screen of a yeast two-hybrid system (Hybrigenics PimRider database), HP0958 also interacts with the sigma factor RpoN (44). HP0958 was defined as a novel flagellar gene in *H. pylori* (42, 48). It contains a zinc-finger motif, suggesting possible interactions with nucleic acids or proteins (48). The deletion of HP0958 impairs the transcription of genes encoding flagellar components, such as the hook protein FlgE, the major flagellin FlaA, and the minor flagellin FlaB (48). Pereira and Hoover showed that HP0958 controls RpoN activity at the protein level by stabilizing RpoN (42). The transcription of the HP0958 gene was not affected in a number of flagellar mutants analyzed in a previous investigation (37).

In the present study, we performed a global transcript analysis of mutants lacking the HP0958 gene. This led us to further investigate the role of the HP0958 protein in flagellin production and to uncover a new regulatory role for this protein in *H. pylori* flagellum biogenesis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in the present study are listed in Table 1. *H. pylori* strain P79 (33), a streptomycin mutant of the P1 wild-type strain, was generously provided by R. Haas. *H. pylori*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
H. pylori		
CCUG17874	Wild-type strain	Culture collection, University of Gothenborg, Sweden
NCTC 26695	Wild-type strain	NCTC, United Kingdom
hp0958 KO[a]	CCUG17874 Δhp0958::Cm^r	48
P1	Wild-type strain	38
P79	P1 Str^r	21
P79-0958KO	P79 Δhp0958::Cm^r	This study
P79-0958/pIR203K04	P79 Δhp0958::Cm^r with pIR203K04 (Kan^r)	This study
P79-0958/pIR1041	P79 Δhp0958::Cm^r with pIR1041 (Kan^r)	This study
P79-0958/pIR1563	P79 Δhp0958::Cm^r with pIR1563 (Kan^r)	This study
P79-0958/pIR1186	P79 Δhp0958::Cm^r with pIR1186 (Kan^r)	This study
E. coli		
One Shot TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Rosetta 2 DE3 pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cm^r)	Novagen, Germany
Plasmids		
pIR203K04	Kanamycin resistance cassette (Kan^r)	32
pIR1041	pIR203K04 with the hp0958 gene under the control of the hp1041 promoter	This study
pIR1563	pIR203K04 with the hp0958 gene under the control of the hp1563 promoter	This study
pIR1186	pIR203K04 with the hp0958 gene under the control of the hp1186 promoter	This study
pGEX-6P-3	N-terminally GST-tagged expression vector (Amp^r)	GE Healthcare, United Kingdom
pDC006	pGEX-6P-3 hp0958 (Amp^r)	This study

[a] KO, knockout.

strains were cultured as previously described (49). A CCUG17874-derivative *H. pylori* mutant lacking the HP0958 gene was previously described (48, 49). The P79 strain-derivative *H. pylori* mutant lacking the HP0958 gene was generated as previously described by Ryan et al. for strain CCUG17874 (48). Transformants from the present study were selected on CBA (Columbia agar base) plates supplemented with antibiotics: 10 μg/ml chloramphenicol (Sigma) and 50 μg/ml kanamycin (Sigma). One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, CA) were propagated on Luria-Bertani (LB) agar plates or LB broth at 37°C supplemented with the following antibiotics: 50 μg/ml kanamycin (Sigma), 100 μg/ml ampicillin (Merck, Germany), and 10 μg/ml chloramphenicol (Sigma).

Extraction of genomic DNA from *H. pylori*. Genomic DNA from 2-day-old *H. pylori* plate cultures was extracted by following the DNeasy tissue kit instructions (Qiagen). The genomic DNA was then concentrated by vacuum-drying and quantified using the Nanodrop ND-1000 (Nanodrop).

Preparation of whole cell fractions. *H. pylori* cells were harvested from 20-h liquid cultures and pelleted for 15 s at 13,000 rpm. The cell pellets were then washed in 1 ml sterile phosphate-buffered saline (PBS). After a further 15-s centrifugation at 13,000 rpm, the supernatant was discarded. The washed cell pellets were resuspended in 250 μl PBS. The optical density at 600 nm of the samples was measured and then standardized as required. Cell preparations were boiled for 5 min and stored at −80°C.

Protein electrophoresis and immunoblotting. A standard protocol was used to perform sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (50) and immunoblotting. Proteins from 12.5% acrylamide gels were transferred onto nitrocellulose membranes by electroblotting (57). Polyclonal antibody directed against *H. pylori* flagellin A and hook protein was used as primary antibody (39). Anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) was used as the secondary antibody. Hydrogen peroxide and 4-chloro-1-naphthol (Sigma) were employed for detection.

RNA isolation from *H. pylori*. Total RNA was extracted from 20-h *H. pylori* liquid cultures using the RNeasy mini kit (Qiagen). Briefly, *H. pylori* cells were harvested and centrifuged for 15 s at 13,000 rpm. Cell pellets were then resuspended in 750 μl RNAprotect bacteria reagent (Qiagen). The remainder of the protocol was performed per the manufacturer's instructions. RNA quality was assessed using a Bioanalyzer 2100 instrument (Agilent Technologies) and quantified by NanoDrop ND-1000 (Nanodrop). Total RNA samples were DNase treated using a DNA-free kit (Ambion).

***H. pylori* microarray.** The design and construction of the *H. pylori* microarray (BμG@S HPv1.0.0; Bacterial Microarray Group at St. George's, University of London) was completed using the approaches described by Hinds et al. (22, 23). In brief, the PCR products were designed to include amplicons representing all 1,576 open reading frames in *H. pylori* NCTC 26695 (56) and all 1,495 open reading frames in *H. pylori* J99 (3) using the approach described by Hinds et al. (23). The microarrays were constructed by robotic spotting of the PCR products in duplicate on UltraGaps amino-silane coated glass slides (Corning) using a MicroGrid II (BioRobotics) (23) and were post-print processed according to the slide manufacturer's instructions.

Type II microarray analysis. To compare the transcriptional profiles of the wild-type and HP0958 mutant strains, the *H. pylori* whole-genome microarray was used in a common reference or type II experimental design, whereby Cy5-labeled cDNA from each strain was cohybridized to an array with a Cy3-labeled genomic DNA reference. Nucleic acid labeling and microarray hybridizations were undertaken according to BμG@S standard protocols (22). In brief, for the common reference, 5 μg of wild-type CCUG17874 genomic DNA was labeled with dCTP Cy3 using random primers (Promega) and a DNA polymerase I large Klenow fragment (Invitrogen). Cy5-labeled cDNA was generated from 4 μg of total RNA during first-strand synthesis using random primers (Promega) and Superscript II reverse transcriptase (Invitrogen). The Cy3- and Cy5-labeled nucleic acid mixtures were then copurified using the MinElute PCR purification kit (Qiagen), mixed in a hybridization solution of 4× saline-sodium citrate buffer (SSC) and 0.3% SDS and hybridized under a LifterSlip (Erie Scientific Company) for 18 h at 65°C. Microarray slides were washed once in 1× SSC, 0.06% SDS at 65°C for 2 min and twice in 0.06× SSC for 2 min, dried by centrifugation, and scanned using a dual laser Affymetrix 428 scanner (Affymetrix, United Kingdom). The images and data were analyzed using the GeneDirector software package, which includes ImaGene and GeneSight v2.0 (Biodiscovery, El Segundo, CA). Genes designated as missing or uncertain in CCUG17874 were filtered out. Array hybridizations for the wild type and for the mutant were performed in triplicate. Ratios for the wild-type DNA versus the wild-type RNA and for the wild-type DNA versus the mutant RNA were exported to Excel (Microsoft). Following the within-slide normalization in GeneSight, the genes containing empty values were discarded. Quantile normalization was then performed to make the entire distribution of the values identical between each array slide (6). The triplicates for the wild type and for the mutant were averaged, and the final $\log_2$ ratios were calculated. One-way analysis of variance was used to calculate statistical confidences. Genes with a P value less than 0.05 and a change greater than 2.00-fold were highlighted as differentially expressed in the mutant.

Quantitative analysis of transcription by RT-PCR. Quantitative real-time PCR (qRT-PCR) was performed as a confirmatory test on selected genes fol-

lowing global transcript analysis by microarray. RT-PCR primers were designed using the Primer3 software package (47) and are listed in Table S1 in the supplemental material. A total of 500 ng of RNA was reverse transcribed using Improm-II reverse transcriptase (Promega) and 500 ng random hexamers as described in the manufacturer's manual. qRT-PCR was performed on flagellar genes using the primers listed in Table S1 in the supplemental material. The reaction mixture was prepared as described in the manufacturer's protocol. Briefly, amplification by qRT-PCR was performed in a final volume of 12.5 μl, including 1 μl cDNA, 50 nM of each primer, 6.25 μl 2× master mix (Biogen, United Kingdom), and 1/60,000 Sybr green I (Biogene, United Kingdom). qRT-PCRs were run and monitored using an ABI 7000 thermocycler and ABI Prism 7000 SDS software (both from Applied Biosystems). The reactions were performed in triplicate (technical replicates) from at least two independent RNA preparations (biological replicates). Relative changes in expression were calculated as described by Pfaffl (43). The *era* gene was used as a housekeeping gene (52). Each transcript abundance was therefore calculated relative to the *era* gene transcript abundance.

Bacterial cytoplasmic protein extract preparation. *H. pylori* cytoplasmic protein extracts were prepared using a modified protocol described by Donahue et al. (11). Cells were grown for 20 h in broth, and cell pellets were resuspended in a buffer containing 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5 mM Na_2EDTA, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Complete Mini; Roche, Germany). *H. pylori* cell suspensions were bead beaten for 1 min and then centrifuged at 13,000 rpm for 5 min. The supernatants were harvested, and the protein concentration was quantified using the BCA protein assay kit (Pierce) and stored at −80°C.

Protein overexpression and purification. The HP0958 gene of *H. pylori* CCUG17874 was overexpressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein using the pGEX-6P-3 vector (GE Healthcare Ltd., United Kingdom). The primers are listed in Table S1 of the supplemental material. *E. coli* Rosetta 2 DE3 pLysS cells (Novagen, Germany) were grown at 37°C to an optical density at 600 nm of 0.4 to 0.6 and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Melford Laboratories Ltd., United Kingdom). After induction, the cells were grown at 18°C for an additional 10 to 15 h. The cells were harvested by centrifugation and resuspended in PBS (Gibco). The cells were then lysed by a freeze/thaw treatment according to the Novagen pET system manual, and the supernatant was harvested following centrifugation at 13,000 × *g* for 20 min. The soluble fraction was incubated with glutathione Sepharose 4B at 4°C overnight. The GST tag was then removed by adding PreScission Protease (GE Healthcare Ltd.). Once the GST tag had been removed, HP0958 was purified on an ÄKTA purifier for fast protein liquid chromatography (GE Healthcare Ltd.), using a HiTrap Q FF column (GE Healthcare Ltd.) in a 20 mM Tris-HCl buffer at pH 9.50. HP0958 was further purified by gel filtration chromatography in 10 mM potassium phosphate buffer at pH 7.0 using a Superdex 75 10/300 GL column (GE Healthcare Ltd.). HP0958 purity was assessed after each purification step by SDS-polyacrylamide gel electrophoresis. The protein concentrations were measured using the BCA protein assay kit (Pierce).

Native RNA agarose gel electrophoresis. RNA samples were resolved using horizontal agarose gel electrophoresis. A 1% (wt/vol) agarose gel was prepared with diethyl pyrocarbonate-treated 1× MOPS buffer, containing 20 mM 3-(*N*-morpholino) propane-sulfonic acid, 5 mM sodium acetate, and 1 mM EDTA at pH 7.0. The native RNA gels were run at 90 V for about 45 min in 1× MOPS running buffer. Following staining with ethidium bromide (Sigma), the samples were visualized under UV irradiation.

RNA-binding assay by EMSA. The binding of the HP0958 protein and RNA was investigated using an electrophoretic gel migration shift assay (EMSA). *flaA* mRNA transcripts and variants were synthesized using a modified protocol of the Riboprobe in vitro transcription system (Promega). DNA templates for in vitro transcription were amplified by PCR, and the T7 promoter was fused to the 5′ end of the DNA fragments in another PCR. The primers are listed in Table S1 in the supplemental material. Next, the linear T7 promoter-attached DNA fragments were used for in vitro transcription per the manufacturer's instructions. The size and quality of the in vitro-transcribed messages were assessed using RNA gel electrophoresis. Different amounts of HP0958 protein were incubated in a total volume of 15 μl binding buffer containing 30 ng *flaA* Riboprobe transcript, 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 5 mM Na_2EDTA, 200 μM *S*-(5-adenosyl)-L-methionine chloride (Sigma), and 40 U RNasin (Promega). The range of the HP0958 protein concentration used was based on previous EMSAs (12). The mixture was incubated at room temperature for 20 min. The RNA was then resolved by native agarose gel electrophoresis. To detect RNA species, Northern hybridization was performed with a labeled PCR probe corresponding to the riboprobe (see below). In a further EMSA, additional EDTA (final concentration, 50 mM) or DTT (final concentration, 10 mM) was added to the incubation mixture.

Northern blotting and hybridization. Separated RNAs were transferred from agarose gels to a positively charged nylon membrane (Hybond; GE Healthcare) by overnight capillary transfer (50). RNAs were covalently bound to the nylon membrane by UV cross-linking using the Stratalinker UV linker (Stratagene). RNA detection was performed using the ECL system (GE Healthcare). All steps were performed per the manufacturer's protocol. DNA probes were amplified by PCR. A 10-μl solution containing 100 ng DNA probe was prepared and boiled for 5 min and incubated for 5 min on ice. Ten microliters of labeling reagent and 10 μl of glutaraldehyde solution were added. The mixture was incubated at 37°C for 20 min. The nylon membrane was prehybridized at 42°C for at least 45 min in ECL hybridization buffer (GE Healthcare) in a hybridization oven. The 30-μl labeled probe was then added to the hybridization buffer. Following overnight hybridization, the membrane was washed once in 5× SSC buffer and three times in wash buffer (6 M urea, 0.4% SDS, 0.5× SSC [pH 7.0]) at 42°C and twice in 5× SSC buffer for 10 min at room temperature. The membrane was drained to remove excess secondary wash buffer. Equal volumes of reagent 1 (GE Healthcare) and reagent 2 (GE Healthcare) were mixed and added directly to the blotted side of the membrane. The blot, completely covered with the developing solution, was incubated for 1 min at room temperature. The excess developing solution was then removed, and the blot was placed in a detection cassette.

Protein stability assay. CCUG17874 and the HP0958 mutant were grown in liquid culture for 20 h in a microaerobic environment. Tetracycline (60 μg/ml) was then added to stop protein synthesis. Aliquots were collected at different times following tetracycline treatment. Whole cell fractions were then prepared as previously described and analyzed by immunoblotting.

RNA stability assay. CCUG17874 and the HP0958 mutant were cultured in liquid culture for 20 h in a microaerobic environment. Rifampin (80 μg/ml) was added to stop transcription. Aliquots were collected at different times following rifampin treatment. Total RNA was then extracted as described above and subsequently analyzed by Northern blotting (50).

Molecular cloning. Standard procedures were used to perform cloning experiments with *E. coli*. The strains, plasmids, and primers used are listed in Table 1 and in Table S1 in the supplemental material. The vector pIR203K04, a generous gift from D. J. McGee, contains a kanamycin resistance marker and was designed to introduce DNA fragments into an intergenic region of the *H. pylori* chromosome (32). The promoter regions of the genes HP1186 (carbonic anhydrase), *flhA*, and *ahpC* (alkyl hydroperoxide reductase) were spliced to the HP0958 gene by single overlapping extension PCR (24). The amplicons were digested with BamHI and ClaI and ligated to the linearized pIR203K04 plasmid. The ligated products were first introduced into *E. coli* as a cloning host and then into the *H. pylori* P79 mutant lacking HP0958. Transformants were selected on kanamycin (50 μg/ml) and analyzed by colony PCR, motility assay, and immunoblotting.

Motility assay. *H. pylori* strains and mutants were grown for 2 days on CBA plates and then stabbed on brucella soft agar plates containing 0.3% (wt/vol) agar and 5% (vol/vol) heat-inactivated fetal bovine serum (Sigma). Motility plates were incubated at 37°C in an atmosphere containing 5% CO_2 and periodically observed.

Bioinformatic analysis and RNA analysis using secondary-structure modeling. The predicted secondary structures were obtained using RNAdraw (35). This prediction program is based on the lowest free energy of formation and models the secondary structures of RNA molecules, such as hairpins. Alignments of the protein sequences with Clustal W (55) were performed using the Institute for Genome Research (TIGR) public database.

Microarray data accession numbers. The BμG@S HPv1.0.0 array design is available in BμG@Sbase (accession no. A-BUGS-18; http://bugs.sgul.ac.uk/A-BUGS-18) and also ArrayExpress (accession no. A-BUGS-18). Fully annotated microarray data have been deposited in BμG@Sbase (accession no. E-BUGS-73; http://bugs.sgul.ac.uk/E-BUGS-73) and also ArrayExpress (accession no. E-BUGS-73).

RESULTS

Transcriptional analysis of the HP0958 mutant. The flagellar apparatus involves more than 40 flagellar proteins in *H. pylori* (37, 56). The disruption of the HP0958 gene was previously shown to impair motility in *H. pylori* and to interfere at different levels in flagellar regulatory circuitry (42, 48). HP0958 was shown to regulate RpoN at a posttranscriptional level by

TABLE 2. Array-based analysis of flagellar gene expression in an HP0958 mutant[a]

Proposed flagellar class	TIGR ORF	Putative gene product (gene)	Change (fold) in the Δhp0958 strain	*P*
Class 1	Hp26695-0019	Chemotaxis protein (*cheV*)	1.071	0.34
	Hp26695-0082	Methyl-accepting chemotaxis transducer (*tlpC*)	0.911	0.24
	Hp26695-0099	Methyl-accepting chemotaxis protein (*tlpA*)	0.831	0.19
	Hp26695-0103	Methyl-accepting chemotaxis protein (*tlpB*)	1.098	0.48
	Hp26695-0173	Flagellar biosynthetic protein (*fliR*)	1.059	0.61
	Hp26695-0244	Signal-transducing protein, histidine kinase (*atoS*)	0.692	0.08
	Hp26695-0246	Flagellar basal-body P-ring protein (*flgI*)	—	—
	Hp26695-0325	Flagellar basal-body L-ring protein (*flgH*)	1.236	0.17
	Hp26695-0326	CMP-*N*-acetylneuraminic acid synthetase (*neuA*)	0.881	0.21
	Hp26695-0327	Flagellar protein G (*flaG*)	0.805	0.28
	Hp26695-0351	Basal-body M-ring protein (*fliF*)	**1.178**	**0.03**
	Hp26695-0352	Flagellar motor switch protein (*fliG*)	**1.329**	**0.01**
	Hp26695-0391	Purine-binding chemotaxis protein (*cheW*)	**1.494**	**0.02**
	Hp26695-0392	Histidine kinase (*cheA*)	1.518	0.07
	Hp26695-0393	Chemotaxis protein (*cheV*)	**1.457**	**0.03**
	Hp26695-0584	Flagellar motor switch protein (*fliN*)	1.098	0.52
	Hp26695-0599	Hemolysin secretion protein precursor (*hylB*)	0.950	0.83
	Hp26695-0616	Chemotaxis protein (*cheV*)	1.209	0.23
	Hp26695-0684	Flagellar biosynthesis protein (*fliP*)	**0.252**	**0.04**
	Hp26695-0685	Flagellar biosynthetic protein (*fliP*)	0.989**	0.49
	Hp26695-0703	Response regulator	0.997	0.99
	Hp26695-0714	RNA polymerase sigma-54 factor (*rpoN*)	1.032*	0.92
	Hp26695-0770	Flagellar biosynthetic protein (*flhB*)	0.754	0.36
	Hp26695-0815	Flagellar motor rotation protein (*motA*)	**1.431**	**0.03**
	Hp26695-0816	Flagellar motor rotation protein (*motB*)	1.281**	0.24
	Hp26695-0840	*flaA1 protein*	0.962	0.80
	Hp26695-1041	Flagellar biosynthesis protein (*flhA*)	0.969	0.59
	Hp26695-1067	Chemotaxis protein (*cheY*)	1.552	0.24
	Hp26695-1092	Flagellar basal-body rod protein (*flgG*)	1.407	0.12
	Hp26695-1286	Conserved hypothetical secreted protein (*fliZ*)	1.832	0.33
	Hp26695-1419	Flagellar biosynthetic protein (*fliQ*)	**0.684**	**0.05**
	Hp26695-1420	Flagellar export protein ATP synthase (*flil*)	0.934	0.65
	Hp26695-1462	Secreted protein involved in flagellar motility	**1.291**	**0.01**
	Hp26695-1575	Homolog of FlhB protein (*flhB2*)	0.964	0.59
	Hp26695-1585	Flagellar basal-body rod protein (*flgG*)	1.014	0.93
Class 2	Hp26695-0114	Hypothetical protein	0.808	0.38
	Hp26695-0115	Flagellin B (*flaB*)	**0.503***	**0.00**
	Hp26695-0295	Flagellin B homolog (*fla*)	0.889	0.25
	Hp26695-0869	Hydrogenase expression/formation protein (*hypA*)	**0.540**	**0.00**
	Hp26695-0870	Flagellar hook (*flgE*)	**0.450**	**0.01**
	Hp26695-0906	Hook length control regulator (*fliK*)	**0.514**	**0.01**
	Hp26695-1076	Hypothetical protein	**0.683**	**0.05**
	Hp26695-1119	Flagellar hook-associated protein 1 (HAP1) (*flgK*)	0.981	0.55
	Hp26695-1120	Hypothetical protein	1.014	0.92
	Hp26695-1154	Hypothetical protein (operon with *murG*)	**0.724**	**0.05**
	Hp26695-1155	Transferase, peptidoglycan synthesis (*murG*)	**0.711**	**0.04**
	Hp26695-1233	Putative flagellar muraminidase (*flgJ*)	**1.617**	**0.00**
Class 3	Hp26695-0472	Outer membrane protein (*omp11*)	1.698*	0.13
	Hp26695-0601	Flagellin A (*flaA*)	**2.12***	**0.01**
	Hp26695-1051	Hypothetical protein	1.175	0.25
	Hp26695-1052	UDP-3-0-acyl-*N*-acetylglucosamine deacetylase (*envA*)	1.134*	0.58
Intermediate class	Hp26695-0165	Hypothetical protein	1.155	0.75
	Hp26695-0166	Response regulator (*ompR*)	1.254	0.41
	Hp26695-0366	Spore coat polysaccharide biosynthesis protein C	0.838	0.37
	Hp26695-0367	Hypothetical protein	0.896	0.20
	Hp26695-0488	Hypothetical protein	**0.789**	**0.04**
	Hp26695-0907	Hook assembly protein, flagella (*flgD*)	**0.715**	**0.01**
	Hp26695-0908	Flagellar hook (*flgE*)	0.794	0.06
	Hp26695-1028	Hypothetical protein	1.196	0.43
	Hp26695-1029	Hypothetical protein	1.037	0.76
	Hp26695-1030	FliY protein (*fliY*)	1.157	0.36
	Hp26695-1031	Flagellar motor switch protein (*fliM*)	1.083	0.22
	Hp26695-1032	Alternative transcription initiation factor, sigma-28 (*fliA*)	1.112*	0.32

Continued on following page

TABLE 2—*Continued*

Proposed flagellar class	TIGR ORF	Putative gene product (gene)	Change (fold) in the Δhp0958 strain	*P*
	Hp26695-1033	Hypothetical protein	0.929	0.45
	Hp26695-1034	ATP-binding protein (*ylxH*)	0.954	0.76
	Hp26695-1035	Flagellar biosynthesis protein (*flhF*)	1.135	0.35
	Hp26695-1122	Anti-sigma-28 factor (*flgM*)	**1.496***	**0.02**
	Hp26695-1440	Hypothetical protein	**0.398***	**0.00**
	Hp26695-1557	Flagellar basal-body protein (*fliE*)	**0.584***	**0.01**
	Hp26695-1558	Flagellar basal-body rod protein (*flgC*) (proximal rod protein)	**0.527***	**0.00**
	Hp26695-1559	Flagellar basal-body rod protein (*flgB*) (proximal rod protein)	**0.644***	**0.00**
	Hp26695-0751	(*flaG*2)	**1.621**	**0.01**
	Hp26695-0752	Flagellar cap protein (*fliD*)	1.492	0.24
	Hp26695-0753	Flagellar chaperone (*fliS*)	**1.760**	**0.00**
	Hp26695-0754	Flagellar chaperone (*fliT*)	—	—
Unassigned	Hp26695-0410	Flagellar sheath-associated protein (*hpaA2*)	1.170	0.48
	Hp26695-0492	Flagellar sheath-associated protein (*hpaA3*)	**0.556**	**0.01**
	Hp26695-0797	Flagellar sheath-associated protein (*hpaA*)	1.278	0.13

[a] Relative expression levels for all known flagellar genes were tabulated. Changes and *P* values were calculated from three independent biological replicates as described in Materials and Methods. Open reading frames (ORFs) and gene annotations are based on the TIGR database (56). Genes were assigned to flagellar classes as previously proposed (37). Changes considered significant (*P* value less than or equal to 0.05) are in bold. Dashes indicate values excluded during array data analysis due to variation or technical problems with array features. *, Confirmatory analysis by qRT-PCR was performed; **, values were missing in the microarray analysis and were investigated by qRT-PCR.

apparently protecting RpoN from proteolysis (42). However, previous studies of HP0958-dependent gene expression have been restricted to candidate approaches and targeted methods (qRT-PCR and gene fusions) and thus were limited to only three selected RpoN-dependent genes, *flgE*, *flaB*, and HP1120, encoding a hypothetical protein (42, 48). To provide a genome-wide understanding of HP0958 involvement in flagellar regulation, we performed type 2 microarray experiments. A total of 44 genes were differentially expressed in the HP0958 mutant compared to those in the wild type (see Table S2 in the supplemental material). Twenty-four genes were downregulated, and 20 genes were upregulated. Seventeen out of the 44 genes had been annotated as hypothetical. In Table 2, we present the relative transcriptional levels in the mutant of all known *H. pylori* flagellar genes by hierarchical class (37). As indicated by these data, HP0958 does not play a major role in regulating class I genes. Pereira and Hoover showed that the mutation of HP0958 caused the downregulation of the class 2 genes *flaB* and HP1120 (42). Our global transcript analysis reveals that most of the RpoN-dependent (class 2) genes were significantly downregulated (Table 2), though *rpoN* expression itself was unaffected in the HP0958 mutant, in agreement with our previous findings (48). Thus, the role of HP0958 in flagellar regulation appeared to be mainly mediated by the protein stabilization of RpoN. Strikingly, however, the class 2 genes HP0114, HP0295 (*flaB* homolog), *flgK* (encoding hook-associated protein HAP1), and HP1120 (encoding a hypothetical protein) were transcribed at the wild-type level, which is in contradiction with a previous study (37). For *flgK* (HP1119) and HP1120, this may be due to read-through from the upregulated *flgM* gene (HP1122). HP1121 was also transcribed at the wild-type level. Another noteworthy finding was that *flgJ*/HP1233, part of the RpoN regulon, was 1.6-fold upregulated in the HP0958 mutant. HP1233 encodes for a putative flagellar muraminidase, present only in the *Helicobacter* genus.

In previous studies, the transcription of the intermediate class genes was shown to be under the control of σ^{54} and σ^{28}. As shown in Table 2, four genes in the intermediate class, HP1440, *fliE*, *flgC*, and *flgB*, were downregulated in the HP0958 mutant but below the twofold-change cutoff. The *fliE*, *flgC*, and *flgB* genes code for flagellar basal-body proteins. The downregulation of *fliE*, *flgC*, and *flgB* was confirmed by qRT-PCR (Fig. 1). Although the microarray data indicated that HP1440 was downregulated, RT-PCR indicated upregulation in the mutant, which we cannot currently explain.

The array experiments indicated that the transcription of *flaA* increased twofold in the HP0958 mutant, and this was confirmed by qRT-PCR (Fig. 1). This upregulation of *flaA* expression was independent of levels of σ^{28}, which was transcribed at the wild-type level. Pereira and Hoover also reported increased *flaA* transcription in an HP0958 mutant, which they suggested could indicate an additional regulatory effect of HP0958 in the broader FliA regulon (42). However, our array data clearly show that the class 3 genes *omp11*, HP1051 (hypothetical), and *envA* were not significantly differentially transcribed in the HP0958 mutant. Despite the increase in *flaA* transcription described herein, previous analyses have reported reduced FlaA protein production in an HP0958 mutant (48). Among other surface-protein-related genes were *omp6* (Fig. 1) and *omp23* that express two outer membrane proteins that we noted were downregulated. *omp6* expresses a

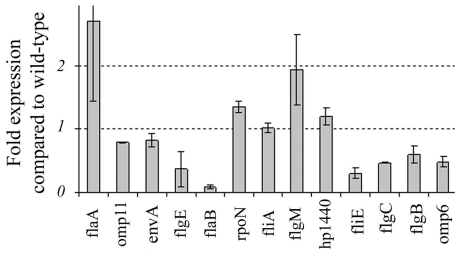

FIG. 1. Confirmation of transcriptional changes in selected flagellar genes in the HP0958 mutant using qRT-PCR. Changes and standard deviations were calculated using the *era* transcript abundance as a reference. qRT-PCRs were performed on at least two biological replicates.

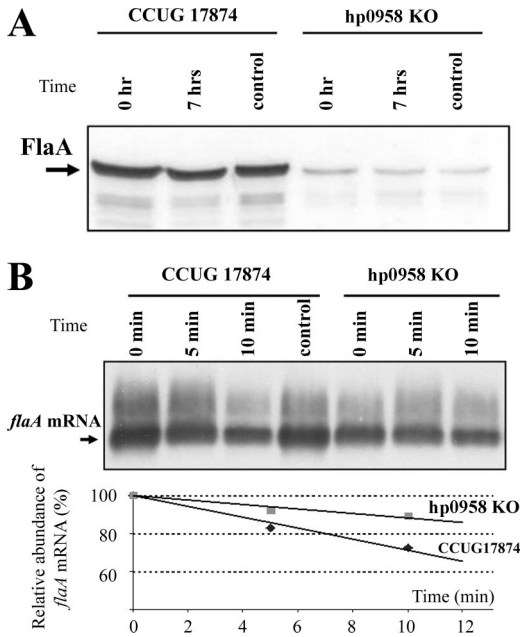

FIG. 2. Stability assays. (A) Mutation of HP0958 does not reduce FlaA protein stability. FlaA protein levels in the HP0958 mutant and the wild type were analyzed by immunoblotting at various time points after the addition of tetracycline. Two independent stability assays were performed. The control samples were treated for 7 h with ethanol, the solvent for tetracycline. (B) Lack of HP0958 increases *flaA* mRNA stability in the HP0958 mutant compared to the wild-type strain. *flaA* mRNA levels in the HP0958 mutant and CCUG17874 were analyzed by Northern blotting at various time points after the addition of rifampin. Time is in min. The control samples were treated with methanol (rifampin solvent) for 10 min. Band intensities were analyzed by densitometry. KO, knockout.

porin of the Hop protein family, involved in the colonization and persistence of *H. pylori* in the gastric mucosa (36, 58).

FlaA stability is unaffected in the HP0958 mutant. A striking contradiction in our findings was the apparent doubling of *flaA* transcription in the HP0958 mutant, despite our previously reported significant reduction in FlaA protein production in this mutant (48). To investigate if the modulation of flagellin production was at the protein level, a protein stability assay was performed. Protein synthesis in *H. pylori* was stopped by adding tetracycline, and aliquots were taken at various time points for analysis by immunoblotting (Fig. 2). After 7 h of treatment with tetracycline, there was no significant difference in stability of the flagellin protein between the wild type and the HP0958 mutant. This indicated that the low abundance of FlaA protein in the HP0958 mutant was not the result of reduced protein stability and that the regulatory effect on *flaA* occurred at a posttranscriptional or translational level.

RNA stability. Since flagellin stability was apparently unaffected by HP0958 mutation, we next investigated the stability of the *flaA* message (Fig. 2). After 10 min of treatment with rifampin, it appeared that the *flaA* message was less stable in the wild type than in the HP0958 mutant. Analysis of the Northern blot by densitometry indicated that the *flaA* message was degraded at a rate approximately 2.5-fold higher in the wild type than in the HP0958 mutant. In the wild type, the half-life of the *flaA* message was calculated at 17.3 min, compared to 42.2 min in the HP0958 mutant. We also confirmed the increased *flaA* mRNA stability in the HP0958 mutant by a qRT-PCR approach (data not shown), whereby the half-life was 19.2 min in the mutant and 28.2 min in the wild type.

The HP0958 protein binds to the *flaA* message. Our previous bioinformatics analyses (48) indicated that HP0958 belongs to the zinc ribbon superfamily of proteins (28), incorporating proteins from COG1579. Proteins in this family typically use the zinc-finger motif to interact with other proteins or nucleic acid (28). This highlighted the potential involvement of HP0958 in flagellar regulatory processes. Multiple alignments of the HP0958 protein sequence and its orthologs highlighted the conservation of two main domains. One of them showed the following pattern: $C\text{-}X_2\text{-}C\text{-}X_{(17\ to\ 20)}\text{-}C\text{-}X_2\text{-}C$, suggesting a zinc-motif type that might interact with nucleic acid. We thus hypothesized that HP0958 may have a regulatory effect on *flaA* mRNA by interacting directly with the *flaA* message. To test for an interaction between the *flaA* transcript and HP0958, EMSAs were performed using in vitro-transcribed mRNA and HP0958. The HP0958 protein was purified to >99% purity, adjudged by silver staining (D. L. Caly, S. A. Moore, and P. W. O'Toole, unpublished data). The synthesis of the in vitro-transcribed *flaA* transcript introduced several nucleotides at the 5′ end, but predictive models suggested that these bases are not involved in secondary-structure formation and would not alter the native conformation of the *flaA* mRNA. Because we used a less-sensitive (nonradiolabeled) detection system than some published studies, we scaled up the components of the EMSA reactions proportionately, e.g., compared to the amounts used for studying FljA-*fliC* mRNA interaction in *Salmonella* (2). Thus, we increased the RNA amount 40-fold from 1.25 fmol to 57 fmol and the protein concentration 50-fold from 264 nM to 13 μM.

Upon the incubation of *flaA* with purified HP0958, a mobility shift of the *flaA* transcript was observed (Fig. 3). The major shift band suggested the direct association of HP0958 with the *flaA* message. A similar experiment using two control RNA species was performed (Fig. 3). Small amounts of a diffuse shift were produced, approximately eightfold less intense than the HP0958-*flaA* shift. The higher affinity of HP0958 for *flaA* RNA suggested that a particular RNA sequence or secondary structure was involved. In *Caulobacter crescentus*, the RNA chaperone FlbT binds to the 5′ untranscribed region of the *fljK* flagellin mRNA to regulate flagellin synthesis posttranscriptionally (4). Following a similar method used to analyze the *C. crescentus* transcript, we identified a potential stem-loop structure located 12 nucleotides upstream of the ribosome binding site of HP0958 (not shown). When the stem-loop was deleted from the in vitro-transcribed *flaA* message, a gel shift pattern identical to the full-length transcript was observed (data not shown). The addition of EDTA to the reaction reduced the HP0958-*flaA* riboprobe association, suggesting that HP0958 requires cations to interact with the *flaA* message. Densitometry analysis indicated that the shifted band was approximately 3.5-fold weaker when incubated with additional EDTA. That supports the hypothesis that HP0958 may be an RNA-binding protein with a zinc-finger motif. DTT can form complexes with zinc ions, and the addition of DTT has been shown to reduce the free zinc ion concentration in reaction mixtures (1). The addition of DTT reduced the amount of the shifted species by

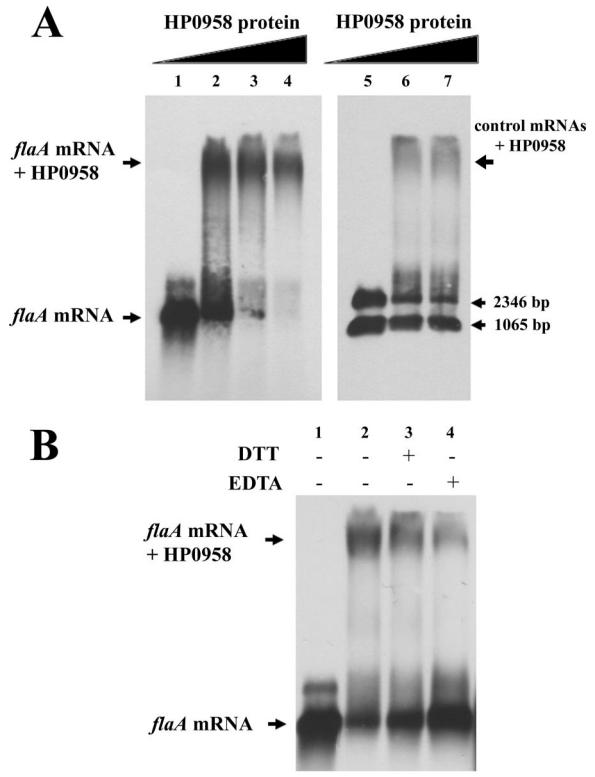

FIG. 3. Gel shift assays of purified HP0958 binding to the *flaA* transcript. (A) EMSA shows that HP0958 binds to the *flaA* transcript. In vitro transcripts added to incubations for EMSA were 30 ng *flaA* mRNA (lanes 1 to 4) and 30 ng control mRNAs (lanes 5 to 7). HP0958 protein concentrations added to EMSAs were 1,200 ng (lane 2), 3,600 ng (lanes 3 and 6), and 6,000 ng (lanes 4 and 7). (B) The *flaA* mRNA and HP0958 association is affected by EDTA and DTT. Lane 1, 30 ng *flaA* transcript; lanes 2 to 4, 30 ng *flaA* transcript and 6,000 ng HP0958 protein. Lanes where additional EDTA (50 mM) or DTT (10 mM) were added are labeled with a plus sign.

1.3-fold compared to that of the control EMSA, supporting the involvement of a zinc-finger motif in the RNA-protein association.

To examine the HP0958-*flaA* mRNA association under more physiological conditions, cytoplasmic fractions from the wild type and the HP0958 mutant were prepared and incubated with the *flaA* riboprobe (Fig. 4). Shifted bands were

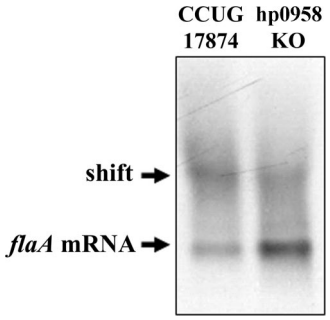

FIG. 4. Loss of HP0958 reduces the *flaA* transcript shift by *H. pylori* cytoplasmic extract. A total of 30 ng *flaA* in vitro transcript and 1 μg total protein were used for both EMSAs. KO, knockout.

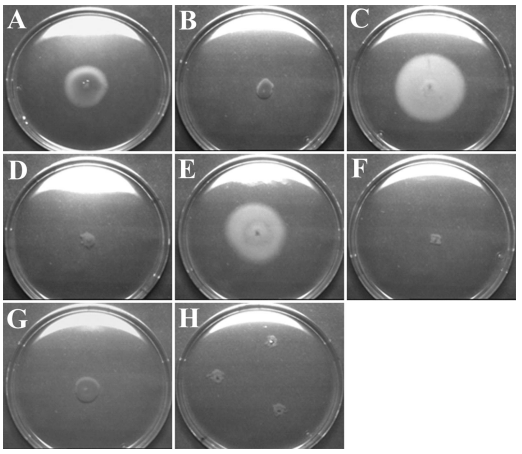

FIG. 5. Restoration of motility by the complementation of an HP0958 mutant, when hp0958 was put under the control of the *ahpC* gene promoter. (A) The CCUG17874 wild-type strain; (B) the CCUG17874-hp0958 KO; (C) the P79 wild-type strain; (D) the P79-hp0958 KO; (E) P79-0958/pIR1563; (F) P79-0958/pIR203K04; (G) P79-0958/pIR1186; (H) P79-0958/pIR1041.

observed in both samples. However, the shifted band obtained with the wild-type cell extract was stronger than that in the HP0958 mutant.

Complementation of the HP0958 mutant. Previous attempts to complement an HP0958 mutant by expressing the gene under the control of the *ureA* promoter were unsuccessful, suggesting that the overexpression of HP0958 interferes with its normal function (42). To eliminate the possible involvement of second-site mutations in our HP0958 mutant, we attempted to complement the mutation by expressing the HP0958 gene under the control of three well-characterized promoters, controlling the expression of *flhA* (a component of the flagellar apparatus), HP1186 (alpha-carbonic anhydrase) (60), and *ahp*C (alkyl hydroperoxide reductase) (34). The complementation plasmids were each introduced into a derivative of the P79 strain in which the HP0958 gene had been insertionally inactivated. We used strain P79 because it supports the replication of the vector used for complementation (21), whereas strain 17874, in which transcription analyses were performed, does not. The phenotypes of the three complemented strain P79 mutants were analyzed by motility assay (Fig. 5) and immunoblotting (Fig. 6). The complemented mutants in which

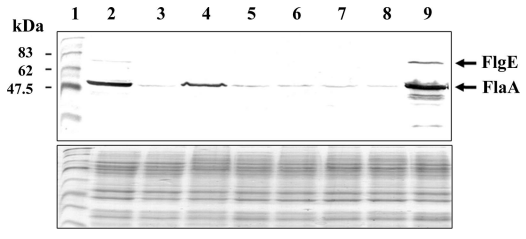

FIG. 6. Restoration of FlaA production by the complementation of an HP0958 mutant is promoter dependent. Lane 1, Protein marker; lane 2, the CCUG17874 wild-type strain; lane 3, the CCUG17874-hp0958 KO; lane 4, the P79 wild-type strain; lane 5, the P79-0958 KO; lane 6, P79-0958/pIR203K04; lane 7, P79-0958/pIR1041; lane 8, P79-0958/pIR1186; lane 9, P79-0958/pIR1563.

the HP0958 gene was under the control of the promoters of *flhA* and the carbonic anhydrase gene were not motile and flagellin synthesis was not restored. The mutant that was transformed with the HP0958 gene under the control of the *ahpC* promoter was motile, and FlaA protein synthesis was restored to the wild-type level or above. This confirms that the mutant phenotype was due only to the loss of the HP0958 gene and that the wild-type phenotype may be restored in an HP0958 mutant in a manner dependent on HP0958 expression levels.

DISCUSSION

HP0958 has been previously identified as an essential gene for flagellum production in *H. pylori* (42, 48), and we aimed to further investigate its role in this process. Pereira and Hoover showed that the stable accumulation of RpoN required HP0958 (42). Consistent with this role, our global transcript analysis indicated that most of the RpoN-dependent genes were downregulated in the HP0958 mutant. The downregulation of the intermediate class genes *fliE*, *flgC*, and *flgB* indicated that they may be under the additional control of RpoN. The position of the HP0958 gene itself, however, in flagellar gene hierarchy, is still unclear. The transcription of class I regulators was unaffected in the HP0958 mutant, showing that HP0958 is below this level or outside the normal hierarchy altogether. The HP0958 gene is in the middle of an operon containing several housekeeping genes (48), which would likely be dependent on the major sigma factor RpoD.

It was previously hypothesized that HP0958 may control the complete FliA flagellar regulon, based on targeted studies with *flaA* (42). The utilization of an array approach allowed us to demonstrate that HP0958 does not regulate the FliA regulon but only the *flaA* gene. The *flaA* gene is transcribed at normal levels in a mutant lacking RpoN (37), ruling out the indirect RpoN-related modulation of *flaA* transcription in the HP0958 mutant. However, in a mutant lacking the RpoN activator FlgR, the transcription of *flaA* was upregulated (54), while the deletion of FlgS also caused an increase in *flaA* transcription but no changes in the expression of other class III genes (37). Thus, it is clear that *flaA* transcription is under some additional controls involving the RpoN regulators but independent of the sigma factor itself.

FlaA protein stability was not affected in the HP0958 mutant, indicating that the modulation of FlaA production was not mediated at the protein level—a possibility that needed to be excluded, given the fact that HP0958 protects RpoN from proteolysis (42). A slight caveat for this interpretation is that the HP0958 mutant lacks flagella (48). Thus, FlaA protein localizes differently in the wild type and in the mutant, likely being cytoplasmic in the latter. Nonetheless, if HP0958 was a FlaA-protective chaperone, one would still expect to see an enhanced degradation of cytoplasmic FlaA protein in the mutant. The present study supports the hypothesis that FlaA protein synthesis is controlled at a posttranscriptional stage. Our data indicate that the HP0958 protein binds to *flaA* mRNA, resulting in a decreased stability of the transcript but higher levels of translation and flagellin production. We know of two relevant precedents for protein-modulated RNA stability and translation efficiency involved in flagellin synthesis. In *Caulobacter crescentus*, the flagellin transcript is posttranscriptionally regulated by an interaction of the protein FlbT with the 5′ untranscribed region of flagellin (FljK) mRNA (4). The binding of FlbT to this transcript was dependent on a predicted loop structure, and mutations in this loop region that prevented FlbT binding resulted in the increased stability of *fljK* mRNA. This system differs from our demonstrated interaction of HP0958 with *H. pylori flaA* mRNA, where the deletion of a predicted stem-loop did not abolish HP0958-*flaA* mRNA interaction. However, the association of the protein-mRNA interaction with decreased mRNA stability is common to our findings for *H. pylori* and the *Caulobacter* posttranscription regulation example. In the latter, it was suggested (4) that FlbT competes with another factor for binding to the *fljK* transcript, and the outcome of this competition determines if the transcript is degraded or translated. Our data from EMSA experiments with *H. pylori* cytoplasmic extracts suggest that a protein(s) other than HP0958 also binds to the *flaA* transcript, since there is a weak shift with extracts from the HP0958 mutant (Fig. 4). A more complex role for the regulatory function of HP0958 in *H. pylori* flagellin synthesis regulation is therefore possible.

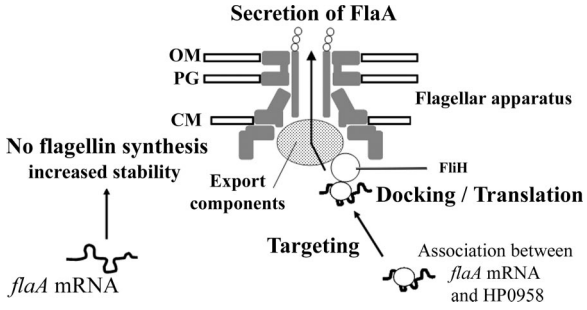

FIG. 7. Proposed mechanistic model for the role of HP0958 in *H. pylori* flagellum biogenesis. The model proposes increased turnover of the *flaA* transcript when in a translation-competent state promoted by HP0958 (right side of the figure) and greater stability of the transcript when HP0958 is absent (left side of the figure). OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane.

Posttranscriptional control of flagellin synthesis is also a feature of flagellar phase variation in *Salmonella enterica* (2, 7, 61), whereby the FljA protein binds to the mRNA for the alternative flagellin protein FliC and blocks its translation (2). However, by analysis of bypass mutations that affected *fliC* mRNA structure, it was discovered that some such mutations block FliC incorporation in the flagellum but still permit its translation. The authors concluded that mRNAs for the alternative flagellins might compete for occupancy at an assembly site and that the outcome of this competition was influenced by the FljA protein (2). Although the *Salmonella* system is much better characterized, permitting such a model to be developed, we think it significant that the yeast two-hybrid interaction network data for *H. pylori* (44) show an interaction of HP0958 with FliH. The FliH protein is a presumptive inhibitor of the flagellar export ATPase FliI (19), and we have recently determined the molecular basis for the *H. pylori* FliH-FliI association (31). The interaction between HP0958 and FliH suggests a possible targeting of the *flaA* message to the flagellar export apparatus, where the translation and secretion of FlaA proteins would jointly occur (Fig. 7). Substrate recognition by the

Yersinia type III secretion system machinery is known to involve signals within the cognate mRNAs (reviewed in reference 45) but which require translation, and some *Yersinia* type III secretion system effector molecules have controversially been suggested to be cotranslationally secreted (45). Following the coupled translation and secretion of FlaA protein, *flaA* mRNA would then be degraded. The disruption of HP0958-mediated addressing of the *flaA* message would leave it biologically inert in the cytoplasm. Compared to mRNA actively being turned over at the FlaA secretion apparatus, this cytoplasmic mRNA might be less susceptible to RNase, potentially explaining its higher stability in the HP0958 mutant than in the wild type. If HP0958 bound with greater affinity to the *flaA* transcript than to RpoN, then commencement of the production of the *flaA* transcript (by relief of FlgM inhibition of FliA after completion of the hook-basal body) would act as a switch to turn off the RpoN regulon. This model thus presents a number of independent lines of approach for experimental testing of its validity.

Pereira and Hoover reported that the overproduction of HP0958 protein interfered with its activity (42). In the present study, the HP0958 mutant was successfully complemented only when put under the control of the *ahpC* promoter (of those tested), confirming that a controlled level of HP0958 protein is important for cellular function. Biochemical analysis in our lab indicates that HP0958 protein forms dimers and hexamers in vitro and that the monomer conformation may be more active than the dimer and hexamer conformations in the *flaA* transcript gel shift assay (data not shown). Further investigation of the physicochemical properties of HP0958 is warranted. The RNA-binding ability of HP0958 was also shown to be sensitive to EDTA, supporting the involvement of the HP0958 zinc-finger motif in the binding, and we have recently confirmed the presence of zinc in the HP0958 protein (Caly, Moore, and O'Toole, unpublished data). An investigation of the HP0958 protein structure will help us to understand its function and to identify the regions of the HP0958 molecule involved in the association with *flaA* mRNA, the σ^{54} sigma factor RpoN, and FliH.

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