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Transcriptome Analysis of the *Vibrio fischeri* LuxR-LuxI Regulon▽

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The *Vibrio fischeri* quorum-sensing signal *N*-3-oxohexanoyl-L-homoserine lactone (3OC6-HSL) activates expression of the seven-gene luminescence operon. We used microarrays to unveil 18 additional 3OC6-HSL-controlled genes, 3 of which had been identified by other means previously. We show most of these genes are regulated by the 3OC6-HSL-responsive transcriptional regulator LuxR directly. This demonstrates that *V. fischeri* quorum sensing regulates a substantial number of genes other than those involved in light production.

Quorum sensing allows a species to measure its population density and control gene expression in a population density-dependent manner. Bacterial quorum sensing involves cell-cell communication mediated by extracellular signal compounds. Various species of proteobacteria use acyl-homoserine lactones (acyl-HSLs) as quorum-sensing signals (2, 12–15, 36, 42). Acyl-HSL quorum sensing was first described in the marine luminescent bacterium *Vibrio fischeri*, where it controls transcription of the luminescence (*lux*) operon (7, 10, 11). The LuxI acyl-HSL synthase and the LuxR transcriptional activator constitute the quorum-sensing system, which controls the *lux* operon directly. The LuxI-generated signal is *N*-3-oxohexanoyl-L-HSL (3OC6-HSL), and LuxR activates the *lux* operon in response to this signal. The LuxR–3OC6-HSL complex binds to a 20-bp inverted repeat centered at −42.5 from the transcriptional start site of the *lux* operon (for reviews, see references 14, 36, and 41).

Vibrio fischeri occurs at low population densities in seawater and at high densities in specific light organ symbioses with certain fish and squid (3, 21, 29). Quorum sensing allows *V. fischeri* to discern its existence in the symbiosis and activate transcription of the *lux* operon (4). Many proteobacteria have acyl-HSL signaling systems, which serve as global regulators of extracellular factor production and are often important for successful interactions with plant or animal hosts (26, 36). Existing evidence shows that besides the *lux* operon, LuxR and 3OC6-HSL activate five other genes (5). However, the search for quorum-controlled genes in *V. fischeri* has been limited to proteomic analyses. We recently reported the complete genome sequence of a strain of *V. fischeri* isolated from a squid light organ (30). Thus, it is now possible to use DNA microarrays to identify LuxR-regulated *V. fischeri* genes on a global scale. We have designed and constructed a microarray and used it to perform an analysis of 3OC6-HSL-regulated genes in *V. fischeri*. We furthered this analysis by examining the activity of relevant promoters in response to 3OC6-HSL and LuxR in recombinant *Escherichia coli* and by analyzing the binding of LuxR to promoter DNA fragments in vitro.

We used *E. coli* DH12S (Invitrogen, Carlsbad, CA) and *V. fischeri* ES114 (3). The genome sequence of *V. fischeri* ES114 is publicly available at http://www.ergo-light.com (30). We used Luria-Bertani broth (31) without added sodium chloride to grow *E. coli*, at 30°C, and seawater-tryptone broth (3) with seawater salts (300 mM NaCl, 10 mM KCl, 50 mM $MgSO_4$, 10 mM $CaCl_2$) to grow *V. fischeri* at 28°C. All cultures were grown with shaking at 250 rpm. For LuxR expression in *E. coli*, we used pHV402, which contains a chloramphenicol resistance gene and contains *luxR* controlled by its own promoter (18). For cloning of promoter elements, we used pPROBE-*gfp*[LVA], which contains a promoterless *gfp* and a kanamycin resistance marker (24). Chloramphenicol and kanamycin were used for plasmid maintenance at 25 and 50 μg/ml, respectively. 3OC6-HSL (Sigma, St. Louis, MO) was added as described elsewhere (33) at a final concentration of 2.3 or 1 μM, as indicated.

In conjunction with Affymetrix (Santa Clara, CA), we designed custom GeneChips (11-μm feature size) representing >99% of the annotated open reading frames in the *V. fischeri* ES114 genome, as well as 125 intergenic regions greater than 150 bp. GeneChips were designed as described by the manufacturer, except that because the *V. fischeri* genome has a low G+C content (38 mol%), we used 16 probe pair sets (25-bp oligonucleotide probe length) per gene or intergenic region. Poly(A) control sequences of *dap*, *lys*, *phe*, and *trp* from *Bacillus subtilis* were included. We compared transcript profiles of *V. fischeri* ES114 grown in the presence versus the absence of added 3OC6-HSL (1 μM). Compared to other strains of *V. fischeri*, strain ES114 produces little 3OC6-HSL in laboratory culture (4). While it produces 3OC6-HSL in amounts insufficient to appreciably induce the *lux* genes (23, 39), strain ES114 produces larger quantities of a second acyl-HSL, C8-HSL, by using an enzyme called AinS (16, 19). C8-HSL can bind LuxR, but C8-HSL does not appreciably activate LuxR (32, 39). However, C8-HSL can affect *lux* gene expression in *V. fischeri* ES114 indirectly (23). Therefore, we were able to add 3OC6-HSL to wild-type cultures and monitor the induction of LuxR-controlled genes. We isolated and purified RNA for GeneChip

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TABLE 1. Differential expression of *V. fischeri* genes in response to 3OC6-HSL

Gene or intergenic region (gene location)[a]	Annotation	Fold regulation at OD_{660}[b] of:	
		0.3	1.5
VF1161 (1290494–1291648)	Periplasmic component of efflux system	25.6	10.3
VF1162 (1291644–1292921)	Outer membrane efflux protein (TolC)	12.5	7.1
VF1163 (1292921–1294135)	Export ABC transporter permease protein	8.9	6.4
VF1164 (1294142–1295422)	Export ABC transporter permease protein	7.2	5.4
VF1165 (1295418–1296140)	ABC transporter ATP-binding protein	7.3	5.4
VF1246 (1390844–1389936)	Putative omptin family serine protease	31.8	12.2
VF1247 (1391269–1391697)	Peptidoglycan-binding protein, LysM	2.6	2.7
VF1348 (1493989–1492961)	Hypothetical cytosolic protein	9.5	6.7
VF1349 (1495924–1494185)	Alkaline serine protease	8.9	6.6
VF1725 (1948180–1949709)	Secretory tripeptidyl aminopeptidase	27.3	16.0
VF1977* (2215928–2215569)	Protein YgiW precursor	6.1	ND
VF1978* (2216620–2215988)	Accessory colonization factor A (AcfA)-like protein	16.1	ND
VF2034 (2270424–2270026)	Hypothetical polypeptide	2.6	ND
VF2035 (2270891–2270424)	Hypothetical polypeptide	2.7	ND
VF2036 (2271435–2270905)	Possible phage regulatory protein	2.7	ND
VFA0090 (99228–98098)	Astacin peptidase	29.2	9.9
VFA0373 (420748–422019)	Mechanosensitive ion channel	9.7	3.5
VFA0622 (702734–702162)	Hypothetical protein	7.2	3.6
VFA0834 (939286–941157)	NirV precursor	ND	3.2
VFA0894 (1014502–1016295)	Bacterial immunoglobulin-like protein	35.9	16.7
VFA0895 (1016495–1016854)	Permease (major facilitator superfamily)	3.3	2.6
VFA0918* (1044437–1043730)	*luxG*	9.0	5.9
VFA0919* (1045577–1044450)	*luxE*	10.0	6.7
VFA0920* (1046636–1045659)	*luxB*	8.1	4.9
VFA0921* (1047714–1046650)	*luxA*	10.6	6.0
VFA0922* (1048660–1047740)	*luxD*	8.9	5.0
VFA0923* (1050115–1048679)	*luxC*	14.4	7.4
VFA0924* (1050725–1050156)	*luxI*	13.9	5.7
VFA1058* (1194722–1195164)	*qsrP*	37.2	10.9
VF1866 to VF1867 (2105494–2106116)	Intergenic region	ND	3.8
VFA0643 to VFA0644 (719637–720412)	Intergenic region	3.5	2.4
VF1615 (1814208–1814837)	Hypothetical protein	ND	−2.7

[a] Genes marked with an asterisk have been identified as 3OC6-HSL regulated elsewhere. VF designates genes on the large chromosome, and VFA designates genes on the small chromosome.

[b] "Fold regulation" refers to transcript levels in the presence versus absence of 3OC6-HSL. ND, no significant difference. All significant differences show a P value of <0.001.

analysis at culture optical density at 600 nm (OD_{600}) values of 0.3 and 1.5, prepared cDNA, and performed hybridizations and scanning as described elsewhere (www.affymetrix.com). Results are the averages from two independent experiments, and the results were analyzed by using the Affymetrix Microarray Suite v.5 and Cyber-T (http://cybert.microarray.ics.uci.edu) (1). We sorted for genes that showed 2.5-fold or greater differential regulation with a P value of <0.001.

Predicted promoter regions of genes identified as 3OC6-HSL-regulated in transcript analyses were amplified by PCR with *V. fischeri* genomic DNA as the template. Fusions were constructed by generation of PCR fragments tailed with EcoRI and BamHI sites, digestion of the PCR fragments with EcoRI and BamHI, and ligation of the digested PCR fragments with EcoRI-BamHI-digested pPROBE-*gfp*[LVA]. The resulting plasmids were used to transform *E. coli* DH12S with or without pHV402 as previously described (31). Promoter activity was measured as follows. Overnight cultures of recombinant *E. coli* were used to inoculate fresh cultures at a starting OD_{600} of 0.1 in medium with or without added 3OC6-HSL (2.3 μM). At an OD_{600} of 1.2 (±0.2), cells from each culture were pelleted by centrifugation and suspended in phosphate-buffered saline and the fluorescence of the suspension was measured with a GENios Pro 96-well plate reader (TECAN, Research Triangle Park, NC). Results are the averages of three independent experiments.

Global analysis of 3OC6-HSL-regulated gene expression. Our microarray analysis revealed 30 genes and 2 intergenic regions with transcript levels that were at least 2.5-fold different when 3OC6-HSL-grown cells were compared to cells grown without added 3OC6-HSL (Table 1). One of the 30 genes (VFA0895) showed a minimal activation, and it overlaps and forms a predicted operon with VFA0896 (http://www.ergo-light.com), which was not regulated by 3OC6-HSL in our experiments. We did not study VFA0895 further. Also, VF1247, VF1615, VF2034, VF2035, VF2036, and VFA0834 showed induction levels just above our 2.5-fold cutoff (2.6- to 3.2-fold). We examined transcript levels of these six genes further by real-time PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 5 ng of cDNA, and 900 nM of each primer. With the exception of VF1247 and VF1615, all genes showed minimal activation by 3OC6-HSL (<2-fold). Thus, we did not examine VF2034 to VF2036 and VFA0834 further.

The 25 3OC6-HSL-controlled genes we continued to study were distributed almost equally between the large and small *V. fischeri* chromosomes. We did not study expression of the intergenic regions differentially regulated by 3OC6-HSL (Table

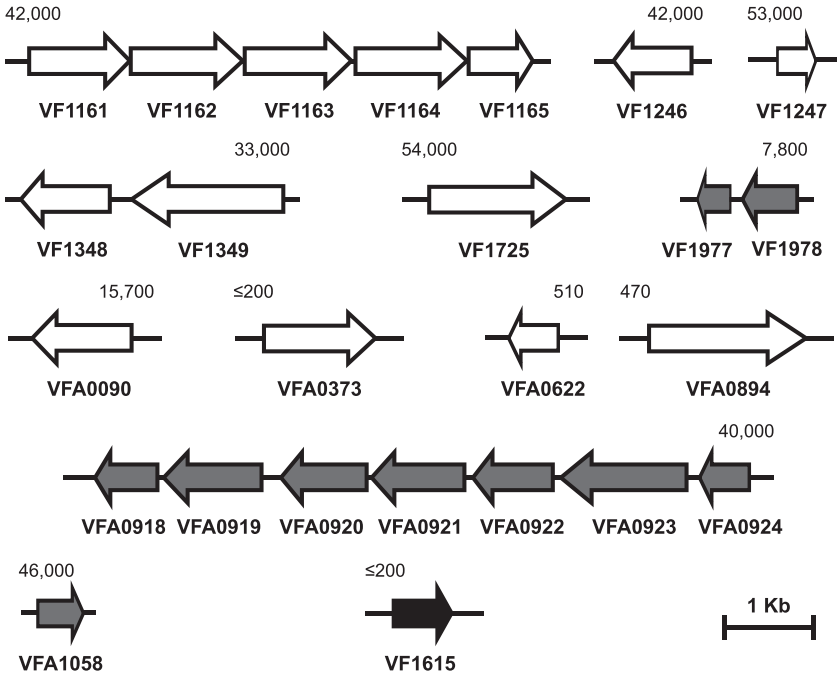

FIG. 1. Genetic organization and transcription activation of 3OC6-HSL-controlled genes. White arrows represent 3OC6-HSL-activated genes, and a black arrow represents the 3OC6-HSL-repressed gene. Genes previously reported as 3OC6-HSL controlled are represented by gray arrows. The directions of the arrows indicate the DNA strand. The values for gene activation (relative units of GFP fluorescence per OD unit) are shown above the first gene in each operon and are from the *E. coli* expression studies described in the text. In all cases, basal levels of expression with LuxR and without 3OC6-HSL or without LuxR and with 3OC6-HSL were <200 fluorescence units. The values are means of three independent experiments, and the ranges were ±28% of the means.

1). Twenty-four of the 25 genes were induced and 1 was repressed by the quorum-sensing signal (Table 1). Ten of these genes have been identified previously as 3OC6-HSL controlled: VF1977, VF1978, and VFA1058 (5) and the 7 genes in the *lux* operon (7, 10, 11). These genes code for functions related to light production or have unknown functions. Activities predicted for the 15 newly discovered genes include protease and peptidase functions, an ABC-type transporter, and a mechanosensitive ion channel.

Many of the 3OC6-HSL-regulated genes we discovered are predicted to be in operons (http://www.cifn.unam.mx/moreno/pub/TUpredictions/Predictions/) (25). There are two clusters of linked genes predicted to consist of two transcriptional units each: VFA0924 to VFA0918 and VF1348 and VF1349. The in silico predictions are based primarily on distances between predicted reading frames and are conservative predictors of operons. In fact, we know from previous work that the VFA0924-to-VFA0918 cluster, *luxICDABEG*, is organized as an operon (10, 11). To determine whether VF1348 and VF1349 might constitute an operon, we performed open reading frame junction-based reverse transcription-PCR, and the analysis supports the contention that these two genes constitute an operon (data not shown). Thus, the 3OC6-HSL-controlled genes identified in the microarray analysis appear to be organized as 13 transcriptional units: 4 multigene operons and 9 single-gene units (Fig. 1).

Evidence for direct LuxR activation of 3OC6-HSL-controlled operons. In the first analysis, we examined the dependence of GFP expression driven by the predicted promoter region of each of the 13 3OC6-HSL-regulated *V. fischeri* transcriptional units in recombinant *E. coli.* Eleven of the 12 3OC6-HSL-activated promoters showed 3OC6-HSL-LuxR dependence (Fig. 1). The 3OC6-HSL-repressed promoter did not show LuxR-dependent control in *E. coli*. We confirmed a direct interaction of LuxR with 7 of the 11 promoters activated by LuxR in *E. coli* (VF1247, VF1349, VF1725, VFA0622, VFA0894, VFA0924, and VFA1058) by showing that they bind to purified LuxR in vitro (Fig. 2). One interpretation of our inability to demonstrate LuxR binding to the other four promoters in vitro is that they are low-affinity LuxR binding targets.

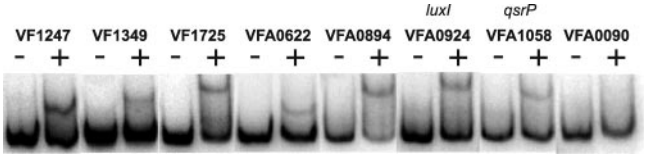

FIG. 2. Gel shift assays showing direct binding of purified LuxR to promoters of 3OC6-HSL-regulated genes in vitro. Experiments were performed as previously described (28, 37). Reaction mixtures contained 1 fmol of DNA, 6 μM 3OC6-HSL, and either no LuxR (−) or LuxR at a final concentration of 6.2 nM (+). DNA probes were generated by PCR amplification of promoter regions using the transcriptional fusion plasmids as templates. Probes are shown side by side to facilitate comparison, even though sizes are different. VF0090 is shown as an example of a promoter fragment that is not shifted by LuxR, and this serves as a negative control.

Search for LuxR binding sites in 3OC6-HSL-controlled promoter regions. The LuxR protein interacts with a 20-bp inverted repeat centered at −42.5 from the start of *luxICDABEG* transcription (6, 8, 9, 37). We sought to assess whether the other LuxR-controlled transcriptional units have similar sequences in their promoter regions by searching for similarities to a relaxed consensus sequence (DVCTGYADKAWNNKA CAGKW) based on multiple *V. fischeri luxI* promoter sequences available from the NCBI database (http://www.ncbi.nlm.nih.gov/) and promoter sequences of *qsrP* from *V. fischeri* MJ-100 and ESR1 and *ribB* from MJ-100 (5) by using the Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be/rsat/) (38). Out of the 11 promoter regions searched, only 2 showed identifiable *lux* boxes. The promoter of VFA0924 (*luxI*) showed the canonical *lux* box, and the promoter of VFA1058 (*qsrP*) presented the sequence ACCTGTAATAAA CGACAGGA, which was described previously as a potential *lux* box (5, 28). Our lack of ability to identify *lux* box elements in the other nine promoters indicates that we do not yet have a clear picture of the elements required for LuxR binding to any given 3OC6-HSL-dependent promoter region. This is not surprising in light of the fact that the *Pseudomonas aeruginosa* LuxR homolog LasR can bind to *lux* box-like sequences called *las* boxes and also to sequences with no obvious similarity to *las* boxes (35). A considerably more detailed analysis is required to identify the LuxR binding determinants in the newly discovered quorum-sensing controlled promoters.

Acyl-HSL quorum sensing was discovered in *V. fischeri*, where it regulates the seven-gene *lux* operon (7, 10, 11). Studies of other proteobacteria have revealed that acyl-HSL signaling often regulates multiple genes that are distributed throughout the genome (for example, see references 34 and 40). In *P. aeruginosa*, a number of acyl-HSL-controlled genes code for the production of extracellular virulence factors (20, 34, 40). A few *V. fischeri* 3OC6-HSL-controlled genes in addition to the *lux* genes have been described previously (5, 28). We used a *V. fischeri* DNA microarray to identify 14 previously unreported 3OC6-HSL-activated genes and 1 3OC6-HSL-repressed gene. The annotation of the *V. fischeri* genome indicates four of the 3OC6-HSL-activated genes we identified code for proteases or peptidases (Table 1). Protease and peptidase genes are regulated by acyl-HSL quorum sensing in other bacteria (34, 40). We know that *V. fischeri* can acquire amino acids from the squid host during growth in the light organ (17). These 3OC6-HSL-induced proteases and peptidases may thus have significant roles in the symbiosis, aiding in nutrient acquisition. The activated genes also include an intimin-like adhesin (VFA0894). In *E. coli*, an intimin is involved in virulence by mediating bacterial attachment to mammalian host cells (22). Therefore, it is easy to imagine that the *V. fischeri* intimin-like protein could be involved in the light organ symbiosis. A gene annotated as coding for an accessory colonization factor A-like protein is also activated by 3OC6-HSL. This gene shows significant sequence similarity to the *V. cholerae acfA* gene, which is required for efficient intestinal colonization by this pathogen (27). Previous studies showed that a LuxR mutant derived from *V. fischeri* ES114 colonized light organs at a reduced level compared to its parent. The colonization defect could be compensated for by provision of the *lux* genes under control of a LuxR-independent promoter (39). These experiments on initial light organ colonization suggest that if any of the genes we have uncovered are involved in symbiotic competence, the involvement is likely to be subsequent to initial colonization.

Microarray data accession number. The microarray data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE7485.

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