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Microarray Analysis of Quorum-Sensing-Regulated Genes in *Porphyromonas gingivalis*

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Quorum sensing is a phenomenon defined as gene regulation in response to cell density that regulates various functions in bacteria. The periodontopathogen *Porphyromonas gingivalis* possesses a *luxS* gene homologue that may encode a quorum-sensing system. In order to identify genes of *P. gingivalis* that are regulated by *luxS*, gene expression analysis was done using microarrays and RNA samples from the W83 wild-type strain and an isogenic *luxS* mutant, LY2001. The results indicated that 17 open reading frames (ORFs) in LY2001 are upregulated and two are downregulated. Real-time PCR was done to confirm the microarray results. Among the upregulated ORFs is a group of stress-related genes, including *htrA*, *clpB*, *groEL*, *dnaK*, and the F subunit of alkyl hydroperoxide reductase. This suggested that *luxS* is involved in stress gene regulation in *P. gingivalis*. Stress response experiments, including high-temperature survival, resistance to hydrogen peroxide (H_2O_2), and survival during exposure to low and high pH, were performed on the *P. gingivalis* wild-type and LY2001 strains. LY2001 had a significantly higher survival rate than did W83 when stressed at 50°C. No difference was found at pH 5, but LY2001 had increased survival compared to W83 at pH 9. LY2001 also survived better than W83 when stressed with 0.35 mM H_2O_2. These results suggest that *luxS* might be involved in promoting survival of *P. gingivalis* in the host by regulating its response to host-induced stresses such as temperature, H_2O_2, and pH.

Quorum sensing was first discovered in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* (3, 4, 37, 38). In response to quorum sensing, these bacteria produce small molecules, called autoinducers, which are secreted into the environment and diffuse into other cells. When the concentration of these autoinducers reaches a threshold level, they bind to a response regulator protein and induce expression of the luciferase gene cluster. There are two classes of quorum-sensing systems in *Vibrio harveyi*. One utilizes an acylhomoserine lactone as the signal, and the other utilizes a *luxS*-encoded signal. Many other bacteria have been found to use autoinducers to regulate various cell functions associated with secondary metabolism, such as antibiotic production (14), conjugation in *Enterococcus faecalis* (16), fruiting body development in *Myxococcus xanthus* (17), biofilm formation (22, 36), and virulence gene expression in *Vibrio cholerae* and *Vibrio vulnificus* (29, 30), *Pseudomonas aeruginosa* (2, 39), *Staphylococcus aureus* (26), and *Escherichia coli* O157:H7 (1, 45).

LuxS functions as the enzyme for producing the autoinducer AI-2 (42). AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps. SAM is used as a methyl donor in the cell, and the methyl group from SAM is transferred to its substrates by SAM-dependent methyltransferases and produces *S*-adenosylhomocysteine (SAH). SAH functions as a potent inhibitor of SAM-dependent methyltransferases and thus is a toxic intermediate (55). Therefore, bacteria rapidly degrade SAH via Pfs. Pfs is a nucleosidase that removes adenine from SAH and produces *S*-ribosylhomocysteine (SRH) in the reaction. Last, SRH is converted to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) by the enzyme LuxS; DPD is thought to give rise to AI-2 by spontaneous cyclization and reaction with borate (9, 42). The homocysteine is then methylated to generate methionine, which can be used by MetK to produce SAM and thus reenter the pathway (9, 42).

The *luxS*-encoded signal AI-2 has been found to regulate various genes in bacteria. For example, in *E. coli*, a total of 242 genes were found to be regulated by the *luxS*-encoded quorum-sensing system (13). These included genes responsible for cell division, DNA processing, virulence, biofilm formation, and motility. In *P. aeruginosa*, 616 genes were reported to be regulated by quorum sensing, including genes involved in nitrogen metabolism, virulence, biofilm formation, central intermediary metabolism, antibiotic resistance, cell division, chaperons and heat shock proteins, and protein secretion (52). This suggests that quorum sensing plays an important role in the physiology of these organisms and their adaptation to their environment. Interestingly, only one operon appears to be regulated by the AI-2 system in *Salmonella enterica* serovar Typhimurium: the *lsr* operon encodes functions similar to those of the ribose transporter (49). The function of the operon is suggested to be transport of AI-2 from the environment into the cells. There are no other genes reported to be regulated by AI-2 in this bacterium.

Porphyromonas gingivalis is a gram-negative, anaerobic, black-pigmented bacterium, which is considered to be a primary etiologic agent of certain periodontal diseases (44). Periodontal diseases, including gingivitis and periodontitis, are potentially serious infections that can lead to tooth loss and have

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been linked to systemic diseases such as cardiovascular disease and adverse pregnancy outcomes (5, 11, 20, 40). Based on genomic analysis and the inability to identify an acylhomoserine lactone signal in culture liquors of *P. gingivalis* (19), this organism does not appear to have an acylhomoserine lactone-regulated quorum-sensing system. However, it does have a *luxS*-encoded quorum-sensing system, as reported in previous studies (6, 10, 19) and shown by our own results described in this study. Two methods have previously been used to study *luxS*-regulated genes in *P. gingivalis* W50 (6) and 33277 (10), i.e., differential display PCR and enzyme assays. However, there are discrepancies between the results obtained by these two methods. For example, using a *luxS* mutant, *rgpA*, encoding the Arg-gingipain, was found to be upregulated by the differential display PCR method but downregulated when the enzyme assay was used.

With the availability of microarrays, it is now feasible to measure transcript levels for thousands of genes simultaneously. Schena et al. (43) first described the use of cDNA microarrays to monitor the parallel expression of 45 *Arabidopsis thaliana* genes. Microarray technology has since been used to monitor gene expression in many organisms (50, 54), including quorum sensing in such species as *V. cholerae* (56), *E. coli* (13), and *Pseudomonas aeruginosa* (52). With the recent availability of *P. gingivalis* cDNA microarrays, it became feasible to study gene regulation at the genomic level. Chen et al. used the *P. gingivalis* microarray slides for comparing the whole genomes of virulent and avirulent strains (8). The samples from each strain were labeled with either Cy3 or Cy5, and the data were normalized using statistics for microarray analysis. They found that expression of at least 7% of the genes in the genome was very low or absent in the avirulent strain. Verification of the array results by PCR indicated that several of the disparate genes were either absent from or variant in the avirulent strain. This study confirmed the quality of the slides for other research groups. The purpose of our study was to use the same microarray techniques to identify genes of *P. gingivalis* that are regulated by quorum sensing and to establish the possible functions of these genes.

MATERIALS AND METHODS

Bacterial and cell culture conditions. *P. gingivalis* strain W83 was grown on blood agar plates (BAPs) consisting of 4% Trypticase soy agar (Difco Laboratories, Detroit, MI), 0.5% yeast extract (Difco), 5% sheep blood (Lampire Biological Laboratories, Pipersville, PA), 5 μg of hemin/ml (Sigma Chemical Co., St. Louis, MO), and 1 μg/ml of vitamin K_1 (Sigma). *P. gingivalis* was also cultured in Trypticase soy broth (TSB) supplemented with 5 μg of hemin and 1 μg of vitamin K_1 per ml. Cells were grown and maintained at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. The *P. gingivalis* W83 *luxS* mutant strain LY2001 was maintained as described for the wild-type strain, except that 5 μg/ml of clindamycin (Sigma) was added to the medium.

Bioluminescence assay. Bioluminescence was measured using previously described methods (47). Briefly, the W83 and LY2001 strains were cultured in supplemented TSB and autoinducer bioassay (AB) medium (47) at a ratio of 1:1 (TSB:AB). The optical density at 600 nm (OD_{600}) of these cultures was measured at regular intervals until it reached 1.0, at which time 4-ml aliquots were taken and passed through 0.2-μm Tuffryn membranes (Gelman laboratory, Pall Corporation, Ann Arbor, MI) prior to storage at −20°C. The day before the assay, cultures of *V. harveyi* indicator strains BB886 (AI-1 indicator strain, BB120 *luxP*::Tn*5*) and BB170 (AI-2 indicator strain, BB120 *luxN*::Tn*5*) (3, 4) were incubated in autoinducer bioassay medium at 30°C for 16 h with agitation. The next day, BB886 and BB170 were diluted 1:2,000 using AB medium. Cell-free culture fluids from the *P. gingivalis* strains were added to a final volume of 10% and incubated with the diluted indicator strain for either AI-1 or AI-2 detection. TSB:AB (1:1) was included as a negative control, and cell-free culture fluids from BB170 were included as a positive control. Chemiluminescence was detected with a Wallac Victor^3 1420 multilabel counter in chemiluminescence mode (Perkin-Elmer, Boston, MA). Chemiluminescence was measured using the samples taken from the previously collected culture fluids, and the data were recorded every 15 min for 11 h.

Mutant construction. PCR was used to generate a 223-bp internal fragment of the *luxS* gene using the primers luxSF1 (5′ AAAAGGATCCCGAATGAAAG AGCCCAATC 3′) and luxSB1 (5′ AAACTGCAGTCTGATGAAGCGGAA AGTC 3′) (underlined sequences indicate BamHI and PstI sites for luxSF1 and luxSB1, respectively). The DNA fragment was then cloned into a *P. gingivalis* suicide vector, pVA3000, using the BamHI and PstI sites. *E. coli* strain S17-1 was used to deliver the vector containing the internal fragment into *P. gingivalis* W83 by conjugation on blood agar. Transconjugants were selected on plates containing 5-μg/ml clindamycin. Homologous recombination of the vector with the chromosome resulted in two copies of the gene, truncated at either the 5′ or the 3′ end. Southern blot analysis was done to confirm the creation of the *luxS* mutant. An ECL detection kit was used according to the manufacturer's protocol for Southern blotting (Amersham Life Science, Little Chalfont, Bucks, England).

***P. gingivalis* microarrays.** *P. gingivalis* microarrays were manufactured and kindly provided by The Institute for Genomic Research (TIGR). PCR amplicons spotted on glass slides (CMT-GAPS; Corning, Corning, N.Y.) were generated from open reading frames (ORFs) predicted by TIGR GLIMMER automated annotation software for the genome sequence of strain W83. Each amplicon for each ORF was spotted at least twice by a microarray robot (Intelligent Automation Systems, Cambridge, Mass.). The amplicons of the represented 2,558 ORFs identified in the genome had mean and median sizes of 486 and 461 bp, respectively. However, due to the number of repeat elements such as insertion sequences, only 1,990 ORFs were unique. The array slides are organized as follows: 12-block rows by 2-block column by 8 rows/block by 32 columns/block = 6,144 spots. *P. gingivalis* genomic DNA was spotted four times on the slides as the positive controls, and Cy3-prelabeled *Arabidopsis* DNA was spotted once on the slide as the negative control. Detailed array information can be viewed at http://www.brop.org.

RNA extraction, cDNA probe generation, and microarray experiments. *P. gingivalis* W83 and the *luxS* mutant LY2001 were cultured in TSB, and then samples were collected at mid-exponential growth phase. The samples were immediately mixed with 2 volumes of RNA Protect bacterial reagent (Qiagen, Valencia, CA) and vortexed for 5 s. The mixtures were centrifuged at room temperature for 10 min at 5,000 × *g*, and the supernatant was discarded. The RNA extraction was done using the RNeasy minikit (Qiagen) following the manufacturer's protocol. During the RNA extraction, DNase was used to remove any DNA contamination (RNase-free DNase set; Qiagen).

cDNA was generated using random primers for reverse transcription (Invitrogen Life Technologies, Carlsbad, CA). The primers were annealed at 70°C for 10 min, followed by snap-freezing in a dry ice-ethanol bath for 30 s and then centrifugation for 1 min. The reaction mixture (Superscript II buffer, 0.1 M dithiothreitol, and amino acid-deoxynucleoside triphosphate mix) was then incubated with Superscript II reverse transcriptase (Invitrogen) at 42°C overnight. Residual RNA was removed by alkaline treatment followed by neutralization, and cDNA was purified with a QIAquick PCR purification kit (Qiagen). Purified cDNAs from the W83 and LY2001 strains were each labeled with indocarbocyanine (Cy3)-dUTP and indodicarbocyanine (Cy5)-dUTP (Amersham Biosciences, Piscataway, NJ) and were processed using a dye-swapping design. A total of six slides were used. Wild-type cDNA was labeled with Cy3 for three slides and with Cy5 for the remaining three slides. This strategy was used to avoid any differences caused by the labeling activity of Cy3 versus that of Cy5. The labeling mixtures were cleaned again using a QIAquick PCR purification kit (Qiagen). Equal amounts of labeled cDNA from the W83 and LY2001 strains were used to hybridize the microarray slides. Hybridization was carried out at 42°C for 18 h in the dark. After hybridization, the slides were washed and scanned using a GenePix scanner (Axon Instruments Inc., Union City, CA) at 532 nm (Cy3 channel) and 635 nm (Cy5 channel), and the images were stored on disks.

Microarray data analysis. Data from six individual experiments were normalized and then analyzed using Spotfinder software (The Institute for Genomic Research, www.tigr.org). The data points with a density below 100,000 were discarded for analysis according to the manufacturer's suggestion. A cutoff ratio of 1.5:1 was used on all the slides. SAM software (Significant Analysis of Microarray, version 1.15; Stanford University, CA) was used to test statistically significant results from the microarray experiments. This statistical analysis in-

TABLE 1. Primer list for real-time PCR[a]

Gene locus	Primer for real-time PCR	Product size (bp)
PG0520	Forward: 5′ CGGAGCCGGAAACAAAGAAGG 3′ Backward: 5′ GCAGCACCCACGTAAAGAACAG 3′	150
PG0538	Forward: 5′ TCTGGGAGGGCAGTACACCTATTCGC 3′ Backward: 5′ AGGCGTCCAGCGTTGTCCCTCG 3′	76
PG0593	Forward: 5′ TCAGCAGTACTTCGATCCGTTC 3′ Backward: 5′ GCCTACTACCTGCCGTGTTTG 3′	85
PG1102	Forward: 5′ TGGCTTTTGGTTGGGACAGAG 3′ Backward: 5′ GAGAGGAGCGTAGCGGAAATC 3′	145
PG1118	Forward: 5′ GCGAGTATCAGGAGAAGTTCAG 3′ Backward: 5′ CACCACGGAATAAGGCTTGC 3′	119
PG1208	Forward: 5′ GTACTTCAGGGTGAGCGTTCTTTGG 3′ Backward: 5′ GCCGTTACATTCAGGATACCGTTGG 3′	146
PG1279	Forward: 5′ GAAGAGGCAGGTCTCGAATTTG 3′ Backward: 5′ TGCGGATAATGATAGCGTTAGC 3′	97
PG1280	Forward: 5′ TGCCGATCAGCTCACAATCATC 3′ Backward: 5′ GCTTGGAGGGGCGATATTCTTC 3′	147
PG1798	Forward: 5′ CCAACGGCATCACACATCTG 3′ Backward: 5′ CGACACCCATTTCCACCTTATC 3′	93
PG16S	Forward: 5′ AGGAACTCCGATTGCGAAGG 3′ Backward: 5′ TCGTTTACTGCGTGGACTACC 3′	108

[a] "Gene locus" refers to the TIGR locus number for the gene or ORF in the *P. gingivalis* W83 database, except for the PG16S gene, which has multiple loci on the chromosome and was not assigned a locus designation. Detailed information for each gene or ORF can be found at http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpg.

volved factoring the change in expression of each gene relative to the standard deviation of all replicates for that gene. Therefore, genes with a small change were not discounted if the ratios were consistent among repeats, thus effectively reducing false negatives. False positives were also avoided when genes had poor reproducibility between replicates. Thus, this method of statistical analysis maximized both the quantity of genes found and the reliability of the results. Spot intensities for all channels were input into the SAM program as paired, unlogged values. Delta values were chosen according to the lowest false-discovery rate (in the SAM program, the lowest false-discovery rate is the *P* value), which for this study was 4.7%. Several other studies have used a 1.5-fold change in expression using microarrays as the cutoff (41, 53), and Hughes et al. reported previously that a less-than-twofold change in relative transcript level can be biologically significant (25). Thus, in our assay, we used the same fold change for the upregulated and downregulated genes and considered the genes with expression ratios of $\geq$1.5 as being biologically significant.

RT-PCR verification and data analysis. Nine genes were selected for verification by real-time PCR (RT-PCR). For each of the genes tested, primers (Table 1) were designed using Beacon Design software (Premier Biosoft International, Palo Alto, CA) to amplify products from 75 to 150 bp. A standard curve was created using serial dilutions of the gel-purified DNA fragment of each gene, and a *P. gingivalis* 16S rRNA gene fragment was used as an internal control. Reverse transcription using Superscript II reverse transcriptase (Invitrogen) was performed. The cDNA was used as a template for RT-PCR. In every run of RT-PCR, two standard curves were created, one for 16S rRNA and one for the target gene. The unknown cDNA samples from the wild-type strain W83 and mutant LY2001 were compared to the standard curve to calculate the starting quantity in each sample. The ratio from real-time PCR for each target gene was calculated for both samples.

Real-time PCR with complemented supernatant. The supernatant of wild-type W83 culture, grown in TSB, was collected by centrifugation when the OD_{600} reached 1.0. It was passed through a 0.2-μm Tuffryn membrane (Gelman laboratory) and stored at −20°C. The RNA was isolated at the same time as described above and stored frozen. Strain LY2001 was grown in TSB until it reached an OD_{600} of 0.8. The cell-free supernatant from the wild type was then added to the mutant culture at a final concentration of 5%. Once the culture reached an OD_{600} of 1.0, RNA samples were collected. Reverse transcription-PCR was done followed by real-time PCR to compare the gene expression difference between the wild type and the mutant complemented with culture liquor from the wild-type strain.

Stress experiments. The W83 and LY2001 strains were cultured in supplemented TSB until they reached the mid-exponential phase of growth.

(i) Temperature stress. One milliliter each of the W83 and LY2001 cultures was centrifuged at 15,600 × *g* for 2 min, and the cell pellets were washed using 1 ml of 0.1 M glycine (pH 7). The cell pellets were then resuspended in 1 ml of the same buffer and incubated at 50°C. Aliquots were removed at 0, 2, 4, 6, 8, and 10 min. Decimal dilutions were made and plated onto blood agar.

(ii) Hydrogen peroxide. Ten milliliters each of W83 and LY2001 cultures was centrifuged at 4,000 × *g* for 10 min at 4°C and then washed with 10 ml of 0.1 M glycine buffer (pH 7) for 10 min. The bacterial pellets were then resuspended in 0.1 mM glycine buffer containing 0.35 mM H_2O_2. Aliquots were taken at 15 min, 30 min, 1 h, and 2 h. Decimal dilutions were made and plated onto BAPs.

(iii) pH stress. Ten milliliters of W83 and LY2001 cultures was centrifuged at 4,000 × *g* for 10 min at 4°C and then washed with 10 ml of 0.1 M glycine buffer (pH 7) for 10 min. The bacterial pellets then were resuspended in either pH 5 or pH 9 0.1 M glycine buffer. Aliquots were taken at different time intervals. Decimal dilutions were made and plated onto BAPs.

Plates from each of the above experiments were incubated in an anaerobic chamber for 7 to 10 days, and CFU were enumerated. The percent survival of the bacteria was calculated by comparison to the inoculum.

(iv) Statistical analysis. Data from the above experiments were analyzed using the Student *t* test. $t \leq 0.05$ is considered statistically significant.

RESULTS

Bioluminescence assay of wild-type W83 and *luxS* mutant LY2001. W83 and LY2001 strains were grown to an OD_{600} of 1.0 in TSB:AB medium, and the filtrates were collected. A luminescence assay was performed as previously described using the cell-free culture fluids. No luminescence was detected from the AI-1 indicator strain BB886 (results not shown), suggesting that the W83 strain does not produce an acylhomoserine lactone signal (AI-1). However, wild-type W83 cell-free culture fluids induced a luminescence signal that was 56-fold higher than that of the negative control when using the AI-2 detector strain BB170 (results not shown). The mutant strain induced a luminescence signal sevenfold greater than that of the negative control, indicating that the mutant had a significant reduction of signal induction compared to the wild-type strain.

Microarray identification of *luxS*-regulated genes or open reading frames. With a cutoff ratio of 1.5 and a false-discovery

TABLE 2. Genes or ORFs differently regulated in the *luxS* mutant[a]

Gene name	Locus in genome	Avg fold difference ± SD	Putative identification
Genes upregulated			
Genes related to stress response			
Chaperonin, 60 kDa, GroEL	PG0520	2.26 ± 0.23	Chaperonin, 60 kDa, GroEL
HtrA protein	PG0593	2.01 ± 0.36	HtrA protein
Alkyl hydroperoxide reductase, F subunit	PG0619	1.85 ± 0.35	Alkyl hydroperoxide reductase, F subunit
ClpB protein	PG1118	6.06 ± 1.65	ClpB protein
DnaK protein	PG1208	4.54 ± 0.72	DnaK protein
Regulatory gene			
RNA polymerase sigma-70 factor, ECF subfamily	PG0985	2 ± 0.41	RNA polymerase sigma-70 factor, ECF subfamily
Putative antigens			
Outer membrane efflux protein	PG0538	1.72 ± 0.13	Outer membrane efflux protein, previously submitted to GenBank by Ross et al. as immunoreactive 52-kDa antigen PG41
Immunoreactive 46-kDa antigen PG99	PG1798	1.65 ± 0.17	Immunoreactive 46-kDa antigen PG99
Hypothetical proteins			
Hypothetical protein	PG0611	1.55 ± 0.1	Hypothetical protein
Hypothetical protein	PG0614	1.58 ± 0.18	Hypothetical protein
Hypothetical protein	PG1102	2.02 ± 0.24	Hypothetical protein
Hypothetical protein	PG1795	1.69 ± 0.21	Hypothetical protein
Hypothetical protein	PG2225	1.71 ± 0.26	Conserved hypothetical protein
Genes of unrelated functions			
Cytochrome *d* ubiquinol oxidase, subunit I	PG0900	1.83 ± 0.31	Cytochrome *d* ubiquinol oxidase, subunit I
ABC transporter, permease protein, putative	PG1664	1.85 ± 0.29	ABC transporter, permease protein, putative, transport and binding proteins: unknown substrate
O-sialoglycoprotein endopeptidase	PG1724	1.63 ± 0.24	O-sialoglycoprotein endopeptidase
Putative epithelial cell attachment protein	PG2224	2.4 ± 0.5	Putative epithelial cell attachment protein
Genes downregulated			
D-Isomer specific 2-hydroxyacid dehydrogenase family protein	PG1279	0.59 ± 0.08	Unknown function: enzymes of unknown specificity
Conserved hypothetical protein	PG1280	0.58 ± 0.07	Hypothetical proteins: conserved

[a] The results of six slides are shown. SD indicates the standard deviation from the six slides for that ORF. The number in the "Average fold difference ± SD" column indicates the expression of that gene in LY2001 versus W83. The cutoff ratio for the fold difference was 1.5. The *P* value for these genes is 0.047. The putative identification from the database for that gene or ORF is also shown.

rate set at 4.7% (*P* value), the microarray experiments indicated that, in the *luxS* mutant, 17 genes or ORFs were upregulated, while two were downregulated (Table 2). These genes were categorized into the following functions: stress response, regulation, putative antigens, hypothetical proteins, and genes of unrelated function. The *clpB* gene (PG1118, TIGR *P. gingivalis* database) exhibited the greatest change in expression between the wild type and the *luxS* mutant. ClpB belongs to the hsp100 chaperon family, which is generally responsible for dissolving protein aggregates that form under conditions of stress. In the *luxS* mutant, *clpB* was upregulated sixfold. Other stress-related genes, including *htrA*, *groEL*, *dnaK*, and alkyl hydroperoxide reductase, also showed a large increase in expression, more so than any other group, indicating that *luxS* may have a primary role of regulation of expression of stress response genes. Among the stress-related group, *htrA* and *clpB* have been reported to be responsible for stress responses in other bacteria (28, 51), although their functions in *P. gingivalis* have yet to be determined. The upregulation of stress-related genes in LY2001 suggests that the *luxS* gene downregulates stress gene expression in the wild-type strain, either directly or indirectly.

Real-time PCR confirmation of microarray-identified genes. Real-time PCR was used to confirm the results obtained using microarray technology. The 16S rRNA was used as an internal control to ensure that the same amount of total RNA from the wild type and the mutant strain was used. The in vitro doubling times of W83 and LY2001 were measured and found to be the same (data not shown), suggesting that 16S rRNA would be a valid internal control. In addition, in each real-time PCR run, the 16S transcripts were compared to the standard curve for either the wild type or the mutant. There was no difference found between them in terms of 16S gene expression, further validating the use of 16S rRNA as an internal control. While

TABLE 3. Real-time PCR analysis of microarray-identified genes[a]

Locus name	Putative identification in TIGR database	Microarray (fold ± SD)	Real-time PCR (fold ± SD)
PG0520	Chaperonin, 60 kDa	2.26 ± 0.23	3.14 ± 1.63
PG0538	Outer membrane efflux protein	1.72 ± 0.13	4.05 ± 1.60
PG0593	HtrA protein	2.01 ± 0.36	1.89 ± 0.15
PG1102	Hypothetical protein	2.02 ± 0.24	3.05 ± 2.30
PG1118	ClpB protein	6.06 ± 1.65	7.78 ± 1.28
PG1208	DnaK protein	4.54 ± 0.72	19.02 ± 0.19
PG1279	D-Isomer specific 2-hydroxyacid dehydrogenase family protein	0.59 ± 0.08	0.70 ± 0.10
PG1280	Conserved hypothetical protein	0.58 ± 0.07	0.52 ± 0.29
PG1798	Immunoreactive 46-kDa antigen PG99	1.65 ± 0.17	1.10 ± 0.30

[a] Real-time PCR for each gene was performed twice, each with four replicates. The "Microarray" and "Real-time PCR" columns in the table indicate the result for gene expression in the mutant versus that in the wild type. The standard deviation for each gene is also shown.

the data were analyzed, the starting quantity of cDNA from the wild type and the mutant was normalized using 16S rRNA. A total of nine genes were selected from the microarray gene list. Seven of the genes were selected from the upregulated group in LY2001, including stress-related genes, hypotheticals, and putative antigens. Both of the downregulated genes were also selected for real-time PCR confirmation. The real-time PCR results confirmed that eight of the nine genes were regulated as indicated by the microarray results (Table 3). One gene, PG1798 (immunoreactive 46-kDa antigen PG99), was not found to be upregulated in LY2001 using real-time PCR. According to the microarray data, this gene was upregulated at a ratio just above the 1.5 cutoff value (Table 2). Since our microarray analysis had a false-discovery rate of 4.7%, allowing one false-positive gene out of 19 genes, it is possible that PG1798 represents a false positive.

Four stress-related genes, including *groEL* (PG0520), *htrA* (PG0593), *clpB* (PG1118), and *dnaK* (PG1208), were upregulated in LY2001. This result, which indicated that *luxS* regulates expression of stress-related genes, led us to perform experiments on W83 and the *luxS* mutant to determine if they responded differently under conditions of stress (see below).

Real-time PCR with complemented supernatant. Real-time PCR for two genes, *dnaK* and *clpB*, was performed on the wild-type strain W83 and supernatant-complemented LY2001 cDNA. These genes were chosen because they showed the greatest difference in expression, compared to the wild-type strain, in the microarray analysis. The results are shown in Table 4. The large fold difference in expression of *dnaK* and *clpB* between W83 and LY2001 was significantly decreased when the supernatant from the wild type was added to the mutant strain culture. These data suggest that the increase in expression of these two genes in the *luxS* mutant was attributable to the *luxS* gene product secreted into the culture, although the role of some other secreted protein cannot be ruled out.

TABLE 4. Real-time PCR with supernatant-complemented LY2001 strain[a]

Locus name	Putative identification in TIGR database	Real-time PCR (fold ± SD)	Complemented mutant strain gene expression (fold ± SD)
PG1208	DnaK protein	19.02 ± 0.19	2.76 ± 1.87
PG1118	ClpB protein	7.78 ± 1.28	0.84 ± 0.28

[a] Real-time PCR for each gene was performed twice using four replicates. The "Real-time PCR" column shows the result for gene expression in the mutant versus that in the wild-type strain by using real-time PCR. The last column indicates the gene expression difference between W83 and LY2001 after LY2001 was supplemented with culture supernatant from strain W83. The standard deviation for each analysis is also shown.

Growth of wild type and LY2001 under different temperatures. There was no difference in the growth rates of W83 and LY2001 at 37°C (results not shown). The doubling times for the wild type and the mutant were the same (120 min), indicating that the *luxS* gene mutation does not have any effect on growth rate under the conditions tested. However, when the strains were stressed at 50°C, the *luxS* mutant exhibited a significantly higher survival rate than did W83 at almost every time point tested, based on *t* values calculated using the Student *t* test (Fig. 1). These results indicate that the mutant is more resistant to heat stress than is the wild type.

Hydrogen peroxide stress. The wild-type and mutant strains were also stressed with 0.35 mM H_2O_2 in 0.1 mM glycine buffer for 15 min, 30 min, 1 h, and 2 h. The *luxS* mutant showed increased survivability over the wild type at each time point tested (Fig. 2). The difference was significant at 15 and 30 min after treatment, indicating that the elevated expression of the stress proteins was related to *P. gingivalis* resistance to H_2O_2. The differences at the 1- and 2-h time points were not signif-

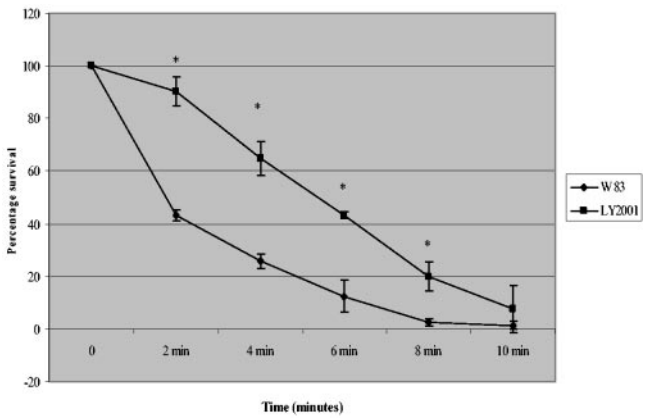

FIG. 1. Survival of W83 and LY2001 at 50°C. The experiments were repeated three times in triplicate. An asterisk indicates that a statistical difference has been found for that time point.

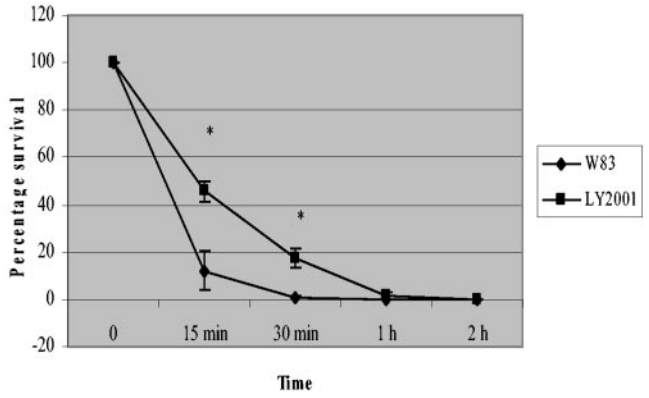

FIG. 2. Percent survival of W83 and LY2001 in H_2O_2 (0.35 mM). This experiment was repeated two times in duplicate. An asterisk indicates that a statistical difference has been found for that time point.

icant, but the percent survival at those two time points was very close to 0% for both strains, indicating that most of the bacteria had already died.

pH stress. The wild-type strain and the *luxS* mutant were treated with glycine buffer (pH 5) for up to 4 h. There were no differences found between these two strains at any time point (results not shown), indicating that genes regulated by *luxS* were not related to survival in an acid environment. Interestingly however, when the bacteria were stressed at pH 9 from 30 min to 2 h in 0.1 M glycine buffer, the *luxS* mutant showed increased resistance over the wild type. Statistically significant differences were found at time points of 30 min, 45 min, and 1 h by using the Student *t* test (Fig. 3).

DISCUSSION

Studies have shown that quorum-sensing systems control not only gene expression related to physiology but also genes associated with virulence in some pathogens. *P. gingivalis* is an etiologic agent of periodontal disease and plays an important role in disease progression. The existence of a *luxS* homologue in the genome database suggested that *P. gingivalis* may have

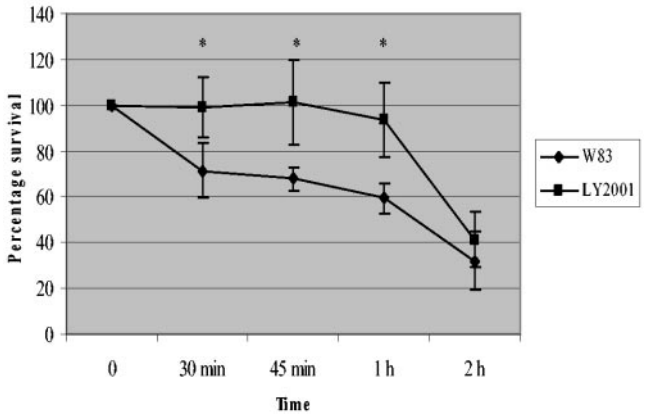

FIG. 3. Percent survival of W83 and LY2001 at pH 9. This experiment was repeated two times in duplicate. An asterisk indicates that a statistical difference has been found for that time point.

an AI-2-regulated quorum-sensing system. Measurement of bioluminescence in *V. harveyi* indicator strains illustrated that the wild-type *P. gingivalis* culture fluid has AI-2 activity and that LY2001 has significantly lower amounts of this activity than does the wild type. The W83 wild-type strain showed 56-fold-higher AI-2 activity than the negative control did. This fold induction was smaller than what has been reported for other bacterial species such as *E. coli* and *Salmonella enterica* serovar Typhimurium (13, 47, 48). Because the autoinducer bioassay was originally developed for *Vibrio* strains, it is possible that the structure of the autoinducer produced by *P. gingivalis* may be modified from that of *V. harveyi* and thus may not be sensed optimally by *Vibrio* strains. Also, unknown chemicals in the environment may interfere with the binding of the autoinducer to the receptor and cause an antagonistic effect. Hjelmgaard et al. (24) showed that a synthetic autoinducer analogue could interfere with quorum sensing in terms of swarming activity in *Serratia liquefaciens* MG1. It has also been shown that furanone 2, an autoinducer antagonist of *V. harveyi*, reduced the bioluminescence to below that of the negative control (35). This suggests that other chemicals in the environment may interfere with quorum sensing and may cause reduced signal recognition and ultimately reduced bioluminescence production. Others have also reported that *P. gingivalis* produced weak AI-2 quorum-sensing signals when periodontal pathogens were screened for signal production (19). Recent observations found that another autoinducer, AI-3, has been identified in *E. coli* (46). The synthesis of AI-3 is dependent on the presence of LuxS. This result adds complexity to this quorum-sensing system. Thus, *Vibrio* strains may have other unidentified autoinducers that are not expressed by *P. gingivalis*, and this could be an additional explanation as to why *P. gingivalis* did not stimulate as much luminescence as *Vibrio* strains did.

We also observed a higher bioluminescence signal (sixfold) in the *luxS*-knockout mutant than in the negative control. This finding made us question the mutant that we constructed. The *luxS* mutant was an insertion mutant. Southern blot analysis showed that the vector used to construct the mutant was inserted into the middle of the gene. In addition, sequencing data confirmed that the vector was inserted at the position of the 111th amino acid of the *luxS* gene. RT-PCR was done in the mutant strain, and a 400-bp band was evident in the wild type but not in the mutant strain sample, indicating that at least the full-length mRNA from *luxS* was not present in the mutant. It is, however, possible that a truncated protein may be produced, which could also explain the sixfold-higher level of activity in the mutant. However, similar weak mutant chemiluminescence has also been detected in *luxS* mutants of other bacteria such as *Streptococcus pyogenes* (33) and a different *luxS* mutant of *P. gingivalis* which was constructed by gene replacement (personal communication). These results suggest the existence of an alternative pathway for producing AI-2 in *S. pyogenes* and in *P. gingivalis* as well. In addition to functioning as an enzyme responsible for producing a bacterial interspecies communication signal, LuxS also has a metabolic function. It recycles SAH to produce SRH. LuxS acts by cleaving SRH to produce homocysteine and DPD. DPD is thought to give rise to AI-2 by spontaneous cyclization and reaction with borate (9, 42). Hauck et al. showed that there is an alternative pathway

for producing DPD from D-ribulose-5-phosphate (23), which can be synthesized from D-ribose-5-phosphate. The enzyme responsible for this conversion is ribose-5-phosphate isomerase (21). During cell lysis, ribose-5-phosphate is released, and the Rpi converts it into ribulose-5-phosphate. Thus, the AI-2 activity in the mutant could be explained by the fact that ribulose-5-phosphate can form DPD nonenzymatically and AI-2 can be formed from DPD.

A previous study (13) that used microarrays based on an *E. coli luxS* mutant indicated that 242 genes, comprising about 5.6% of the *E. coli* genome, exhibited significant transcriptional changes (either induction or repression) in response to a 300-fold AI-2 signaling differential, with many of the identified genes displaying high induction levels with the cutoff ratio of 2.3. The genes regulated by *luxS* included genes involved in cell division, DNA processing, morphology, virulence, biofilm formation, motility, and surface and outer membrane-associated functions, among others. This indicates that *luxS* is a strong global regulator for *E. coli*. In contrast, in our study, only 19 genes or ORFs appeared to be regulated by *luxS*. This corresponds to about 1% of the *P. gingivalis* genome. This suggests that the *luxS* gene may not be a global regulator in *P. gingivalis* as it is in *E. coli*. Another example of quorum-sensing regulation of a small number of genes occurs in *Streptococcus pneumoniae*. In this case, a total of 16 genes that encode potential bacteriocin peptides and immunity proteins are regulated by BlpC, a signal peptide of the strain (15).

In our microarray experiments, the *clpB* gene showed the greatest fold difference between wild type and mutant, which is about sixfold. In comparison with the *E. coli* results, these differences were smaller. The explanations for this could be the following. (i) In *E. coli*, the results were obtained based on a 300-fold luminescence signal difference, while for our study, the difference was only about 56-fold. (ii) The conditions that we used for detecting the transcript levels may not be those that will result in the largest difference between the wild type and the mutant. (iii) Microarray analysis is a relatively qualitative technique for detecting relative transcript levels. Real-time PCR is a more sensitive technique in terms of quantitation. Thus, the lower expression ratios that were detected in microarray assays may minimize the differences. Thus, real-time PCR was performed to confirm the microarray experiments, and the majority of the tested genes showed greater differences than those obtained with the microarray technique.

A group of stress-related genes, including *dnaK*, *groEL*, *clpB*, and *htrA*, were found to be regulated by *luxS* in *P. gingivalis*. It is known that these genes are intimately involved in the clearance of misfolded aggregates and premature polypeptides produced during stress. This result indicated that there is some correlation between quorum sensing and stress response. DeLisa et al. (12) imposed different stresses on chemostat-grown *E. coli* cultures, including heat shock, ethanol, and H_2O_2, and found that the AI-2 levels were decreased for heat shock and ethanol treatments. When the cultures were treated with H_2O_2, the AI-2 level decreased at first and then increased. By measuring the bioluminescence of stress gene mutants, it was found that mutant strains produced altered bioluminescence relative to the parent strain. This provides a direct link between AI-2 signaling and σ^{32}-mediated proteins, suggesting that a chaperon-mediated folding pathway exists that directly affects the accumulation of extracellular AI-2. The aberrant protein folding pattern in the stress gene mutant may somehow have adverse effects on proteins or enzymes that are related to AI-2 signal production, so that the autoinducer produced from these mutants would change in response to the stress gene mutation. In stress gene mutants of other bacterial species, a relationship between several stress and starvation genes and known quorum-sensing genes has also been found (12). In *Vibrio vulnificus*, a *luxR* homologue mutant (*smcR*) was compared with the wild type for resistance to H_2O_2. The mutant showed a decrease in survival compared to the wild type. This result indicated that quorum sensing may regulate stress responses in the cell (35). In this study we tested the wild-type strain and a *luxS* mutant with regard to resistance to stress. The stresses used were high temperature, H_2O_2, and pH. Our *luxS* mutant showed enhanced resistance to each kind of stress. This suggests that there is coordinate regulation of these stress responses. Since expression of genes related to stress responses, including the genes *clpB*, *htrA*, *dnak*, *groEL*, and the F subunit of alkyl hydroperoxide reductase, is elevated in the *luxS* mutant, it is possible that some or all of these genes play a role in stress resistance.

Since *luxS* and *smcR* are both components of the same signaling system, it might be expected that a *luxS* mutant would have a phenotype similar to that of an *smcR* mutant. However, the *smcR* (*luxR* homologue) mutant of *V. vulnificus* showed decreased resistance to H_2O_2 (35), while our W83 *luxS* mutant showed increased resistance to H_2O_2. The reasons for this difference could be that they may have different functions or regulate different groups of genes in the two species. Also, it appears that the LuxR homologues are most likely regulated by other factors in addition to the AI-2 autoinducer (27, 34, 57). These LuxR homologues may in fact be global regulators with the AI-2 signal system being one of their inducers. Thus, the decreased resistance of the *smcR* mutant may be the result of other regulatory factors.

Previous studies have shown that expression of *dnaK* and *groEL* was increased when *P. gingivalis* was stressed at 42°C, but expression of these genes did not change in response to oxidative stress or pH (32). These data suggest that *dnaK* and *groEL* are related to temperature stress, but not oxidative or pH stress. The increased resistance of LY2001 to oxidative and pH stress may thus be the result of an elevated expression of other stress-related genes, possibly *clpB* or *htrA*, or both.

When bacteria invade host cells, they encounter a variety of stresses, including high temperature, nutrient loss, and oxidation, all of which must be overcome by the bacteria in order to survive. Thus, stress proteins are considered to be related to virulence. For example, Johnson et al. showed that transposon mutagenesis of the *htrA* gene resulted in an avirulent strain of *S. enterica* serovar Typhimurium (28). It has also been shown that an *S. enterica* serovar Typhimurium heat shock protein is involved in mucus-mediated interaction of the bacterium with the host (18). Additionally, other stress gene mutants (*clpB*) were found to have decreased virulence in *Salmonella enterica* serovar Typhimurium (51) and *Listeria monocytogenes* (7). Further, protection against a variety of infectious agents is thought to be due to antibodies directed against specific stress proteins that often involve conserved epitopes. For example, Lopatin et al. found that patients with higher anti-Hsp (Hsp90, DnaK,

and GroEL) antibody concentrations tended to have significantly ($P \leq 0.05$) healthier periodontal tissues (31). Collectively, these data support the importance and relationship of stress proteins and virulence.

Our microarray data showed that at least five stress response genes were upregulated in the *P. gingivalis luxS* mutant. This might suggest that the *luxS* mutant may have increased virulence compared to the wild-type strain. However, Burgess et al. reported that overall virulence of a *luxS* mutant of *P. gingivalis* strain W50 was not changed using a murine lesion model (6). The reasons for this could be as follows. (i) The mouse abscess model is not totally relevant for studying virulence in humans. Thus, the *luxS* mutant of *P. gingivalis* may have an avirulent phenotype in mice but a phenotype related to virulence in humans (6). (ii) *luxS* may have different regulatory roles in various strains of *P. gingivalis*. Thus, the avirulent phenotype of one *luxS* mutant strain may not necessarily occur with other strains. Without the availability of a more appropriate animal model that more closely mimics the human disease, it is not possible to definitely test these hypotheses.

Our results indicated that a list of stress-related genes is downregulated by AI-2 signal. This appears contradictory because bacteria within biofilms are more resistant to stresses at a relatively high local concentration of AI-2. However, the environment of planktonic cultures is different from that of biofilms. In a biofilm, the bacteria are organized in specific ways so that each member will have access to nutrients and the advantages of physical barriers. Thus, bacteria living within a biofilm are less likely to confront stresses than are those in planktonic cultures. Even though the local AI-2 concentration is high and the stress genes are suppressed, since the bacteria are in the biofilm, they likely would not be compromised significantly by the lack of expression of the stress-related genes. Thus, our data obtained from planktonic cultures cannot simply and directly be applied to biofilm biology. The more relevant data in terms of quorum sensing in a biofilm can be obtained only by actually performing the experiments, which are planned as a follow-up to these experiments. For the work reported here, our goal was to obtain fundamental information on quorum sensing and related gene regulation in *P. gingivalis*.

In summary, the *luxS* mutant of *P. gingivalis* W83 was found to have decreased bioluminescence and increased expression of some stress-related genes. The *luxS* mutant showed increased resistance to a variety of stresses, including high temperature, pH, and H_2O_2. This suggests that *luxS* is involved in stress gene responses in *P. gingivalis*. Future experiments will address the mechanism responsible for this regulation, perhaps in other bacterial species as well.

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REFERENCES

1. **Anand, S. K., and M. W. Griffiths.** 2003. Quorum sensing and expression of virulence in *Escherichia coli* O157:H7. Int. J. Food Microbiol. **85:**1–9.
2. **Arevalo-Ferro, C., M. Hentzer, G. Reil, A. Gorg, S. Kjelleberg, M. Givskov, K. Riedel, and L. Eberl.** 2003. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. Environ. Microbiol. **5:**1350–1369.
3. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol. Microbiol. **9:**773–786.
4. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273–286.
5. **Beck, J., R. Garcia, G. Heiss, P. S. Vokonas, and S. Offenbacher.** 1996. Periodontal disease and cardiovascular disease. J. Periodontol. **67:**1123–1137.
6. **Burgess, N. A., D. F. Kirke, P. Williams, K. Winzer, K. R. Hardie, N. L. Meyers, J. Aduse-Opoku, M. A. Curtis, and M. Camara.** 2002. LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence. Microbiology **148:**763–772.
7. **Chastanet, A., I. Derre, S. Nair, and T. Msadek.** 2004. *clpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. J. Bacteriol. **186:**1165–1174.
8. **Chen, T., Y. Hosogi, K. Nishikawa, K. Abbey, R. D. Fleischmann, J. Walling, and M. J. Duncan.** 2004. Comparative whole-genome analysis of virulent and avirulent strains of *Porphyromonas gingivalis*. J. Bacteriol. **186:**5473–5479.
9. **Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545–549.
10. **Chung, W. O., Y. Park, R. J. Lamont, R. McNab, B. Barbieri, and D. R. Demuth.** 2001. Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. J. Bacteriol. **183:**3903–3909.
11. **Dasanayake, A. P., S. Russell, D. Boyd, P. N. Madianos, T. Forster, and E. Hill.** 2003. Preterm low birth weight and periodontal disease among African Americans. Dent. Clin. N. Am **47:**115–125.
12. **DeLisa, M. P., J. J. Valdes, and W. E. Bentley.** 2001. Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. J. Bacteriol. **183:**2918–2928.
13. **DeLisa, M. P., C. F. Wu, L. Wang, J. J. Valdes, and W. E. Bentley.** 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. J. Bacteriol. **183:**5239–5247.
14. **Derzelle, S., E. Duchaud, F. Kunst, A. Danchin, and P. Bertin.** 2002. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **68:**3780–3789.
15. **de Saizieu, A., C. Gardes, N. Flint, C. Wagner, M. Kamber, T. J. Mitchell, W. Keck, K. E. Amrein, and R. Lange.** 2000. Microarray-based identification of a novel *Streptococcus pneumoniae* regulon controlled by an autoinduced peptide. J. Bacteriol. **182:**4696–4703.
16. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75:**3479–3483.
17. **Dworkin, M., and D. Kaiser.** 1985. Cell interactions in myxobacterial growth and development. Science **230:**18–24.
18. **Ensgraber, M., and M. Loos.** 1992. A 66-kilodalton heat shock protein of *Salmonella typhimurium* is responsible for binding of the bacterium to intestinal mucus. Infect. Immun. **60:**3072–3078.
19. **Frias, J., E. Olle, and M. Alsina.** 2001. Periodontal pathogens produce quorum sensing signal molecules. Infect. Immun. **69:**3431–3434.
20. **Genco, R., S. Offenbacher, and J. Beck.** 2002. Periodontal disease and cardiovascular disease: epidemiology and possible mechanisms. J. Am. Dent. Assoc. **133**(Suppl.)**:**14S–22S.
21. **Hamada, K., H. Ago, M. Sugahara, Y. Nodake, S. Kuramitsu, and M. Miyano.** 2003. Oxyanion hole-stabilized stereospecific isomerization in ribose-5-phosphate isomerase (Rpi). J. Biol. Chem. **278:**49183–49190.
22. **Hammer, B. K., and B. L. Bassler.** 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol. Microbiol. **50:**101–104.
23. **Hauck, T., Y. Hubner, F. Bruhlmann, and W. Schwab.** 2003. Alternative pathway for the formation of 4,5-dihydroxy-2,3-pentanedione, the proposed precursor of 4-hydroxy-5-methyl-3(2H)-furanone as well as autoinducer-2, and its detection as natural constituent of tomato fruit. Biochim. Biophys. Acta **1623:**109–119.
24. **Hjelmgaard, T., T. Persson, T. B. Rasmussen, M. Givskov, and J. Nielsen.** 2003. Synthesis of furanone-based natural product analogues with quorum sensing antagonist activity. Bioorg. Med. Chem. **11:**3261–3271.
25. **Hughes, T. R., M. J. Marton, A. R. Jones, C. J. Roberts, R. Stoughton, C. D. Armour, H. A. Bennett, E. Coffey, H. Dai, Y. D. He, M. J. Kidd, A. M. King, M. R. Meyer, D. Slade, P. Y. Lum, S. B. Stepaniants, D. D. Shoemaker, D. Gachotte, K. Chakraburtty, J. Simon, M. Bard, and S. H. Friend.** 2000. Functional discovery via a compendium of expression profiles. Cell **102:**109–126.
26. **Ji, G., R. C. Beavis, and R. P. Novick.** 1995. Cell density control of staphy-

lococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA **92:**12055–12059.
27. **Jobling, M. G., and R. K. Holmes.** 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene hap, and its identification as a functional homologue of the *Vibrio harveyi* luxR gene. Mol. Microbiol. **26:**1023–1034.
28. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
29. **Kim, S. Y., S. E. Lee, Y. R. Kim, C. M. Kim, P. Y. Ryu, H. E. Choy, S. S. Chung, and J. H. Rhee.** 2003. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Mol. Microbiol. **48:**1647–1664.
30. **Kovacikova, G., W. Lin, and K. Skorupski.** 2003. The virulence activator AphA links quorum sensing to pathogenesis and physiology in *Vibrio cholerae* by repressing the expression of a penicillin amidase gene on the small chromosome. J. Bacteriol. **185:**4825–4836.
31. **Lopatin, D. E., C. E. Shelburne, N. Van Poperin, C. J. Kowalski, and R. A. Bagramian.** 1999. Humoral immunity to stress proteins and periodontal disease. J. Periodontol. **70:**1185–1193.
32. **Lu, B., and B. C. McBride.** 1994. Stress response of *Porphyromonas gingivalis*. Oral Microbiol. Immunol. **9:**166–173.
33. **Lyon, W. R., J. C. Madden, J. C. Levin, J. L. Stein, and M. G. Caparon.** 2001. Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*. Mol. Microbiol. **42:**145–157.
34. **McCarter, L. L.** 1998. OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. J. Bacteriol. **180:**3166–3173.
35. **McDougald, D., S. Srinivasan, S. A. Rice, and S. Kjelleberg.** 2003. Signal-mediated cross-talk regulates stress adaptation in *Vibrio* species. Microbiology **149:**1923–1933.
36. **Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi.** 2003. Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. Infect. Immun. **71:**1972–1979.
37. **Nealson, K. H., and J. W. Hastings.** 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol. Rev. **43:**496–518.
38. **Nealson, K. H., T. Platt, and J. W. Hastings.** 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J. Bacteriol. **104:** 313–322.
39. **Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell.** 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149:**1311–1322.
40. **Offenbacher, S., H. L. Jared, P. G. O'Reilly, S. R. Wells, G. E. Salvi, H. P. Lawrence, S. S. Socransky, and J. D. Beck.** 1998. Potential pathogenic mechanisms of periodontitis associated pregnancy complications. Ann. Periodontol. **3:**233–250.
41. **Schachter, P. P., S. Ayesh, T. Schneider, M. Laster, A. Czerniak, and A. Hochberg.** 2002. Expression of kinase genes in primary hyperparathyroidism: adenoma versus hyperplastic parathyroid tissue. Surgery **132:**1094–1098.
42. **Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler.** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. **41:**463–476.
43. **Schena, M., D. Shalon, R. W. Davis, and P. O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:**467–470.
44. **Socransky, S. S., and A. D. Haffajee.** 1992. The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol. **63:**322–331.
45. **Sperandio, V., A. G. Torres, J. A. Giron, and J. B. Kaper.** 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. **183:**5187–5197.
46. **Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper.** 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. USA **100:**8951–8956.
47. **Surette, M. G., and B. L. Bassler.** 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **95:**7046–7050.
48. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA **96:**1639–1644.
49. **Taga, M. E., J. L. Semmelhack, and B. L. Bassler.** 2001. The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. Mol. Microbiol. **42:**777–793.
50. **Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow.** 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. Infect. Immun. **71:**2643–2655.
51. **Turner, A. K., M. A. Lovell, S. D. Hulme, L. Zhang-Barber, and P. A. Barrow.** 1998. Identification of *Salmonella typhimurium* genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. **66:**2099–2106.
52. **Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080–2095.
53. **Wang, H., Y. Z. Zhu, P. T. Wong, J. M. Farook, A. L. Teo, L. K. Lee, and S. Moochhala.** 2003. cDNA microarray analysis of gene expression in anxious PVG and SD rats after cat-freezing test. Exp. Brain Res. **149:**413–421.
54. **Wilson, M., J. DeRisi, H. H. Kristensen, P. Imboden, S. Rane, P. O. Brown, and G. K. Schoolnik.** 1999. Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. Proc. Natl. Acad. Sci. USA **96:**12833–12838.
55. **Winzer, K., K. R. Hardie, N. Burgess, N. Doherty, D. Kirke, M. T. Holden, R. Linforth, K. A. Cornell, A. J. Taylor, P. J. Hill, and P. Williams.** 2002. LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone. Microbiology **148:**909–922.
56. **Zhu, J., and J. J. Mekalanos.** 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev. Cell **5:**647–656.
57. **Zhu, J., M. B. Miller, R. E. Vance, M. Dziejman, B. L. Bassler, and J. J. Mekalanos.** 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **99:**3129–3134.

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