

## The Quorum-Sensing Molecule Autoinducer 2 Regulates Motility and Flagellar Morphogenesis in *Helicobacter pylori*<sup>∇</sup>

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**The genome of the gastric pathogen *Helicobacter pylori* contains a homologue of the gene *luxS*, which has been shown to be responsible for production of the quorum-sensing signal autoinducer 2 (AI-2). We report here that deletion of the *luxS* gene in strain G27 resulted in decreased motility on soft agar plates, a defect that was complemented by a wild-type copy of the *luxS* gene and by the addition of cell-free supernatant containing AI-2. The flagella of the *luxS* mutant appeared normal; however, in genetic backgrounds lacking any of three flagellar regulators—the two-component sensor kinase *flgS*, the sigma factor  $\sigma^{28}$  (also called *flhA*), and the anti-sigma factor *flgM*—loss of *luxS* altered flagellar morphology. In all cases, the double mutant phenotypes were restored to the *luxS*<sup>+</sup> phenotype by the addition of synthetic 4,5-dihydroxy-2,3-pentanedione (DPD), which cyclizes to form AI-2. Furthermore, in all mutant backgrounds loss of *luxS* caused a decrease in transcript levels of the flagellar regulator *flhA*. Addition of DPD to *luxS* cells induced *flhA* transcription in a dose-dependent manner. Deletion of *flhA* in a wild-type or *luxS* mutant background resulted in identical loss of motility, flagella, and flagellar gene expression. These data demonstrate that AI-2 functions as a secreted signaling molecule upstream of FlhA and plays a critical role in global regulation of flagellar gene transcription in *H. pylori*.**

Many bacterial species regulate aspects of their behavior through an intercellular signaling system called quorum sensing (4). Bacteria produce small molecules, termed autoinducers (AIs), that travel across the bacterial membrane and accumulate in the environment. Increases in cell density result in corresponding increases in AI concentration in the extracellular environment and cell cytoplasm. At threshold AI concentrations, the interaction between the AI and its transduction machinery results in the coordinated transcription of target genes required for specific group behaviors (52).

A quorum-sensing system that is widespread across the bacterial kingdom and has been proposed to function as a “bacterial Esperanto,” uses a collection of molecules collectively called AI-2 (4). AI-2-dependent processes include biofilm formation, motility, and type III secretion (18). AI-2 is synthesized by the LuxS protein (47), and *luxS* genes have been identified in at least 250 bacterial genomes to date (50). LuxS functions to cleave *S*-ribosylhomocysteine, an intermediate in the *S*-adenosylmethionine (SAM) pathway, into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (40). Through processes of dehydration and cyclization, DPD exists in equilibrium with several molecules, some or all of which function as the molecular signal AI-2 (40). AI-2 has been extensively studied in *Vibrio harveyi*, where it has been shown to regulate bioluminescence and other traits (5, 20, 21, 28), and a *V. harveyi* reporter strain that expresses bioluminescence in response to the addition of AI-2 has become the standard

method for detecting AI-2 production by other bacterial species (3).

Although AI-2 production is common among bacterial species, it remains controversial as to how many of these bacteria utilize AI-2 as a signaling molecule, as opposed to producing it as a metabolic by-product of the SAM pathway (15, 50). One such controversial case is *Helicobacter pylori*, a gram-negative pathogen of the human stomach. Two independent groups demonstrated that *H. pylori* secretes AI-2 into its extracellular environment by a *luxS*-dependent mechanism (19, 25). A subsequent study reported that an *H. pylori luxS* mutant exhibited reduced production of the flagellin protein FlaA and reduced expression of an *flaA* transcriptional reporter construct (29). In addition, an *H. pylori luxS* mutant is reported to form biofilms more efficiently than wild-type cells (10). Recently, two groups reported that mutation of the *luxS* genes in *H. pylori* strains SS1 and TK1402 exhibited motility defects on soft agar plates and colonization defects in mouse and gerbil models of infection, respectively (27, 37). However, Lee et al. found that a *luxS* mutant in a third *H. pylori* strain, X47, did not exhibit these defects. Based on the phenotypic variability they observed between strains, these authors concluded that *luxS* mutant phenotypes were the result of fitness defects due to the disruption of the SAM metabolic pathway, and they argued that AI-2 was unlikely to function as a quorum-sensing molecule in *H. pylori*. Evidence for a signaling function for AI-2 would require a demonstration that purified extracellular AI-2 is sufficient to reverse a *luxS*-associated phenotype. To date, besides the demonstration that AI-2-containing cell-free supernatant could modestly increase *flaA* reporter gene expression (29), no such evidence has been reported.

The possibility that *H. pylori* flagellar gene expression is

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regulated by an extracellular signaling molecule is intriguing in light of a long-standing proposal that *H. pylori* may regulate the composition of its flagellar filament in response to environmental cues (44). Unlike the regulation of flagellar gene expression in the model organisms *Salmonella* and *Caulobacter crescentus*, in which a single master regulator controls transcription of an entire hierarchy of genes in response to signals such as growth phase, in *H. pylori* the top-tier regulators of flagellar gene transcription, as well as chemotaxis- and motor protein-encoding genes, are constitutively expressed under the control of the housekeeping sigma factor,  $\sigma^{80}$ , and are referred to as class 1 genes (36). Class 2 genes are controlled by  $\sigma^{54}$  (RpoN) and contain “middle” structural flagellar genes that encode components of the rod, hook, and sheath and the minor flagellin FlaB. Class 3 genes are controlled by  $\sigma^{28}$  (FliA) and the anti-sigma factor FlgM (11, 24), and this group contains “late” flagellar structural genes such as the major flagellin FlaA. A final intermediate class, including both structural and regulatory genes, is controlled by both  $\sigma^{54}$  and  $\sigma^{28}$  (36). The closest candidate for a master regulator in this hierarchy is FlhA, a component of the basal body export apparatus, that is required for flagellum formation and for expression of most class 2 and class 3 genes and the intermediate class of flagellar genes (36, 41). A possible point through which environmental signals could be channeled into the flagellar transcriptional hierarchy is the unconventional cytoplasmic sensor kinase FlgS (HP0244), which coregulates  $\sigma^{54}$ -dependent genes of class 2 and the intermediate classes (6, 36).

Motility is an important prerequisite for *H. pylori* colonization of the stomach, as has been demonstrated for mutants lacking flagellar genes in multiple animal models (reviewed in reference 38). Thus, dissecting regulation of motility in *H. pylori* is important for understanding this pathogen’s virulence strategies. By characterizing flagellar morphology and flagellar gene transcription in wild-type, *luxS* mutant, and AI-2-supplemented cells lacking key flagellar regulators, we demonstrate that AI-2 signaling influences the flagellar regulon through modulation of the transcription of the top-tier regulator gene *flhA*. Our data provide strong support for the model that *H. pylori* uses AI-2 as an intercellular signaling molecule.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *H. pylori* strain G27 (14) was used in this study as the wild-type strain from which all mutants were derived, as listed in Table 1. All strains were maintained on blood agar plates consisting of Columbia agar (Difco) and 5% defibrillated horse blood (Hemostat) supplemented with 0.02 mg ml<sup>-1</sup>  $\beta$ -cyclodextrin (Sigma), 8 mg ml<sup>-1</sup> amphotericin B (Sigma), and 20  $\mu$ g ml<sup>-1</sup> vancomycin (Sigma) and incubated at 37°C and 10% CO<sub>2</sub>. Liquid medium (hereafter, BB medium) for *H. pylori* growth consisted of filter-sterilized Brucella broth (Difco) supplemented with 10% fetal bovine serum (Gibco) and 20  $\mu$ g ml<sup>-1</sup> vancomycin (Sigma). Liquid cultures were grown in 50-ml conical tubes with loosened lids (BD Falcon) and shaking at 37°C in anaerobic jars (Oxoid) with CampyGen microaerobic sachets (Oxoid). Selective plates were made with 10  $\mu$ g ml<sup>-1</sup> kanamycin (Fisher), 0.08 M sucrose, or 18  $\mu$ g ml<sup>-1</sup> metronidazole (Sigma).

**Construction of the  $\Delta luxS$ , *luxS*\*, and flagellar mutant strains.** All primers used for strain construction are given in Table 2. The *luxS* gene (HP0105) was PCR amplified using the primers LuxSSpeR and LuxSEcoF and ligated into the TOPO vector using a TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) to create the vector pBR003. The primers TopoXbaF and TopoBglR were used to amplify outward from HP0105 and around pBR003. A *kan-sacB* construct (12) encoding kanamycin resistance (Kan<sup>r</sup>) and sucrose sensitivity (Suc<sup>s</sup>) was PCR amplified using the primers FBgIIpKan+ and PFA3

TABLE 1. Strains used in this study

<i>H. pylori</i> strain	Description	Reference or source
Wild type	G27	14
<i>luxS</i> strain	<i>luxS::kan sacB</i>	This study
$\Delta luxS$ strain	Deletion of HP0105	This study
<i>luxS</i> * strain	<i>rdx::luxS</i> and deletion of <i>luxS</i>	This study
<i>fliA</i> strain	<i>fliA::kan</i>	This study
<i>fliA</i> $\Delta luxS$ strain	<i>fliA::kan</i> and deletion of <i>luxS</i>	This study
<i>flgS</i> strain	<i>flgS::kan</i>	This study
<i>flgS</i> $\Delta luxS$ strain	<i>flgS::kan</i> and deletion of <i>luxS</i>	This study
<i>flgM</i> strain	<i>flgM::kan</i>	This study
<i>flgM</i> $\Delta luxS$ strain	<i>flgM::kan</i> and deletion of <i>luxS</i>	This study
<i>flhA</i> strain	<i>flhA::kan</i>	This study
<i>flhA</i> $\Delta luxS$ strain	<i>flhA::kan</i> and deletion of <i>luxS</i>	This study

(12). The pBR003 and Kan-SacB PCR products were digested and ligated together to make the vector pBR007. pBR007 was purified and used to disrupt the HP0105 locus in strain G27 through natural transformation and selection of Kan<sup>r</sup> colonies. The resulting *luxS::kan-sacB* strain was verified by PCR and sequencing of the genomic locus. To create the  $\Delta luxS$  deletion strain, a deletion construct consisting of 265 bp immediately 5' of the HP0105 open reading frame fused to 255 bp 3' of HP0105 was created by PCR amplifying these sequences from G27 genomic DNA and then stitching them together in a subsequent PCR using primers listed in Table 2. This PCR product was used to transform the *luxS::kan-sacB* mutant strain, and Suc<sup>s</sup> colonies were isolated and validated as above. The *luxS* gene is not located in an apparent operon in either of the two *H. pylori* strains for which the genome has been sequenced (1, 49), and thus the  $\Delta luxS$  deletion is unlikely to have polar effects.

The complemented *luxS*\* strain was constructed by inserting a wild-type copy of the *luxS* gene in the *rdx* locus of the  $\Delta luxS$  chromosome, as described previously (43). PCR products corresponding to the first 56 bp of the *rdxA* gene, 988 bp of the full-length *luxS* gene (including 265 bp of upstream sequence), and the final 94 bp of the *rdxA* gene were amplified in three separate PCRs and then stitched together in two subsequent PCRs using the primers listed in Table 2. The final PCR product was used to disrupt the *rdx* locus of  $\Delta luxS$  by selecting for metronidazole-resistant colonies, which were validated as above.

The *flhA* (HP1041) gene was amplified using the FlhAXho2 and FlhABgl2 primers and ligated into the TOPO vector using a TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) to create the vector KHR1. A Kan<sup>r</sup> nonpolar cassette with two internal ribosomal binding sites from pUC18 K-2 to ensure expression of downstream genes (33) was used to disrupt the *flhA* locus. The Kan<sup>r</sup> nonpolar cassette was amplified by the primers M13Asc1 and M13Nhe1, digested along with KHR1, and ligated into the vector. The *flhA::Kan*<sup>r</sup> construct was PCR amplified and introduced into the *flhA* locus through natural transformation. The *flgS* (HP0244), *flgM* (HP1122), and *fliA* (HP1032) mutants were all constructed using the Kan<sup>r</sup> nonpolar cassette as described previously (35). In each case the 5' and 3' ends of the flagellar gene and Kan<sup>r</sup> nonpolar cassette were PCR amplified in separate reactions and then stitched together in two subsequent PCRs using primers listed in Table 2.

**Motility assays.** Motility assays were performed on 0.4% agar plates consisting of Brucella broth, 0.02 mg ml<sup>-1</sup>  $\beta$ -cyclodextrin (Sigma), 8 mg ml<sup>-1</sup> amphotericin B (Sigma), and 20  $\mu$ g ml<sup>-1</sup> vancomycin (Sigma). Plates were seeded with 1  $\mu$ l of liquid culture grown to an optical density at 600 nm (OD<sub>600</sub>) of 1.0, corresponding to early stationary phase growth (24 h as shown in Fig. 1), and incubated for 5 to 8 days under microaerophilic conditions. The area of outward migration was recorded with a digital camera and calculated relative to a standard size reference area on the plate using Metamorph imaging software.

**Bacterial growth curve and bioluminescence bioassay.** Bacterial cultures were started from an overnight liquid culture, diluted to an OD<sub>600</sub> of 0.05 in BB medium, and at various time points samples were taken and plated to determine the number of CFU per milliliter. At those same time points cell suspensions were harvested and filtered through a 0.2- $\mu$ m-pore-size filter in SpinX columns and centrifuged at 6,000  $\times$  g (Nalgene Nunc International). Cell-free supernatant (CFS) was collected from all strains and assayed for AI-2 activity with the *V. harveyi* BB170 bioassay as previously described (45, 46). Briefly, an overnight *V. harveyi* culture was diluted 1:10,000 in fresh AB medium (22a). CFS preparations were added to the diluted *V. harveyi* to a 10% (vol/vol) final concentration, and these cultures were shaken at 30°C for 3 h, after which 3-ml aliquots were assayed

TABLE 2. Primers used for cloning and RT-PCR

Primer	Sequence (5'-3')
LuxSSpeR.....	AAAATCATTCCCAACCAATGATCAGGG
LuxSEcoF.....	CCGGAATTCAGAGCAAGCGTTCGCTAAAA
TopoXbaF.....	GCTCTAGACTGAGATCATCCGCAACCAT
TopoBglR.....	GAAGATCTTTGGCATGTCCATGTGATCT
FBglIIPkan+.....	GAAGATCTTCCCGGGCGAACCATTTGAGGTGA
PFA3.....	GCTCTAGACCCGGGTATAAGCCCATTTTCATGC
FLS2Bam.....	CGGGATCCGAGGCGGTTGCTTTTGTAGA
LuxS3AB.....	CGACATAACTGGCATGGTTGGCGAACGCTTGCTCTAAA
RLS2Xho2.....	TTTATCTTCTCGAGCGCCCATGAATGTCTGAAGT
RdxA1F.....	TAAACGAGCGCCATTCTTG
RdxA1LR.....	GATTAAAAGCGCTTCAGCGTGAGGCGGTTGCTTTTGTAGA
RdxA2LF.....	CTTCAGACATTCATGGGCGATTCAACCACAGCATGCAAA
RdxA2R.....	CGATCAAGCATGCGATTTTA
FliaAF.....	TGCTTCGTTTCAACCATGAG
FliaAK.....	AAGCAACAACACCACATCACAGGAAACAGCTATGACA
FliaBK.....	CTGGCCGTCGTTTACTCGCGCATTTCTCAAATC
FliaBR.....	ACTCACACGATTAGGGCGTTT
HP0244AF.....	CGATGAGATTTATGCGTCCA
HP0244AK.....	CCTTATTTGAAGCGTTCCACAGGAAACAGCTATGAC
HP0244BK.....	ACTGGCCGTCGTTTACGAGAGCGAACAGGGTCAA
HP0244BR.....	GCTAACCCCTAGGCCGTTACC
FlgMAF.....	GTTGTGGTTGCTCATGTTTCG
FlgMAK.....	AATGCCGTTTCTTCTTTCAGGCAACAGCTATGA
FlgMBK.....	ACTGGCCGTCGTTTACTCTCACAAAATGGCAAAGGA
FlgMBR.....	TCCTAACTTCATGATTGA
FlhAIntF.....	CCTAGTGCGGTGAGCGATATTATC
FlhAIntR.....	GCTCACGCTAAATTCGCCAA
UreAIntF.....	AAAGACTGCGGCTGAATTG
UreAIntR.....	CCATCACATCATCCGGTTT
FlhABgl2.....	GAAGATCTACCTCACAGAACCCGTATCGT
FlhAXho2.....	ATTCGGCTCGAGCAATGGAAACTCTCAGCCACTTC
M13Nhe1.....	CTAGCTAGCGTAAAACGACGGCCAGT
M13Acs1.....	TTGGCGCGCCAGGAAACAGTTATGAC
1320f.....	CCATGAAGTCGGAATCGCTAG
1431r.....	ACTCCCATGGTGTGACGG

in triplicate for light production using an Optocomp I luminometer (MGM Instruments, Inc.).

**AI-2 complementation experiments.** To test for complementation of the  $\Delta luxS$  motility phenotype, soft agar plates consisting of 0.4% agar were made using 70% BB medium and 30% CFS collected from wild-type or  $\Delta luxS$  cultures at an OD<sub>600</sub> of 1.0. Synthetic DPD was prepared as described previously (42). DPD activity was quantified with the *V. harveyi* BB170 bioassay and compared to CFS from wild-type *H. pylori* cultures grown to an OD<sub>600</sub> of 1.0, the point in the growth curve at which we measured maximal AI-2 activity. DPD was added to *H. pylori* liquid cultures of various genotypes to a final concentration of 0.01 mM, 0.1 mM, or 1.0 mM. Cultures were incubated for 4 h, after which RNA was extracted or bacterial cells were visualized by transmission electron microscopy (TEM).

**RNA collection and extraction.** RNA was collected from *H. pylori* strains grown in BB medium to an OD<sub>600</sub> of 1.0. Cells were passed onto 0.45- $\mu$ m-pore-size cellulose nitrate membrane filters (Whatman, Maidstone, England) by vacuum. Filters were placed in 50-ml conical tubes and stored at -80°C. To extract RNA, filters were thawed on ice, appropriate amounts of Trizol were added, and RNA was extracted following the manufacturer's instructions (Gibco). Contaminating genomic DNA was removed using a Turbo DNA Free kit (Ambion).

**RT-PCR.** Primers used for reverse transcription-PCRs (RT-PCRs) are listed in Table 2. All RT reactions were performed using 1  $\mu$ g of RNA, Superscript II (Invitrogen), and gene-specific primers, and mixtures were incubated for 50 min at 42°C. Quantitative RT-PCR (qRT-PCR) reaction mixtures contained 5  $\mu$ l of cDNA template, gene-specific primers, 12.5  $\mu$ l of SYBR Green Master Mix (Applied Biosystems), and distilled H<sub>2</sub>O to a 25- $\mu$ l total volume. qRT-PCR assays were performed in triplicate with an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Transcript levels were normalized to *ureA* or the 16S rRNA gene in each sample (using  $\Delta\Delta C_T$  analysis as described in ABI User Bulletin 2, available at <http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf>). The *ureA* transcript was chosen for normalization because it amplified consistently across different genotypes at the same growth phase. The *flhA* mutants were an exception in that they expressed reduced levels of *ureA* tran-

script, consistent with a previous report implicating *flhA* in regulation of *ureA* transcription (32); for these mutants the 16S rRNA gene was chosen for normalization, using primer sequences 1320f and 1431r as described previously (13). Other primers used for qRT-PCR were designed using Primer Express software (Applied Biosystems); all primer sequences are listed in Table 2.

**TEM.** All samples for TEM were grown overnight in BB medium from a starting OD<sub>600</sub> of 0.02. A 10- $\mu$ l sample of each strain was applied to copper grids (400 mesh) with Butvar support film coated with carbon, rinsed twice, and negatively stained for 1 min with 1% phosphotungstate (pH 6.97). Grids were visualized and photographed in a Philips CM12 TEM at an acceleration voltage of 80 kV. Bacterial counts for each genotype and treatment group were conducted on three separate grids, counting a minimum of 100 cells, for at least two independent experiments.

## RESULTS

***luxS* is responsible for AI-2 production in *H. pylori* strain G27.** To investigate whether AI-2 functions as a signaling molecule in *H. pylori*, we generated a clean deletion of the *luxS* gene in the laboratory strain G27 (hereafter referred to as the  $\Delta luxS$  strain). To verify that any phenotypes we observed with the  $\Delta luxS$  strain were due to the loss of the *luxS* gene product, we constructed a genetically complemented strain (hereafter referred to as the *luxS*\* strain) by placing the full-length *luxS* gene and upstream sequences in the  $\Delta luxS$  mutant chromosome at the *rdx* locus, which encodes a nonessential gene that, when mutated, results in metronidazole resistance without causing a growth disadvantage (2, 43). We isolated CFS from



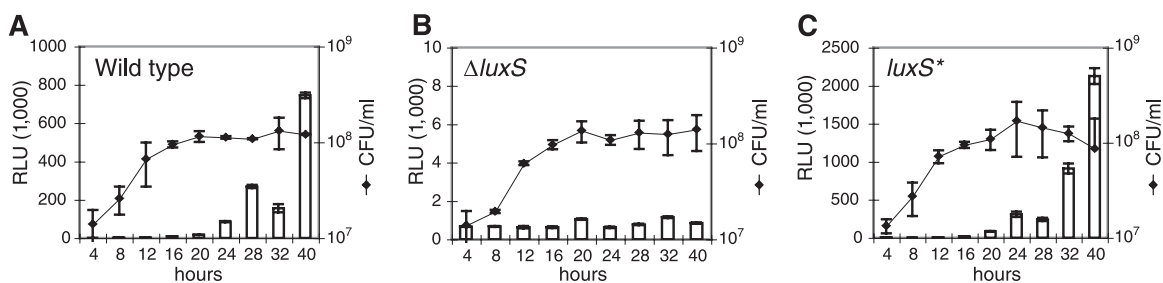


FIG. 1. AI-2 production in *H. pylori* is *luxS* dependent. AI-2 production in the wild-type strain G27 (A), the  $\Delta luxS$  mutant (B), and the complemented *luxS*\* strain (C) was measured as the ability of CFS harvested at the indicated times after inoculation of liquid *H. pylori* cultures to induce bioluminescence in the *V. harveyi* reporter strain BB170 (relative light units [RLU]; bars). The cell densities of the cultures across the growth curves were determined by viable plate counts (CFU/ml; diamonds). Error bars indicate standard deviations of triplicate or duplicate samples for the bioluminescence and cell density measurements, respectively.

wild-type,  $\Delta luxS$ , and *luxS*\* cultures at different time points during growth in liquid medium and assayed their abilities to induce bioluminescence when incubated with the *V. harveyi* reporter strain BB170. The wild-type strain produced AI-2 in a growth-stage-dependent manner, with significant AI-2 activity accumulating during stationary phase (Fig. 1A). The  $\Delta luxS$  mutant, unlike the wild-type parent strain, was not able to activate bioluminescence (Fig. 1B, note difference in y-axis scale). The *luxS*\* strain activated bioluminescence in the reporter at levels higher than the wild-type strain (Fig. 1C), suggesting that the insertion of the *luxS* gene and its promoter at a heterologous position in the chromosome resulted in elevated expression of the gene product. The complementation experiment confirmed that LuxS is responsible for AI-2 production in *H. pylori*. Neither the interruption of the *luxS* gene nor the insertion of *luxS* at the *rdx* locus caused any growth defect under the conditions examined (Fig. 1).

**The  $\Delta luxS$  mutant has reduced motility.** Based on the report that *luxS* may regulate *flaA* expression (29), we measured motility of the *H. pylori* wild-type,  $\Delta luxS$ , and *luxS*\* strains. Liquid bacterial cultures growing at early stationary phase were spotted onto the center of soft agar plates and incubated for 6 days, and the resulting swarm halo areas were quantified. The  $\Delta luxS$  mutant consistently produced a reduced swarm halo compared to the wild-type strain in repeated trials (one representative

trial is shown in Fig. 2). Interestingly, the *luxS*\* strain, which produces higher levels of AI-2 than the wild type (Fig. 1), also produced a larger swarm halo than the wild-type strain. To test whether the  $\Delta luxS$  motility defect could be complemented by a secreted factor produced by wild-type but not  $\Delta luxS$  mutant cells, we prepared soft agar plates composed of 70% fresh BB medium and 30% CFS from each strain. Both the wild-type and *luxS*\* strains formed halos approximately 25% smaller when grown on wild-type CFS-supplemented plates than on normal medium (Fig. 2), which could be due to AI-2, depletion of an essential nutrient, or to the presence of a noxious by-product of bacterial metabolism in the spent medium, or a combination of all three. Both strains formed halos approximately 50% smaller when grown on  $\Delta luxS$  CFS relative to normal medium, which could be due to similar noxious effects of the wild-type CFS without the positive effect of AI-2 or to differences in the chemical composition of the CFS from the two strains, as illustrated by the global metabolic difference between wild-type and  $\Delta luxS$  *E. coli* (51). Notably, the  $\Delta luxS$  mutant formed halos over 600% larger on wild-type CFS-supplemented plates than on normal medium or  $\Delta luxS$  CFS-supplemented plates (Fig. 2). These data indicate that a substance in the wild-type but not  $\Delta luxS$  CFS, likely AI-2, can complement the motility defect of the  $\Delta luxS$  mutant.

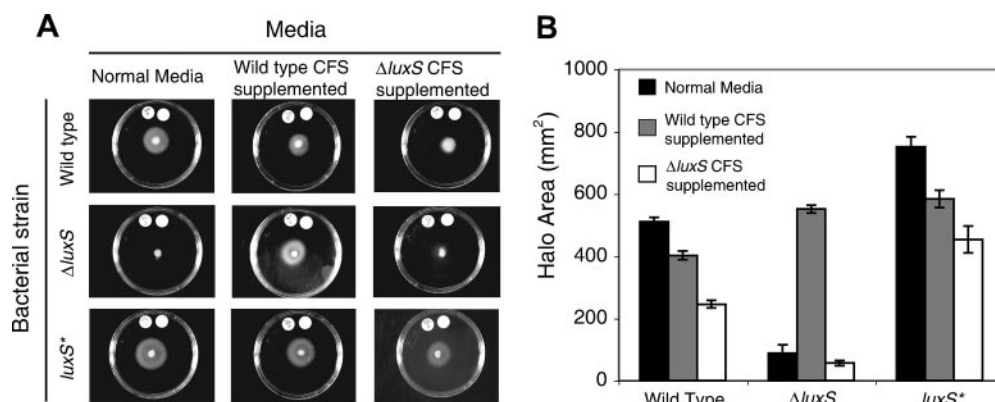


FIG. 2. The  $\Delta luxS$  mutant has a motility defect that is rescued by wild-type CFS. Wild-type,  $\Delta luxS$ , and *luxS*\* bacteria were seeded onto soft agar plates composed of normal medium or supplemented with CFS from wild-type or  $\Delta luxS$  cultures. After 6 days of incubation, the halos of migration were visualized (A) and quantified (B). Error bars indicate the standard deviation of halo sizes of triplicate cultures in one experiment.

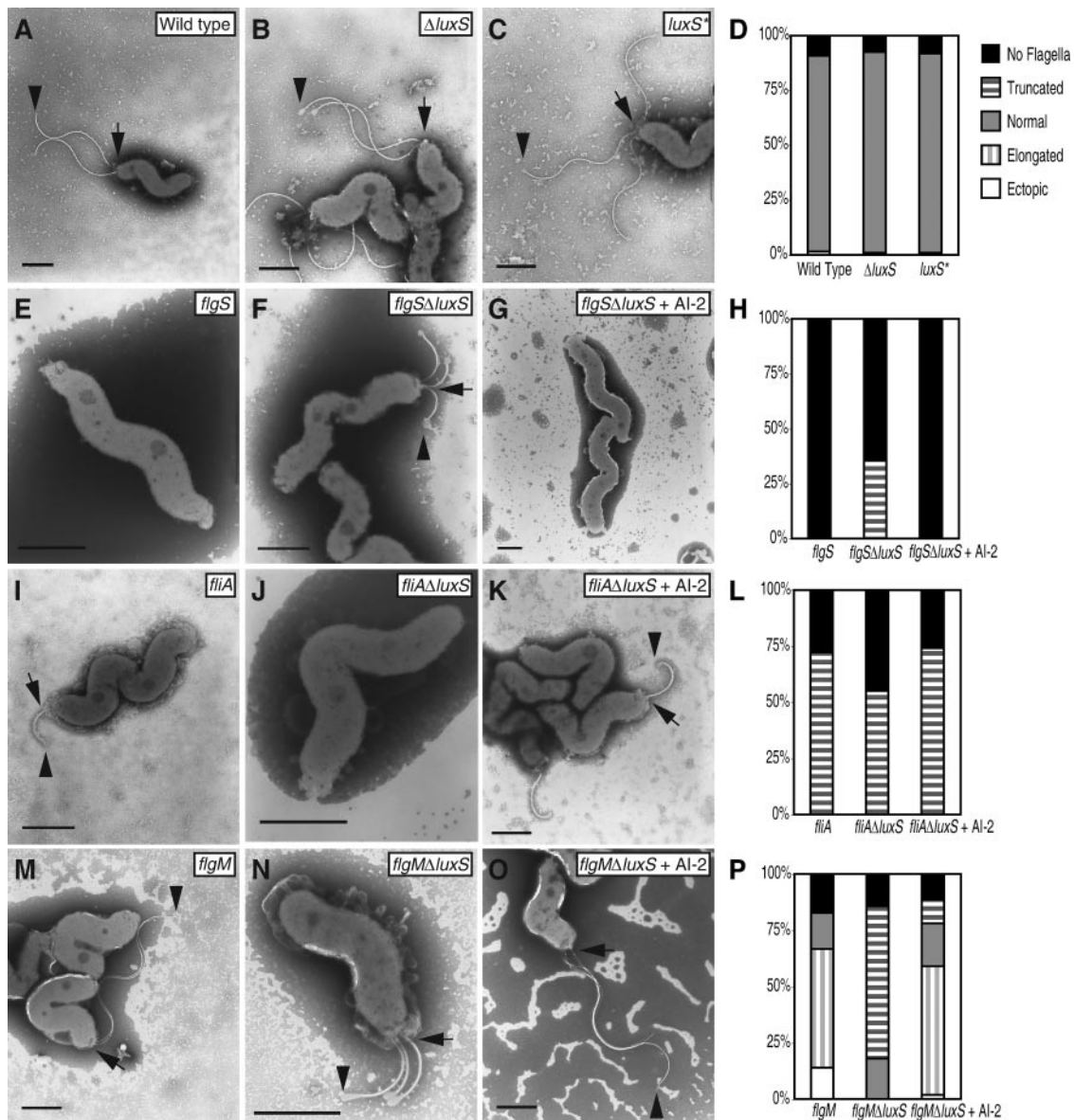


FIG. 3. AI-2 modulates flagellar morphogenesis. Representative flagella, as visualized by TEM, are shown for each of the genotypes indicated grown in normal medium (A to C, E, F, I, J, M, and N) or for 4 h in the presence of 0.1 mM DPD (G, K, and O). In all cells with flagella, the base is marked by an arrow, and the tip is marked by an arrowhead. Scale bars, 1  $\mu$ m. The flagellar morphologies were scored for at least 100 cells for each treatment group (D, H, L, and P).

**AI-2 influences flagellum morphogenesis.** We used TEM to examine the flagellar morphology of the wild-type,  $\Delta luxS$  mutant, and  $luxS^*$  strains. In cell populations of all three genotypes, the flagella appeared normal, as scored for at least 100 cells per genotype (Fig. 3A to D). We reasoned, however, that the flagellum might be especially sensitive to the presence of AI-2 in genetic backgrounds in which its integrity was already compromised. To test this idea, we constructed mutations in four key flagellar regulators in both the wild-type and the  $\Delta luxS$  mutant genetic backgrounds. Using a nonpolar cassette designed to allow translation of downstream genes in the operon (33), we created insertion mutations in *flgS* (HP0244), encoding the sensor kinase that regulates class 2 and the in-

termediate class gene transcription (6, 36); in *fliA*, the  $\sigma^{28}$  responsible for class 3 and the intermediate class gene expression; in *flgM*, the anti-sigma factor for *fliA* (11, 24); and in *flhA*, the flagellar master regulator (36, 41). All flagellar single and  $\Delta luxS$  double mutants were tested for motility on soft agar plates. In all cases, the mutants were nonmotile, as expected based on previous reports, with no differences between the single and double mutants (data not shown).

We assessed the flagellar morphologies of the single and double mutants by TEM (at least 100 cells of each genotype in at least two independent experiments). In the cases of *fliA*, *flgM*, and *flgS*, we found that the flagellar  $\Delta luxS$  double mutants had phenotypes that differed from the single mutant

phenotypes as described below. The results for one representative experiment are shown in Fig. 3. The *flgS* single mutant did not produce any flagella (Fig. 3E), as reported previously (36). In contrast, 36% of the *flgS*  $\Delta luxS$  double mutant population produced truncated flagella (Fig. 3F and H). The *fliA* single mutant exhibited truncated flagella (Fig. 3I), as has been previously described (11, 24). In the *fliA*  $\Delta luxS$  double mutant population, the proportion of cells completely lacking flagella increased, with a corresponding decrease in the fraction of cells carrying truncated flagella (Fig. 3J and L). As previously reported, the *flgM* single mutant produces abnormally long flagella (11), and a small proportion displays ectopic flagella at both cell poles (24) (Fig. 3M). In contrast, the majority of the *flgM*  $\Delta luxS$  double mutant cells exhibited truncated flagella (Fig. 3N and P). As previously reported, the *flhA* mutant did not produce flagella (41). The *flhA*  $\Delta luxS$  double mutant cells were also completely aflagellated (data not shown).

To test whether the *flgS*  $\Delta luxS$ , *fliA*  $\Delta luxS$ , and *flgM*  $\Delta luxS$  double mutant flagellar phenotypes were due to the loss of AI-2 signaling, we treated each of the double mutant cultures with synthetic DPD, which spontaneously converts to AI-2. For these experiments we used a final concentration of 0.1 mM DPD that corresponded to the maximal concentration of AI-2 produced by *H. pylori* cultures (see Fig. 4C). Cultures were incubated with DPD for 4 h and visualized by TEM. In all cases, the addition of DPD completely reversed the  $\Delta luxS$  double mutant flagellar phenotypes (Fig. 3G, K, and O). These results demonstrate that AI-2, acting as an extracellular signal, modulates flagellar morphogenesis.

**AI-2 regulates flagellar gene transcription by modulating *flhA* expression.** To determine the molecular mechanism underpinning the changes in flagellar morphology in the  $\Delta luxS$  mutants, we used semiquantitative RT-PCR to screen for alterations in gene expression of representative genes of the four flagellar classes in the 10 genotypes described above. The genes that showed marked differences in transcript levels between the genotypes were reanalyzed by qRT-PCR in an independent experiment. Transcript abundances were normalized to *ureA*, which displayed consistent levels across the different genotypes at the same growth phase. The *flhA* and *flhA*  $\Delta luxS$  mutants were exceptions and exhibited marked reduction in *ureA* transcription, consistent with a previous report of reduced urease activity in *flhA* mutants (32); for these mutants 16S rRNA transcript was used for normalization. The class 1 genes involved in chemotaxis and motor function (*cheW*, *cheA*, *motA*, *motB*, *tlpA*, and *hpaA*, encoding a flagellar sheath-associated protein) did not exhibit differences in expression levels between the genotypes. In contrast, dynamic changes were observed across the genotypes in multiple genes involved in flagellar biosynthesis, including genes regulated by all three sigma factors (Fig. 4A). The transcriptional differences between the various mutants demonstrate the complexity of the flagellar gene transcription hierarchy and its sensitivity to perturbations. For example, the *flgS*  $\Delta luxS$  double mutant, which formed some truncated flagella in contrast to the aflagellated *flgS* mutant, exhibited elevated levels of *flaA* transcript encoding the major flagellin. Whereas elimination of *flgS* in a  $\Delta luxS$  background derepressed flagellar production, *flgS* levels were elevated in both the *fliA*  $\Delta luxS$  and *flgM*  $\Delta luxS$  double mutants relative to the *luxS*<sup>+</sup> single mutants. We note

that elevation of *flgS* transcription was associated with loss or truncation of flagella in the double mutants, suggesting a role for FlgS as a negative regulator of flagellar biosynthesis.

Notably, the top tier regulator *flhA* showed a consistent pattern of reduced transcription in the  $\Delta luxS$  genotypes (Fig. 4B). We demonstrated that *flhA* was required for expression of the flagellar genes *flgS*, *fliA*, *flgM*, and *flaA* (Fig. 4C), confirming its role as a key activator of the flagellar regulon. The *flhA*  $\Delta luxS$  double mutant exhibited a pattern of flagellar gene transcription identical to that of the *flhA* single mutant (Fig. 4C). To test whether loss of AI-2 signaling could account for the reduction in *flhA* transcript in the *flgS*  $\Delta luxS$ , *fliA*  $\Delta luxS$ , and *flgM*  $\Delta luxS$  double mutants, we added back an equivalent amount of synthetic DPD to  $\Delta luxS$  cultures to restore the normal level of AI-2 in the medium. To do this, we compared the level of AI-2 activity in CFS derived from early stationary phase wild-type *H. pylori*, which we had shown to produce maximal AI-2 (Fig. 1A), to various concentrations of synthetically prepared DPD. We found that a 0.1 mM concentration of DPD and wild-type CFS caused roughly equivalent levels of expression of bioluminescence in the *V. harveyi* BB170 reporter strain (Fig. 4D). Indeed, incubation of the  $\Delta luxS$  mutant with this concentration of DPD was sufficient to restore *flhA* transcript levels to that of wild-type cells (Fig. 4E). Furthermore, exposure of wild-type cells to 0.1 mM DPD resulted in elevated *flhA* transcript levels (Fig. 4E). To further characterize the dose responsiveness of *flhA* transcription to AI-2, we exposed  $\Delta luxS$  mutant cultures, which are devoid of endogenous AI-2, to a range of DPD concentrations. *flhA* transcript levels increased in a dose-dependent manner between 0.01 and 0.1 mM DPD (Fig. 4F). No additional increase in *flhA* transcript levels was observed at 1 mM DPD, suggesting that the effects of DPD on *flhA* transcription were saturable or possibly even inhibitory. These data show that AI-2 alone is sufficient to modulate flagellar gene expression in a dose-dependent manner.

## DISCUSSION

In this study we show that AI-2 signaling is necessary for normal levels of motility in *H. pylori* strain G27. The basis of the  $\Delta luxS$  mutant phenotype could be due to a defect in the morphogenesis or function of the motility apparatus. We observed no abnormalities in the morphology of the  $\Delta luxS$  mutant flagella by TEM or of the *luxS*<sup>\*</sup> strain that produces elevated levels of AI-2, and the mutant cells were motile when observed in wet mount, although we did observe some differences in their swimming behavior, which is the focus of a separate study (B. A. Rader and K. Guillemin, unpublished data). However, when we deleted the *luxS* gene in mutants lacking any of three key flagellar regulatory genes—the sensor kinase *flgS*, the flagellar sigma factor *fliA*, and its anti-sigma factor, *flgM*—we observed strikingly different flagellar phenotypes in the double versus the single mutants. All of the double mutant phenotypes were reversed to that of the *luxS*<sup>+</sup> single mutants by the addition of purified AI-2, indicating that the regulation of flagellar morphology is mediated by this extracellular signal. We confirmed *flhA*'s role as a top-tier regulator of flagellar gene transcription and demonstrated that an *flhA* mutant and an *flhA*  $\Delta luxS$  double mutant had identical phenotypes, consistent with the idea that the AI-2 signal acts up-

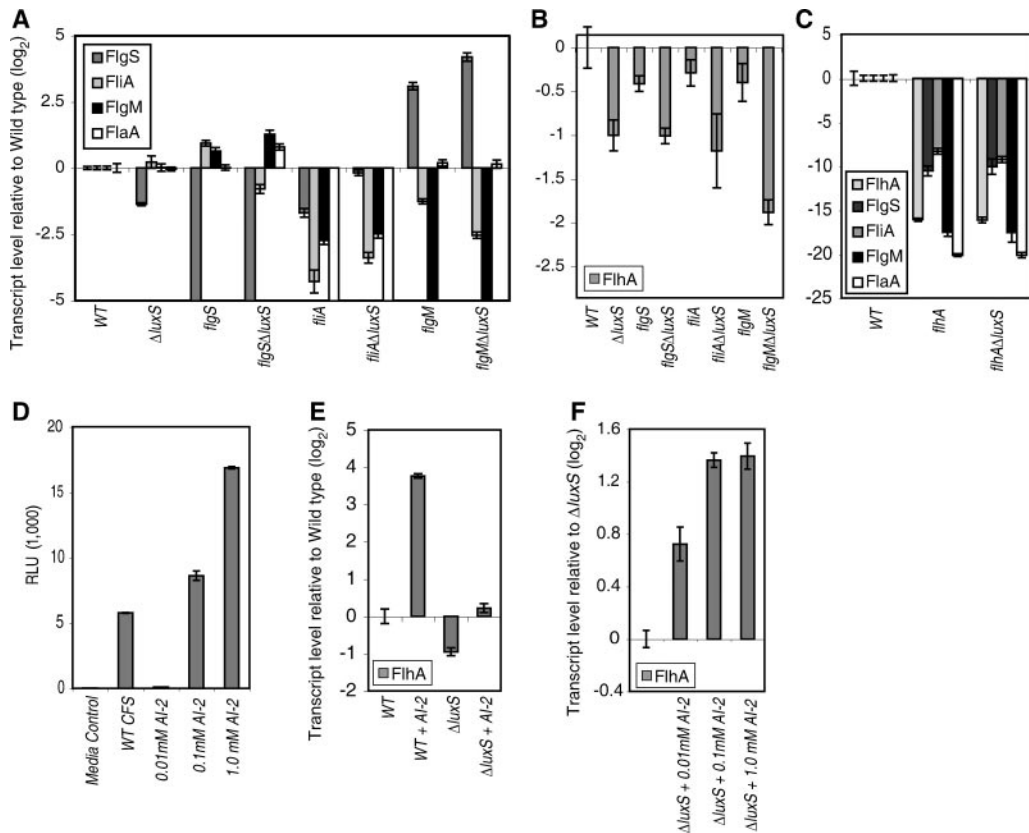


FIG. 4. AI-2 modulates flagellar gene transcription. Transcript levels of *flgS*, *fliA*, *flgM*, and *flaA* (A) and of *flhA* (B) were determined by qRT-PCR normalized to the levels of *ureA* in each of the genotypes indicated and are represented as the log<sub>2</sub> difference relative to the levels in the wild-type strain. Levels of the same transcripts were determined in the indicated genotypes and normalized to the levels of the 16S rRNA gene in each sample (C). AI-2 activity in CFS from wild-type early-stationary-phase *H. pylori* was measured relative to different concentrations of DPD (D). *flhA* transcript levels were measured as in panel B in wild-type and  $\Delta luxS$  cultures untreated or treated for 4 h with 0.1 mM DPD (E) and in  $\Delta luxS$  cultures treated with a range of DPD concentrations (F). Error bars indicate the standard deviations for three triplicate samples.

stream of FlhA. We further demonstrated that purified AI-2 is sufficient to induce expression of *flhA*.

Our finding that AI-2 regulates flagellar gene expression in *H. pylori* argues that this molecule functions as an intercellular signaling molecule in this species. In addition, our data suggest that AI-2 signaling regulates motility, as indicated by our finding that wild-type but not  $\Delta luxS$  CFS can restore spreading of  $\Delta luxS$  mutants on soft agar plates. It is unlikely, however, that AI-2 regulation of flagellar gene expression explains the motility defect of the  $\Delta luxS$  mutants, given the similarity in the wild-type and mutant flagellar morphologies. Our data do not rule out additional metabolic requirements for *luxS* in this species that could explain the colonization defects observed with *luxS* mutant *H. pylori* strains (27, 37). The finding that a *luxS* mutant in the X47 strain did not exhibit motility or colonization defects (27) indicates that AI-2 signaling or LuxS metabolic activities are not required in all *H. pylori* strains, reflecting a phenotypic heterogeneity in this species that we have also observed (2).

**Integration of environmental signals into the flagellar gene transcription hierarchy.** Our genetic and molecular data suggest a model, shown in Fig. 5, in which AI-2 regulates flagellar morphology by influencing initial steps in the flagellar gene transcription hierarchy. Consistent with the model that the

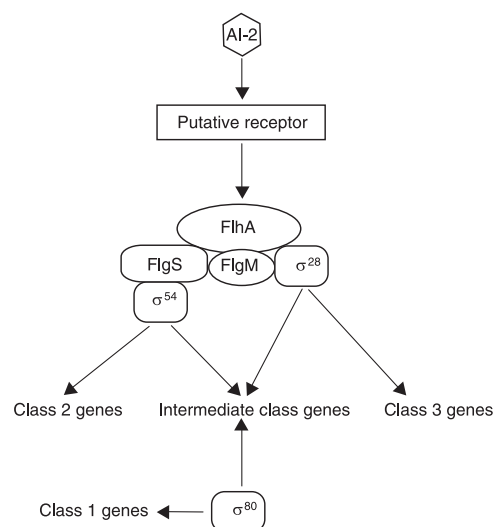


FIG. 5. Model of AI-2 modulation of the *H. pylori* flagellar regulon. The role of AI-2 in transcriptional regulation of components of the flagellar gene hierarchy of *H. pylori* is shown, based on Neihus et al. (36). AI-2 signals upstream of FlhA, which, in turn, regulates branching pathways of gene transcription regulated by FlgS, FlgM, and FliA/ $\sigma^{28}$ .



AI-2 signal functions exclusively upstream of FlhA, we observed identical phenotypes in the *flhA* single and *flhA*  $\Delta$ *luxS* double mutants in their lack of motility and flagella and reduced expression of flagellar genes. In contrast, the flagellar morphology of the  $\Delta$ *luxS* double mutants in combination with flagellar regulators in parallel pathways that branch downstream of FlhA differed from those of the corresponding single mutants, consistent with the model that the AI-2 signal functions both upstream and in parallel to FlgS, FliA, and FlgM. In addition, our finding that AI-2 is sufficient to activate expression of *flhA* places it upstream of this top-tier flagellar regulator.

The comparisons between the wild-type strain and mutants in *flgS*, *fliA*, and *flgM* revealed interdependence between these regulators: in every case in which one of the regulators was removed, the transcript levels of the other two were altered. In the absence of *luxS*, loss of *flgS* resulted in increased transcription of the major flagellin, *flaA*, and increased flagellum formation, whereas elevated *flgS* levels in the *fliA*  $\Delta$ *luxS* and *flgM*  $\Delta$ *luxS* mutants correlated with shorter flagella than with the *luxS*<sup>+</sup> genotypes. All three regulators were also required for wild-type levels of *flhA* expression, and their effects were at least additive with that of *luxS*. Since *flhA* is required for expression of most of the flagellar gene regulon (36), AI-2 regulation of *flhA* transcription is likely to contribute to the differences in flagellar gene expression and flagellar morphology observed in the different  $\Delta$ *luxS* mutants, although the complexity of this regulatory network and the importance of post-transcriptional regulatory events make it difficult to formulate specific predictions about the consequence of reducing *flhA* levels in each mutant background analyzed. Our finding that AI-2 itself is sufficient to increase *flhA* transcript levels in a dose-dependent manner represents a new signal that can influence *H. pylori* flagellar biosynthesis.

Multiple complex and diverse hierarchical regulatory pathways of flagellar gene expression have been described in bacteria (31), many of which are regulated by master regulators whose expression is, in turn, influenced by environmental conditions such as nutrient availability and cell cycle (7, 30). In several systems, AI-2 has been implicated in regulating flagellar biosynthesis. An *luxS* mutant of *Campylobacter jejuni*, a close relative of *H. pylori*, also exhibits decreased motility on soft agar plates and regulates *flaA* expression in a *luxS*-dependent manner (17, 23). Treatment of *Escherichia coli* strain K-12 with purified AI-2 has been shown to increase this bacterium's motility on soft agar plates through a mechanism that involves a novel motility quorum-sensing regulator, MqsR, regulating *qseBC* and *flhDC* expression (22).

**Environmental regulation of *H. pylori* flagellar morphogenesis.** Motility is an absolute requirement for *H. pylori* colonization in several animal models (16, 38, 39) and is thus considered an important virulence trait of this pathogen. Little is known, however, about the regulation of *H. pylori* flagellar biosynthesis within the gastric environment. Unlike the flagellar gene hierarchies of the enteric *Gammaproteobacteria* or *Caulobacter*, which possess the master regulators FlhCD and CtrA, respectively, responsible for all flagellar gene expression, many of the *H. pylori* flagellar and chemotaxis genes (the class 1 genes) are under the control of the housekeeping sigma factor,  $\sigma^{80}$ , and are constitutively expressed (36). Environmen-

tal factors, however, clearly modulate expression of flagellar genes. Growth of *H. pylori* in acidic medium induces expression of many of the class 2 and intermediate genes and is associated with increased motility (34), whereas growth on cultured gastric epithelial cells represses expression of the class 3 gene *flaA* (26). *flaA* expression increases in the switch from exponential growth to stationary phase (48), during the period when we observe significant AI-2 accumulation, and AI-2-containing CFS induces transcription of an *flaA* reporter (29). We report here that AI-2 regulates expression of the class 1 gene *flhA* and influences flagellar morphogenesis. In addition, the transcript levels of another class 1 gene, *flgS*, fluctuated dramatically between the different genotypes we analyzed, indicating that expression of these top-tier regulators is sensitive to concentrations of an environmental signal.

*H. pylori* may use AI-2, which we have shown it produces in a cell density-dependent manner, in conjunction with other environmental cues to regulate flagellar structures as a survival strategy within the human stomach. At high cell densities and high AI-2 concentrations, it may be beneficial for *H. pylori* to increase flagellum production as a strategy to promote motility and dispersal throughout the stomach. Also, despite the textbook picture of the stomach as devoid of bacteria, a recent 16S rRNA enumeration study demonstrated a diverse gastric microbiota (8), which may contribute to AI-2 levels in the stomach and influence *H. pylori* motility in this habitat. Patterns of *H. pylori* dispersal within the gastric environment are correlated with disease outcome: ulcers are associated with concentrated bacterial populations in the distal stomach and gastric cancer arises from infections in which the bacteria are distributed throughout the stomach (9). An understanding of the environmental cues, both bacterial and host derived, that regulate *H. pylori* flagellar morphogenesis in the human stomach will contribute to our ability to predict and control *H. pylori*-associated disease outcomes.

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