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Analysis of LuxR Regulon Gene Expression during Quorum Sensing in *Vibrio fischeri*▽

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The regulation of the *lux* operon (*luxICDABEG*) of *Vibrio fischeri* has been intensively studied as a model for quorum sensing in proteobacteria. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis previously identified several non-Lux proteins in *V. fischeri* MJ-100 whose expression was dependent on LuxR and 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL). To determine if the LuxR-dependent regulation of the genes encoding these proteins was due to direct transcriptional control by LuxR and 3-oxo-C6-HSL or instead was due to indirect control via an unidentified regulatory element, promoters of interest were cloned into a *lacZ* reporter and tested for their LuxR and 3-oxo-C6-HSL dependence in recombinant *Escherichia coli*. The promoters for *qsrP*, *acfA*, and *ribB* were found to be directly activated via LuxR-3-oxo-C6-HSL. The sites of transcription initiation were established via primer extension analysis. Based on this information and the position of the *lux* box-binding site near position −40, all three promoters appear to have a class II-type promoter structure. In order to more fully characterize the LuxR regulon in *V. fischeri* MJ-100, real-time reverse transcription-PCR was used to study the temporal expression of *qsrP*, *acfA*, and *ribB* during the exponential and stationary phases of growth, and electrophoretic mobility shift assays were used to compare the binding affinities of LuxR to the promoters under investigation. Taken together, the results demonstrate that regulation of the production of QsrP, RibB, and AcfA is controlled directly by LuxR at the level of transcription, thereby establishing that there is a LuxR regulon in *V. fischeri* MJ-100 whose genes are coordinately expressed during mid-exponential growth.

The term quorum sensing describes the ability of a microorganism to recognize and respond to other microorganisms in a population by detecting the concentration of self-produced intercellular molecules commonly known as autoinducers. When an autoinducer reaches a critical threshold concentration, often at high cell densities, it triggers a signal transduction pathway leading to an alteration of gene expression patterns. There are a number of important bacterial processes regulated in this manner, including antibiotic production, release of exoenzymes, production of virulence factors, induction of genetic competency, conjugative plasmid transfer, biofilm formation, and bioluminescence (for reviews, see references 11, 24, 38, and 41).

In the gram-negative bioluminescent marine bacterium *Vibrio fischeri* a complex signal transduction system controls expression of bioluminescence (for a review, see reference 35). However, it is the products of *luxI* and *luxR* that directly activate *lux* operon transcription. LuxI, the autoinducer synthase, produces the diffusible autoinducer 3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (8). As the levels of 3-oxo-C6-HSL rise, complexes form between it and an autoinducer-dependent activator of transcription, LuxR. The complex can then activate transcription of the *lux* operon by binding to the *lux* box in the promoter region, leading to an increase in bioluminescence (33). The 252-amino-acid, two-domain LuxR polypeptide is one of the most-studied members of a family of over 50 transcriptional regulator proteins involved in acyl homoserine lactone-mediated quorum sensing (for reviews, see references 11, 21, and 40).

In LuxR, the N-terminal domain is believed to be membrane associated and to function as a receptor for the 3-oxo-C6-HSL ligand (15, 17). In response to binding the 3-oxo-C6-HSL, the N-terminal domain modulates the activity of the C-terminal domain (CTD). Binding of 3-oxo-C6-HSL permits multimerization of LuxR and subsequent activation of transcription of the *lux* operon, which is carried out by the CTD (3, 4). The CTD of LuxR has a helix-turn-helix motif and binds to a region of DNA termed the *lux* box, which is 20 bp long and has a dyad symmetry centered at a position −42.5 bp upstream of the transcription start site for the *lux* operon (5, 9). When LuxR functions as an activator of transcription at the *luxI* promoter, it is proposed to function as a homodimer in an ambidextrous manner similar to the manner observed for the cyclic AMP receptor protein at a class II-type promoter (1, 25) contacting both the alpha and sigma subunits of RNA polymerase (10, 16). Full-length LuxR protein was purified in the presence of 3-oxo-C6-HSL, which permitted its binding to the *lux* box to be demonstrated in vitro. Binding of 3-oxo-C6-HSL to LuxR appeared to be reversible in this system (34). In this study we examined the ability of LuxR to bind to the promoters of additional genes in *V. fischeri* MJ-100, leading to activation of transcription.

While the global quorum-sensing response in some organisms is substantial (e.g., *Pseudomonas aeruginosa* has over 400 quorum-sensing-controlled genes [29, 36]), little is known about the extent of the quorum-sensing response in *V. fischeri* (2, 19). Previously, two-dimensional sodium dodecyl sulfate-

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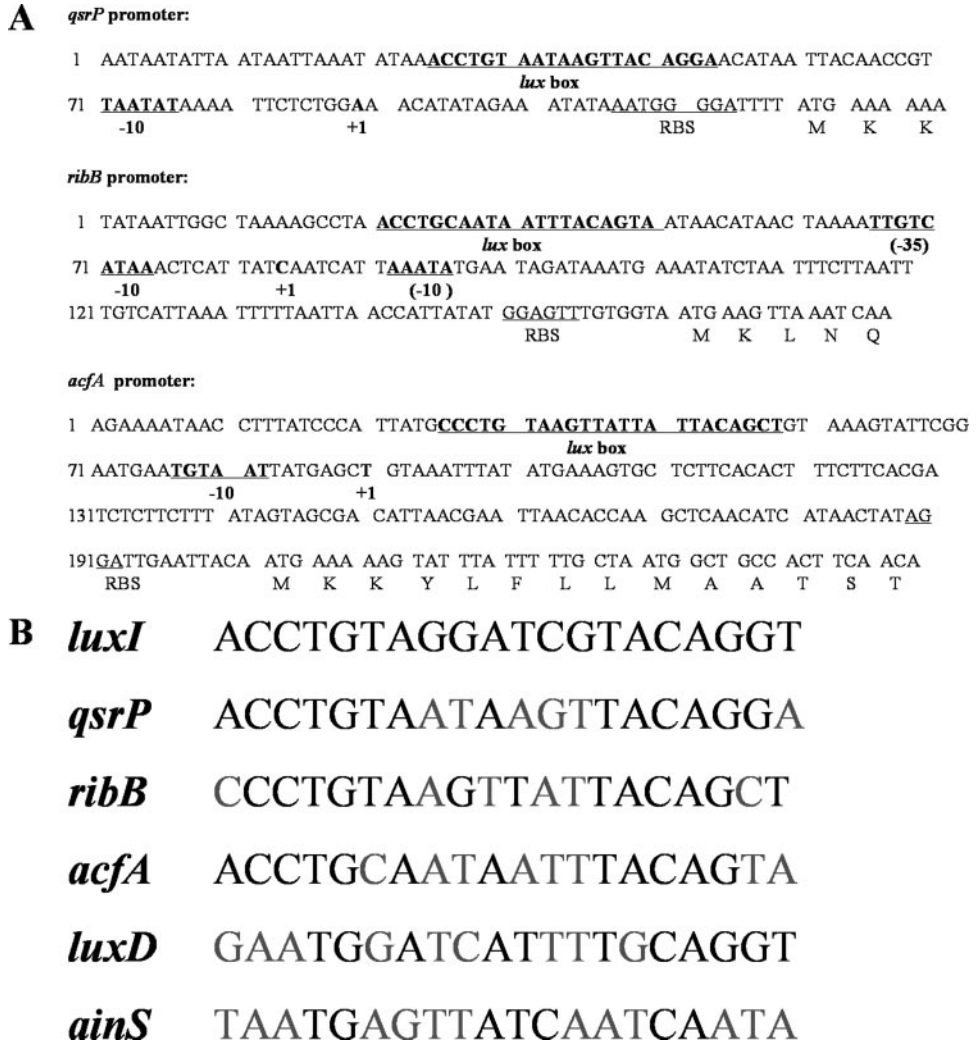

FIG. 1. (A) Nucleotide sequences of the promoter regions of the *V. fischeri* MJ-100 *qsrP*, *ribB*, and *acfA* genes. The positions of putative *lux* boxes, −10 and −35 regions, and a ribosome-binding site (RBS) are indicated; +1 sites are indicated by bold type. A second nonfunctional promoter sequence upstream of *ribB* is indicated by (−10) and (−35). (B) Comparison of *V. fischeri* MJ-100 *lux* box sequences from different genes. The gray nucleotides differ from the nucleotides in the *luxI* box.

polyacrylamide gel electrophoresis (2D SDS-PAGE) analysis of protein profiles produced from quorum-sensing mutants of *V. fischeri* MJ-100 defective in production of acyl-homoserine lactones and LuxR was performed (2). Strain MJ-100 (6, 7) is a spontaneous nalidixic acid-resistant variant of the MJ-1 strain (27) in which the regulation of the *lux* operon by LuxR has been extensively studied. Five quorum-sensing-regulated proteins other than Lux proteins were identified in this study. Four of the proteins were identified via amino acid sequencing, and two of them (AcfA and QsrV) appear to be encoded by an operon. Therefore, three putative LuxR-regulated promoters were identified. Based on sequence similarity, the genes adjacent to these promoters are *qsrP*, *ribB*, and *acfA*. Neither the *qsrP* nucleotide sequence nor the deduced amino acid sequence exhibited significant similarity with known genes or gene products. Therefore, QsrP is considered to be a novel periplasmic protein that plays a role in the ability of *V. fischeri* to colonize the sepiolid squid *Eurpyrmna scolopes* (2). In *Escherichia coli*, RibB is a 3,4-dihydroxy-2-butanone-4-phosphate synthase, which catalyzes a key step in riboflavin (vitamin B_2) synthesis (26). AcfA has a *Vibrio cholerae* homologue which is believed to affect the ability of *V. cholerae* to colonize the mouse intestinal epithelium (22). While *qsrP* and *acfA* have been found in *V. fischeri* MJ-100 and ES114, the only strain of *V. fischeri* whose genome has been sequenced (28), *ribB* was not found in the ES114 strain, which demonstrates that there is strain-to-strain variation in quorum-sensing-controlled outputs in *V. fischeri*. The results of the 2D SDS-PAGE study, however, did not discriminate between direct and indirect regulation of these genes, leaving open the possibility of indirect quorum-sensing control that is mediated by an unidentified regulatory element. To test this possibility and to definitively establish the presence of a quorum-sensing regulon in *V. fischeri*, expression from the *qsrP*, *ribB*, and *acfA* promoters and from the *qsrQ* and *qsrRST* promoters that are divergently expressed from *qsrP* and *acfA*, respectively, was analyzed in

recombinant *E. coli*. Two other promoters, upstream of *ainS* and *arcA*, were also included in these experiments based on the presence of putative *lux* boxes identified through sequence analysis (12; unpublished results).

After this initial screening, the promoters directly activated by LuxR-acyl homoserine lactone, the *qsrP*, *ribB*, and *acfA* promoters (Fig. 1), were more intensively studied. Primer extension analysis was performed to identify the transcription start sites so that the distances from them to the *lux* box could be established with certainty and the promoter structure could thereby be deduced. To determine if there is any hierarchy in the order of gene expression of the LuxR regulon, real-time reverse transcription-PCR (RT-PCR) was used to examine the temporal expression of these three genes. Electrophoretic mobility shift assays (EMSA) were used to establish direct binding and to quantitate the relative binding affinities of LuxR to the promoters under investigation. These studies provided insights into the role that transcriptional regulation and posttranscriptional regulation play in the control of the *V. fischeri* MJ-100 LuxR regulon.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in this study are described in Table 1. Luria-Bertani broth (LB) was used to grow *E. coli* strains. It was supplemented, where indicated below, with ampicillin (Ap) (100 μg/ml), chloramphenicol (Cm) (20 μg/ml), 200 nM *N*-(β-ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) (Sigma, St. Louis, MO), or 1 mM isopropyl-β-D-1-thiogalatopyranoside (IPTG) (Sigma). Seawater complete (SWC) medium with instant ocean substituted for natural seawater (Aquarium Systems, Mentor OH) (20) or LBS medium (6) was used to grow *V. fischeri* MJ-100.

Plasmid construction. The *acfA* and *luxI* genes were previously cloned into pSUP102 (2). *V. fischeri* chromosomal DNA was purified as a template (37), and *qsrP* and *ribB* were amplified using the primers indicated in Table 2 and cloned into pSUP102. The promoter regions of a number of genes containing putative *lux* boxes (*luxI*, *qsrP*, *ribB*, *acfA*, *ainS*, *qsrQ*, *qsrRST*, and *arcA*) were PCR amplified using the primers indicated in Table 2, cloned into pGEM-T (Promega, Madison, WI), and sequenced to confirm their integrity (Virginia Bioinformatics Institute Core Laboratory, Virginia Tech, Blacksburg). The promoter sequences were subsequently subcloned into the promoter-probe β-galactosidase reporter pSP417 (23) using EcoRI/BamHI sites to construct pNQ101 to pNQ110 (Table 1). Two versions of the P_{qsrQ} and P_{qsrRST} promoters were cloned, one with the 5′ end just upstream of the putative *lux* box and the other with additional upstream sequences containing the divergently transcribed promoters P_{qsrP} and P_{acfA}, respectively. For the RT-PCR analysis, internal fragments of *qsrP* and *nadB* were PCR amplified and cloned into pGEM-T, creating pNQqsrP and pNQnadB.

β-Galactosidase assays. The pNQ101 to pNQ110 constructs were transformed individually into *E. coli* JM109 competent cells containing pAMS121 (32) containing a *luxR* gene that could be induced by IPTG. Overnight cultures were initially inoculated from freezer stocks into 5 ml LB medium with appropriate antibiotics and incubated at 30°C. A subculture of each strain was prepared by transferring the correct amount of the overnight culture into 5 ml LB medium plus Ap (100 μg/ml) and Cm (20 μg/ml) with either 200 nM 3-oxo-C6-DL-HSL or 1 mM IPTG, both 3-oxo-C6-DL-HSL and IPTG, or nothing to obtain an optical density at 600 nm (OD_{600}) of 0.025. When the OD_{600} reached 0.5, 5 μl of cells was diluted 1:200 in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) with 400 μM dithiothreitol and lysed with 50 μl of chloroform. Chemiluminescent β-galactosidase assays (Tropix, Bedford, MA) were performed with 5 μl of cell lysate using an LD 400S luminescence detector (Beckman Coulter, Fullerton, CA) and a 20-s integration time. All assays were performed in three trials with triplicate samples in each trial, and the results were expressed in relative light units.

RNA purification. For primer extension, overnight cultures were initially inoculated from freezer stocks into 5 ml LB medium plus Ap (100 μg/ml) and Cm (20 μg/ml) (for *E. coli*) or SWC medium with no antibiotics (for *V. fischeri*) and incubated at 30°C. A subculture of each strain was prepared by transferring an appropriate amount of the overnight culture into 5 ml LB medium plus Ap (100 μg/ml) and Cm (20 μg/ml) with and without 200 nM 3-oxo-C6-HSL or SWC medium with and without 200 nM 3-oxo-C6-HSL to obtain an OD_{600} of 0.025. Then the cells were grown to an OD_{600} of 1.0. Five milliliters of cells was harvested and resuspended in TE buffer (10 mM Tris, 2 mM EDTA) and passed through 19- and 26-gauge needles (Becton Dickinson & Co, Franklin Lakes, NJ) four times each to improve lysis. Subsequent steps were performed using the QIAGEN RNeasy kit protocol (QIAGEN, Valencia, CA). After purification, the RNA concentration was determined using a spectrophotometer. The sample was then dried in a Speedvac instrument and resuspended in RNase-free water.

For real-time RT-PCR, *V. fischeri* MJ-100 was grown in LBS medium to an OD_{600} of 0.25, 0.5, 1.0, or 2.0. In addition to the QIAGEN RNeasy kit, QIAGEN RNeasy Protect bacterial reagent and QIAGEN RNase-free DNase I were also used to stabilize the RNA and remove the residual DNA. The RNA was sent to the Virginia Bioinformatics Institute Core Laboratory (QIAGEN) to check the quantity and quality of the RNA before use in real-time RT-PCR protocols.

Primer extension. Each primer was 5′ end labeled using [γ-^{32}P]dATP (Amersham, Piscataway, NJ) and a Primer Extension System kit (Promega, Madison, WI). Primer extension was performed using this kit according to the manufacturer's instructions, except that an ethanol precipitation step was used to remove unincorporated [γ-^{32}P]dATP from the primer. For ethanol precipitation 90 μl RNase-free water, 11 μl 3 M RNase-free sodium acetate, and 220 μl ethanol were added, mixed by vortexing, and kept at room temperature for 1 h. DNA sequences were obtained using a plasmid template purified with a QIAGEN Miniprep kit (QIAGEN) and were diluted to obtain a concentration of 100 ng/μl. Sequencing reactions were performed using an *fmol* DNA cycle sequencing system kit (Promega) according to the manufacturer's instructions.

Real-time RT-PCR. An Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) was used first to make sure that there was no DNA or other contaminants in the

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference(s) or source
E. coli strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*lacZ*Δ*M15*]	42
DH5α	*supE44 lacU169* ϕ80d*lacZ*Δ*M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	14
V. fischeri strains		
MJ-100	Spontaneous Nx^r variant of MJ-1	6, 7
MJ-211	Wild type, Δ*luxI* (nonpolar)	18
Plasmids		
pGEM-T	Multiple cloning sites in *lacZ*	Promega
pAMS121	P_{tac}-*luxR, Cm*^{r}	32
pSP417	Cloning vector with a promoterless *lacZ*	23
pNQ101	pSP417 with *luxI* promoter	This study
pNQ102	pSP417 with *qsrP* promoter	This study
pNQ103	pSP417 with *ribB* promoter	This study
pNQ104	pSP417 with *acfA* promoter	This study
pNQ105	pSP417 with *ainS* promoter	This study
pNQ106	pSP417 with *qsrQ* promoter	This study
pNQ107	pSP417 with *qsrQ* and *qsrP* promoters	This study
pNQ108	pSP417 with *qsrR* promoter	This study
pNQ109	pSP417 with *qsrR* and *acfA* promoters	This study
pNQ110	pSP417 with *arcA* promoter	This study
pSUP102	pACYC184 based, RP4, Cm^r Tc^r Mob^r	31
pSC300	P_{tac} *luxR* in pKK233-3	4
pNQ1	pSUP102 with *ribB*	This study
pSUP102-acfA	pSUP102 with *acfA*	2
pSUP102-luxI	pSUP102 with *luxI*	2
pSUP102-qsrP	pSUP102 with *qsrP*	This study
pNQqsrP	pGEM-T with part of *qsrP*	This study
pNQnadB	pGEM-T with *nadB*	This study
pSH202	*luxR-luxI*, Cm^r	3

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)	Product amplified
LacZ assays		
QSRPF	GAATTCCAGAAGTAGACTAAAGGTGC	*qsrP* promoter
QSRPR	GGATCCGGTGATATAAGTGTGAAAAGTAATAACGCTG	
ACFAF	GAATTCGTATTACTATCGTATTGAATTAG	*acfA* promoter
ACFAR	GGATCCGCTGTTGAAGTGGCAGCCATTAGC	
RIBBF	GAATTCGTGGCCTAACGCCTGCATAACACG	*ribB* promoter
RIBBR	GGATCCCGACACGCTCTATAGGAGATCC	
AINSF	GAATTCGTGCCTTCACAGGCGTCAATTAACTTC	*ainS* promoter
AINSR	GGATCCCTTGTACTGATTAGTTGCTGGAG	
LUXIF	GAATTCCGCTGGGAATACAATTAC	*luxI* promoter
LUXIR	GGATCCTTTATACTCCTCCGATGGAATTGCC	
qsrQSF	GAATTCCGGTTGTAATTATGTTCCTG	Two versions of *qsrQ* promoter
qsrQLF	GAATTCGGTGATATAAGTGTGAAAAGTAATAACGCTG	
qsrQR	GGATCCCGACGAGATAAACGAGCATTCACC	
qsrRSF	GAATTCCTGTTGAAGTGGCAGCCATTAGC	Two versions of *qsrR* promoter
qsrRLF	GAATTCCATTCATTCCGAATACTTTTACAGC	
qsrRR	GGATCCCCAATGTTGCGAATAGATCTTTTGCC	
arcAF	GAATTCCAGCTAAGGTAGCAACTTGATCACC	*arcA* promoter
arcAR	GGATCCCATTGTAGCCTTCAGCTTCAAAAATGC	
Gene cloning for primer extension		
qsrPForward	AAGAAACCTCGACGAGATAAACG	*qsrP* from MJ-100
qsrPReverse	GTCCTAAAGAGGAAATGCTAAGTGGT	
ribBForward	CCACTTAATACGGTAAACTC	*ribB* from MJ-100
ribBReverse	CACGATTTGCTACATTTGGT	
Primer extension		
acfAPE	CAATCCTATAGTTATGATG	
qsrPPE	GGTGGTGATATAAGTGTGAAAAG	
ribBPE	CTTCATTACCACAAACTCC	
Real-time RT-PCR		
RTnadBF	AAGAAACCGATATCTCCGATCC	Part of *nadB*
RTnadBR	CGTAATGTTTGGGTGGTTAAGC	
RTluxIF	GTGGATGCTGGCGTTTATTAC	Part of *luxI*
RTluxIR	TTTGGGAGCACTCTGTTGAC	
RTqsrPF	ACAGAACTTTTTTGTTGGGAG	Part of *qsrP*
RTqsrPR	TCGACATAAATAGGAGGAATGG	
RTribBF	AGGACATACAGAAGGGACTG	Part of *ribB*
RTribBR	AATTATTTCAGGGGTTTTAGCC	
RTacfAF	AGCGTATTCGGCGGTATTG	Part of *acfA*
RTacfAR	ACTTGGTAGCTTGCTGATGC	
EMSA		
PluxIF	GAATTCCGCTGGGAATACAATTAC[a]	*luxRI* (control)
PluxIR	GGATCCTTTATACTCCTCCGATGGAATTGCC[a]	
AMS4	CGCTGGGAATACAATTAC[b]	*luxI* promoter
LuxR2A	AAAAAATCCGATTTTTTTATCAT[b]	
qsrPF135	GGAGATGACGATTTTATTGCG	*qsrP* promoter
qsrPR135	CTATATGTTTCCAGAG	
acfAF130	CACGTAAGACCAAGATTAAATG	*acfA* promoter
acfAR130	CAGCTCATAATTACATTCATTCCG	
ribBF125	GTAAACCTAGAATGCCGAATGTAGC	*ribB* promoter
ribBR125	GAGTTTATGACAATTTTAGTTATG	
EMSAluxDF	GGGAAACACTCCCTAAAAAGAACGTACC	Part of *luxD* contaning *luxD* box
EMSAluxDR	GCGAATGACATGAAAACCGTTAGTAG	

[a] See reference 34.
[b] See reference 10.

RNA samples and to determine the quantities of RNA that had been purified. cDNA was made from 1 μg of total RNA using random hexamer primers and an iScrip cDNA synthesis kit (Bio-Rad, Hercules, CA). The resulting cDNA was diluted 1:10 and used as a template in RT-PCR with SYBR green detection using the Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad). The starting quantity of template for each sample was determined using a five-point standard curve generated by amplification of PCR products containing portions of the genes of interest from known quantities of plasmid templates. Melting curves were analyzed at the end of each PCR run, and controls included PCRs without total RNA and without the reverse transcriptase. RT-PCR was performed using the cDNA of each set of RNA in triplicate. The mean quantities of *luxI*, *qsrP*, *ribB*, and *acfA* transcripts were normalized based on the mean quantity of the control gene, *nadB*.

EMSA. The EMSA protocol used was based on the method of Urbanowski et al. (34). A DNA probe that served as both positive and negative controls was derived from a 400-bp PCR fragment containing the *luxI-luxR* regulatory region using template pSH202 (3). This PCR product was labeled at both ends using [γ-^{32}P]ATP plus T4 nucleotide kinase and was subsequently cleaved with MwoI, resulting in 160- and 240-bp fragments. Other DNA probes were generated via PCR and contained approximately 130 bp of the *luxI*, *qsrP*, *acfA*, or *ribB* promoter region or the *lux* box of *luxD* (Table 2). Protein-DNA binding reaction mixtures contained 1 fmol of each DNA probe in 20 μl (final volume) of DNA binding buffer (20 mM Tris-HCl [pH 7.4 at 22°C], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg of bovine serum albumin/ml, 5% glycerol). Purified LuxR and 6 μM 3-oxo-C6-L-HSL were added as indicated below, and the reaction mixtures were incubated at 26°C for 20 min. One microliter of loading dye (0.1% xylene cyanol in 50% glycerol) was added, the reaction mixtures were loaded onto a native 5% Tris-glycine-EDTA gel on ice, and electrophoresis was performed at 10 V/cm for 2 h at 4°C.

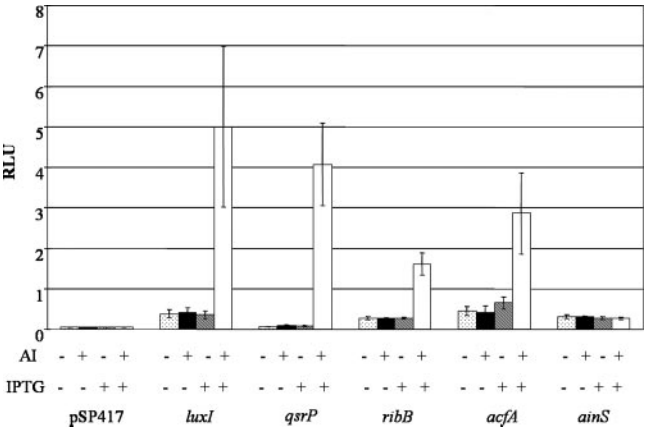

FIG. 2. LuxR-dependent LacZ expression driven by *V. fischeri* promoters. The *luxI*, *qsrP*, *ribB*, *acfA*, and *ainS* promoters were fused to LacZ in pRS417, and β-galactosidase levels in recombinant *E. coli* JM109 were determined. Three independent trials were conducted, with triplicate assays for each trial. The error bars indicate standard deviations from the means. The presence (+) and absence (−) of the autoinducer (AI) and IPTG are indicated at the bottom. RLU, relative light units.

RESULTS AND DISCUSSION

LuxR dependence of *V. fischeri* promoters in recombinant *E. coli*. β-Galactosidase assays were performed with recombinant *E. coli*/pAMS121 cells containing pNQ101 to pNQ110 individually (Table 1) in order to establish the presence of LuxR-dependent gene expression from the constructs. The reporter constructs contained the promoters for *luxI*, *qsrP*, *ribB*, *acfA*, *ainS*, *qsrQ* (two versions), *qsrRST* (two versions), and *arcA* from *V. fischeri* MJ-100 fused to *lacZ*. Cells were grown to the mid-exponential phase (OD_{600}, 0.5), when the LuxR-dependent quorum-sensing response is normally upregulated. Growth media with four different combinations of IPTG and 3-oxo-C6-HSL levels were used to influence the expression and activity of the LuxR encoded on pAMS121. Four promoters controlling *luxI*, *qsrP*, *ribB*, and *acfA* expression were shown to be activated in a LuxR-dependent manner, but only in the presence of both LuxR and 3-oxo-C6-HSL (Fig. 2). The levels of β-galactosidase expressed from these constructs were not equal; the *luxI* and *qsrP* constructs had the highest β-galactosidase levels, the *ribB* construct had the lowest β-galactosidase levels, and *acfA* expression was intermediate compared to the expression for the other genes in recombinant *E. coli*. This range suggests that there are differences in promoter strength and message stability between these four genes. The remaining promoters that were examined for LuxR-dependent expression did not drive β-galactosidase expression at levels above the background level, as shown for P_{ainS} (Fig. 2 and data not shown). Therefore, we concluded that in recombinant *E. coli*, *ainS*, *qsrQ*, *qsrRST*, and *arcA* are not upregulated by LuxR under the conditions tested here. To test if these promoters were repressed by LuxR, additional β-galactosidase assays were performed to compare expression (relative light units/OD_{600}) at an OD_{600} of 0.25 and the expression at an OD_{600} of 0.75; no repression was observed (data not shown). Therefore, no direct induction or repression of *ainS*, *qsrQ*, *qsrRST*, and *arcA* by LuxR was found in these studies or in the previous 2D SDS-PAGE analysis (2). These findings may not exclude the possibility that there is quorum-dependent regulation under circumstances where the environment within the native host, *V. fischeri*, plays an additional regulatory role. The genes that were definitively determined to be in the LuxR regulon, *qsrP*, *ribB*, and *acfA*, were used for further analysis.

Analysis of the sites of transcription initiation. To define the promoter structure of *qsrP*, *ribB*, and *acfA*, the transcriptional start sites were identified. TraR, which is a LuxR homolog in *Agrobacterium tumefaciens*, can activate transcription from either class I or class II promoters (39). In a class I promoter, the activator binding site is located at approximately position −60 and the activator interacts with the C-terminal domain of the alpha subunit of RNA polymerase (13). In a class II promoter, the activator binding site is centered near position −40, overlapping the −35 recognition site for RNA polymerase and placing the activator in a position to potentially have multiple protein-protein interactions with the alpha and sigma subunits of RNA polymerase (1). In *V. fischeri*, the *luxI* promoter has a class II organization (10, 16). Based on the initial sequence analysis of the *qsrP* and *acfA* promoters, the *lux* box was predicted to overlap the −35 site (Fig. 1). Therefore, these two promoters were hypothesized to have a class II structure, similar to the *luxI* promoter. However, the *ribB* promoter contained two putative −10 sites, two putative −35 sites, and a *lux* box overlapping one of the −35 regions (Fig. 1A), indicating that it could potentially have either a class I-type structure or a class II-type structure.

Primer extension analysis was performed with RNA extracted from *E. coli*/pSC300, a strain expressing *luxR* under control of P_{tac}, and the vectors encoding *qsrP*, *ribB*, or *acfA*. RNA was also extracted from the *V. fischeri luxI* mutant strain MJ-211 (Table 1). Strains were grown either in the presence or in the absence of 3-oxo-C6-HSL to ascertain the dependence

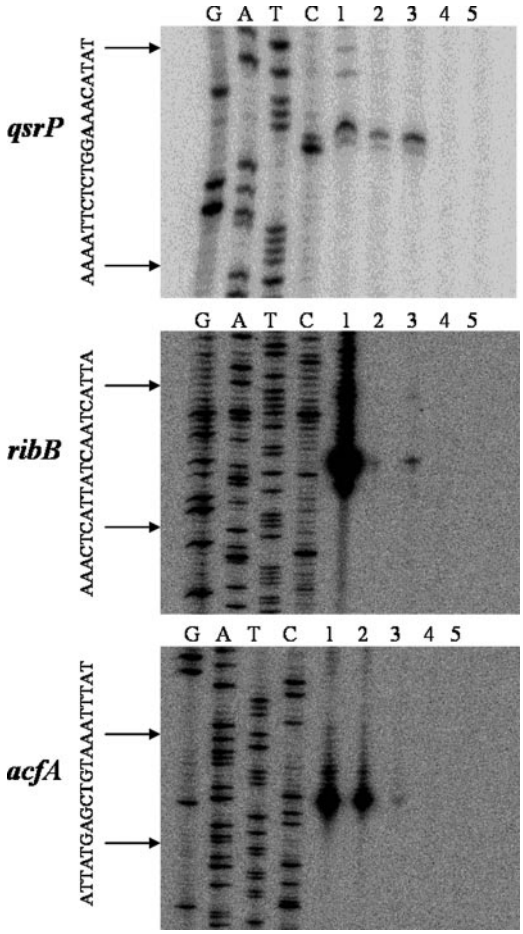

FIG. 3. Primer extension analysis of the *qsrP*, *acfA*, and *ribB* promoters. Lanes G, A, T, and C, sequencing ladder from plasmid DNA; lane 1, RNA from *E. coli* JM109/pSC300 (*luxR*)/pSUP102 carrying the promoter of interest with 3-oxo-C6-HSL; lane 2, RNA from *E. coli* JM109/pSC300 (*luxR*)/pSUP102 carrying the promoter of interest without 3-oxo-C6-HSL; lane 3, RNA from *V. fischeri* MJ211 (Δ*luxI*) with 3-oxo-C6-HSL; lane 4, RNA from *V. fischeri* MJ211 (Δ*luxI*) without 3-oxo-C6-HSL; lane 5, no RNA (negative control). The results are representative of the results of assays done in duplicate.

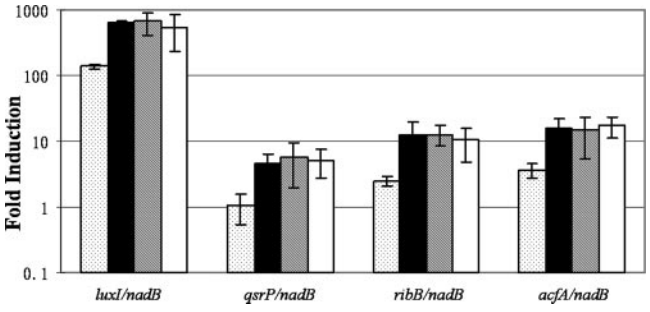

FIG. 4. Results of real-time RT-PCR performed during the exponential and stationary phases of growth with *V. fischeri* MJ-100. The SQ ratios of *luxI*, *qsrP*, *ribB*, and *acfA* mRNA to *nadB* mRNA are shown at four points during growth. OD_{600} of 0.25, 0.5, 1.0, and 2.0 are indicated by light gray, black, dark gray, and white bars, respectively. The results are representative of the results for triplicate samples from two trials, and the error bars indicate the standard deviations from the means. Note the exponential scale.

of the transcript on the quorum-sensing response. Since a higher copy number of the target transcript was present in *E. coli*, it was anticipated that more primer extension product would be generated than was generated in *V. fischeri*, and that is what was observed. The primer extension results for *qsrP* (Fig. 3) show both a major site and a minor site of transcription initiation under three of the four conditions tested; no transcripts were recovered from *V. fischeri* in the absence of 3-oxo-C6-HSL. The strongest transcripts were produced from either *E. coli* or *V. fischeri* strains in the presence of 3-oxo-C6-HSL. Based on these results, the position of the dominant transcriptional start site of *qsrP* was identified (Fig. 1A and 3), and the promoter was confirmed to have a class II-type structure.

For *ribB* and *acfA*, a series of bands were observed upon analysis of the primer extension products from *E. coli*. However, in *V. fischeri*, there was only one band for each gene, which corresponded to the dominant band from the *E. coli* samples. The strongest primer extension products were detected in samples from *E. coli* that had been exposed to 3-oxo-C6-HSL. Very weak *ribB* and *acfA* primer extension products were detected in *V. fischeri* cells that had been exposed to 3-oxo-C6-HSL. Therefore, for *ribB* and *acfA*, the amount of mRNA produced in *V. fischeri* appeared to be close to the threshold of detection for primer extension analysis. Nevertheless, the transcriptional start sites for *ribB* and *acfA* were identified (Fig. 1A and 3), and we concluded that both promoters have a class II-type architecture.

Timing of LuxR-dependent gene expression in *V. fischeri*. In order to ascertain the expression pattern of the LuxR regulon in its native host, *V. fischeri*, a real-time RT-PCR analysis was carried out with RNA samples extracted from cells grown from the early exponential phase to the stationary phase. In *P. aeruginosa*, the timing of quorum-sensing gene expression is on a continuum, which means that some genes are induced early in growth and others are induced at the transition from the exponential phase to the stationary phase or during the stationary phase (29). In this study, we used two RNA sets, both purified under the same conditions from *V. fischeri* cells grown to four different OD_{600} (0.25, 0.5, 1.0, and 2.0). The luminescence outputs of these cells were checked when the cells were harvested for RNA purification. At these four OD_{600}, the ratios of relative light units to OD were 27, 583, 643, and 829 (average data from the two trials). Thus, the quorum-sensing phenotype of bioluminescence was activated by mid-exponential-phase growth, as has been observed previously.

Using fragments of *nadB* and *luxI* as controls, the expression of the three putative LuxR-dependent genes was determined in two independent experiments using real-time RT-PCR. The *nadB* gene, which encodes L-aspartate oxidase in *E. coli*, was found to be expressed constitutively based on the work of other researchers (E. P. Greenberg, personal communication), and our initial analysis supported this finding. The starting quantity (SQ) of transcript was used to plot the RT-PCR data because it showed an elevated response due to quorum sensing, which is intuitively easier to understand. However, the threshold cycle, which is inversely proportional to the log SQ, also confirmed our findings. The SQ of *luxI*/*nadB* transcripts increased significantly at an OD_{600} of 0.5, and expression was maintained at OD_{600} of 1.0 and 2.0 (Fig. 4). This pattern of expression is

what would be expected for a LuxR-dependent gene activated by the quorum-sensing response of the cells.

The SQ of *qsrP*/*nadB*, *ribB*/*nadB*, and *acfA*/*nadB* were much lower than that of *luxI*/*nadB* (note the exponential scale in Fig. 4). However, the former three transcripts were expressed in a pattern similar to that of *luxI*. The amount of the transcripts was significantly greater than the background amount at an OD_{600} of 0.5, and the level of expression was roughly maintained at OD_{600} of 1.0 and 2.0. Hence, similar to the results obtained with the β-galactosidase reporters in recombinant *E. coli*, the RT-PCR analysis demonstrated that *qsrP*, *ribB*, and *acfA* are temporally expressed in a pattern like that of *luxI*. However, the expression of these three genes was found to be significantly lower than that of *luxI* in the RT-PCR analysis. This was not predicted based on the β-galactosidase assays performed with the heterologous host *E. coli*. The apparent discrepancy may be resolved by considering that in the β-galactosidase assays, it is not only the rate of transcription but also the stability of the transcript, the rate of translation of *lacZ* mRNA, and the stability of LacZ that contribute to the final output measured. In the case of real-time RT-PCR, the pool of a particular mRNA of interest is measured and is dependent on the rate of transcription and the stability of the mRNA. To help further determine the relative expression levels of the LuxR regulon genes, EMSA were performed to ascertain whether differences in the DNA binding affinity of LuxR to the *lux* box of these genes were a key parameter in controlling expression levels.

Affinity of LuxR for the *lux* boxes of the genes in the LuxR regulon. EMSA were used to determine the relative LuxR binding affinities for DNA fragments containing the *lux* boxes from the *luxI*, *qsrP*, *acfA*, and *ribB* promoters (Fig. 1B). In addition, the *luxD* box, a proposed *lux* box internal to *luxD* within the *lux* operon that has been hypothesized to be involved in negative regulation of the *lux* operon (30), was also used as a binding site for LuxR. The promoter of *luxR* itself served as a negative control. In the presence of 6 μM autoinducer, LuxR could bind to the promoter regions of four of the five *lux* box sequences examined (Fig. 5). A direct interaction between LuxR and the *luxI*, *qsrP*, *acfA*, and *ribB* promoters was definitively established. However, a direct association of LuxR with the *luxD* box could not be established under the conditions that were utilized, even when the ratio of protein to probe was increased 10- to 20-fold (data not shown). Therefore, in contrast to a previous proposal (30), this suggests that the *luxD* box may not play a major role in regulation of the *lux* genes. The dissociation constant (K_D) of LuxR for the DNA was roughly calculated by determining the protein concentration that shifted 50% of the probe. K_D values of 0.55 nM for P_{luxI}, 1.96 nM for P_{qsrP}, 0.60 nM for P_{ribB}, and 1.22 nM for P_{acfA} demonstrated that LuxR has higher affinities for P_{luxI} and P_{ribB} and lower affinities for P_{qsrP} and P_{acfA}. However, all of the K_D values were within fourfold of one another, suggesting that the weaker expression of *qsrP*, *acfA*, and *ribB* in *V. fischeri* measured via real-time RT-PCR was not due to dramatic differences in the affinity of LuxR for the promoters under study. Thus, the difference in expression is most likely due to the altered efficiencies in the interactions between LuxR and RNA polymerase, the rate of open complex formation, or the stability of the mRNA.

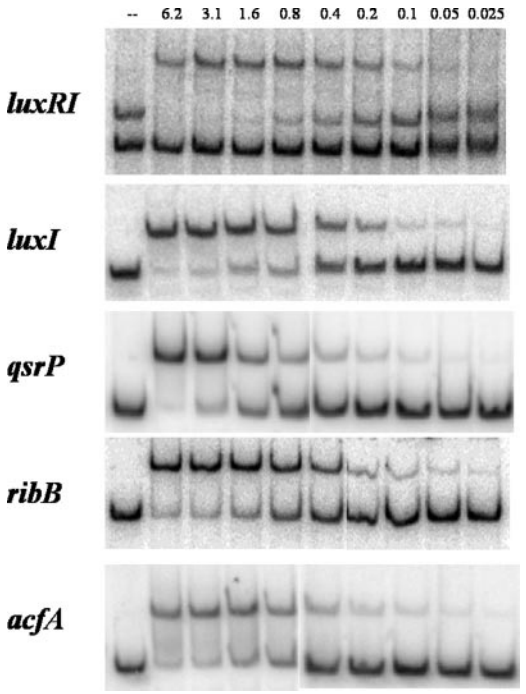

FIG. 5. EMSA demonstrating LuxR binding to *luxRI*, *luxI*, *qsrP*, *ribB*, and *acfA* promoter DNA. Each lane contained approximately 1 fmol of probe (for *luxRI*, 1 fmol of both probes) and the concentration of LuxR indicated at the top (nM) with 6 μM 3-oxo-C6-HSL. The results are representative of the results of assays done in duplicate.

Conclusions. Direct LuxR-dependent transcription of *qsrP*, *acfA*, and *ribB* from *V. fischeri* MJ-100 has been demonstrated. Activation of transcription of these genes occurs during mid-exponential growth, similar to temporal expression of the *lux* operon. However, real-time RT-PCR analysis indicated that the amounts of the *qsrP*, *acfA*, and *ribB* transcripts present in the cell's mRNA pool are significantly lower than the amount of *luxI*. EMSA demonstrated that LuxR has a slightly higher affinity for the *lux* boxes in the *luxI* and *ribB* promoters than for those in the *qsrP* and *acfA* promoters. LuxR binding to the *luxD* box, however, could not be demonstrated. The establishment here of a LuxR regulon in *V. fischeri* may provide insights into the optimal binding site for LuxR and lead to the identification of other LuxR-dependent genes.

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