

# Intercellular Communication in *Helicobacter pylori*: *luxS* Is Essential for the Production of an Extracellular Signaling Molecule

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**Individual bacteria of numerous species can communicate and coordinate their actions via the production, release, and detection of extracellular signaling molecules. In this study, we used the *Vibrio harveyi* luminescence bioassay to determine whether *Helicobacter pylori* produces such a factor. Cell-free conditioned media from *H. pylori* strains 60190 and 26695 each induced >100-fold-greater luminescence in *V. harveyi* than did sterile culture medium. The *H. pylori* signaling molecule had a molecular mass of <10 kDa, and its activity was unaffected by heating to 80°C for 5 min or protease treatment. The genome sequence of *H. pylori* 26695 does not contain any gene predicted to encode an acyl homoserine lactone synthase but does contain an orthologue of *luxS*, which is required for production of autoinducer-2 (AI-2) in *V. harveyi*. To evaluate the role of *luxS* in *H. pylori*, we constructed *luxS* null mutants derived from *H. pylori* 60190 and 26695. Conditioned media from the wild-type *H. pylori* strains induced >100-fold-greater luminescence in the *V. harveyi* bioassay than did conditioned medium from either mutant strain. Production of the signaling molecule was restored in an *H. pylori luxS* null mutant strain by complementation with a single intact copy of *luxS* placed in a heterologous site on the chromosome. In addition, *Escherichia coli* DH5 $\alpha$  produced autoinducer activity following the introduction of an intact copy of *luxS* from *H. pylori*. Production of the signaling molecule by *H. pylori* was growth phase dependent, with maximal production occurring in the mid-exponential phase of growth. Transcription of *H. pylori vacA* also was growth phase dependent, but this phenomenon was not dependent on *luxS* activity. These data indicate that *H. pylori* produces an extracellular signaling molecule related to AI-2 from *V. harveyi*. We speculate that this signaling molecule may play a role in regulating *H. pylori* gene expression.**

In the past decade, there has been considerable progress in our understanding of intercellular communication among bacteria. In one form of intercellular communication, termed quorum sensing, bacteria release extracellular signaling molecules (autoinducers), which accumulate as the population grows. When the extracellular signaling molecule reaches a critical threshold concentration, a signal transduction cascade is triggered within each cell of the population, the final result being an alteration in gene expression (for reviews, see references 15, 19, and 21). This altered pattern of gene expression presumably increases the capacity of bacteria to survive environmental changes that accompany increased cell density. In various bacterial species, autoinducer-mediated regulation of gene expression controls diverse processes, including sporulation (27), genetic competence (2, 28), antibiotic synthesis (40), alternative sigma factor synthesis (26), the production of virulence determinants (11, 22, 48), and even the production of other quorum-sensing molecules (35).

One of the best studied of the autoinducer-controlled gene expression systems is the density-dependent bioluminescence of marine vibrios (*Vibrio fischeri* and *V. harveyi*). *V. fischeri* exists free in seawater, as well as in a symbiotic relationship within the light organ of squid (38). *V. fischeri* produces and releases an *N*-acyl homoserine lactone (AHL) signaling molecule. Transcription of the luciferase operon (*luxCDABEGH*) of *V. fischeri* is repressed in the absence of a threshold level of

AHL. When cell density, and hence AHL level, reaches a threshold concentration, the LuxR regulator is inactivated, and bioluminescence occurs (6). Many gram-negative bacterial species produce related AHL molecules, but the AHL-mediated quorum-sensing systems are typically quite species specific. The production of AHL molecules in gram-negative bacteria is catalyzed by AHL synthases, which are usually encoded by *luxI* orthologues.

*V. harveyi*, a free-living marine bacterium, produces two different quorum-sensing molecules, designated autoinducer-1 (AI-1) and AI-2, which are each capable of regulating luciferase activity. *V. harveyi* AI-1 has been identified as hydroxybutanoyl-L-homoserine lactone (13). The chemical structure of the second signaling molecule (AI-2) is not known, but the *luxS* gene is required for its production (44). The presence of AI-2 is detected by a sensory histidine kinase (encoded by *luxQ*) located within the cytoplasmic membrane of *V. harveyi* (6). Many strains of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium contain *luxS* orthologues and produce autoinducer molecules that are functionally similar to *V. harveyi* AI-2 (42–44). Surette et al. (44) have noted that *luxS* orthologues are present in many additional bacterial species and have suggested that many of these species may produce extracellular signaling molecules.

*Helicobacter pylori* is a curved, gram-negative bacterium found associated with the gastric epithelium of humans and other primates. Colonization of the human stomach with *H. pylori* consistently results in the development of gastric mucosal inflammation and is a risk factor for the development of peptic ulcer disease and gastric adenocarcinoma (10, 14, 25, 33). In the present report, we describe the production of an

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TABLE 1. *H. pylori* strains used in this study

Strain (parent strain)	Genotype	Urease activity	Source or reference
60190	Wild type	+	ATCC 49503
26695	Wild type	+	46
L60-1 (60190)	<i>luxS::CAT</i>	+	This study
L60-2 (60190)	<i>luxS::CAT ureA::luxS-aphA3</i>	-	This study
L26-1 (26695)	<i>luxS::CAT</i>	+	This study
L26-2 (26695)	<i>luxS::CAT ureA::luxS-aphA3</i>	-	This study
60190 VX-1	<i>vacA::xylE-aphA3</i>	+	17
60190 VX-1/LC-1	<i>vacA::xylE-aphA3 luxS::CAT</i>	+	This study

extracellular signaling molecule by *H. pylori* and report that *H. pylori luxS* is essential for this activity.

#### MATERIALS AND METHODS

**Bacteria and culture conditions.** Wild-type *H. pylori* strains and all mutant strains used in this study are listed in Table 1. *H. pylori* strains were routinely cultured at 37°C in ambient air containing 5% CO<sub>2</sub>. *V. harveyi* BB152 (*luxL::Tn5*) is capable of producing AI-2 but not AI-1, and strain BB170 (*luxN::Tn5*) is capable of sensing AI-2 but not AI-1 (7, 42). *V. harveyi* strains were grown at 25°C on Luria-marine agar plates (5) or in autoinducer bioassay (AB) medium (20). *V. harveyi* strains were kind gifts of B. Bassler (Princeton University).

**Generation of cell-free CM and *V. harveyi* luminescence bioassay.** *H. pylori* strains were cultured in brucella broth supplemented with either 5% fetal bovine serum (FBS) or 0.5% charcoal (12). Cell density was monitored by readings of optical density at 600 nm (OD<sub>600</sub>). Cell-free conditioned media (CM) were prepared by centrifuging *H. pylori* cultures at 8,000 × g followed by filtration of the supernatant through 0.2-μm-pore-size filters. CM preparations were routinely stored frozen at -70°C. CM preparations from *V. harveyi* strains were prepared in the same manner, except that the cells were cultured in AB medium at 25°C.

The *V. harveyi* luminescence bioassay was performed essentially as described by Surette and Bassler (42). Briefly, an overnight culture of *V. harveyi* BB170 was diluted 1:5,000 into fresh AB medium. Experimental CM preparations were added to the diluted *V. harveyi* culture at a 10% (vol/vol) final concentration. Aliquots of 1 ml were removed at various time points, and total luminescence was quantified using a luminometer (ALL 2010; Analytical Luminescence Laboratory). Viable cell counts (CFU per milliliter) of *V. harveyi* were determined at each time point by serial dilution, and relative light units were calculated as total luminescence per 10<sup>6</sup> *V. harveyi* cells.

**Molecular biology techniques.** Extraction of *H. pylori* genomic DNA, cloning, plasmid preparation, restriction enzyme digestion, repair of 5' and 3' overhangs, and PCR protocols were performed essentially as previously described elsewhere (4, 17, 18).

**Generation of *H. pylori luxS* null mutants.** *luxS* from *H. pylori* 26695 genomic DNA was amplified using primers 5' GCGGACATTGTGGCACATAGCGGC and 5' CTATTGCTTGAACAATAATCCCCGC, which were derived from the sequence of *H. pylori* 26695 (46). The 1,490-bp amplicon was cloned into pGEM-T (Promega). The chloramphenicol acetyltransferase (CAT) gene from *Campylobacter coli* (47) was ligated into the unique *XcmI* site within the cloned *H. pylori* 26695 *luxS* sequence. Disruption of the chromosomal *luxS* gene in *H. pylori* 26695 or 60190 was accomplished by natural transformation, allelic exchange, and screening for chloramphenicol-resistant *H. pylori* clones as previously described (17). The resulting *luxS* mutant strains derived from *H. pylori* 26695 and 60190 were designated L26-1 and L60-1, respectively. PCR analysis of genomic DNA indicated the orientation of the CAT cassettes and demonstrated that a double-crossover event had occurred in each mutant (data not shown). To assay for the presence of intracellular autoinducer, *luxS H. pylori* cells were disrupted by sonication (Virsonic 60; Virtis Company, Gardiner, N.Y.), using three 30-s pulses on ice with 1-min cooling intervals between pulses. The membrane fraction was removed by centrifugation at 10,000 × g for 30 min, and the resulting cytoplasmic fraction was assayed for autoinducer activity.

***luxS* complementation studies.** For complementation studies, we used suicide plasmid pAD-1, which the *H. pylori ureA* sequence with unique restriction sites for *XbaI* and *SmaI* in close proximity to the *ureA* ribosome binding site and initiation codon (3). This plasmid is designed such that cloned sequences can be placed within the urease locus on the *H. pylori* chromosome (resulting in a urease-negative phenotype), and the cloned sequence will then be transcribed under the control of the *ureA* promoter and translated using the *ureA* ribosome binding site (3). *luxS* was amplified from *H. pylori* genomic DNA using primers 5' TGCTTAGATGAAAACACAAAATGAATGTAGAGAG and 5' TCCC CCGGGTCAAACCCCACTTCAGACCAC, which contain restriction sites for *XbaI* and *SmaI*, respectively, at the 5' ends. The amplicon, which contained the entire *luxS* open reading frame (ORF), was digested with *XbaI* and *SmaI* and

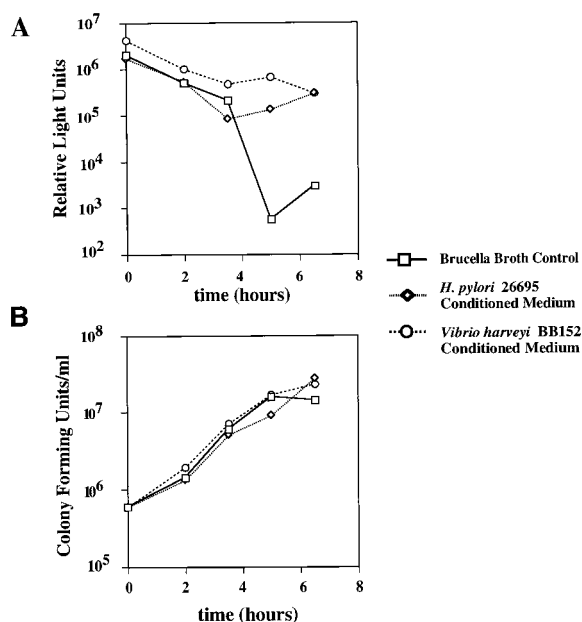


FIG. 1. Production of an extracellular signaling molecule by *H. pylori*. *V. harveyi* BB170 was inoculated into AB medium containing 10% CM from *V. harveyi* BB152, 10% CM from *H. pylori* 26695, or 10% sterile brucella broth. Cultures were incubated at 25°C with constant agitation, and aliquots were removed at serial time points for measurement of luminescence (A) and viable cell count (B). Luminescence is expressed in relative light units (luminescence per 10<sup>6</sup> viable *V. harveyi* BB170 cells). At the 5-h time point, CM from *V. harveyi* BB152 and *H. pylori* 26695 induced >100-fold-greater luminescence than did sterile brucella broth.

cloned into pAD-1. A selectable marker, *aphA3* from pUC4K (Amersham Pharmacia), was then cloned into the *SmaI* site, and the resulting plasmid (pAD/*luxS-aphA3*) was used to transform *H. pylori luxS* null mutant strains (L60-1 and L26-1). *H. pylori* colonies were screened for both chloramphenicol and kanamycin resistance. The resulting urease-negative mutants were designated L60-2 and L26-2, respectively.

#### RESULTS

***H. pylori* produces an extracellular signaling molecule.** Many strains of *E. coli* and *S. enterica* serovar Typhimurium produce and release autoinducers that can induce bioluminescence in *V. harveyi* (7). This led us to speculate that *H. pylori* may produce a similar signaling molecule. To test this hypothesis, we used a *V. harveyi* reporter strain, BB170 (kind gift from B. Bassler), which does not have a functional sensor for AI-1 (AHL) but has an intact sensor for AI-2. *V. harveyi* BB152 is capable of producing AI-2, but not AI-1, and was thus used as a source of homologous AI-2 in the bioassay. Figure 1A shows the result of a typical experiment. When inoculated into AB medium containing 10% sterile brucella broth, *V. harveyi* BB170 produces a strong luminescent signal for about 4 h, and the levels of luminescence decrease markedly by the 5-h time point. In contrast, when inoculated into AB medium containing 10% CM from *V. harveyi* BB152 (a supplemental source of AI-2), *V. harveyi* BB170 maintains a high level of luminescence at the 5-h time point. These results confirm data previously reported by Surette and Bassler (42) and illustrate that the 5-h time point is appropriate for monitoring the effects of exogenous AI-2 in this assay.

To determine whether *H. pylori* produces an extracellular signaling molecule, we inoculated *V. harveyi* BB170 into AB medium containing 10% CM from *H. pylori* 26695. Under these conditions, the level of *V. harveyi* luminescence was

<i>H. pylori</i>	LuxS	1	MKTPKMNVESFNL DHTKVKAPYVVRVADRKKGVNGDLIVKYDVRFKQPNQD	50
<i>S. aureus</i>	LuxS	1	MTKMNVESFNL DHTKVVAPFIRLAGTMEGLNGDVIHKYDIRFKQPNKE	48
<i>B. subtilis</i>	LuxS	1	MPSVESFELDHNAVVPYVRHCGVHKVGTG DVVKNKFDIRFCQPNKQ	46
<i>C. perfringens</i>	LuxS	1	MVKVESFELDHTKVKAPYVRKAGIKIGPKGDIVSKFDLRFVQPNKE	46
<i>V. harveyi</i>	LuxS	1	MPLLDSTVDHTRMNA PAVRVAKTMQTPKGDITVFDLRF TAPNKD	46
			* * * * *	
<i>H. pylori</i>	LuxS	51	HMDMPSLHSL EHLVAEIIRNHASY-----VVDWSPMG CQTGFYLTVLNHD	95
<i>S. aureus</i>	LuxS	49	HMDMPGLHSL EHLMAENIRNHSDK-----VVDLSPMG CQTGFVVSFINHD	93
<i>B. subtilis</i>	LuxS	47	AMKPDTHIHTLEHLLAFTIRSHA EKYDHFIDIISP MG CQTGYIYLVVSGEP	96
<i>C. perfringens</i>	LuxS	47	LLSDKGMHTLEHFLAGFMREK LDD-----VIDISP MG CKTGFYLTSTFGDI	91
<i>V. harveyi</i>	LuxS	47	ILSEKGIHTLEHLYAGFMRNHLNG-DSVEI IDISP MG CRTGFYMSLIGTP	95
			* * * * * * * * *	
<i>H. pylori</i>	LuxS	96	NYTEILEVLEKTMQDVLKAT---EVPASNEKQCGWA ANHTLEGAKDLARA	142
<i>S. aureus</i>	LuxS	94	NYDDVLNIVEATLNDVLNAT---EVPACNEVQCGWA ASHSLEGAKTIAQA	140
<i>B. subtilis</i>	LuxS	97	TSAEIVD LLEDTMKEAVEIT---EIPAANEKQCGQ AKLHDLEGAKRLMRF	143
<i>C. perfringens</i>	LuxS	92	DVKDII EALEYSLSKVLEQE---EIPAANELQCGS AKLHSLLEAKSHAKQ	138
<i>V. harveyi</i>	LuxS	96	SEQQVADAWIAAMEDVLKVENQNKIPELNEYQCGT AMHSLDEAKQIAKN	145
			* * * * * * * * *	
<i>H. pylori</i>	LuxS	143	FLDK-RAEWSEVGV	155
<i>S. aureus</i>	LuxS	141	FLDK-RNEWHDVFGTK	156
<i>B. subtilis</i>	LuxS	144	WLSQDK EELLKVFG	157
<i>C. perfringens</i>	LuxS	139	VLENGISDKFYVE	151
<i>V. harveyi</i>	LuxS	146	ILEVGVAVNKNDELALPESMLRELRID	172
			*	

FIG. 2. Alignment of the deduced *H. pylori* LuxS sequence with deduced LuxS sequences from four other bacterial species. LuxS sequences from *H. pylori* 26695 (GenBank accession no. AE000532), *S. aureus* (preliminary sequence data obtained from The Institute for Genomic Research website at <http://www.tigr.org/>), *B. subtilis* (accession no. Z9919), *C. perfringens* (accession no. AB028629), and *V. harveyi* (accession no. AAD 17292) were aligned using the ClustalW algorithm. *H. pylori* LuxS is most closely related to LuxS from *S. aureus* (67% amino acid identity; 15% similarity). Positions of amino acid identity are indicated by asterisks.

maintained at the 5-h time point (Fig. 1A). Similar results were obtained when *V. harveyi* BB170 was inoculated into AB medium containing 10% CM from *H. pylori* 60190 (data not shown). Regardless of whether prepared from brucella broth containing 5% FBS or 0.5% charcoal, *H. pylori* CM stimulated *V. harveyi* luminescence (data not shown). Differences in the luminescence of *V. harveyi* cultures assayed in Fig. 1A could not be attributed to variation in growth rates or bacterial cell densities (Fig. 1B). These data suggest that *H. pylori* produces an extracellular signaling molecule that acts on *V. harveyi*.

***H. pylori* contains a luxS orthologue.** The recent discovery by Surette et al. (44) that the *luxS* gene is essential for AI-2 production in *V. harveyi*, *S. enterica* serovar Typhimurium, and *E. coli*, prompted us to search the genomes of two sequenced strains of *H. pylori* (26695 and J99) for *luxS* orthologues. Both genomes contain *luxS* orthologues, designated ORF HP 0105 and JHP 0097, respectively (1, 46). The predicted primary structures of LuxS proteins from these two *H. pylori* strains are 95% identical. LuxS from *H. pylori* is most closely related to LuxS proteins found in gram-positive species (*Staphylococcus aureus* [67% amino acid identity], *Bacillus subtilis* [45% amino acid identity], and *Clostridium perfringens* [44% amino acid identity]) (Fig. 2). The flanking gene arrangements are widely divergent among most of these species. However, in both *H. pylori* and *C. perfringens*, *luxS* is linked to two genes (*cysK* and

*metB*) that are involved in amino acid biosynthesis. Neither of these genes is linked to *luxS* loci in the other species examined to date.

To investigate the function of the *H. pylori luxS* gene product, we constructed *H. pylori* mutants in which the *luxS* gene was disrupted by insertional mutagenesis. Figure 3 shows a *V. harveyi* bioassay comparing CM from wild-type *H. pylori* 60190 and CM from an isogenic *luxS* mutant, L60-1. CM from wild-type *H. pylori* 60190 induced >100-fold-higher levels of luminescence than did CM from *H. pylori* L60-1. Similarly, CM from wild-type *H. pylori* 26695 induced >100-fold-higher levels of luminescence than did CM from *H. pylori* 26695 containing a null mutation in *luxS* (data not shown).

In an effort to rescue the signaling phenotype in an *H. pylori luxS* null mutant strain under single-copy replacement conditions, we used a recently developed *H. pylori* suicide vector that allows the replacement of single-copy genes under the control of the strong *ureA* promoter (3). As shown in Fig. 3, CM from the complemented mutant strain 60190 *luxS::CAT/ureA::luxS-aphA3* (L60-2) induced nearly 100-fold-greater luminescence than did CM from L60-1 or medium controls. Thus, the *luxS* gene is essential for the synthesis of an extracellular signaling molecule in *H. pylori*. The CAT cassette used for insertional mutagenesis of *luxS* in *H. pylori* L60-1 contains a sequence that is predicted to be a rho-independent terminator. Therefore,

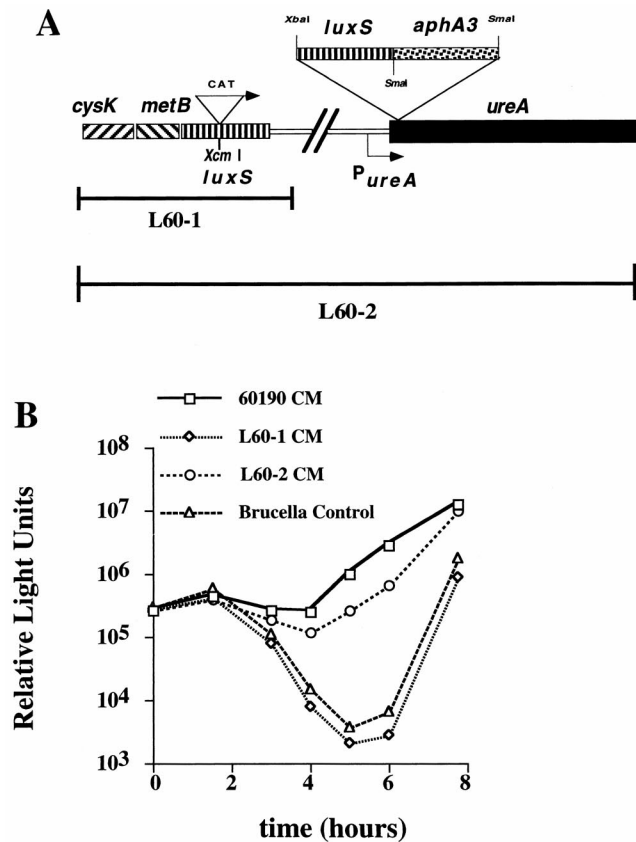


FIG. 3. Role of *H. pylori luxS* in the production of an extracellular signaling molecule. (A) *H. pylori* L60-1 is a *luxS* null mutant strain in which *luxS* has been disrupted by the insertion of a CAT cassette. *H. pylori* L60-2 contains the same *luxS* mutation as in L60-1, but an intact copy of *luxS* has been inserted within the *ureA* gene. (B) *V. harveyi* BB170 was inoculated into AB medium containing 10% CM from *H. pylori* 60190 (wild type), 10% CM from *H. pylori* L60-1 (60190 *luxS*::CAT), 10% CM from *H. pylori* L60-2 (60190 *luxS*::CAT *ureA*::*luxS-aphA3*), or 10% brucella broth. Comparison of *V. harveyi* luminescence values at the 5-h time point demonstrates that wild-type *H. pylori* 60190 CM induces 100-fold-greater luminescence than does either *H. pylori* L60-1 CM or the brucella broth control. At the 5-h time point, CM from the *luxS*-complemented *H. pylori* strain, L60-2, induces approximately 100-fold-greater luminescence than does CM from *H. pylori* L60-1.

these data also suggest that genes immediately downstream from *luxS* are not required for production of the signaling molecule.

To test whether *H. pylori luxS* mutants might be defective in secretion of the signaling molecule, *H. pylori* wild-type and *luxS* mutant cells were each lysed by sonication, membrane fractions were removed by centrifugation, and the cytoplasmic fractions were tested for autoinducer activity in the *V. harveyi* BB170 bioassay. Autoinducer activity was detectable in the wild-type *H. pylori* cytoplasmic extracts but not in extracts from the *luxS* mutant cells (data not shown). This suggests that a functional *H. pylori* LuxS is required for synthesis of the signaling molecule, rather than for its secretion or release.

**Characteristics of the *H. pylori* signaling molecule.** Experiments utilizing a 10-kDa-cutoff ultrafiltration membrane (Amicon) demonstrated that the <10-kDa fraction of *H. pylori* CM induced luminescence in *V. harveyi* BB170, whereas the >10-kDa fraction had no effect (data not shown). Incubation of *H. pylori* CM preparations with proteinase K (Sigma) had no effect on the autoinducer activity. The autoinducer activity in CM from *H. pylori* was almost completely abrogated by treat-

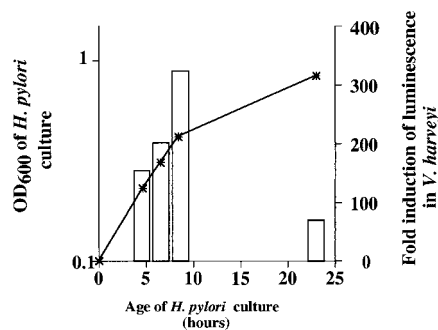


FIG. 4. Kinetics of *H. pylori* signaling molecule production. *H. pylori* 60190 was cultured in brucella broth containing 5% FBS, and aliquots were removed at serial time points for measurement of cell density (OD<sub>600</sub>) (asterisks) and the capacity of CM to induce luminescence in the *V. harveyi* BB170 bioassay system at the 5-h time point (open bars). Luminescence induction is reported as a ratio of relative light units in the presence of *H. pylori* 60190 CM compared to relative light units in the presence of uninoculated brucella broth. The results shown here are representative of results obtained in three independent experiments.

ment at 100°C for 5 min. In contrast, treatment at 80°C for the same length of time had no effect on the activity of the signaling molecule (data not shown). These data indicate that the *H. pylori* signaling molecule is a small (<10-kDa) heat-resistant molecule that is likely not proteinaceous.

**Production of the *H. pylori* signaling molecule is growth phase dependent.** To examine the kinetics of autoinducer production in *H. pylori*, we prepared CM at serial time points reflecting all phases of growth from broth cultures of *H. pylori* 60190. The maximal autoinducer activity was detected in CM preparations from mid-logarithmic-phase cultures (Fig. 4). Levels of autoinducer activity were markedly reduced in CM prepared from stationary-phase *H. pylori* cultures (Fig. 4). This pattern of results was very reproducible, but the absolute values of luminescence varied considerably among individual experiments. The loss of autoinducer activity in stationary-phase cultures suggests that either the *H. pylori* signaling molecule is labile or *H. pylori* cells are capable of degrading the autoinducer.

**Growth phase-dependent regulation of *vacA* transcription.** In previous studies (M. H. Forsyth and T. L. Cover, unpublished data), we have noted that the transcription of *H. pylori vacA* (encoding a vacuolating cytotoxin) is dependent on the bacterial growth phase. Figure 5 shows the results of a typical experiment in which *vacA* transcription was monitored using a reporter strain (60190 VX-1) containing a *vacA*::*xylE* transcriptional fusion (17). Levels of XylE activity are low during the early, low-cell-density portions of the growth curve. XylE activity progressively increases as cultures proceed into exponential, higher-cell-density phases of growth. Peak levels of *vacA* transcription are reached at the onset of stationary phase and subsequently decline over time.

Because this pattern of *vacA* transcription so closely mirrored the demonstrated kinetics of the *H. pylori* signaling molecule production (Fig. 4), we investigated the possibility that *vacA* transcription may be regulated through a *luxS*-dependent signaling mechanism. To test this possibility, we introduced the *luxS*::CAT mutation into *H. pylori* 60190 VX-1 (thus creating 60190 VX-1/LC-1). Both 60190 VX-1 and 60190 VX-1/LC-1 grew at approximately the same rate and to approximately the same density (Fig. 5B). XylE specific activities of these two strains did not differ significantly (Fig. 5A), which indicated that the growth phase regulation of *vacA* transcription is not dependent on LuxS activity.

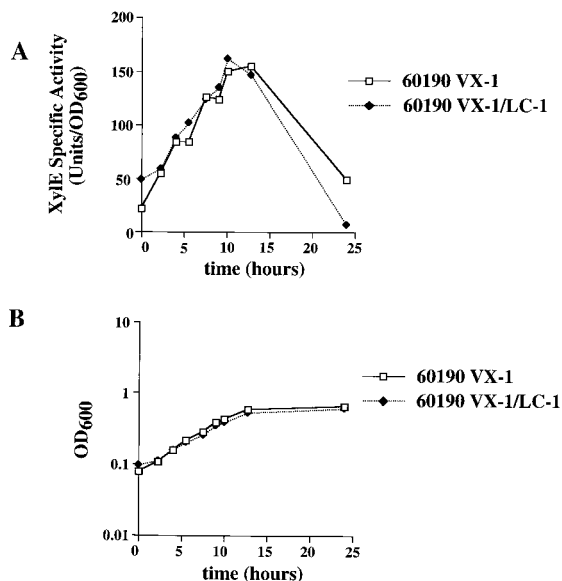


FIG. 5. Growth phase regulation of *H. pylori vacA* transcription. *H. pylori* 60190 VX-1 (containing a *vacA::xylE* transcriptional fusion) and *H. pylori* VX-1/LC-1 (containing both a *vacA::xylE* transcriptional fusion and a *luxS::CAT* mutation) were grown in brucella broth containing 5% FBS. Aliquots were removed at serial time points for measurement of XylE specific activity (A) and cell density ( $OD_{600}$ ) (B). Levels of *vacA* transcription, as evidenced by XylE activities, were growth phase dependent, but this phenomenon was not dependent on LuxS function.

**Activity of recombinant *H. pylori* LuxS expressed in *E. coli* DH5 $\alpha$ .** Many pathogenic *E. coli* strains produce an AI-2-like activity (42) that can be detected using the *V. harveyi* bioassay. However, the commonly used laboratory strain of *E. coli*, DH5 $\alpha$ , fails to produce measurable amounts of autoinducer due to a frameshift mutation in the 3' portion of the *luxS* ORF (44). To examine the function of *H. pylori luxS* in *E. coli*, we cloned the *luxS* orthologue of *H. pylori* into pGEM-T (Promega) and introduced this plasmid (pLuxS) into *E. coli* DH5 $\alpha$ . Conditioned media from *E. coli* DH5 $\alpha$  containing pGEM-T (without *H. pylori* sequence) lacked autoinducer activity in the *V. harveyi* bioassay, whereas CM from *E. coli* DH5 $\alpha$  containing pLuxS induced much higher levels of luminescence (Fig. 6). As expected, disruption of the cloned *H. pylori luxS* gene, accomplished by inserting a CAT cassette into the unique *XcmI* site, resulted in the loss of autoinducer production. These data are further confirmation that *H. pylori luxS* plays an essential role in the production of an extracellular signaling molecule.

## DISCUSSION

The capacity of individual bacteria to regulate gene expression in response to changes in bacterial cell density is known as quorum sensing. Several different families of bacterial quorum-sensing molecules have been described, including AHL derivatives (AI-1 molecules), small peptides, and AI-2 molecules (2, 15, 19). In the present study, we describe the production of a quorum-sensing molecule (autoinducer) by *H. pylori*. Several lines of evidence suggest that the *H. pylori* autoinducer is closely related to the AI-2 family of signaling molecules: (i) like the AI-2 molecules of *V. harveyi*, *E. coli*, and *S. enterica* serovar Typhimurium, the *H. pylori* autoinducer is a low-molecular-mass molecule that is relatively heat resistant; (ii) the *H. pylori* autoinducer is detectable by a *V. harveyi* reporter

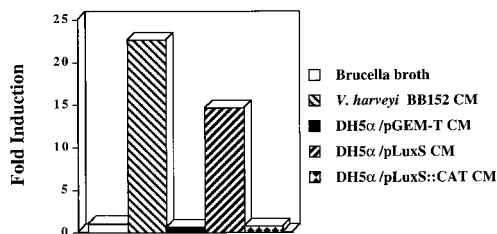


FIG. 6. *H. pylori luxS* complements the *luxS* frameshift mutation of *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$ , which bears a nonfunctional *luxS* (44), was transformed with a plasmid containing the intact *H. pylori luxS* gene (pLuxS), a plasmid containing a disrupted *H. pylori luxS* gene (pLuxS::CAT), or the cloning vector alone (pGEM-T). Each strain was grown in brucella broth to mid-logarithmic phase ( $OD_{600}$  of ~0.5), and cell-free CM were prepared. The CM were tested for the capacity to induce luminescence in the *V. harveyi* bioassay at the 5-h time point. Sterile, pristine brucella broth was used as a control, and all induction values are relative to this sample. CM from *V. harveyi* BB152 was used as a positive control for the bioassay. The DH5 $\alpha$  strain bearing a functional *H. pylori luxS* (pLuxS) induced high levels of luminescence in *V. harveyi* BB170. In contrast, DH5 $\alpha$  strains bearing either a nonfunctional *luxS*<sub>H<sub>sp</sub></sub> (pLuxS::CAT) or the vector alone were incapable of inducing luminescence in this bioassay. Results shown are representative of three independent experiments.

strain that responds to AI-2, but not AI-1, molecules; and (iii) a functional *luxS* orthologue is essential for the production of the *H. pylori* signaling molecule, as well as for production of AI-2 molecules in *V. harveyi*, *E. coli*, and *S. enterica* serovar Typhimurium.

The AI-2 family of quorum-sensing molecules has only been recently described (44), and there are many features of this quorum-sensing pathway that are not yet completely understood. In particular, the molecular composition of AI-2 has not yet been elucidated for any bacterial species. The *luxS* gene clearly seems to be essential for the production of this novel autoinducer (44), but it is not known whether LuxS possesses enzymatic activity or whether it serves some other function. *H. pylori* LuxS does not exhibit any obvious homology to known enzymes. If LuxS is an enzyme required for AI-2 synthesis, it is not known whether *luxS* alone is sufficient for synthesis of AI-2 or whether other genes are required. Moreover, nothing is known about the mechanisms whereby AI-2 is released or secreted into the extracellular milieu. As noted in this study, *H. pylori luxS* null mutants do not contain detectable cell-associated autoinducer activity, which suggests that LuxS is not required for secretion of the autoinducer.

In both *E. coli* and *S. enterica* serovar Typhimurium (42), as well as *H. pylori*, production of AI-2 is influenced by the growth phase of the bacteria. The mechanism of growth phase regulation of AI-2 production is not known. However, we speculate that there may be transcriptional regulation of *luxS* (or possibly other genes in the AI-2 biosynthetic pathway). In addition, there may be regulation of genes in an AI-2 degradative pathway.

Many bacterial species contain *luxS* orthologues (Fig. 2), and production of an AI-2 molecule has now been experimentally demonstrated in *V. harveyi*, *E. coli*, *S. enterica* serovar Typhimurium, and *H. pylori*. Whether these AI-2 molecules are chemically identical or exhibit some variation is not yet known. However, the capacity of AI-2 molecules from multiple species to induce luminescence in *V. harveyi* suggests that there is considerable structural similarity. If LuxS is indeed a unique synthase required for AI-2 production, potentially the expression of recombinant LuxS from various bacterial species in *E. coli* DH5 $\alpha$  will facilitate the characterization and comparison of these various AI-2 autoinducers.

Although it seems likely that many bacterial species produce AI-2 molecules, the function of these molecules remains poorly understood. At present, these autoinducers are known to regulate the expression of luciferase in *V. harveyi* (6) and the expression of a type III secretion system in *E. coli* O157:H7 (41). However, the full complement of genes that might be regulated by AI-2 has not yet been explored. We consider it likely that AI-2 molecules may regulate gene expression in each of the bacterial species that produce them. If this hypothesis is correct, then mechanisms must be present for sensing and responding to these autoinducers. In *V. harveyi*, AI-2 is sensed by a histidine kinase (LuxQ) located within the cytoplasmic membrane (6). It seems likely that similar sensing systems may be operable in *E. coli*, *S. enterica* serovar Typhimurium, and *H. pylori*, but these systems have not yet been identified. The *H. pylori* genome sequence predicts the existence of several histidine kinases, but it is not known which, if any, of these has a functional activity corresponding to *V. harveyi* LuxQ.

The transcription of *vacA* (encoding *H. pylori* vacuolating cytotoxin) is growth phase dependent (Forsyth and Cover, unpublished data; this study), and the growth phase induction of *vacA* transcription seems to resemble the production kinetics of *H. pylori* AI-2. To test whether the growth phase regulation of *vacA* is dependent on *H. pylori* AI-2, we compared the transcription of *vacA* in wild-type and isogenic *luxS* mutant *H. pylori* strains. Inactivation of *luxS* did not alter *vacA* transcription, and therefore the mechanism of *vacA* growth phase regulation remains unknown. Growth phase regulation has been documented for virulence determinants in multiple bacterial species (24, 31, 32, 36) and is likely to be important in pathogenesis. We speculate that yet another quorum-sensing system may operate in *H. pylori* and that this system is the mediator of the observed induction of *vacA* transcription.

Quorum-sensing systems mediated by AHL signaling molecules are typically quite species specific. In contrast, the AI-2 quorum-sensing systems of *E. coli*, *S. enterica* serovar Typhimurium (42–44), and *H. pylori* do not appear to be species specific. One possible role for such a nonspecific bacterial sensing system could be detection of total viable bacterial biomass as an indication of competition for resources. For example, alteration of gene expression in response to increasing bacterial density might be advantageous if it resulted in decreased utilization of nutrients or if it induced utilization of alternate nutrients.

Alternatively, this *H. pylori* signaling molecule may not regulate *H. pylori* gene expression, but may act on various other bacterial species. For example, some *S. aureus* strains produce signal molecules which interfere with quorum sensing in other *S. aureus* strains (23), and *S. aureus* may also produce a signaling molecule identical to that produced by *Enterococcus faecalis* (16, 34). Yet another alternative target for *H. pylori* signal molecules may be the cells of the gastric system itself. For example, *Pseudomonas aeruginosa* produces a signal molecule, OdHL, *N*-(3-oxododecanoyl)-*L*-homoserine lactone, that possesses immunomodulatory activity (45) and alters the expression of P2Y2 and P2Y4 receptors in tracheal gland cells (39). If gastric epithelial cells were responsive to an *H. pylori* signaling molecule, this might represent a bacterial strategy for altering the gastric environment to allow persistent *H. pylori* colonization (8, 9).

*H. pylori* is perhaps unique in terms of its place within the gastrointestinal ecosystem. While nearly the entire length of the gastrointestinal tract has an abundant and diverse microbial flora, *H. pylori* is often the sole species colonizing the gastric mucosa. Given the vast number of bacteria from count-

less species which pass through the stomach, many of which can colonize other regions of the alimentary canal, it is remarkable that *H. pylori* is nearly the only species to successfully colonize this niche. This may be due to unique properties that allow *H. pylori* to enter the gastric mucus layer and escape the very acidic pH of the gastric environment (29, 30). In addition, *H. pylori* produces bacteriocins that may act to prevent colonization by other bacterial species (37). We speculate that the capacity of *H. pylori* to produce and detect AI-2 may be an important component of the process by which bacterial overgrowth in the gastric mucosa is prevented.

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