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Signaling System in *Porphyromonas gingivalis* Based on a LuxS Protein

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The *luxS* gene of quorum-sensing *Vibrio harveyi* is required for type 2 autoinducer production. We identified a *Porphyromonas gingivalis* open reading frame encoding a predicted peptide of 161 aa that shares 29% identity with the amino acid sequence of the LuxS protein of *V. harveyi*. Conditioned medium from a late-log-phase *P. gingivalis* culture induced the luciferase operon of *V. harveyi*, but that from a *luxS* insertional mutant did not. In *P. gingivalis*, the expression of *luxS* mRNA was environmentally controlled and varied according to the cell density and the osmolarity of the culture medium. In addition, differential display PCR showed that the inactivation of *P. gingivalis luxS* resulted in up-regulation of a hemin acquisition protein and an arginine-specific protease and reduced expression of a hemin-regulated protein, a TonB homologue, and an excinuclease. The data suggest that the *luxS* gene in *P. gingivalis* may function to control the expression of genes involved in the acquisition of hemin.

Quorum sensing, the density-dependent regulation of gene expression, is widespread among both gram-negative and gram-positive bacteria. Quorum sensing involves the synthesis and detection of extracellular signaling molecules termed autoinducers (AIs) (2, 13). Quorum sensing in gram-negative bacteria was first described for the marine symbiotic organism *Vibrio fischeri*. The number of acyl homoserine lactone (HSL) AI molecules in a given culture of *V. fischeri* increases as the cell density increases, and once a critical concentration of AI is reached, a signal transduction cascade that leads to the production of bioluminescence by cells is initiated (15). Components of this system include LuxI, an acyl HSL synthase that directs synthesis of 3-oxo-hexanoyl-HSL (*V. fischeri* AI-1); AinS, an acyl HSL synthase that catalyzes the synthesis of octanoyl-HSL (*V. fischeri* AI-2); and LuxR, a transcriptional activator necessary for responses to *V. fischeri* AI-1 (10). Homologues of the *luxI* and *luxR* genes of *V. fischeri* have been described now for a range of gram-negative bacteria and are responsible for the density-dependent regulation of quite diverse physiological functions (1, 2, 13, 30, 41). Light production by *Vibrio harveyi* is similarly under the control of quorum-sensing systems, however the bioluminescence genes are not regulated by homologues of the *V. fischeri* LuxI and LuxR proteins (3, 4). Rather, in *V. harveyi,* quorum sensing involves two parallel regulatory systems. Signaling system 1 is dependent on two genes, *luxL* and *luxM*, for the synthesis of *N*-3-hydroxybutanoyl-L-HSL (*V. harveyi* AI-1), and signal detection is mediated by the sensor kinase LuxN (3, 26). LuxM shows sequence homology to *V. fischeri* AinS (10). Signaling system 2 requires the *luxS* gene for the synthesis of *V. harveyi* AI-2, a non-HSL AI, the structure of which is unknown (41, 42). The primary sensor for *V. harveyi* AI-2 is thought to be LuxP, and the LuxP–AI-2 complex interacts with LuxQ to initiate signal transduction (4, 26). Signals from both LuxN and LuxQ feed into the LuxU phosphorelay protein that then transmits the signal to the response regulator LuxO (4, 26). Whereas the *V. harveyi* AI-1 quorum-sensing circuit is species specific, the AI-2 system can be used for interspecies cell-cell signaling and may confer upon bacterial cells the ability to monitor the total bacterial density of mixed populations (2, 40).

luxS-based signaling has recently been described for *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Helicobacter pylori,* and *Shigella flexneri* (9, 12, 21, 42). In *S. enterica* serovar Typhimurium, the expression of the *luxS* gene is controlled by environmental factors. AI production and signaling activity increase at high osmolarity and low pH levels and during the mid-to-late-exponential-growth phase. Since these conditions are relevant to *S. enterica* serovar Typhimurium as an enteric pathogen, the *luxS* gene is thought to play an important role in the virulence of the organism (41). However, the full extent of the role of the *luxS* gene in different organisms is still a matter of conjecture.

Porphyromonas gingivalis, a gram-negative anaerobe, is an etiologic agent of severe adult periodontitis (38). The environmental niche of this organism is within a mixed-species biofilm that exists in the gingival crevice, an area that experiences fluctuations in temperature, pH, osmolarity, and nutrient availability (17, 43). Additionally, *P. gingivalis* can invade, replicate, and persist at high density within gingival epithelial cells (5, 23). Thus, there is a potential role for density-dependent gene regulation in *P. gingivalis*. In this study, we identified a gene of *P. gingivalis* encoding a peptide exhibiting 29% identity with LuxS of *V. harveyi*. We also show that conditioned culture medium of *P. gingivalis* 33277, but not that of a *luxS* insertional mutant, induced luciferase expression in *V. harveyi*. Inactiva-

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TABLE 1. Plasmids used and constructed in this study

Plasmid	Description[a]	Source or reference
pCRII-TOPO	3.9-kb cloning vector	Invitrogen
pCR-LUX	409-bp *luxS* PCR product cloned into pCRII-TOPO	This study
pVA3000	Suicide vector for *Bacteroides*; Em^r	25
R751	IncP plasmid used to mobilize vectors from *E. coli* to *Bacteroides* recipient; Tp^r, Tra^+	29
pLR409	pVA3000 containing an insertion of *Bam*HI-*Xba*I fragment from pCR-LUX	This study

[a] Abbreviations: Em^r, erythromycin resistant; Tp^r, trimethoprim resistant; Tra^+, self-transferable.

tion of *luxS* also influenced the expression of several genes involved in hemin uptake, suggesting that LuxS may play a role in the acquisition of hemin by *P. gingivalis.*

MATERIALS AND METHODS

Bacteria and culture conditions. *P. gingivalis* 33277 and its derivative (see below) were grown from frozen stocks in Trypticase soy broth (TSB) (BBL) supplemented with 1 mg of yeast extract per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml. For AI assays, *P. gingivalis* was cultured in AI bioassay (AB) medium (16) modified by the addition of 0.5 mg of yeast extract per ml, 2.5 μg of hemin per ml, and 0.5 μg of menadione per ml. *P. gingivalis* was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C. *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco) under aerobic conditions at 37°C. *Streptococcus gordonii* DL1 was grown in Trypticase-peptone broth supplemented with 5 mg of yeast extract per ml and 0.5% glucose under aerobic conditions at 37°C. *V. harveyi* reporter strain BB170 (sensor 1^-, sensor 2^+) was kindly provided by B. Bassler (Princeton University) and was grown in AB medium overnight at 30°C.

Autoinducer assay. Cell-free culture supernatants from *P. gingivalis* parent and mutant strains (see below) were prepared by centrifugation (10,000 × *g* for 10 min) and filtration (filter pore size, 0.22 μm) and tested for the induction of signaling system 2 in *V. harveyi* BB170 by the previously described luminescence assay (16, 40). Briefly, an overnight culture of BB170 was diluted 1:2,000 in AB medium, and 100 μl of cell-free *P. gingivalis* culture fluid was added to 900 μl of diluted *V. harveyi* cells. Cell-free culture fluid of *V. harveyi* BB170 was included as a positive control, and sterile medium was included as a negative control. The reaction was carried out at 30°C, and light production was monitored with a Bio-Orbit 1251 luminometer.

Oligonucleotides and PCR conditions for the *luxS* gene. The oligonucleotides for *luxS* PCR were luxS1 (5′-CCGTCGCTACATCGAGTACC-3′) and luxS2 (5′-CGAGGCATATATGTCTCCCG-3′ the antisense primer). The oligonucleotides used for testing the cotranscription of *luxS* and the upstream open reading frame (ORF) were luxpro1 (5′-GAGGATCTTCTCGCCCTTTT-3′) and luxS2. For testing cotranscription with the downstream ORF, the primers luxS1 and luxdwn2 (5′-GTGCCGTCTGATTCACATT-3′) were used. Reverse transcription was performed in the presence of 2 μg of total RNA, 50 ng of antisense primer, 50 U of reverse transcriptase (RT) (Ambion), 13 U of RNase inhibitor, 10 mM deoxynucleoside triphosphate (dNTP), and 1× RT buffer. Annealing of the primer and template was carried out at 72°C for 2 min and then at 48°C for 1 h. Controls without RT were included in all experiments. The resulting cDNA was amplified, with each 100 μl of PCR mixture containing 1× PCR buffer, 3 μl of cDNA, 1.5 mM $MgCl_2$, 10 mM dNTP, 100 ng of each primer, and 2.5 U of *Taq* DNA polymerase. The amplification conditions were denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and elongation at 72°C for 2 min for 35 cycles.

Construction of a *luxS* mutant. An insertional mutation of the *luxS* gene was constructed using standard recombinant DNA technology (34). The plasmids used are listed in Table 1. Plasmid DNA was prepared by using the Wizard Plus Miniprep kit (Promega) according to the manufacturer's instructions. The 409-bp PCR product containing the *luxS* gene was cloned into the *Bam*HI-*Xba*I sites of plasmid pCRII-TOPO using the TOPO TA cloning kit (Invitrogen). The 502-bp *Bam*HI-*Xba*I region of the resulting pCR-LUX was then cloned into the *Bam*HI-*Xba*I sites of suicide plasmid pVA3000 carrying the erythromycin resistance gene cassette *ermAM-ermF* to create pLR409. *E. coli* DH5α containing R751 was transformed with pLR409 to create the donor strain for mating with *P. gingivalis*. An overnight culture of the donor was used to inoculate LB medium and cultured aerobically for 2 to 3 h, until the culture reached an A_{600} of 0.2. An overnight culture of the recipient, *P. gingivalis* 33277, was used to inoculate TSB and cultured anaerobically for 6 h, until the culture reached an A_{600} of 0.3. The donor and the recipient were mixed at a ratio of 1 to 5 and spotted onto HAWP filters (pore size, 0.45 μm; Millipore). The mating was performed initially under aerobic conditions for 16 h and then under anaerobic conditions for 8 h. Transconjugants were selected on Trypticase-soy-blood plates supplemented with erythromycin (20 μg/ml) and gentamicin (100 μg/ml).

Confirmation of integration events. To ensure that the correct fusion had occurred on the *P. gingivalis* chromosome, a Southern blot analysis was performed. Chromosomal DNA from six transconjugants was digested sequentially with *Bam*HI, *Pvu*I, *Hin*dIII, and *Sst*I and probed with the PCR-amplified *luxS* that was biotin labeled with the Bionick labeling kit (Gibco BRL). The hybridized probe was detected by using the avidin peroxidase detection system (KPL). Failure to produce *luxS* mRNA by mutant strains was confirmed by RT-PCR using the primers lxmut1 (5′-CAGCACTTGTGCTTCTCCAA-3′) and lxmut2 (5′-GAGGAGCAGGACTTTGTTCG-3′) under the conditions described above. One transconjugant with the appropriate chromosomal integration and with loss of mRNA production was designated PLM1 and selected for further study. The growth rates of the parent and PLM1 mutant strains were comparable.

Biofilm formation. Biofilm formation by the parent and mutant strains of *P. gingivalis* with *S. gordonii* was determined as described previously (7). *S. gordonii* DL1 cells (10^7 cells/ml) were labeled with hexidium iodide and passed over a saliva-coated glass slide in a flow chamber for 4 h at a flow rate of 2 ml/h. Following the deposition of streptococci, *P. gingivalis* cells (10^7 cells/ml) were labeled with fluorescein and passed through the flow cell at 2 ml/h for 4 h. The *P. gingivalis*-streptococcal biofilm was examined with a confocal microscope (Bio-Rad MRC600). Fluorescent optical sections were collected, and confocal assistant software was used to format and merge images (7).

Invasion of epithelial cells. Invasion of *P. gingivalis* strains was quantitated by the standard antibiotic protection assay, as previously described (23). Primary cultures of gingival epithelial cells were obtained from gingival explants and maintained in tissue culture in keratinocyte growth medium (Clonetics). *P. gingivalis* cells were reacted with gingival epithelial cells at a multiplicity of infection of 100 for 90 min. External, adherent bacteria were killed by incubation for 1 h with gentamicin (300 μg/ml) and metronidazole (200 μg/ml), and internal bacteria were released by lysis of the cells in sterile distilled water for 20 min and enumerated by plate counting.

RNA isolation and DD-PCR. Total RNA was isolated from the parent and mutant strains cultivated in TSB by using a total RNA isolation kit (Totally RNA; Ambion) and then was subjected to reverse transcription. The reaction mixture, containing 2 μg of RNA, 1 μl of 10 mM dNTP, and 100 pmol of random hexamers, was incubated at 80°C for 10 min and put on ice. The enzyme mixture, containing 40 U of Moloney murine leukemia virus RT (Ambion), 1× RT reaction buffer, and 1 μl of anti-RNase (Ambion), was added to a final volume of 20 μl. The reaction was performed at 42°C for 1 h, followed by inactivation of the enzyme at 92°C for 10 min. Differential display PCR (DD-PCR) was performed using 5 μl of the synthesized cDNA in 100 μl of a solution containing 1 U of *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 100 pmol of arbitrary primers. The arbitrary primers used were act1 (5′-GGCATG GGTCAGAAGGATT-3′), act2 (5′-CTCAAGTTGGGGGACAAAAA-3′), kgp1 (5′-CGGAACAGCTTCTTCCAATC-3′), and kgp2 (5′-AATCTTGCTCC GCCCTTATT-3′). The thermal cycling parameters were 50 cycles of 94°C for 1 min, 34°C for 1 min, and 72°C for 2 min. Differentially expressed PCR products were excised from the gel and cloned into pCRII-TOPO, and DNA sequencing was done by the University of Washington DNA Sequencing Service. The DD-PCR results were further investigated by RT-PCR using RNA preparations identical to those described above and primers derived from the sequences of cloned products. The primers used were Exinuc1 (5′-TACAAGGAGCACGCA GACAG-3′), Exinuc2 (5′-TCCCGTGGACGATATGTAGG-3′), Hemreg1 (5′-TACCGCTGTACCATTGACGA-3′), Hemreg2 (5′-TAACACTCCTCTCGCC GACT-3′), OMP1 (5′-ATACGGAGGAGGTGAGCGTA-3′), OMP2 (5′-AGT GATGCAATGCTCTGACG-3′), RGP1 (5′-TGTTCGGTTCTGCAGTTGTC-3′), RGP2 (5′-TAATCGCTTCCACCACCTTC-3′), TonB1 (5′-CGGCCAAAT CTGTCTTGACT-3′), and TonB2 (5′-ACCGTCGTTCATACCCGTAG-3′).

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V. harveyi        --MPLL--DSFTVDHTRMNAPAVRVAKTMQTPKGDTITVFDLRFTAPNKD-ILSEKGIHT
E. coli           --MPLL--DSFTVDHTRMEAPAVRVAKTMNTPHGDAITVFDLRFCVPNKE-VMPERGIHT
S. typhimurium    --MPLL--DSFAVDHTRMQAPAVRVAKTMNTPHGDAITVFDLRFCIPNKE-VMPEKGIHT
H. pylori         MKTPKMNVESFNLDHTKVKAPYVRVADRKKGVNGDLIVKYDVRFKQPNQD-HMDMPSLHS
P. gingivalis     --MEMEKIPSFQLDHIRLKR-GIYVS-RKDYIGGEVVTTFDIRMKEPNREPVLGAPELHT
                            ** :** :::   : *:   .   *: :. :*:*:  **::  :    :*:

V. harveyi        LEHLYAGFMRNHLNGDSVEIIDISPMGCRTGFYMSLIGTPSEQQVADAWIAAMEDVLKVE
E. coli           LEHLFAGFMRNHLNGNGVEIIDISPMGCRTGFYMSLIGTPDEQRVADAWKAAMEDVLKVQ
S. typhimurium    LEHLFAGFMRDHLNGNGVEIIDISPMGCRTGFYMSLIGTPDEQRVADAWKAAMADVLKVQ
H. pylori         LEHLVAEIIRNHAS----YVVDWSPMGCQTGFYLTVLNHDNYTEILEVLEKTMQDVLKAT
P. gingivalis     IEHLAATYLRNHPLYKD-RIVFWGPMGCLTGNYFLMRGDYVSKDILPLMQETFRFIRDFE
                  :*** *  :*:*       ::  .**** ** *: : .      :      ::  : .

V. harveyi        NQNKIPELNEYQCGTAAMHSLDEAKQIAKNILEVGVAVNKNDELALPESMLRELRID
E. coli           DQNQIPELNVYQCGTYQMHSLQEAQDIARSILERDVRINSNEELALPKEKLQELHI-
S. typhimurium    DQNQIPELNVYQCGTYQMHSLSEAQDIARHILERDVRVNSNKELALPKEKLQELHI-
H. pylori         ---EVPASNEKQCGWAANHTLEGAKDLARAFLDKRAEWS---EVGV-----------
P. gingivalis     G--EVPGTEPRDCGNCLLHNLPMAKYEAEKYLREVLDVATEENLNYPD---------
                     ::*  :  :**    *.*  *:  *.  *           ::
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FIG. 1. Alignment of the deduced *P. gingivalis* LuxS sequence (obtained from the database of the The Institute for Genomic Research [http://www.tigr.org]) with deduced LuxS sequences from other bacteria. Sequences from *V. harveyi* (GenBank accession no. AAD17292), *E. coli* (GenBank accession no. P45578), *S. enterica* serovar Typhimurium (GenBank accession no. AAF73475), and *H. pylori* (GenBank accession no. AAD07175) were aligned using the ClustalW algorithm. The amino acid residues of these sequences that are identical appear in boldface. Symbols: *, identity; :, strong similarity; ., weak similarity.

RESULTS

Presence of *lux* homologues in *P. gingivalis*. A BLAST search of the *P. gingivalis* genomic database of The Institute for Genomic Research (http://www.tigr.org) revealed several predicted open reading frames with significant identity to various Lux proteins of *V. harveyi.* In particular, an ORF of *P. gingivalis* exhibited 29% identity (49 of 167 amino acid residues were conserved) and 49% similarity (82 of 167 amino acid residues were similar) with the LuxS protein of *V. harveyi*. The sequences were also compared to those of the human pathogens (*S. enterica* serovar Typhimurium and *H. pylori*) that have been shown to produce a functional signaling molecule. Regions of identity occurred within those portions of LuxS that demonstrated the greatest conservation among species (Fig. 1). In addition, similar regions of identity with the *E. coli* LuxS protein that is 100% homologous to LuxS of *S. flexneri* were observed (9). Moreover, a direct comparison of the *P. gingivalis* LuxS protein with the *Borrelia burgdorferi* LuxS protein (which has yet to be demonstrated to be functional) showed 50% identity.

Interrogation of the *P. gingivalis* genome database revealed that *luxS* was separated by only 18 bp from an upstream in-frame ORF with homology to the 5-methylthioadenosine nucleosidase–*S*-adenosylhomocysteine nucleosidase gene (Fig. 2). RT-PCR data indicated that these two genes are cotranscribed (data not shown). The region upstream of the putative 5-methylthioadenosine nucleosidase–*S*-adenosylhomocysteine nucleosidase gene contained prokaryote promoter consensus sequences identified using the Promoter Predictions search tool of the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org) and by visual comparison with a set of predicted *P. gingivalis* consensus promoter sequences (20). Thus, transcription of *luxS* may require the promoter of the upstream ORF. In addition, a 438-bp ORF with no identifiable homology spans

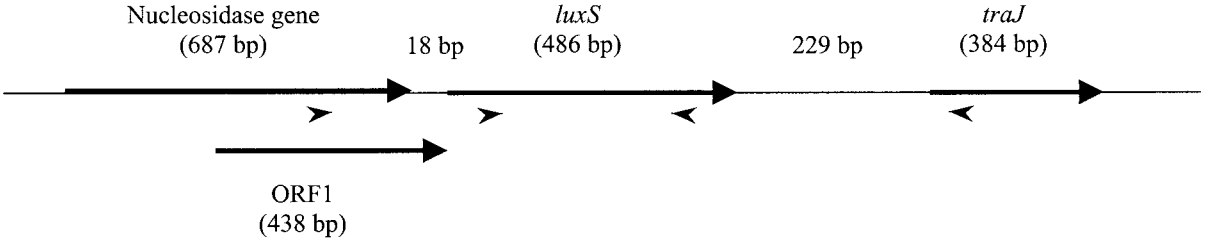

FIG. 2. Schematic arrangement of the *luxS* ORF and those upstream (nucleosidase) and downstream (*traJ*) of it. The ORFs themselves are indicated by arrows, with the direction of the arrow indicating the direction of transcription. The genes for LuxS and the nucleosidase are cotranscribed and encompass an additional ORF in a different reading frame. The primers used for RT-PCR are indicated with arrowheads.

TABLE 2. Identification of *P. gingivalis* ORFs that share significant sequence identity[a] with Lux proteins of *V. harveyi*

Lux protein[b]	Function	Identity of *P. gingivalis* ORF[c]
LuxS (AAD17292)	AI-2 synthase	49/167 (29)
LuxQ (AA20838)	AI-2 sensor	107/411 (26)
LuxO (S49540)	Response regulator	167/469 (35)
LuxN (S37350)	AI-1 sensor	94/356 (26)

[a] *e* value, $<10^{-10}$.
[b] GenBank accession numbers appear in parentheses.
[c] Each value is the number of identical amino acid residues/the total number of amino acid residues. Values in parentheses are percentages.

the intergenic region overlapping both the LuxS and 5-methylthioadenosine nucleosidase–*S*-adenosylhomocysteine nucleosidase genes (Fig. 2). Downstream (229 bp) of *luxS* is an ORF with homology to the *traJ* gene of *Bacteroides thetaiotaomicron* that comprises the transfer region of a conjugative transposon. RT-PCR revealed that this gene is not cotranscribed with *luxS* (data not shown).

P. gingivalis also possesses putative ORFs that demonstrate significant sequence identity with *V. harveyi* proteins LuxN and LuxQ, the sensor kinases of signaling systems 1 and 2, respectively, and with LuxO (Table 2). Lux O of *V. harveyi* is thought to be a negative regulator of luminescence and to integrate sensory inputs from AI-1 and AI-2 signaling systems. The presence of homologues to *luxS* and *luxQ* on the *P. gingivalis* chromosome suggests that *P. gingivalis* possesses a signaling system similar to the AI-2 circuit in *V. harveyi.* Some differences between the AI-2 systems in *P. gingivalis* and *V. harveyi* can be expected, however, as homologues of LuxP (the primary AI-2 sensor) and LuxU (the phosphorelay protein for AI-2 and AI-1) were not present in *P. gingivalis*. The absence of homologues of LuxU and of LuxLM indicates that *P. gingivalis* does not possess a functional homoserine lactone-dependent signaling pathway similar to the *V. harveyi* AI-1 circuit. In addition, *P. gingivalis* does not appear to possess a quorum-sensing pathway similar to either the AI-1 or the AI-2 circuit of *V. fischeri,* as homologues of LuxI, LuxR, and AinS were not detected.

AI-2 activity in *P. gingivalis*. To test whether *P. gingivalis* exhibits functional signaling activity related to the *V. harveyi* AI-2 system, cell-free culture media from late-log-phase cultures of *P. gingivalis* 33277 and PLM1 (in which *luxS* was insertionally inactivated) were assayed for induction of luminescence in *V. harveyi* BB170. After 3 h of incubation, conditioned medium from *P. gingivalis* 33277 induced luminescence by 120-fold in BB170 (Fig. 3). In contrast, luminescence induced by the PLM1 mutant was less than twofold higher than the level induced by media only (Fig. 3). Comparable results were found after 4 h of incubation (Fig. 3). Plate counts showed that the growth of the *V. harveyi* reporter was similar whether stimulated with supernatant from the parent or with that from the mutant (data not shown). The level of induction by *P. gingivalis* was almost one log unit lower than that induced by control *V. harveyi* culture supernatants. This may indicate that the AI molecule of *P. gingivalis* differs structurally from that of *V. harveyi*, resulting in less efficient recognition. Alternatively, or additionally, there may be less AI in *P. gingivalis* culture supernatants.

Environmental control of *luxS* gene expression in *P. gingivalis*. Growth phase-dependent LuxS expression is a feature of the AI-2 systems of bacteria other than *P. gingivalis* (12, 40, 41). To investigate whether *luxS* expression in *P. gingivalis* is controlled by environmental cues, *luxS* transcripts were examined by RT-PCR using RNA from cells grown under various conditions (Fig. 4). As a control, the *P. gingivalis fimA* gene was also amplified under the same conditions, using primers described previously (44). The levels of *luxS* mRNA (quantitated by NIH Image software) in *P. gingivalis* grown to late log phase were over three times higher than those in the same strain grown to early log phase. The NaCl concentration of the growth media also affected expression of *luxS* mRNA which was highest at the normal osmolarity of TSB (80 mM, approximately half physiological). At NaCl concentrations of 160 and

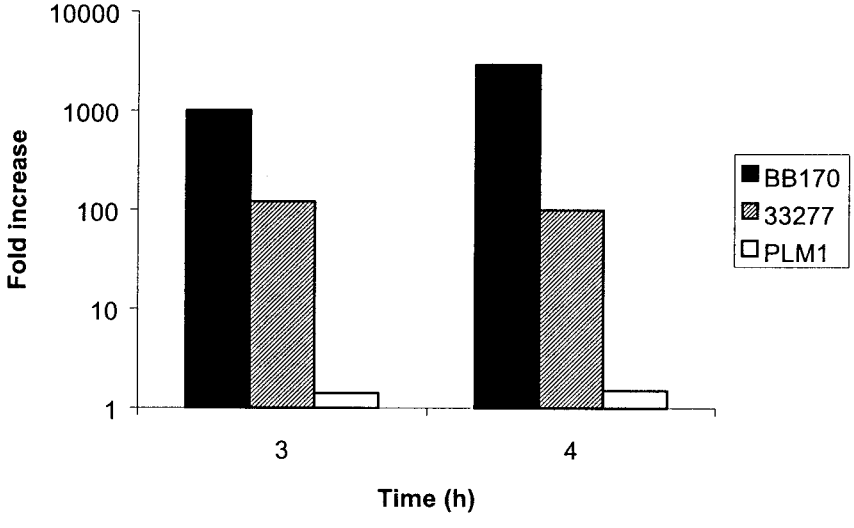

FIG. 3. Induction of *V. harveyi* BB170 luminescence by cell-free supernatants of 33277 (parent strain), PLM1 (mutant), and *V. harveyi* BB170. Activation was measured by comparing the level of luminescence induced by the test strain to that induced by sterile medium.

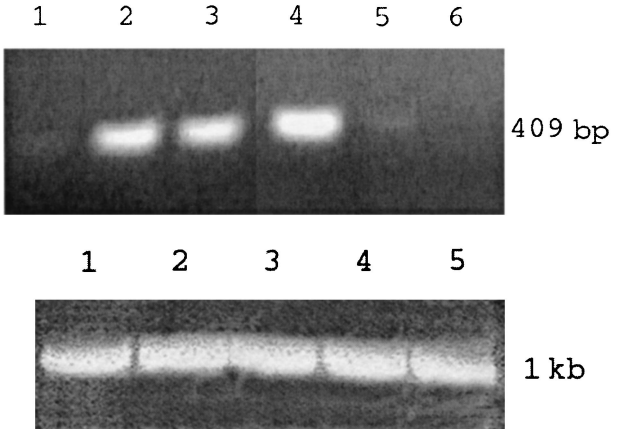

FIG. 4. RT-PCR using RNA from cells grown under various conditions. (Top) RT-PCR of mRNA of *luxS* of *P. gingivalis* grown under various conditions. Lanes: 1, early log growth; 2, late log growth; 3, late log growth at 34°C; 4, late log growth at 80 mM NaCl; 5, late log growth at 160 mM NaCl; 6, late log growth at 240 mM NaCl. (Bottom) RT-PCR of mRNA of *fimA* of *P. gingivalis* grown under various conditions (as a control for total RNA levels). Lanes: 1, early log growth; 2, late log growth; 3, late log growth at 80 mM NaCl; 4, late log growth at 160 mM NaCl; 5, late log growth at 240 mM NaCl. Note that no constitutively expressed *P. gingivalis* gene that would be a more appropriate control has been reported and that *fimA* mRNA levels vary according to growth temperature (44).

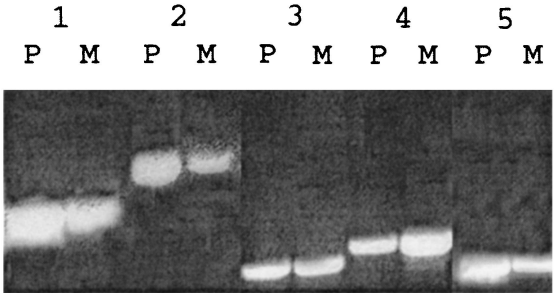

FIG. 5. RT-PCR to confirm differential expression of genes of parent strain 33277 (P) and mutant PLM1 (M). Lanes: 1, excinuclease ABC homologue; 2, HemR; 3, RgpA; 4, *P. fluorescens* hemin acquisition protein homologue; 5, TonB homologue.

240 mM, *luxS* expression was significantly reduced and absent, respectively (Fig. 4). Expression of *luxS* was affected neither by growth temperature nor by pH, which was tested between pH 6.5 and 8.5 (data not shown).

Functional role of *P. gingivalis* LuxS. *P. gingivalis* can form a mixed-species biofilm with *S. gordonii* and can invade gingival epithelial cells. Both processes could require that *P. gingivalis* assess the local environment through a quorum-sensing system. Therefore, we examined wild-type and mutant strains for biofilm formation in conjunction with *S. gordonii* and for invasion of gingival epithelial cells. However, no differences in either biofilm structure or invasion efficiency were observed (data not shown). To determine if *luxS* plays a role in regulating gene expression in *P. gingivalis*, parent and mutant strains were analyzed by DD-PCR. Five amplification products that were differentially present or absent in parent and mutant samples were sequenced and identified by a BLAST search of the GenBank database (http://www.ncbi.nlm.nih.gov). As shown in Table 3, genes encoding two previously described *P. gingivalis* proteins were identified: the expression of the gene encoding hemin-regulated protein (HemR) (22) was reduced in the *luxS* knockout strain PLM1, whereas that of the gene encoding arginine-specific protease (RgpA) (31) was increased. In addition, a gene homologous to an outer membrane hemin acquisition protein of *Pseudomonas fluorescens* (19) was up-regulated in PML1; while the expression of genes homologous to those encoding TonB (33) and excinuclease ABC (6) was down-regulated. RT-PCR confirmed the differential expression of these genes with the exception of *rgpA* (Fig. 5). Discrepancies between the results obtained by DD-PCR and RT-PCR are frequently reported and may be due to differences in the dynamic ranges of the two techniques (11). Moreover, in this case, a further degree of variability could result from the inability of the RT-PCR primers to distinguish between *rgpA* and the closely related gene *kgp*, which encodes a lysine-specific protease.

DISCUSSION

P. gingivalis will encounter fluctuations in environmental conditions as it traverses the oral fluids, colonizes oral surfaces, and interfaces with oral tissues. Concomitantly, cell density changes will occur as the organism establishes a subgingival infection and thrives in the multispecies biofilm that exists in the periodontal pocket. Therefore, it is possible that *P. gingivalis* might monitor these diverse environments by quorum sensing. A BLAST search for *lux* genes in the *P. gingivalis* genomic sequence database identified ORFs relating to *V. harveyi* signaling system 2, namely, LuxS, LuxQ, and LuxO.

TABLE 3. Characterization of genes differentially regulated in *P. gingivalis* PLM1

Gene product (GenBank accession no.)	Homologous protein[a] (reference)	Function	Expression in *luxS* mutant
HemR (AAC44980)	*P. gingivalis* hemin-regulated outer membrane protein (22)	TonB-dependent receptor	Absent
Excinuclease ABC homologue (P14951)	*B. subtilis* excision nuclease (6)	DNA repair	Absent
Outer membrane protein (BAA88494)	*P. fluorescens* heme acquisition protein (19)	Hemin uptake	Present
RgpA (A55426)	*P. gingivalis* arginine-specific protease (31)	Multiple, including provision of hemin by degradation of hemin-sequestering proteins	Present
TonB homologue (E82955)	*Pseudomonas* siderophore acquisition protein (33)	Energy transducer	Absent

[a] Determined by comparison with sequences of the GenBank database.

Interestingly, homologues of LuxLM, which are required for the *V. harveyi* AI-1 pathway, were not found in *P. gingivalis*, and neither were homologues of the *V. fischeri* quorum-sensing components LuxI, LuxR, and AinS. Thus, of the described quorum-sensing systems, a pathway related to *V. harveyi* signaling system 2 would appear to be the only potentially operative circuit in *P. gingivalis*. Homologues of *V. harveyi* signaling system 2 components LuxP, LuxU, and LuxR were not detected in *P. gingivalis*. This could indicate that there are mechanistic differences in the signaling circuits between the two species or that the *P. gingivalis* functional equivalents have diverged to the extent that they no longer exhibit significant sequence homology with *V. harveyi*.

The flanking gene arrangements vary among species in which *luxS* has been identified, and no operon arrangements have been reported (12). In *P. gingivalis,* the *luxS* gene was cotranscribed with an upstream nucleosidase homologue. In addition, this mRNA will also encompass a third ORF in a different reading frame. The significance of this arrangement, which appears, thus far, to be unique to *P. gingivalis*, remains to be investigated. Downstream of *luxS*, and not cotranscribed, is a gene homologous to the transfer region of a *Bacteroides* conjugative transposon. Similarly, *Yersinia pestis* has a gene for a transposase approximately 200 bp upstream of a *luxS* gene. This design may have implications for horizontal transfer of the *luxS* gene, which is present in at least 30 species.

As some components of the *V. harveyi* AI-2 circuit may not be present in *P. gingivalis*, we initiated a series of experiments to investigate whether the *P. gingivalis luxS* gene is expressed and is functional. Analysis of mRNA by RT-PCR demonstrated that the *luxS* gene is transcribed in *P. gingivalis*. Moreover, conditioned culture medium of *P. gingivalis* induced bioluminescence in *V. harveyi*, indicating that the signal molecule produced by *P. gingivalis* is recognized by the AI-2 receptor of *V. harveyi*. However, the level of bioluminescence induced by *P. gingivalis* was significantly lower than the control levels obtained using conditioned broth from overnight cultures of *V. harveyi*. This suggests that the *P. gingivalis* signaling molecule may be functionally and structurally distinct from that of *V. harveyi*. Consistent with this, conditioned broth from *Actinobacillus actinomycetemcomitans*, which possesses a *luxS* homologue exhibiting significantly greater similarity to the *V. harveyi luxS* gene, induced bioluminescence comparable to that of the *V. harveyi* control (D. R. Demuth, unpublished data).

The production of LuxS-dependent AI-2 in other gram-negative organisms, such as *E. coli*, *S. enterica* serovar Typhimurium, and *H. pylori,* is regulated by metabolic conditions and environmental stimuli such as growth phase, pH, and osmolarity (12, 41). Similarly, *P. gingivalis luxS* was expressed at higher levels as the cell density increased, during log-phase growth, or when the osmolarity of the growth medium was reduced to approximately half of the physiological level. Such conditions of low osmolarity may be relevant to growth in the oral cavity since saliva is a very hypotonic fluid. However, unlike the situation with *S. enterica* serovar Typhimurium, changes in pH did not appear to regulate *luxS* expression in *P. gingivalis*. This may simply reflect the different environmental conditions encountered by organisms indigenous to the human oral cavity and gastrointestinal tract.

Quorum-sensing systems serve to regulate a variety of physiological responses (3, 13), the production of light being only one example. Given that *P. gingivalis* is not capable of bioluminescence, we embarked on a series of studies to define the role of *luxS* in *P. gingivalis*. Both biofilm formation and intracellular invasive properties are associated with quorum sensing in other species (8, 39); however, the *P. gingivalis luxS* mutant was not impaired in these activities in our assay systems. Differential display of mRNA from parent and mutant strains revealed a potential novel role for *P. gingivalis* LuxS in regulating genes involved in the acquisition of hemin. The loss of LuxS activity resulted in up-regulation of a putative hemin-acquisition protein and the arginine-specific protease, RgpA. Interestingly, the RgpA protease has been suggested to play a role in increasing hemin availability by degradation of host hemin-sequestering proteins (32, 36). The mutant strain also demonstrated down-regulation of a TonB homologue and of HemR, a *P. gingivalis* outer membrane protein that is negatively regulated by hemin and is TonB-dependent. In *P. gingivalis*, hemin levels can control expression of various virulence-associated genes (14, 27, 37). In addition, the proteolytic and hemagglutination activities in *P. gingivalis* are intricately interconnected and affect levels of available hemin (18, 24, 28, 35). This raises the possibility that LuxS is a component of a complex virulence-associated cascade that regulates iron acquisition, which, in turn, influences the expression of specific virulence genes of *P. gingivalis*. Such an ability to potentially modulate virulence factors through iron acquisition mechanisms sets apart the role of LuxS in *P. gingivalis* from any other species with a known *V. harveyi*-like AI-2 signaling pathway.

Inactivation of *luxS* also resulted in down-regulation of a putative ABC excinuclease, a DNA repair enzyme that catalyzes the excision of UV-damaged nucleotide segments (6). Therefore, the *luxS* gene may also play a role in stress response in *P. gingivalis*.

In summary, we have identified in *P. gingivalis* a functional *luxS*-dependent signaling pathway that can activate a quorum-sensing circuit in *V. harveyi*. This system is regulated in response to specific growth and environmental stimuli and may play a role in regulating the expression of specific genes involved in hemin acquisition by *P. gingivalis*.

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