Evidence for a Signaling System in Helicobacter pylori: Detection of a luxS-Encoded Autoinducer

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Helicobacter pylori possesses a homolog of the luxS gene, initially identified by its role in autoinducer production for the quorum-sensing system 2 in Vibrio harveyi. The genomes of several other species of bacteria, notably Escherichia coli, Salmonella enterica serovar Typhimurium, and Vibrio cholerae, also include luxS homologs. All of these bacteria have been shown to produce active autoinducers capable of stimulating the expression of the luciferase operon in V. harveyi. In this report, we demonstrate that H. pylori also synthesizes a functional autoinducer (AI-2) that can specifically activate signaling system 2 in V. harveyi. Maximal activity is produced during early log phase, and the activity is diminished when cells enter stationary phase. We show that AI-2 is not involved in modulating any of the known or putative virulence factors in H. pylori and that a luxS null mutant has a two-dimensional protein profile identical to that of its isogenic parent strain. We discuss the implications of having an AI-2-like quorum-sensing system in H. pylori and suggest possible roles that it may play in H. pylori infection.

Since its discovery in 1982, Helicobacter pylori has attracted a great deal of attention, both because it occupies the unusual and inhospitable niche of the gastric lumen and because of the diversity of clinical outcomes associated with this infection. Research efforts have focused on identifying genes and the functions of their protein products that allow this organism to survive and persist in the gastric lumen and because of the unique ability to colonize and cause disease in humans. In addition, much effort has been dedicated to investigating how expression of these genes is regulated in the host.

Only a handful of the genes characterized in H. pylori have been definitively shown to be transcriptionally regulated. Studies of the H. pylori flagellar loci indicate that these genes are transcriptionally controlled by a regulatory hierarchy including sigma 54 and sigma 28 homologs and FlgR, an NtrC homolog (33). The pattern of flagellar transcription shows similarities to those of both the family Enterobacteriaceae and Caulobacter (30, 33). There have also been reports suggesting that environmental conditions, such as pH, temperature, metal concentrations, and iron availability (7, 8, 26, 57), can influence H. pylori transcription. One surprising feature of the genetic makeup of H. pylori revealed by the full genome sequence is the absence of most of the regulatory genes present in other gastrointestinal bacteria (38). Since the genome of H. pylori differs significantly from those of other organisms occupying the human gastrointestinal tract, we were interested in identifying and studying mechanisms utilized by H. pylori that contribute to its unique ability to survive and persist in the gastric lumen.

Despite the relative lack of regulatory-gene homologs, the H. pylori genome does contain putative homologs of some sensor kinase and response regulator proteins belonging to the family of two-component systems. These proteins are widespread in other organisms and are central to the transcriptional regulation of a variety of processes that respond to changing environmental conditions (22).

The H. pylori genome contains a homolog of luxS, a gene involved in autoinducer (AI) production in one of the two quorum-sensing systems of Vibrio harveyi (36). Quorum sensing involves the production and detection of membrane-permeating signaling molecules, or AIs, that function to communicate information to a population of cells about their external environment and cell density (19). The interaction of an AI with its cognate sensor protein results in activation of a signal transduction cascade that ultimately leads to changes in gene expression (19). In V. harveyi, quorum sensing controls the expression of bioluminescence.

Quorum sensing was first characterized in the regulation of bioluminescence in the marine bacterium Vibrio fischeri (reviewed by Dunlap and Greenberg [13]). In V. fischeri, quorum sensing depends on the action of two regulatory proteins, LuxI and LuxR. The product of the luxI gene directs the synthesis of the AI, an acylated homoserine lactone derivative. As cell density increases, the amount of AI also increases. At a critical concentration, the accumulated AI interacts with LuxR, the AI-binding protein. When bound to AI, LuxR acts as a transcriptional activator of the luciferase structural operon, and the bacteria emit light.

Since its discovery in V. fischeri, quorum sensing has been recognized as a widespread mechanism utilized by many gram-negative bacteria for the control of gene expression. Many elegant studies of cell-cell signaling in bacteria have recently been published (2, 9, 10, 12, 15, 16, 21, 23, 34, 36). The results of these studies underscore the importance of microbial cell-cell signaling in a variety of processes, including the development of disease in Pseudomonas aeruginosa- and Staphylococcus aureus-associated infections (1, 18, 29, 39, 40), the formation of microbial biofilms (27), and the production of light in luminous bacteria (3, 14, 24).

The expression of bioluminescence in V. harveyi is similarly under the control of a quorum-sensing mechanism. However, in this case, the regulated expression of luciferase is not mediated by homologs of the V. fischeri LuxI and LuxR proteins. Rather, V. harveyi accomplishes quorum sensing through the integration of two parallel two-component regulatory systems...
The recently described \textit{V. harveyi} luxS gene is required for the production of the AI (AI-2) in signaling system 2 (36), and luxS homologs exist in over 25 other bacterial species, including *Vibrio cholerae*, *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *H. pylori*. Surette et al. (36) assessed several different genera possessing the luxS homolog and found that AI-2 activity was produced by many of these organisms. In this study, we present data indicating that *H. pylori* possesses AI-2 activity and that this activity can specifically activate luxS in *V. harveyi* through signaling system 2.

**MATERIALS AND METHODS**

**Bacterial strains and recombinant DNA techniques.** The bacterial strains used were the clinical isolate \textit{H. pylori} Alston, a generous gift from David Cave (St. Elizabeth’s Hospital, Boston, Mass.), \textit{V. harveyi} BB170 (14), and \textit{E. coli} DH5α (Bio-Rad Laboratories). \textit{EJ103} is a luxS deletion derivative of \textit{H. pylori} Alston constructed by the following procedure. One kilobase of sequence directly flanking both the upstream and downstream regions of luxS in \textit{H. pylori} was PCR amplified with Tag polymerase (Gibco BRL, Grand Island, N.Y.) using the following primers: upstream region, A (5′ TCTAGAGGGCGTTTCTGTTTCTAACTC) and C (5′ GGTACCTCTATGCGTTATAGGAAGG); downstream region, C (5′ GGCGGCCGCTTTGCCAGATAAG) and D (5′ TCTAGACTGGTCTGAAGTGGGG). A directed ligation containing the resulting two PCR fragments, a kanamycin (Tnp93) cassette, and plBAscruII 2K vector resulted in the plasmid pEJ32, in which the kanamycin cassette is flanked by the two PCR fragments such that it replaces the complete luxS gene was amplified with the primers XP1 (5′ ATGAAAACACCAAAAACTGAGCG) and XP2 (5′ ATGAAAACACCAAAAACTGACGAGCGG), using both the upstream and downstream regions of luxS in the plasmid pEJ32, in which the kanamycin cassette is flanked by the two PCR fragments such that it replaces the complete *luxS* gene. pEJ32 was electro-ported into \textit{H. pylori} Alston, resulting in the luxS mutant strain EJ103. PCR analysis with primers B and C gave rise to a 1,300-bp product, confirming that luxS had been replaced by the kanamycin cassette. To express LuxS, the \textit{H. pylori} luxS luxS mutant strain \textit{EJ103} was grown in LB broth supplemented with 100 μg/ml of ampicillin and incubated at 37°C overnight. The presence of vacuoles and the ability to produce vacuoles in a \textit{Hep}2 epithelial monolayer using a method adapted from Cover et al. (11). Cell supernatant volumes from \textit{H. pylori} grown under conditions that result in AI-2 activity in BBH were concentrated 30- to 40-fold using Centriprep30 and Centricon30 concentrators (Amicon, Beverly, Mass.). \textit{Hep}2 cells were seeded in 24-well tissue culture plates to a density of 10⁵ cells/ml and incubated overnight in a 5% CO₂ atmosphere at 37°C. Following incubation, the supernatant was removed, centrifuged at 15,000 rpm for 5 min, and filtered as described above. The resulting cell-free culture fluid was assayed for IL-8 by enzyme-linked immunosorbent assay (catalog no. EH2-IL8-10; Endogen, Woburn, Mass.) following the manufacturer’s instructions.

**RESULTS**

**Identification of AI-2 activity in \textit{H. pylori}.** Analysis of the \textit{H. pylori} genome revealed a hypothetical protein with 40% identity and 60% homology to LuxS of \textit{V. harveyi} (Fig. 1). To determine if \textit{H. pylori} possessed an AI-2-like activity, we tested \textit{H. pylori} Alston cell-free supernatants derived from late-log-phase growth for the ability to induce luminescence in the \textit{V. harveyi} reporter strain BB170 (sensor 1 sensor 2) (4). The results shown in Fig. 2A demonstrate that the addition of 10% \textit{H. pylori} cell-free supernatant stimulates luminescence in the \textit{V. harveyi} reporter strain BB170 equal to or greater than of the positive control AI-2 from \textit{serovar Typhimurium}. Specifically,
cell-free supernatants from both *H. pylori* and serovar Typhimurium induced signaling system 2 in the *V. harveyi* reporter strain approximately 500-fold. To determine whether *H. pylori* produces this activity, cell-free supernatants obtained from early-, middle-, and late-log-phase cultures were assayed with BB170. These results, shown in Fig. 3, suggest that AI-2 activity per cell reaches a maximum during early log phase and is greatly diminished in late-log-phase cultures. Activation of luminescence was not observed when *H. pylori* supernatants were tested in the *V. harveyi* reporter strain BB886 (sensor 1 promoter in pEJ30). Neither growth medium nor supernatants prepared from *E. coli* DH5α alone caused stimulation of bioluminescence expression in *V. harveyi* BB170.

**AI-2 activity in *H. pylori* does not affect expression of known virulence factors.** Only a few genes in *H. pylori* have been demonstrated to play a role in virulence. Mutation of the luxS homolog in *E. coli* O157 results in a loss of virulence, suggesting that cell-cell communication plays a role in pathogenesis (32, 36). By analogy, we wanted to test whether the expression of any of the known or putative virulence factors described for *H. pylori* was under the regulatory control of AI-2. The parental strain, Alston, and the luxS deletion strain, EJ103, were grown under conditions that result in AI-2 activity and compared for any differences in the growth kinetics in BBH medium, motility, urease activity, and ability to induce IL-8 production in HEp-2 cells. The growth kinetics of the two strains in BBH medium were similar, and microscopic examination of these cultures showed that both strains were motile. There were no discernible differences between Alston and EJ103 in urease activity or in the ability to induce IL-8 production. In addition, when equivalent amounts of concentrated supernatants isolated from Alston and EJ103 were used in a vacuolating cytotoxic assay (11, 28), the luxS mutant was as effective as the parent strain in causing accumulation of vacuoles in HEp-2 cells (data not shown). Taken together, these data indicate that luxS is not involved in the in vitro expression of these factors previously shown to play a role in virulence in *H. pylori*.

**2-D gel analysis of proteins from the wild-type *H. pylori* strain and the ΔluxS mutant EJ103.** We next examined the 2-D protein profiles of both strains grown under conditions that result in AI-2 activity. This experiment was designed to identify candidate proteins that could be under AI-2 regulatory control in *H. pylori*. The resulting profiles showed a complex pattern of approximately 400 to 500 expressed proteins with high resolution. Under the conditions used, no apparent differences between the parent strain and EJ103 were detectable (data not shown).

**DISCUSSION**

The identification of luxS and examination of its critical role in signaling system 2 quorum sensing in *V. harveyi* led to the discovery of luxS homologs in a variety of other bacteria, including many in which quorum-sensing systems had not been characterized.

![Alignment of the *V. harveyi* and putative *H. pylori* LuxS proteins.](Image 135x550 to 468x714)
predicted protein product has such high homology to the V. harveyi times. (A) Wild-type Aliquots were taken, and both cell density and light production were determined. reporter strain in microtiter plates and incubated at 30°C on a rotary shaker. V. harveyi DH5 luxS while the V. harveyi tants were tested for the ability to induce luminescence expression in

V. harveyi LuxS homolog that shares 40% identity and 60% similarity H. pylori -dependent AI-2 activity. Cell-free supernatants were tested for the ability to induce luminescence expression in V. harveyi BB170. Ten percent cell-free supernatants or sterile media were mixed with the reporter strain in microtiter plates and incubated at 30°C on a rotary shaker. Aliquots were taken, and both cell density and light production were determined. Activity is reported as fold activation of luminescence of BB170 over the level of luminescence when sterile media were added. Assays were repeated at least two times. (A) Wild-type H. pylori and Typhimurium 14028 supernatants contain signaling substances that induce expression of luminescence in V. harveyi BB170, while the luxS deletion strain EJ103 does not. (B) H. pylori luxS expression in E. coli DH5α restores AI-2 activity. The error bars indicate standard deviations.

FIG. 2. H. pylori produces a luxS-dependent AI-2 activity. Cell-free supernatants were tested for the ability to induce luminescence expression in V. harveyi. In an effort to undertake a global examination of luxS-mediated gene expression in H. pylori, we performed 2-D gel analysis of the parental strain and an isogenic luxS deletion strain, EJ103. This analysis suggested that a luxS deletion has little effect on protein complexity and abundance during steady-state analysis, since no obvious and reproducible differences in protein profiles between the two strains were detected. There are several potential explanations for this result. Several studies have reported that levels of target gene activation in response to quorum-sensing signals are as little as two- or threefold (20, 25, 27). In these cases, 2-D gel analysis may not be sensitive enough to detect modest changes in protein synthesis.

One limitation of all studies of expression of pathogenic factors in vitro is the difficulty in approximating an in vivo environment. Controlling for multiple environmental factors that may simultaneously influence gene expression is difficult, if not impossible, to accomplish. In this regard, it is also possible that sensory information from several environmental cues may be channeled through other, AI-2-independent, pathways that converge at some critical junction. Thus, a combination of transmitted signals may be required to influence downstream gene expression. If so, elimination of any single pathway may not necessarily result in an obvious phenotype. Our burgeoning understanding of the variety and combinations of molecular mechanisms employed by bacteria to establish quorum sensing as a critical mode of communicating information suggests that this is very likely to be the case. For instance, in V. harveyi, the expression of bioluminescence is only partially due to density-dependent signaling. Other environmental cues, such as the availability of iron, oxygen, and carbohydrates, also contribute to the regulation of bioluminescence in an AI-2-independent fashion.

The initial identification of luxS in V. harveyi took advantage of the facility of luxS-dependent signaling to establish quorum sensing. Several studies have reported that levels of target gene activation in response to quorum-sensing signals are as little as two- or threefold (20, 25, 27). In these cases, 2-D gel analysis may not be sensitive enough to detect modest changes in protein synthesis.

Previously described. One of these organisms, H. pylori, has a LuxS homolog that shares 40% identity and 60% similarity with the V. harveyi LuxS protein (Fig. 1). Homology exists over the entire length of the protein. Despite its clear role in quorum sensing in V. harveyi, an exact function in AI-2 production has not been assigned. Furthermore, there are no motifs in the protein that suggest a particular enzymatic or regulatory activity. Since H. pylori possesses an open reading frame whose predicted protein product has such high homology to the V. harveyi LuxS, we wanted to determine if H. pylori could synthesize AI-2 activity and investigate whether this activity might be associated with a potential quorum-sensing system. Our results indicate that H. pylori (luxS+) does produce an active signaling molecule (AI-2) that activates expression of bioluminescence in V. harveyi in a system-2-specific manner (Fig. 2). Activation of luminescence by supernatants harvested at various times during the growth of H. pylori revealed that AI-2-specific activity reaches a maximum early in log phase and diminishes as cells enter late log phase. This suggests that the activity of the molecule is density dependent and, specifically, that conditions of low cell density favor activity (Fig. 3). The loss of activity in stationary phase indicates that early-log-phase activity is degraded when H. pylori reaches stationary phase, an observation that mirrors the findings in serovar Typhimurium (34).

Despite the association between infection with H. pylori and gastric disease, very few gene products have been conclusively shown to play a role in H. pylori-mediated pathogenesis. Since LuxS has been implicated in pathogenesis (32), we wanted to test whether our luxS null mutation would have an effect on the expression or activities of any of the confirmed or putative virulence factors described for H. pylori. Our results indicate that none of the factors or processes that we examined, including growth, motility, urease activity, vacuolating cytotoxin activity, and induction of IL-8 response in epithelial cells, were affected in EJ103. It is possible that any impact that the H. pylori system 2 may have on regulating gene expression involves processes other than those directly involved in pathogenesis. In addition to several reports implicating a role for quorum sensing in pathogenesis (20, 25, 32, 34–36, 39), it is known to control a variety of other phenotypes not directly associated with pathogenesis, such as biofilm formation, luminescence, sporulation, and natural competence. Nevertheless, as the molecular mechanisms and factors underlying H. pylori-mediated pathogenesis become more fully understood and characterized, a necessity for cell density-dependent regulation via luxS may be revealed.

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of the observation that *E. coli* DH5α was phenotypically LuxS− by complementing the LuxS defect with a *V. harveyi* library (34). Subsequently, the *E. coli* DH5α luxS gene was found to have a frameshift mutation. The observation that luxS genes from many other organisms complement the defect in *E. coli* DH5α suggests that LuxS is a highly conserved protein. The availability of several bacterial genome sequences reveals that LuxS homologs exist in at least 25 different species. Our data from the trispecies complementation experiment shown in Fig. 2B demonstrating that expression of the *H. pylori* luxS gene in *E. coli* DH5α results in active AI-2 molecules that complement the AI-2 defect in *V. harveyi* underscores the high level of LuxS conservation among species. There is mounting evidence from a few organisms that have a luxS homolog for the existence of LuxS-homologous signaling systems (32). Taken together, while it is currently unknown what functions are controlled by AI-2 in *H. pylori*, these observations suggest that a LuxS signaling system is present in *H. pylori*. Understanding how this complex microbial communication process contributes to the biology of *H. pylori* will necessitate developing a facility for "bacterial lexicology."

Since *H. pylori* is unique in its ability to colonize the gastric mucosa, it likely has developed specialized strategies allowing it to exploit, grow, and persist in the restrictive niche of the stomach. The lack of any other characterized reservoir may imply that the organism spends most of its life cycle in this unique environment, the gastric lumen of its host. This raises the possibility that *H. pylori* has little need to regulate gene expression to survive the low pH of the stomach. However, because *H. pylori* depends on a host organism for its survival, it must strike a dynamic balance between growing in the gastric niche and avoiding the host immune response in order to successfully persist. Density-dependent cell signaling could provide a mechanism for *H. pylori* to keep its population size in check in response to changes in its host’s environment in order to avoid alerting the host immune system to the infection.

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