Endorsement of LuxS plays a role in the synthesis of an extracellular signaling molecule, autoinducer 2 (AI-2). To analyze a possible role of AI-2 in regulating Helicobacter pylori gene expression, we constructed a panel of transcriptional reporter strains. We show that the expression of H. pylori flaA is growth phase dependent and that flaA transcription increases in association with increased culture density. Mutating the luxS gene eliminates growth-phase-dependent control of flaA, and this growth phase dependence is restored when the luxS mutant strain is complemented with the wild-type luxS gene.

Quorum sensing is a mechanism used by bacteria to regulate expression of genes in response to changes in population density (reviewed in reference 21). Quorum-sensing processes depend on the production, secretion, and recognition of diffusible signaling molecules that accumulate with bacterial growth. Among the gram-negative bacteria, two quorum-signaling mechanisms have been identified. The LuxI/LuxR system uses an acetylated homoserine lactone signal molecule (reviewed in reference 16), and the LuxS system uses a signal molecule termed autoinducer 2 (AI-2) (4).

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Synthesis of AI-2 is dependent on the activity of the LuxS protein, which functions as an AI-2 synthase (31). The pathway for AI-2 production has been linked to the methyl metabolic cycle (25), which allows recycling between S-adenosyl methionine and methionine. A by-product of this pathway is the toxic metabolite S-ribosylhomocysteine, which LuxS converts to 2,3-pentanedione (DPD) and homocysteine. DPD cyclizes in the presence of boron, forming AI-2 (24, 25). To date, the best characterized LuxS-based quorum-sensing system is that of Vibrio harveyi. In this organism, AI-2 regulates gene expression by activating an elaborate network of two-component signal transduction systems (14, 15, 21).

Various gram-negative and gram-positive bacterial species, including Helicobacter pylori (13, 18, 20), Campylobacter jejuni (10), enterohemorrhagic Escherichia coli (EHEC) (27), Salmonella enterica serovar Typhimurium (30), Clostridium perfringens (23), and Bacillus anthracis (19) produce AI-2 molecules that can stimulate luminescence in an AI-2-specific reporter strain. AI-2 plays a role in the regulation of multiple genes that are reportedly transcribed in a growth-phase-independent manner (33). To construct these reporter strains, the coding regions of the following genes from H. pylori strain J99, a strain for which the entire genome sequence is known (1), were PCR amplified and cloned into plasmid pGEMT (Promega) with the following oligonucleotide primers: for flaA (i.e., gene designations HP0601 and HP0658), 5′-GGCGCA AACTACGGAAATCTCG-3′ and 5′-GGCGGCGGTTTTTC AATAAATCC-3′; for HP0609 (JHP0548), 5′-CTTCTTAGG GCTAGGTTTCGGT-3′ and 5′-GGGTGTGTTATGG GAAGTGGC-3′; and for HP0689 (JHP0628), 5′-CCACCC AGTGGAGATGAGACCG-3′ and 5′-GCCGCAATAGA GCTGCTATCAC-3′.

Transcriptional reporters for flaA, HP0609, and HP0689 were generated by insertion of blunt-ended HindIII fragments harboring either a promoterless E. coli chloramphenicol acetyltransferase (cat) or β-galactosidase (lacZ) cassettes were introduced into flaA. As controls, we constructed reporter strains to monitor expression of two genes (i.e., HP0609 and HP0689) that are reportedly transcribed in a growth-phase-independent manner (33). To construct these reporter strains, the coding regions of the following genes from H. pylori strain J99, a strain for which the entire genome sequence is known (1), were PCR amplified and cloned into plasmid pGEMT (Promega) with the following oligonucleotide primers: for flaA (i.e., gene designations HP0601 and HP0658), 5′-GGCGCA AACTACGGAAATCTCG-3′ and 5′-GGCGGCGGTTTTTC AATAAATCC-3′; for HP0609 (JHP0548), 5′-CTTCTTAGG GCTAGGTTTCGGT-3′ and 5′-GGGTGTGTTATGG GAAGTGGC-3′; and for HP0689 (JHP0628), 5′-CCACCC AGTGGAGATGAGACCG-3′ and 5′-GCCGCAATAGA GCTGCTATCAC-3′.

Transcriptional reporters for flaA, HP0609, and HP0689 were generated by insertion of blunt-ended HindIII fragments harboring either a promoterless E. coli chloramphenicol acetyltransferase (cat) gene from plasmid pCM7 (American Type Culture Collection no. 37173) (6) or aphA-3 (encoding kanamycin resistance) and a promoterless lacZ gene from plasmid pBW (9) into unique restriction sites within the coding regions of the cloned genes (i.e., NheI for flaA, BglII for HP0609, and BamHI for HP0689). These insertions are predicted to disrupt the encoded proteins at amino acids 404 (FlaA), 342 (HP0609), and 167 (HP0689). Introduction of the transcriptional reporters into the H. pylori chromosome was accomplished by natural transformation and allelic exchange...
as previously described (9, 12). Transformants were initially selected on the basis of antibiotic resistance, and insertion of reporters into the desired sites was confirmed by PCR analyses of genomic DNA. The cat transcriptional reporters were successfully introduced into H. pylori 399 and 26695. Multiple attempts to introduce the lacZ transcriptional fusions into H. pylori 399 were unsuccessful. These fusions were, however, successfully introduced into H. pylori 1061 (17), a strain in which the lacZ reporter has been used previously (9, 35).

H. pylori luxS sequences were PCR amplified with the primers 5′-CGATCAAGCAGACAAAGTGTGGG-3′ and 5′-AACT CATCGGCTCTAAATCTCC-3′. Cloning of the H. pylori J99 luxS gene (i.e., JHP0097) and the strain 26695 luxS gene (i.e., HP0105) into pGEMT resulted in plasmids pGEMTluxS1 and pGEMTluxS2, respectively. The luxS genes were disrupted via introduction of the aph3 cassette from pUC4K (Pharmacia) into a unique XcmI site, resulting in pGEMTluxSKm1 and pGEMTluxSKm2, respectively. These insertions are predicted to disrupt the encoded LuxS proteins at amino acid 75. Transformation of H. pylori strains 399, 26695, and 1061 with plasmids pGEMTluxSKm1, pGEMTluxSKm2, and pGEMTluxSCAT (13), respectively, resulted in the luxS mutants 399luxSKm, 26695luxSKm, and 1061luxSCAT.

Additional luxS mutant plasmids were constructed by XcmI/NheI restriction digestion of pGEMTluxS2, followed by insertion of a sacB-kan cassette (7) into the blunt-ended XcmI/NheI site (yielding pGEMTluxSN396) or by recircularization of the XcmI/NheI-digested pGEMTluxS2 (yielding pGEMTluxSNX). pGEMTluxSNX codes for a C-terminal-truncated LuxS protein (i.e., J99luxSXN). pGEMTluxSXN codes for a C-terminal truncated LuxS protein (i.e., J99luxSXN). pGEMTluxSXN396, and introduction of the disrupted luxS gene into the chromosome resulted in the luxS mutant 26695luxSXN1 (kanamycin resistant and sucrose [5% sucrose] sensitive). Strain 26695luxSXN1 was then transformed with either plasmid pGEMTluxSNX (encoding LuxSΔ75-156) or with pGEMTluxS2 (encoding wild-type LuxS), and transformants in which sacB-kan had been replaced with the introduced luxS sequences were selected based on sucrose resistance and kanamycin sensitivity. These transformations yielded strains 26695luxSXN1 (wild-type luxS restored in its original locus) and 26695luxSXN (encoding LuxSΔ75-156). To introduce a functional luxS gene into a luxS mutant strain while leaving the nonfunctional truncated luxS allele intact (complementation), strain 26695luxSNX was transformed with plasmid pADluxS (13), a plasmid designed to introduce an intact luxS gene in the ureA locus of H. pylori, yielding strain 26695luxSXNPAD-1.

For our initial studies of growth-phase-dependent transcription in H. pylori, broth cultures were inoculated with each of the reporter strains and aliquots were removed at serial time points to measure optical density and to quantify the levels of reporter activity. As shown in Fig. 1A, the expression of the flaA-cat transcriptional fusion progressively increased as the culture density increased. In contrast, the expression of HP0609 and HP0689 transcriptional fusions remained essentially unchanged (Fig. 1B and C). Growth-phase-dependent changes in flaA were also detected in the lacZ transcriptional reporter strain (Fig. 2) and were similar to those detected in strain 399 by the cat transcriptional fusions. No growth-phase-dependent changes in expression of HP0609 or HP0689 were detected in the lacZ reporter strains (Fig. 2).

To determine if growth phase regulation of flaA in H. pylori is dependent on the AI-2 quorum-sensing system, the flaA-cat and flaA-lacZ fusions were introduced into luxS mutants of H. pylori 399 (i.e., 399luxSKm) and H. pylori 1061 (i.e., 1061luxSCAT), respectively. In contrast to the growth-phase-dependent expression of flaA observed in the wild-type strains,
expression of flaA in the luxS mutant strains was not growth phase dependent (Fig. 1A and 2). Expression of HP0689 and HP0609 was not altered by mutation of the luxS gene (Fig. 1B and C). These data provide evidence that growth phase regulation of flaA expression is dependent on luxS.

To further investigate a potential role of luxS in regulating flaA expression, luxS mutant strains containing either the flaA-cat or flaA-lacZ reporter were cultured in the presence of conditioned medium derived from either wild-type H. pylori J99 or its luxS mutant derivative (i.e., J99luxSKm). Conditioned medium from J99 induced 1,000-fold greater luminescence in V. harveyi BB170 (3, 29) than did conditioned medium from J99luxSKm (data not shown). However, the levels of flaA expression were only slightly higher when the flaA reporter strains were grown in wild-type conditioned medium than when grown in conditioned medium lacking AI-2 (P < 0.05, paired Student’s t test) (Fig. 3). In these experiments, flaA expression was analyzed following a 15-h induction period, when culture optical densities at 600 nm (OD_{600}) were in the range (0.8 to 1.0) where maximal flaA expression is observed (see Fig. 1). No stimulatory effect of wild-type conditioned medium on flaA reporter expression was observed when the reporter strains were incubated with conditioned medium for shorter time periods. While the lack of significant induction at early time points was surprising, this result was, nonetheless, consistently reproduced in experiments with both H. pylori J99 and H. pylori 1061 reporter strains. Conditioned medium had no effect on expression of either HP0609 or HP0689 following a 15-h induction period (data not shown).

To more rigorously test the role of luxS in growth phase regulation of flaA, we analyzed expression of flaA in a wild-type H. pylori (26695) strain, two isogenic luxS mutant strains, and derivatives of these luxS mutant strains into which a functional copy of luxS was introduced. Schematic illustrations of these strains are shown in Fig. 4A. Conditioned media from 26695luxSXN1 (i.e., sacB/kan luxS mutant) and 26695luxSXN (i.e., LuxSA75-156) failed to induce luminescence in V. harveyi BB170 (data not shown). In contrast, conditioned media from derivatives of these luxS mutant strains, in which luxS was restored in its original locus (i.e., 26695luxSC-1) or inserted in the ureA locus (i.e., 26695luxSPAD-1), induced luminescence in V. harveyi in a manner similar to the effects of conditioned media from wild-type H. pylori 26695 (data not shown). Only strains 26695luxSC-1 and 26695luxSPAD-1 showed growth-phase-dependent expression of flaA similar to that of the 26695 wild-type strain (Fig. 4B). No growth-phase-dependent regulation of flaA was found in the luxS mutants 26695luxSXN1 and 26695luxSXN (Fig. 4B).
Taken together, our present work clearly indicates that luxS plays a role in regulating the growth-phase-dependent expression of flaA. A previous study demonstrated that luxS also upregulates expression of flagellar genes in EHEC and E. coli K-12 (8, 27). Notably, there are some features of luxS-dependent regulation of H. pylori flaA expression that are atypical compared to the features of AI-2-mediated regulation of gene expression in several other bacterial species. In particular, H. pylori-conditioned medium (containing extracellular AI-2) produced only a small effect on flaA transcription.

One possible explanation for the weak effects of H. pylori-conditioned medium on flaA expression is that specific proteins required for AI-2 signal transduction may not be present in H. pylori. This possibility is supported by results of BLAST searches of the H. pylori genome (34) which fail to identify homologs of various AI-2 signaling components found in V. harveyi (e.g., LuxQ, LuxU, LuxO, LuxP [4]), EHEC (QseBC [28]), and S. enterica serovar Typhimurium (Lsr transporter [32]). Alternatively, in H. pylori an intact luxS gene may be required for the synthesis of a putative AI-2 signal receptor. A third possibility would be that, rather than function as an extracellular signaling molecule in H. pylori, AI-2 may function primarily as an intracellular signal. In this case, the minimal induction of flaA expression by conditioned medium could be a result of AI-2 diffusing inefficiently back into the cell. Winzer et al. (36) proposed that besides contributing to quorum signaling, LuxS also has an important metabolic function. In support of this, the production of AI-2 has been linked to intracellular levels of S-adenosylmethionine, a molecule closely tied to the metabolic state of the cell. In this model, AI-2 signals the metabolic status of a bacterial population, leading to the appropriate up- or downregulation of target genes. In support of this notion, AI-2 is a global regulator in EHEC and regulates genes with diverse functions, including type III secretion, production of flagellar components, cell division and growth, and the SOS response (27). In H. pylori, Thompson et al. (33) found, using microarray studies, that the transcription of about 20% of H. pylori genes varied in association with changes in growth phase. A large proportion of these genes are known to be involved in cell maintenance and growth. Based on the
model proposed by Winzer et al. (36), we speculate that AI-2 may serve to identify the metabolic status of an *H. pylori* population, leading to appropriate expression of target genes. Using AI-2, *H. pylori* may thus be able to modulate expression of various genes, including flaA, in response to different growth environments it would encounter within its host.

In conclusion, we have demonstrated that growth phase regulation of flaA expression in *H. pylori* is dependent on luxS. Future studies will seek to identify additional genes that are regulated by a luxS-dependent pathway as well as components of AI-2-dependent signaling pathways in *H. pylori*.

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