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Quorum Sensing in *Vibrio anguillarum*: Characterization of the *vanI/vanR* Locus and Identification of the Autoinducer *N*-(3-Oxodecanoyl)-L-Homoserine Lactone

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Certain gram-negative pathogens are known to control virulence gene expression through cell-cell communication via small diffusible signal molecules termed autoinducers. This intercellular signal transduction mechanism termed quorum sensing depends on the interaction of an *N*-acylhomoserine lactone (AHL) autoinducer molecule with a receptor protein belonging to the LuxR family of positive transcriptional activators. *Vibrio anguillarum* is a gram-negative pathogen capable of causing a terminal hemorrhagic septicemia known as vibriosis in fish such as rainbow trout. In this study, we sought to determine whether *V. anguillarum* employs AHLs to regulate virulence gene expression. Spent *V. anguillarum* culture supernatants stimulated bioluminescence in a recombinant *lux*-based *Escherichia coli* AHL biosensor strain, whereas they both stimulated and inhibited AHL-mediated violacein pigment production in *Chromobacterium violaceum*. This finding suggested that *V. anguillarum* may produce multiple AHL signal molecules. Using high-performance liquid chromatography and high-resolution tandem mass spectrometry, we identified the major *V. anguillarum* AHL as *N*-(3-oxodecanoyl)-L-homoserine lactone (ODHL), a structure which was unequivocally confirmed by chemical synthesis. The gene (*vanI*) responsible for ODHL synthesis was cloned and sequenced and shown to belong to the LuxI family of putative AHL synthases. Further sequencing downstream of *vanI* revealed a second gene (*vanR*) related to the LuxR family of transcriptional activators. Although deletion of *vanI* abolished ODHL synthesis, no reduction of either metalloprotease production or virulence in a fish infection model was observed. However, the *vanI* mutant remained capable of weakly activating both bioluminescence and violacein in the *E. coli* and *C. violaceum* biosensors, respectively, indicating the existence of additional layers of AHL-mediated regulatory complexity.

Many different bacteria exploit a cell-cell communication device to regulate transcription of multiple target genes in concert with cell density. This communication device, termed quorum sensing, depends on the production of one or more diffusible signal molecules termed autoinducers or pheromones which enable a bacterium to monitor its own population density (for reviews, see references 15, 45, 55, and 57). Quorum sensing is thus an example of multicellular behavior in prokaryotes and is now known to regulate diverse physiological processes including bioluminescence (29, 30), swarming (13), antibiotic biosynthesis (1, 2, 58), plasmid conjugal transfer (14, 41, 59), and the production of exoenzyme virulence determinants in human and plant pathogens (4, 23, 38). In gram-negative bacteria, the autoinducer molecules are *N*-acylhomoserine lactones (AHLs) which vary predominantly in the presence or absence of an acyl chain C-3 substituent (oxo- or hydroxy-) and in the length of the *N*-acyl side chain (molecules with from 4 to 14 carbons have been characterized [2, 5, 11, 19, 39, 48, 53, 56]). In gram-positive bacteria, all of the pheromones identified so far have been either γ-butyrolactones or posttranslationally modified peptides (22, 57).

The bioluminescent phenotype of the symbiotic marine bacterium *Vibrio* (*Photobacterium*) *fischeri* is perhaps the most intensively studied quorum sensing system (29, 30). At high cell densities such as those achieved within the light organs of certain marine animals, *V. fischeri* is highly bioluminescent. When free-living, at low cell densities, cultures of *V. fischeri* emit little light. As the cell density increases, the level of the autoinducer, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), accumulates until it reaches a critical threshold concentration at which transcription of the luminescence (*lux*) operon is triggered. OHHL is thought to bind directly to, and activate, the transcriptional activator protein LuxR such that the LuxR-OHHL complex stimulates transcription of the *luxICDABE* operon. Since *luxI*, the autoinducer synthase, is the first gene in this operon, this leads to increased levels of LuxI and hence more OHHL, setting up an autoregulatory loop. The DNA binding site for LuxR (termed the *lux* box) has been shown by in vivo and in vitro experiments to be a 20-bp inverted repeat located between the *luxR* and *luxICDABE* operons and centered around −40 bp from the transcriptional start site (10, 51). Since OHHL freely diffuses (24) across the cell membrane, the induction of one cell leads directly to the induction of the surrounding cells creating a positive feedback circuit that can generate a rapid response to a small initial stimulus. LuxR/LuxI/OHHL therefore constitutes a quorum sensing circuit which enables *V. fischeri* to sense its own population density.

Recent work from this and other laboratories has led to the

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identification of superfamilies of both the LuxR and LuxI proteins and their cognate AHLs in bacteria such as *Erwinia carotovora* (1, 2, 28, 42, 52), *Erwinia stewartii* (4), *Yersinia enterocolitica* (53), *Agrobacterium tumefaciens* (14, 41, 59), *Serratia liquefaciens* (13), *Enterobacter agglomerans* (52), *Rhizobium leguminosarum* (19, 48), *Pseudomonas aureofaciens* (58), and *Pseudomonas aeruginosa* (1, 16, 26, 38, 39, 40, 56). AHL-mediated, cell density-dependent regulation of gene expression and consequently cell-cell communication, therefore, appear to be common among gram-negative bacteria.

Apart from *V. fischeri* and the related but free-living marine bacterium *Vibrio harveyi*, there is little information on quorum sensing in other *Vibrio* species such as the fish pathogen *Vibrio anguillarum*. This bacterium is responsible for a terminal hemorrhagic septicemia known as vibriosis. Although the disease was first described almost a century ago, not much is known of the pathogenesis of vibriosis. To date, the siderophore-mediated iron-sequestering system is the best-characterized *V. anguillarum* virulence determinant (for a review, see reference 8). In addition, lipopolysaccharide (36), chemotactic motility (37), and rainbow trout serum resistance (54) have all been shown to contribute to virulence. Interestingly, an extracellular metalloprotease with 47% identity at the amino acid level to the elastase of *P. aeruginosa* has been cloned (32). However, virulence analysis of strains lacking the metalloprotease was inconclusive, suggesting that this enzyme may not be directly involved in the pathogenesis of *V. anguillarum* infection.

To gain further insights into the regulation of virulence gene expression in *V. anguillarum*, we sought to determine whether this fish pathogen produced AHLs. Preliminary experiments using the AHL reporter systems described by Swift et al. (52) and by Winson et al. (56) indicated that stationary-phase culture supernatants from *V. anguillarum* strains contained AHLs capable of activating the *V. fischeri* LuxR protein. In the present study, we describe the identification and characterization of a quorum sensing circuit in *V. anguillarum* consisting of the LuxI homolog VanI, the LuxR homolog VanR, and the autoinducer molecule *N*-(3-oxodecanoyl)-L-homoserine lactone (ODHL). Furthermore, we show that deletion or disruption of *vanI* or *vanR*, respectively, abolished ODHL synthesis. However, when evaluated in a fish infection model and compared with the wild-type strain NB10, these mutants exhibited no apparent reduction in virulence.

MATERIALS AND METHODS

Strains, phage, plasmids, and media. *V. anguillarum* NB10 (serotype O1) is a clinical isolate from the Gulf of Bothnia outside the Norrby Laboratory, Umeå, Sweden (35). *Escherichia coli* SY327 [Δ(*lac pro*) *argE*(am) *rif malA recA56*] (31) was used for transformation after subcloning fragments into either the pNQ705-1 or pDM4 vector. All plasmids to be conjugated into *V. anguillarum* were transformed into *E. coli* S17-1 (*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7) (50), which was used as the donor strain. Plasmid transfers from *E. coli* to *V. anguillarum* were done as previously described (33).

E. coli XL1-Blue (46) (*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*]) was used for bacteriophage lambda infections and for most transformations. *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)] (46) was used to harbor the bioluminescence sensor plasmid pSB401 that was used to screen the plasmid library for *luxI* homologs as described by Swift et al. (52).

pBSVanI was derived from a positive bioluminescent plasmid isolated from the *V. anguillarum* NB10 gene library. After *Cla*I digestion, the fragment containing the vector and a 3.2-kb portion of the chromosomal insert was purified from a 0.7% agarose gel and then ligated to itself to form pBSVanI. pVanI, pVanR, and pVanIR are pSup202P (32) derivatives which contain the *vanI*, *vanR*, and *vanIR* genes, respectively. Fragments containing the desired genes were obtained by PCR. A restriction enzyme site was added to the 5′ end of each PCR primer to aid cloning of the PCR products (*vanI*, residues 1 to 1080; *vanR*, residues 601 to 1860; *vanIR*, residues 1 to 1860 [see Fig. 5]). pDM4 and pNQ705-1 are derivatives of pNQ705 (32), which is a chloramphenicol-resistant derivative of the suicide vector pGP704, which requires the *pir* gene for replication. pDM4 (33) and pNQ705-1 (27) have previously been described.

The medium routinely used for *E. coli* was TYS broth, which contains Bacto Tryptone (10 g/liter), Bacto Yeast Extract (5 g/liter), and sodium chloride (10 g/liter). For *V. anguillarum*, Trypticase soy medium (BBL) was used for routine growth. For purification and identification of AHLs, *V. anguillarum* was grown at 20°C, and Trypticase soy medium was replaced with M9 medium (46) supplemented with 2% (wt/vol) NaCl. Supplemented M9 medium avoids the major problems associated with rich laboratory media which contain many compounds that strongly absorb UV light. These compounds are extracted along with AHLs during the preliminary purification stages and make the identification of the lactones difficult since they absorb UV light poorly. For purification of AHLs produced via VanI, *E. coli*(pBSVanI) was grown at 30°C in M9 medium containing ampicillin.

Antibiotics and enzymes. Antibiotic concentrations for all *E. coli* strains were as follows: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml. Antibiotic concentrations for *V. anguillarum* were as follows: tetracycline, 5 μg/ml; and chloramphenicol, 5 μg/ml. Restriction enzymes were purchased from a variety of sources, and KGB buffer (46) was used for all digests. Reaction conditions for the DNA-modifying enzymes were as suggested by the manufacturers.

AHL reporter assays. Autoinducers were detected by using a Tn*5*-generated *Chromobacterium violaceum* mutant termed CV026 which responds to a range of AHLs by inducing the synthesis of the purple pigment violacein (26, 56). For this assay, AHL-producing strains are cross-streaked vertically to a horizontal streak of CV026 on Trypticase soy agar plates. Alternatively, spent culture supernatants, solvent extracts, or high-pressure liquid chromatography (HPLC) fractions (see below) were added to wells cut in nutrient agar plates seeded with CV026, incubated at 30°C overnight, and then examined for the presence of violacein haloes. CV026 is capable of producing violacein in response to AHLs with *N*-acyl side chains between four and eight carbons in length. Longer-chain AHLs (e.g., with acyl chain lengths of 10 to 14 carbons) can be detected by their ability to inhibit violacein production stimulated by shorter-chain AHLs (unpublished data). In this assay, nutrient agar plates are seeded with CV026 plus 5 μg of OHHL per ml (equivalent to 23.47 μM OHHL). The concentration of OHHL was optimized experimentally for the detection of the inhibitory activity of ODHL in the violacein activation test (unpublished data). Samples added to wells cut in the agar were then examined after overnight incubation for the presence of white haloes in a purple background; ODHL was used as a positive control. In addition to CV026, we used a bioluminescent *E. coli*-based reporter plasmid system for the sensitive detection of AHLs. This biosensor, termed pSB401, couples the *luxR* and promoter region from *V. fischeri* to the entire *lux* structural operon (comprising *luxCDABE*) from *Photorhabdus luminescens* (to be described in detail elsewhere). When expressed in *E. coli*, pSB401 responds to a range of AHLs at 37°C and, in contrast to the bioluminescent reporters that we previously described (52), does not require the addition of exogenous aldehyde.

Isolation, purification, and chemical characterization of AHLs. Spent supernatants (4 liters) from stationary-phase cultures of *V. anguillarum* NB10 or *E. coli*(pBSVanI) were extracted with dichloromethane (700:300 supernatant-dichloromethane). Dichloromethane was removed by rotary evaporation, and the residue was reconstituted in 1.0 ml of acetonitrile and applied to a C_8 reverse-phase semipreparative HPLC column (Kromasil KR100-5C8 [250 by 8 mm] column; Hichrom, Reading, United Kingdom). Fractions were eluted with a linear gradient of acetonitrile in water (20 to 95%) over a 30-min period at a flow rate of 2 ml/min and monitored at 210 nm. Depending on their activity in the CV026 violacein reporter assays, fractions were rechromatographed by using isocratic mobile phases of acetonitrile-water (35%:65%) or acetonitrile-water (50%:50%). Active peaks from this fractionation were then rechromatographed on an analytical HPLC attached to a photodiode array system (Waters 996 PDA system operating with a Millenium 2010 Chromatography Manager; Watford, Herts, United Kingdom), and both retention times and spectral properties were compared with those of a series of synthetic AHL standards. The major active fraction was collected and pooled after preparative HPLC and analyzed by mass spectrometry (MS). Mass spectra were obtained on a V.G. 70-SEQ instrument of EBqQ geometry (Fisons Instruments, VG Analytical, Manchester, United Kingdom). Samples were ionized by positive-ion fast atom bombardment (FAB). The molecular ion (M + H) peaks recorded by FAB-MS were further analyzed by tandem mass spectrometry (MS-MS) and shown to be identical to the MS-MS spectra of the respective authentic synthetic material.

Synthesis of AHLs. The general method described by Chhabra et al. (7) for the synthesis of a series of AHLs was used to produce ODHL (see Fig. 3B), *N*-octanoyl-L-homoserine lactone (OHL), *N*-(3-oxooctanoyl)-L-homoserine lactone (OOHL), and OHHL. Each compound was purified to homogeneity by semipreparative HPLC, and its structure was confirmed by MS and proton nuclear magnetic resonance spectroscopy as described before (2, 7, 56).

Construction and screening of the plasmid library. A *V. anguillarum* genomic plasmid library was constructed by excising the pBluescript plasmids containing chromosomal inserts from a previously described (32) genomic library in the Lambda Zap II system (Stratagene). Excision was done as described earlier (32) except that approximately 10,000 pooled lambda recombinant plaques were used instead of a single plaque.

To screen the *V. anguillarum* gene library for *luxI* homologs, the biolumines-

cent *E. coli*-based reporter plasmid system pSB401 was used as described by Swift et al. (52). The genomic library was transformed into *E. coli* JM109(pSB401), and transformants were analyzed for light production by exposing colonies to X-ray film for 1 min.

DNA techniques and sequencing. Oligonucleotides for primers were synthesized by using an Applied Biosystems model 394 DNA/RNA synthesizer. Unless otherwise stated, all conditions for the various DNA techniques were as described by Sambrook et al. (46). Double-stranded DNA sequencing was performed by the dideoxy-chain termination method with T7 DNA polymerase (Pharmacia Biotech). By using pBSVanI plasmid DNA, both strands of the 3.2-kb fragment containing *vanI* and *vanR* were sequenced by primer walking in two directions. Purification of DNA fragments from agarose gels was done with a Sephaglas BandPrep kit from Pharmacia Biotech.

PCR conditions. PCR was performed as previously described (27). To optimize some PCRs, 2 to 5 μg of the single-stranded DNA binding protein T4 gene 32 protein (Boehringer Mannheim) was added to the reaction.

Construction of the *vanI*, *vanR*, and *vanIR* mutations. Two types of mutations were made, a full-gene in-frame deletion and a chromosomal insertion. Both methods have previously been described (32, 33). The chromosomal insertion was made by integrating a plasmid into the *vanR* gene, and the *vanI* in-frame deletion was made by allelic exchange. For the *vanI* full-gene deletion, the suicide vector pDMVanI, a derivative of pDM4 that contains a recombinant PCR fragment (residues 1 to 259 joined by overlap PCR to residues 839 to 1080 [see Fig. 5]), was used to create the mutant DM21. This region from the DM21 chromosome was amplified by PCR and sequenced to confirm an in-frame deletion. For the *vanR* insertional mutation, the suicide vector pNQVanR2, a derivative of pNQ705-1 that contains a PCR fragment (residues 1321 to 1500 [see Fig. 5]) from the 5′ end of *vanR*, was used to create the mutant DM25. The double mutant DM26 was created by making an insertional mutagenesis of *vanR* in DM21, using pNQVanR2. In both DM25 and DM26, the insertion of the plasmid was check by PCR analysis using a previously described primer (32) complementary to a region on the plasmid just outside the polylinker region and a primer complementary to a chromosomal DNA region just outside the PCR fragment cloned into pNQVanR2. The PCR fragments obtained from the mutants were analyzed by restriction mapping to ensure that the PCR fragments obtained were from the correct region of the chromosome. Stability of the two insertion mutants was tested. Each strain was grown for 30 generations in the absence of chloramphenicol. Of 100 colonies tested, no loss in chloramphenicol resistance was seen.

Computer analysis. Database searches were done with the Genetics Computer Group Sequence Analysis software (9) of the Genetics Computer Group, Inc. (University of Wisconsin).

Fish infections. Rainbow trout (*Oncorhynchus mykiss*) with an approximate weight of 10 to 15 g were infected with *V. anguillarum* either by intraperitoneal injections or by immersion of the fish in seawater containing *V. anguillarum* as previously described (35). The immersion and intraperitoneal infections were done at least two times. Five fish were infected for each bacterial dilution used. The 50% lethal doses (LD_{50}s) were calculated as described by Reed and Muench (44). The LD_{50}s recorded represent an averaged number of all infections for each strain.

Nucleotide sequence accession number. The DNA sequence of the entire 3.2-kb fragment has been submitted to GenBank, and its accession number is U69677.

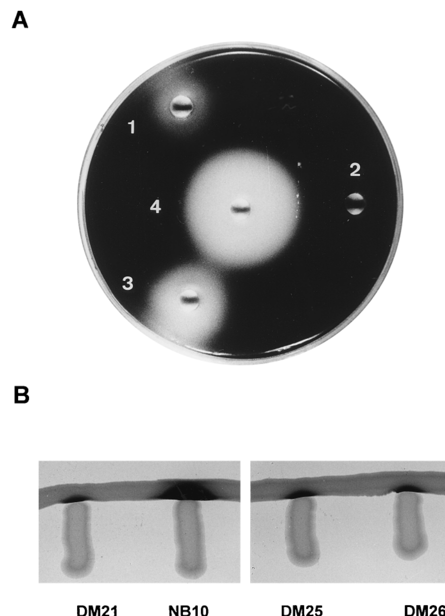


FIG. 1. (A) Restoration of ODHL production in *V. anguillarum* strains with mutations in *vanR* (DM25) and in both *vanR* and *vanI* (DM26) and complemented with plasmid-borne copies of *vanR* or *vanIR*, respectively. For this assay, stationary-phase, cell-free supernatants from DM25(pVanR) (well 1) DM25 (well 2), DM26(pVanIR) (well 3), and ODHL (well 4) were added to wells cut in nutrient agar seeded with *C. violaceum* CV026 plus OHHL (5 μg/ml). Plates were incubated for 24 h at room temperature. A white halo around the well in a violet background is indicative of ODHL synthesis. (B) Stimulation of violacein synthesis in *C. violaceum* CV026 by cross-streaking against *V. anguillarum*. CV026 was streaked horizontally and the *V. anguillarum* strains were streaked vertically on Trypticase soy agar. Plates were incubated at room temperature for 48 h. NB10, wild type; DM21, *vanI* mutant; DM25, *vanR* mutant; DM26, *vanIR* mutant.

RESULTS

Screening *V. anguillarum* for AHLs. We have previously described several different reporter assays for the detection of AHLs in bacterial culture supernatants. These assays depend either on the stimulation of bioluminescence in a recombinant *lux E. coli* reporter strain or on the induction of violacein pigment production in CV026, an AHL-negative white mutant of *C. violaceum*. The violacein production reporter assay also differentiates between AHLs with different *N*-acyl side chain lengths. AHLs with *N*-acyl side chain lengths of 4 to 8 carbons induce violacein production, whereas AHLs with *N*-acyl side chain lengths of 10 or more carbons cannot. Although AHLs with *N*-acyl side chains of 10 or more carbons in length are unable to induce violacein production, their presence can be detected via their ability to antagonize the production of violacein by compounds with C_6 acyl side chains. When cross-streaked against CV026, *V. anguillarum* NB10 activated violacein production (Fig. 1B). However, while cell-free supernatants from NB10 also activated the *E. coli lux* reporter, such supernatants failed to induce violacein production in CV026 (Fig. 2A). The lack of violacein production by the cell supernatants could be due to the dilution of an AHL into the culture medium as opposed to growth on a plate. When tested for their ability to antagonize the OHHL-mediated induction of violacein synthesis, *V. anguillarum* cell-free supernatants were found to be positive (Fig. 2B). These data suggested that *V. anguillarum* produces multiple AHLs with various *N*-acyl side chain lengths.

Purification and identification of *N*-acyl homoserine lactones from *V. anguillarum*. *V. anguillarum* was grown to stationary phase in M9 medium supplemented with sodium chloride, and the cell-free supernatant was extracted with dichloromethane and fractionated by HPLC as described in Materials and Methods. Using a linear gradient of acetonitrile in water (20 to 95%), we collected over a 30-min period six fractions (F1 to F6) covering each 5-min interval. When assayed using the two CV026 assays, none of the fractions collected induced violacein production, but F5 eluting at 20 to 25 min strongly inhibited OHHL-mediated activation of violacein production. F5 contained a single peak eluting with a retention time of 20.1 min which matched the spectral properties and retention time of a synthetic ODHL standard. To establish that ODHL is the major *V. anguillarum* autoinducer, the peak eluting at 20.1 min was collected and analyzed by high-resolution tandem MS. Figure 3 reveals that the putative AHL displayed a molecular ion peak at m/z 270 (M + 1), which is consistent with that of ODHL.

Cloning and sequencing of the *luxI* and *luxR* homologs *vanI* and *vanR*. We have previously described a strategy for the isolation of *luxI* homologs (52) which is based on complemen-

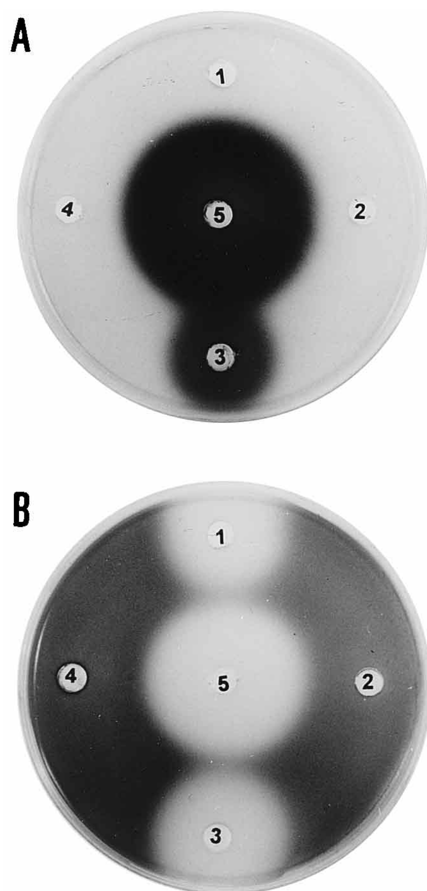


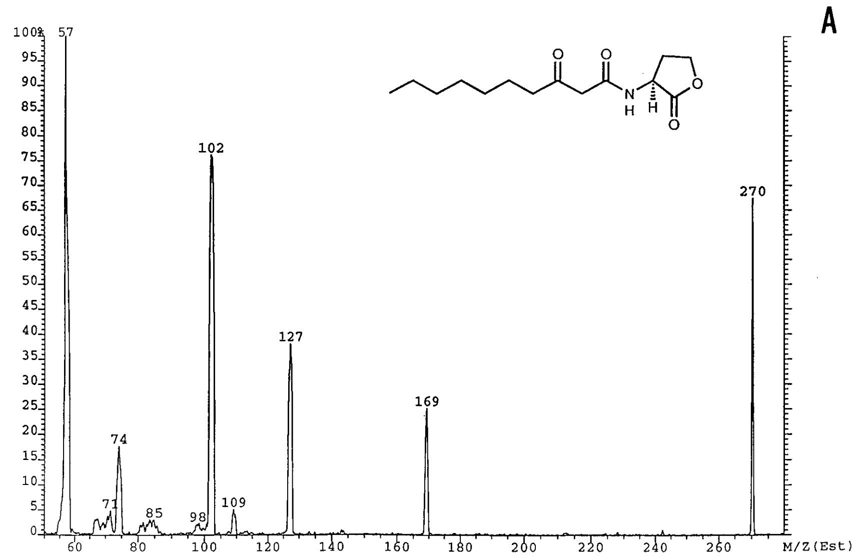
FIG. 2. Induction (A) and inhibition (B) of violacein synthesis in *C. violaceum* CV026 by *V. anguillarum* supernatants. (A) Stationary-phase cell-free culture supernatants from *V. anguillarum* NB10 (wild type; well 1), DM21 (*vanI* mutant; well 2), *E. coli* carrying pBSVanI (well 3), uninoculated culture medium (as a control; well 4), or OHHL (5 μg/ml; well 5) were added to wells cut in agar seeded with CV026 and incubated overnight. The induction of a violet halo is indicative of the presence of AHLs with *N*-acyl side chains of four to eight carbons. (B) Plates were seeded with CV026 plus OHHL (5 μg/ml), and to the wells cut in the agar were added stationary-phase cell-free culture supernatants from *V. anguillarum* NB10 (wild type; well 1), DM21 (*vanI* mutant; well 2), *E. coli* carrying pBSVanI (well 3), uninoculated culture medium (as a control; well 4), or ODHL (well 5). A white halo around the well in a violet background is indicative of the presence of AHLs with *N*-acyl side chains of greater than eight carbons.

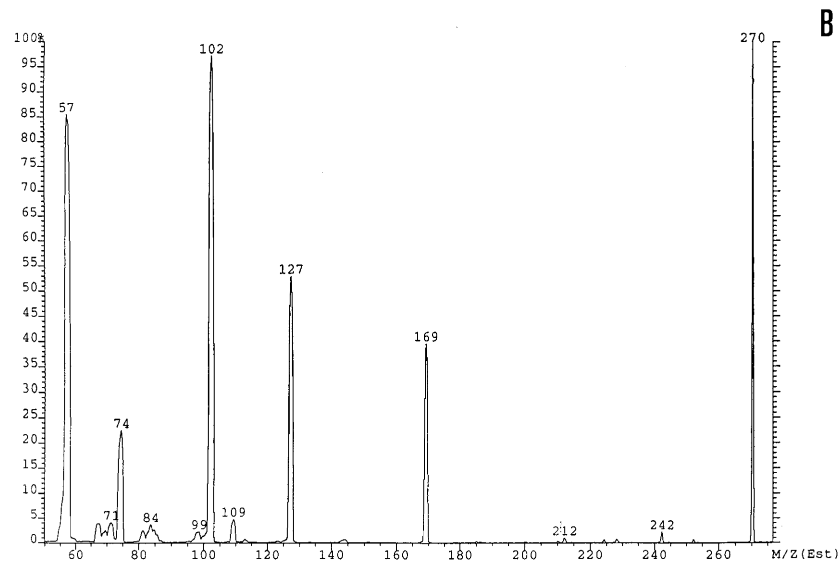
tation in *trans* of a *lux* AHL sensor deleted for *luxI*. A plasmid library of *V. anguillarum* NB10 genomic DNA was constructed and transformed into *E. coli* JM109(pSB401). Three bioluminescent colonies were picked, and the plasmid DNA from each was analyzed by restriction endonuclease digestion. All were shown to contain a fragment of approximately 8.2 kb. Digestion of this fragment with *Cla*I yielded two fragments, 5 and 3.2 kb, of which the smaller was still capable of activating the *lux* biosensor. The plasmid containing the 3.2-kb fragment was designated pBSVanI and was used for DNA sequence analysis.

Analysis of the DNA sequence revealed four open reading frames (ORFs), for which the genetic organization is shown in Fig. 4. The translated amino acid sequence of ORF1 has 31% identity with a hypothetical 14.2-kDa protein located in the 3′ region following the *tonB* gene of *E. coli* (43). The function of this gene is unknown. ORF2 was only partially sequenced, and the deduced amino acid sequence showed strong similarity to numerous repressor proteins from *E. coli*, with the highest homology to the glycerol-3-phosphate repressor. The third and the fourth ORFs are convergently transcribed and are separated by 3 bp. The DNA and deduced protein sequences for these ORFs are shown in Fig. 5. One protein sequence exhibited significant homology to members of the LuxI family of AHL synthases, while the second clearly belongs to the LuxR family of transcriptional activators. These ORFs were therefore designated *vanI* and *vanR*, respectively. Compared with other members of the LuxI family, VanI is most closely related to the *V. fischeri* homolog LuxI, with which it is 47% identical (68% similarity). Among the LuxR family, VanR is also most closely related to LuxR (38% identical and 58% similarity). In addition, a DNA structural motif termed the *lux* box has been proposed to function as the target site for LuxR homologs (10, 51). A *lux* box-like sequence has been found upstream of some but not all genes regulated by LuxR homologs (10, 14, 18, 26). Approximately 70 bp upstream of the ATG start of *vanI* (Fig. 5), there is a near-consensus *lux* box-like sequence (the consensus sequence is RNSTGYAXGATNXTRCASRT, where N = A, T, C, or G; R = A or G; S = C or G; Y = T or C; and X = N or a gap).

Identification of the AHLs synthesized via VanI. To determine whether VanI is responsible for ODHL synthesis, cell-free supernatants from *E. coli*(pBSVanI) were evaluated in the CV026 bioassays. Two phenotypes were seen: a strong inhibition of OHHL-mediated activation of violacein synthesis, which is indicative of the presence of ODHL (Fig. 2), and a violacein-inducing activity. The presence of ODHL was unequivocally confirmed by HPLC and MS (Fig. 3), and *E. coli*(pBSVanI) clearly produced more ODHL than *V. anguillarum* NB10. The violacein-inducing activity from *E. coli*(pBSVanI) was located to two fractions (F3 and F4, eluting at 10 to 15 and 15 to 20 min, respectively). To identify the violacein-activating compound(s), we rechromatographed F3 and F4, using an isocratic mobile phase of acetonitrile-water (35 to 65%, vol/vol). Two peaks with retention times of 7.3 and 11.1 min, respectively, were identified, collected, and shown to induce violacein production in CV026. When the retention times and spectral properties of these compounds were compared with those of a series of AHL standards, the more polar compound was tentatively identified as OOHL, and the more hydrophobic compound was identified as OHL. As these AHLs were present at very low levels, we were unable to obtain sufficient material to confirm their identities by MS.

Mutational analysis of *vanI* and *vanR*. To examine the roles of *vanI* and *vanR* in AHL synthesis, in-frame deletion mutagenesis was carried out. For *vanI*, the full gene was deleted from the start codon up to the stop codon (residues 260 to 838 [Fig. 5]), resulting in mutant DM21. Analysis of cell-free culture supernatants of DM21 by using the CV026 bioassays (Fig. 2) and HPLC analysis (data not shown) revealed that production of ODHL had been abolished. To make a VanR-negative strain, a suicide plasmid containing the chloramphenicol resistance gene was successfully inserted 23 amino acids downstream of the methionine start for *vanR*, resulting in mutant DM25. Cell-free supernatants from DM25, as with DM21, did not contain ODHL, indicating that VanR functions as the transcriptional activator of *vanI* and hence controls ODHL synthesis (Fig. 1A). Interestingly, when cross-streaked against CV026, DM21 and DM25 are both still capable of weakly inducing violacein production (Fig. 1). This finding implies that *V. anguillarum* may possess an additional LuxI homolog responsible for the synthesis either of low levels of additional AHLs or of an AHL which is capable of only very weak activation of pigment production in CV026. To confirm that a second DNA locus is responsible for this activity, we con-





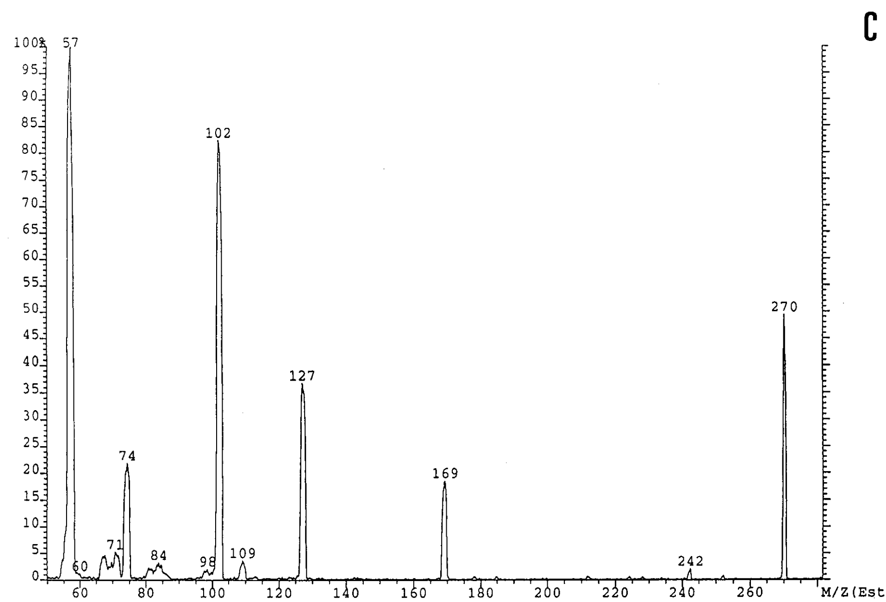


FIG. 3. Daughter-ion spectrum (MS-MS) of autoinducer compound 1 (*m/z* 270 peak) purified from spent culture supernatant of *V. anguillarum* NB10 (A) is indistinguishable from that of synthetic ODHL (B) or *E. coli* transformed with the *vanI* gene on pBSVanI (C).

FIG. 4. Schematic representation of the *vanI*/*vanR* locus. The horizontal arrows indicate the direction of transcription. ORF1 was completely sequenced, and the predicted product showed similarity to a hypothetical 14.2-kDa protein located in the 3′ region following the *tonB* gene of *E. coli*. ORF2 was partially sequenced, and the predicted product showed similarity to the glycerol-3-phosphate repressor from *E. coli*.

structed a third mutant, DM26, which combined the above two mutations. This mutant retained the weak violacein-inducing activity observed with DM21 and DM25 (Fig. 1).

To confirm that the lack of ODHL synthesis was due to the loss of either *vanI* or *vanR*, vectors for transcomplementation analysis of the mutations were constructed and introduced into mutants DM21, DM25, and DM26 by conjugation. Restoration of ODHL synthesis was demonstrated by using the CV026 bioassays. Compared to the wild-type strain NB10, strains DM21(pVanI) (data not shown), DM25(pVanR), and DM26(pVanIR) antagonized OHHL-mediated violacein production (Fig. 1A).

Effect on metalloprotease production. Previously, the gene for the major extracellular metalloprotease, *empA*, was sequenced, and the deduced protein sequence showed 47% identity with elastase, the gene product of the *lasB* gene from *P. aeruginosa* (32). The *lasB* gene is regulated via the LuxR homologs LasR (16) and VsmR (RhlR) (26, 56), and a consensus palindromic *lux* box-like sequence was found upstream of *lasB* (15, 18). We reasoned that since *empA* is similar to *lasB* and since the metalloprotease is expressed late in the growth cycle of *V. anguillarum*, then there may be a *lux* box-like sequence upstream of the *empA* gene. Indeed, there is a potential *lux*

```
   1 aagcgttaac caaactgcaa tattattttg aatgcagcag atataaaaaa tcctcttagt tgcctaagag gatttttaaa gccgagcggt acgagttatt

 101 tactttatgt tgtaccttat tgagccaact gagcgttagt tattcagttg cgttaataat agtttaaaca gtatttcgac gtaactgttc gatcgaacag
                                                                        →  vanI
 201 gttctatctc tgatgagatt gtttagcgtg atttctccaa taacctaagg agaagtcaca tgactatttc aatttattca catacctttc aaagtgtccc
   1                                                                    M  T  I  S   I  Y  S   H  T  F  Q   S  V  P

 301 tcaagctgat tatgtgtcat tgctgaagtt acgctataaa gtttttttcgc aacgcttgca gtgggagcta aaaacaaatc gaggaatgga gactgatgag
  15  Q  A  D   Y  V  S  L   L  K  L   R  Y  K   V  F  S  Q   R  L  Q   W  E  L   K  T  N  R   G  M  E   T  D  E

 401 tacgacgttc cagaagcaca ctatttgtat gccaaagagg aacagggtca tttagtgggg tgttggcgaa ttttgccaac gacgtcgcgt tatatgctta
  49 Y  D  V  P   E  A  H   Y  L  Y   A  K  E  E   Q  G  H   L  V  G   C  W  R  I   L  P  T   T  S  R   Y  M  L  K

 501 aagatacttt ttcggaatta ctcggtgtgc agcaagcacc caaagcaaag gagatttatg agctgagtcg ttttgcggtc gataaagatc attcggcgca
  83  D  T  F   S  E  L   L  G  V  Q   Q  A  P   K  A  K   E  I  Y  E   L  S  R   F  A  V   D  K  D  H   S  A  Q

 601 attgggcggc gtgagtaatg ttacgctgca gatgtttcag tcgctgtatc atcacgccca acaatatcac atcaatgcct atgtaacggt cacatcggcc
 116  L  G  G   V  S  N  V   T  L  Q   M  F  Q   S  L  Y  H   H  A  Q   Q  Y  H   I  N  A  Y   V  T  V   T  S  A

 701 agtgtggaaa aattgattaa gcggatgggg atcccatgcg aaagactcgg tgataaaaaa gtgcatcttt tgggaagtac acgttctgtc gctttgcata
 149 S  V  E  K   L  I  K   R  M  G   I  P  C  E   R  L  G   D  K  K   V  H  L  L   G  S  T   R  S  V   A  L  H  I

 801 ttccaatgaa tgaagcatat cgtgcaagtg tcaatgcata agccttataa atagggattg atatagccac ctaaaatagc tttagtgatc gcttggtagc
 183  P  M  N   E  A  Y   R  A  S  V   N  A  *        *  L   Y  P  N   I  Y  G   G  L  I  A   K  T  I   A  Q  Y

 901 gattcgtcgc gccaagcttt ttacaagcat tactgaaatg gaacttcacg gtacgttctg acgtattaat aatcgtggca atttcccatg cgcttttgcc
 223 R  N  T  A   G  L  K   K  C  A   N  S  F  H   F  K  V   T  R  E   S  T  N  I   I  T  A   I  E  W   A  S  K  G

1001 ttcggccgcc caagctaagc attgtacttc acgtgcggtg agcaccgctc tcggttttgc gtctttatgg taacgggtga tgttgccaat attatgagcc
 189  E  A  A   W  A  L   C  Q  V  E   R  A  T   L  V  A   R  P  K  A   D  K  H   Y  R  T   I  N  G  I   N  H  A

1101 agtaacggta caatgagctg tgaggtatgg atactttgtt gattcaaatc gtaagacttg gtatctgagg ttgcaaaact tatcatgcca aattcccccc
 156  L  L  P   V  I  L  Q   S  T  H   I  S  Q   Q  N  L  D   Y  S  K   T  D  S   T  A  F  S   I  M  G   F  E  G

1201 taagtccatg gattggaatg ctaaatcccg ctttgagtcc attgcaacgc gcctcttcga aaatgactcg tccatcatta ttgactcgtt tagcgtcatc
 123 R  L  G  H   I  P  I   S  F  G   A  K  L  G   N  C  R   A  E  E   F  I  V  R   G  D  N   N  V  R   K  A  D  D

1301 ccagcgaatg ggcaaaaaat tggtaatgct gtatttgaca atcggatcga tatgcatgaa gcctgattca tcatattgct gtcgccagct attggggtaa
  89  W  R  I   P  L  F   N  T  I  S   Y  K  V   I  P  D   I  H  M  F   G  S  E   D  Y  Q   Q  R  W  S   N  P  Y

1401 ttgtccgtga caagcgtttc actggttttt aacgtgggtt gaaaagagag gccaaagagg aaaaattcat gaccaattaa gttattgagc ccattgagta
  56  N  D  T   V  L  T  E   S  T  K   L  T  P   Q  F  S  L   G  F  L   F  F  E   H  G  I  L   N  N  L   G  N  L

1501 cattttccaa atcatcgtgg ctagtgattt gttggttctc ttggatcagt ctcagaattt tgtacataag ccgaaagttc taaattgcgt cgtactatat
  23 V  N  E  L   D  D  H   S  T  I   Q  Q  N  E   Q  I  L   R  L  I   K  Y  M
                                                                    vanR  ←
1601 tggcacttat atcaaaaaaa accgtaacag tacgagcaaa taatgaaata gtttatttat ttggcgggaa aatctagttc aaatgtcggt tttggcctca

1701 atctatctat accacctgaa tggttacttc attttcacga gcgaatgcat caatttcttc tggtggttga gcattggtaa tgatcaaatc tacgtcggca

1801 attgtgccta gcttaaccat ggcattacgt ccgaatttgc tgtggtcgac accgagataa
```

FIG. 5. DNA sequences of the *vanI* and *vanR* genes. The deduced amino acid sequences are indicated for both genes. A potential *lux* box-like sequence with dyad symmetry is underlined.

TABLE 1. Virulence analysis

Strain	Mutation	Intraperitoneal injection (LD_{50})	Immersion (bacteria/ml)
NB10	None (wild type)	58	4×10^3
DM21	*vanI* gene deletion	74	1×10^4
DM25	*vanR* plasmid insertion	273	1×10^3
DM26	*vanI* gene deletion, *vanR* plasmid insertion	36	2×10^4

box-like sequence (5′-GAGCGaATcATTcGCAGtc-3′; lowercase letters indicate nonconsensus sequence) with dyad symmetry approximately 70 bp upstream of the translational start codon. Thus, DM21, DM25, and DM26 were tested for proteolytic activity on Trypticase soy agar plates containing 1% skim milk to determine if the *vanI* and *vanR* gene products regulate the expression of the metalloprotease. The zone of clearing for all mutants was the same as that for the wild type (data not shown), indicating that the metalloprotease expression is not regulated by *vanI* and *vanR*.

Virulence analysis. To determine the effects of the mutations on virulence, the *vanI*, *vanR*, and *vanIR* mutants were evaluated in a fish infection model. The LD_{50}s were determined for infection via immersion in infected seawater and via intraperitoneal injection of the mutant strains (Table 1). In each case, there was no significant difference between the virulence of the parent and mutant *V. anguillarum* strains.

DISCUSSION

The results presented in this study add *V. anguillarum* to the rapidly expanding group of gram-negative bacteria which employ AHLs and possess LuxI/LuxR-type regulatory circuits. By exploiting an assay in which AHL-mediated stimulation of violacein pigment production in *C. violaceum* is inhibited, we were able to identify and chemically characterize a novel AHL, ODHL. Autoinducer molecules with a C_{10} *N*-acyl side chain have not previously been identified; therefore, ODHL extends the list of known AHL molecules which now contains compounds with C_4, C_6, C_8, C_{10}, C_{12}, and C_{14} *N*-acyl side chains. Apart from *N*-(3-hydroxybutanoyl) homoserine lactone (HBHL) produced by *V. harveyi* and OHL produced by *V. fischeri*, each of the other AHLs for which a synthase has been identified is synthesized via a LuxI homolog. In *V. anguillarum*, ODHL is generated via the LuxI homolog VanI, which is also responsible for the production of small amounts of two additional AHLs in *E. coli*. These molecules have been tentatively identified as OHL and OOHL since they migrate on HPLC with the same retention times as synthetic standards. Interestingly, at the amino acid sequence level, VanI shares the greatest similarity with LuxI even though the *V. fischeri* protein is responsible primarily for the production of the C_6 AHL, OHHL, together with small amounts of *N*-hexanoyl-L-homoserine lactone (HHL). Two LuxI homologs responsible for generating longer-chain AHLs, namely, LasI from *P. aeruginosa* (C_{12} acyl chain; *N*-[3-oxododecanoyl]-L-homoserine lactone [OdDHL]) and TraI from *A. tumefaciens* (C_8 acyl chain; OOHL) are only around 30% identical to VanI at the amino acid level. Thus, although some 12 LuxI homologs have now been sequenced, it is not possible to predict, from the sequence data, the likely identity of the AHL molecule(s) synthesized via a given homolog. However, following on from the preliminary work of Eberhard et al. (12), recent work from two laboratories focusing on TraI (34) and LuxI (20, 47) have shown that these proteins are indeed AHL synthases. OOHL and HHL are synthesized, respectively, by TraI and LuxI from *S*-adenosylmethionine and the appropriately charged acyl carrier protein (either the 3-oxooctanoyl acyl carrier protein or hexanoyl acyl carrier protein). Furthermore, it is apparent that introduction of the *V. fischeri luxI* gene, the *Y. enterocolitica yenI* gene, the *P. aeruginosa lasI* and *vsmI* (*rhlI*) genes, and the *S. liquefaciens swrI* gene into a heterologous genetic background, i.e., *E. coli*, results in the synthesis of the same AHL molecules observed in the original heterologous organism. In this report, we have demonstrated that in both *V. anguillarum* and *E. coli*, *vanI* directs the synthesis of ODHL. These observations suggest that all of the substrates and accessory proteins must be cross-functional and shared by these different gram-negative bacteria and that specificity is determined by the nature of the LuxI homolog. Presumably, different LuxI homologs are capable of extracting the correct acyl side chain from the fatty acid biosynthetic machinery, since Cao and Meighen (6), Moré et al. (34), and Schaefer et al. (47) have obtained evidence to indicate that AHLs are produced via fatty acid biosynthesis rather than degradation.

Approximately 70 bp upstream of *vanI*, we located a 20-bp region of dyad symmetry which is closely related to the *V. fischeri lux* box. Mutational analysis of the *V. fischeri* palindrome indicated that it serves as the LuxR binding site (10). Recently, Stevens et al. (51) have demonstrated that the LuxR C-terminal domain binds to the *lux* box in vitro but only in the presence of RNA polymerase. Related *lux* box-like elements have been identified upstream of the *P. aeruginosa lasB* (18) and *vsmI* (*rhlI*) genes (28) and in *A. tumefaciens* upstream of both the *traA* and *traI* genes (14, 15). By analogy with previously characterized LuxR/LuxI-type regulatory circuits, we therefore presumed that in *V. anguillarum*, the *lux* box-like sequence upstream of *vanI* is likely to function as the target for a LuxR homolog. By sequencing the DNA downstream of *vanI*, we located an ORF termed VanR, the translated amino acid sequence of which has significant homology with the LuxR family of transcriptional activators. VanR is most closely related to LuxR (38% identical) and is only around 25% identical with other members (e.g., LasR, VsmR, CarR, and YenR) of this family of transcriptional activators. Unlike *luxR* and *luxI* in *V. fischeri*, which are divergently transcribed, *vanI* and *vanR*, like *yenR* and *yenI* (*Y. enterocolitica* [51]), *esaR* and *esaI* (*Erwinia stewartii* [4]), and *phzR* and *phzI* (*P. aureofaciens* [58]), are convergently transcribed. The close linkage of *vanI* and *vanR* and the presence of a *lux* box-like sequence upstream of *vanI* implied that there is a regulatory connection between these two genes. In many acyl homoserine lactone signaling systems, the LuxI homologs are controlled by a positive autoregulatory loop (14, 21, 29, 30, 49), and this is likely to be the case for VanI in *V. anguillarum*. Thus, VanR/ODHL is likely to be responsible for the autoinducible activation of *vanI*, and mutations in either *vanI* or *vanR* should lead to the loss of ODHL synthesis. To test this hypothesis, we constructed mutations in *vanR*, in *vanI*, and in both *vanI* and *vanR*. None of these mutants were capable of synthesizing ODHL, indicating that *vanI* is indeed subject to ODHL-mediated autoinduction. However, when cross-streaked against the *C. violaceum* mutant CV026, all three mutants remained capable of weakly stimulating violacein pigment production. This finding suggests that *V. anguillarum* may possess additional LuxI homologs. This would not be unprecedented since *P. aeruginosa* PAO1, for example, has two pairs of LuxRI homologs, LasRI and VsmRI (RhlRI) (16, 26, 38). In this human pathogen, LasI is responsible for OdDHL synthesis (39) and VsmI (RhlI) is responsible for *N*-butanoyl-L-homoserine lactone (BHL)/HHL synthesis (56). It is therefore conceivable that, like *P. aeruginosa*, *V.*

anguillarum produces both long-chain (ODHL) and short-chain AHL molecules which are generated via two different LuxI homologs. The ability of the *V. anguillarum vanRI* mutants to stimulate violacein production is consistent with the presence of a shorter-chain (<8 carbons) AHL molecule. Alternatively, it is possible that the genetic locus responsible for the synthesis of this additional AHL molecule(s) does not contain a LuxI homolog but is related either to the recently described *ainS* locus of *V. fischeri* (17) or to the *luxLM* locus of *V. harveyi* (3). Both the *ainS* and *luxLM* loci direct the synthesis of AHL molecules. AinS, the C-terminal region of which is related to LuxM, directs the synthesis of OHL in *V. fischeri* and appears to be the sole gene required for OHL synthesis in *E. coli* (17). Work is currently under way to characterize the genetic locus responsible for the production of the additional AHL(s) in *V. anguillarum*.

From the data presented in this report, it is evident that *V. anguillarum* employs AHLs for regulating gene expression, and we therefore sought to identify structural genes regulated via the *vanIR* locus. Although the *V. fischeri luxR* and *luxI* genes are linked to those they regulate, in other organisms this is not always the case (45). In *V. anguillarum*, the ORF (ORF1) adjacent to *vanI* is unlikely to form part of the same transcriptional unit since it is divergent, and therefore the *vanIR* locus is probably not linked directly to a structural operon. Although expression of the *V. anguillarum* metalloprotease gene *empA* is growth phase dependent and the gene possesses a *lux* box-like sequence around 70 bp upstream of the translational start codon, neither the *vanI* nor the *vanR* mutant exhibited any apparent reduction in protease production. Furthermore, these mutants exhibited no loss of virulence in an experimental fish infection model. In this context, in both *Enterobacter* (52) and *Y. enterocolitica* (53), we have encountered considerable difficulties in linking a LuxR/LuxI-type regulatory locus with a phenotype. In *V. fischeri*, OHL produced via AinS has been proposed to modulate bioluminescence by suppressing or delaying LuxR/OHHL-mediated activation of the *lux* operon at low cell densities (25). It is therefore possible that ODHL modulates temporal expression of target genes which are regulated via the additional and as yet unidentified autoinducer system in *V. anguillarum*.

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