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VanT, a Homologue of *Vibrio harveyi* LuxR, Regulates Serine, Metalloprotease, Pigment, and Biofilm Production in *Vibrio anguillarum*

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Vibrio anguillarum **possesses at least two *N*-acylhomoserine lactone (AHL) quorum-sensing circuits, one of which is related to the *luxMN* system of *Vibrio harveyi*. In this study, we have cloned an additional gene of this circuit, *vanT*, encoding a *V. harveyi* LuxR-like transcriptional regulator. A *V. anguillarum* Δ*vanT* null mutation resulted in a significant decrease in total protease activity due to loss of expression of the metalloprotease EmpA, but no changes in either AHL production or virulence. Additional genes positively regulated by VanT were identified from a plasmid-based gene library fused to a promoterless *lacZ*. Three *lacZ* fusions (*serA*::*lacZ*, *hpdA-hgdA*::*lacZ*, and *sat-vps73*::*lacZ*) were identified which exhibited decreased expression in the Δ*vanT* strain. SerA is similar to 3-phosphoglycerate dehydrogenases and catalyzes the first step in the serine-glycine biosynthesis pathway. HgdA has identity with homogentisate dioxygenases, and HpdA is homologous to 4-hydroxyphenylpyruvate dioxygenases (HPPDs) involved in pigment production. *V. anguillarum* strains require an active VanT to produce high levels of an L-tyrosine-induced brown color via HPPD, suggesting that VanT controls pigment production. Vps73 and Sat are related to *Vibrio cholerae* proteins encoded within a DNA locus required for biofilm formation. A *V. anguillarum* Δ*vanT* mutant and a mutant carrying a polar mutation in the *sat-vps73* DNA locus were shown to produce defective biofilms. Hence, a new member of the *V. harveyi* LuxR transcriptional activator family has been characterized in *V. anguillarum* that positively regulates serine, metalloprotease, pigment, and biofilm production.**

Diverse gram-negative and gram-positive bacteria have been shown to use intercellular communication mechanisms to regulate the transcription of multiple target genes in concert with cell density. This type of communication, termed quorum sensing, is mediated through the production of diffusible signal molecules, termed autoinducers or pheromones, which effectively enable a bacterium to monitor its own population density (for reviews, see references 18, 24, 25, and 56). In gram-negative bacteria, the best-studied autoinducer molecules are *N*-acylhomoserine lactones (AHLs), which vary in the length, saturation state, and C3 substitutions of the *N*-acyl side chain (25).

The cell density-dependent regulation of bioluminescence in *Vibrio (Photobacterium) fischeri* (38, 39) is frequently used as the paradigm for quorum sensing. In this marine symbiont, as the bacterial cell population density increases, the level of the autoinducer *N*-(3-oxohexanoyl)homoserine lactone (3-oxo-C6-HSL) (19) accumulates until a critical threshold concentration is reached. 3-Oxo-C6-HSL then binds to the transcriptional activator LuxR, and the resulting LuxR/3-oxo-C6-HSL complex triggers transcription of the luminescence (*lux*) operon, resulting in the emission of light (39).

Similar to *V. fischeri*, the free-living marine bacterium *Vibrio harveyi* also regulates bioluminescence in a cell density-dependent manner through the production and sensing of *N*-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL) (for a review, see reference 39). However, the regulation of light production in *V. harveyi* is very different and appears to be more complex than in *V. fischeri*. Based on genetic analyses, Freeman and Bassler (21, 22) have proposed a model for the regulation of bioluminescence in *V. harveyi* that involves two signaling systems and two autoinducer molecules. The first quorum-sensing system relies on 3-hydroxy-C4-HSL (10), the synthesis of which is directed by *luxM*. Interestingly, the gene product of *luxM* shows no homology to the LuxI family of AHL synthases (6). Regulation of bioluminescence via the second quorum-sensing system utilizes another, as yet unidentified, signal molecule (AI-2), which is chemically distinct from AHLs and is synthesized via LuxS (64).

The sensors for 3-hydroxy-C4-HSL and AI-2, named LuxN and LuxQ, respectively, resemble proteins belonging to two-component signaling systems (6, 8), and each possesses a conserved histidine kinase and a response regulator domain. At low cell densities, in the absence of signal molecules, LuxN and LuxQ are suggested to work in parallel by relaying the phosphates from their response regulator domains to a shared phosphorelay protein, LuxU (22). The phosphate from LuxU is then transferred to the response regulator domain of the σ^{54}-

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dependent activator LuxO, which, when phosphorylated, represses bioluminescence, together with σ^{54}, by activating the expression of an as yet unidentified repressor (7, 21, 31). In contrast, at high cell densities, the signal molecules accumulate and are believed to bind to their respective sensors (21, 22). It is thought that 3-hydroxy-C4-HSL may bind directly to LuxN, whereas AI-2 is postulated to bind to LuxQ via interaction with a putative periplasmic protein, LuxP. Binding of the signals is suggested to switch the sensor kinase activities of LuxN and LuxQ into phosphatases, leading to the dephosphorylation of LuxO and derepression of the *lux* operon. This then allows LuxR, the transcriptional activator, to positively activate the bioluminescence genes. LuxR from *V. harveyi* has no similarity to LuxR from *V. fischeri*.

The nonbioluminescent salmonid fish pathogen *Vibrio anguillarum* contains multiple quorum-sensing systems (46, 47). A *V. fischeri* LuxI homologue, VanI, was characterized and shown to be required for the production of *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) (46). A null mutant for *vanI* showed a loss of 3-oxo-C10-HSL production. However, the *vanI* mutant was still capable of weakly activating bioluminescence gene biosensors in *Escherichia coli*. Two additional AHLs were identified, *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-hydroxy-C6-HSL), along with the gene, *vanM*, responsible for their synthesis (47). VanM was shown to be homologous to LuxM of *V. harveyi*. Moreover, a second gene, *vanN*, which encodes a *V. harveyi* LuxN homologue, was found downstream of *vanM* (47).

To further characterize the *V. harveyi*-like quorum-sensing system in *V. anguillarum*, we wanted to determine if a homologue of the *V. harveyi* LuxR transcriptional activator is also present in *V. anguillarum*. To date, three homologues (HapR, OpaR, and SmcR) of the *V. harveyi* LuxR transcriptional activator have been identified in *Vibrio* spp. HapR from *V. cholerae* is the positive regulator of a hemagglutinin protease encoded by the *hap* gene (27). OpaR from *V. parahaemolyticus* is the positive regulator of an opacity phenotype that appears to be correlated with capsular polysaccharide production (20, 33). SmcR from *V. vulnificus* is thought to positively regulate the metalloprotease gene (*vvp)* and to negatively regulate the cytolysin gene (*vvhA)*, alkaline phosphatase production, motility, fimbria production, and biofilm formation (35, 36, 58).

This work describes the cloning and characterization of *vanT* from *V. anguillarum*, which codes for a homologue of *V. harveyi* LuxR. Functional analyses suggest that VanT positively regulates the biosynthesis of serine, glycine, and one-carbon-unit molecules, the expression of the metalloprotease gene *empA*, pigment production, and biofilm formation.

MATERIALS AND METHODS

Strains, phage, plasmids, and media. Bacterial strains and plasmids are described in Table 1. *E. coli* SY327 (λ*pir*) was used for transformation after subcloning fragments into either the pNQ705-1 or pDM4 suicide vector. All plasmids to be conjugated into *V. anguillarum* were transformed into *E. coli* S17-1 (λ*pir*), which was used as the donor strain. Plasmid transfers from *E. coli* to *V. anguillarum* were done as previously described (45). *E. coli* XL1-Blue was used for bacteriophage lambda infections and for most transformations. *E. coli* DH5αPRO was used for the two-plasmid screening. *E. coli* JM109 was used to harbor the bioluminescence sensor plasmid pSB1075.

E. coli was routinely grown in Luria 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 broth medium (TSB) from BBL was used for routine growth. For selection against *E. coli* after conjugation, two *Vibrio* selective media were used, TCBS agar (Difco Laboratories) and VAM medium (4). Biofilm growth medium was minimal M63 salts (60) supplemented with 1% (wt/vol) NaCl, 1.5% (wt/vol) Casamino Acids, 1% (wt/vol) glucose, 1 mM $MgSO_4$, and 10 μg of thiamine per ml.

Antibiotic concentrations for all *E. coli* strains were ampicillin at 100 μg/ml, tetracycline at 10 μg/ml, kanamycin at 30 μg/ml, and chloramphenicol at 25 μg/ml. Antibiotic concentrations for *V. anguillarum* in the various media were: TSB and TCBS, tetracycline at 5 μg/ml and chloramphenicol at 5 μg/ml; VAM, chloramphenicol at 1 μg/ml.

Isolation and detection of AHLs. AHLs were purified and characterized as described by Cámara et al. (9). Essentially, spent supernatants from stationary-phase cultures of *V. anguillarum* NB10 and AC10 grown overnight in TSB were extracted with dichloromethane, and solvent extracts were separated by thin-layer chromatography (TLC) as previously described (9, 34). For analysis of 3-hydroxy-C6-HSL and C6-HSL, reverse-phase aluminum-backed RP18 F_{254S} TLC plates (20 cm by 20 cm; Merck) and a mobile phase of 60% (vol/vol) methanol in water were employed. In contrast, 3-oxo-C10-HSL was analyzed on aluminum-backed silica gel 60 F_{254} normal-phase TLC plates (20 cm by 20 cm; Merck) using a 45%:55% (vol/vol) hexane-acetone mix as the mobile phase. AHL synthetic standards (see below) were used as markers. Detection of 3-hydroxy-C6-HSL and C6-HSL was done by overlaying the TLC plates with soft top agar seeded with the *Chromobacterium violaceum* CV026 reporter using both the AHL activation and inhibition violacein assays (34). For the detection of 3-oxo-C10-HSL, a bioluminescent *E. coli lux*-based AHL biosensor termed *E. coli* JM109(pSB1075), which contains an intact *lasR* gene and the *lasI* promoter from *Pseudomonas aeruginosa* fused to *luxCDABE* from *Photorhabdus luminescens*, was used (70).

The AHL standards, 3-oxo-C10-HSL, 3-hydroxy-C6-HSL, and C6-HSL, were synthesized, purified, and characterized as described previously (9, 13). Each compound was subjected to mass spectrometry (MS), proton nuclear magnetic resonance spectroscopy, and infrared spectroscopy. For 3-hydroxy-C6-HSL and C6-HSL, spectroscopic data are provided by Chhabra et al. (13), and Milton et al. (46) give data for 3-oxo-C10-HSL.

DNA techniques and sequencing. Oligonucleotides were synthesized using Applied Biosystems DNA/RNA synthesizer model 394. Unless otherwise stated, all conditions for the various DNA techniques were as described by Sambrook et al. (57). Reaction conditions for the DNA-modifying enzymes and DNA restriction enzymes were performed as suggested by the manufacturers. Double-stranded DNA sequencing was performed using the dideoxy chain termination method with T7 DNA polymerase (Pharmacia Biotech) and by primer walking in two directions from known regions of DNA sequence. The T7 and T3 primers were used for sequencing fragments in pBluescript. For sequencing the gene fusions to *lacZ*, a primer (5′-GATTAAGTTGGGTAACGC-3′) whose site is located at the 5′ end of the *lacZ* gene was used.

PCR conditions. PCR was performed as previously described (37) except that instead of the standard reaction buffer, a buffer containing 1% Thesit was used (52). To obtain the initial 175-bp *V. anguillarum* PCR fragment using the degenerate primer, the PCR optimization kit from Stratagene (buffer 8) was employed. When a PCR fragment required minimal errors, the high-fidelity *Pfu* polymerase (Stratagene) was used.

Cloning of the *vanT* DNA locus. Protein alignments of LuxR from *V. harveyi* (59), OpaR from *Vibrio parahaemolyticus* (33), and HapR from *Vibrio cholerae* (27) indicated that these proteins were most homologous at the amino terminus. Two oligonucleotides were designed based on complementation to the *V. harveyi luxR* sequence (59). HarveyiR-3 [5′-GGCACTAGT(CT)TGICGIACIAC(AG)T G(AG)TT-3′] is a degenerate, inosine-containing oligonucleotide that is complementary to *V. harveyi* residues 522 to 539 that encode amino acids NHV VRQF (59) and that has an *Spe*I site at its 5′ end. HarveyiR-4 [5′-GGTGAGC TCAAACGTAAACAGCAACTGATGGA-3′] was designed to be directly complementary to *V. harveyi luxR* residues 363 to 385 that encode amino acids KRKQQLME (59) and that has a *Sac*I site at the 5′ end. A 175-bp fragment was amplified from the chromosome of *V. anguillarum*, purified from 1% agarose using Ultrafree-DA spin columns (Millipore), digested overnight with restriction enzymes *Sac*I and *Spe*I, cloned into similarly digested pBluescript (Stratagene), creating pBSVanT-175, and sequenced. The deduced protein sequence of this fragment was 92% similar to LuxR of *V. harveyi* (59).

The 175-bp fragment was used as a probe to screen a previously described (44) gene bank of chromosomal DNA from *V. anguillarum* in the Lambda Zap II bacteriophage (Stratagene). Probe labeling, plaque hybridization, and excision of the recombinant plasmids were done as previously described (44, 45). For all

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant markers	Reference(s) or source
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	57
SY327	Δ*(lac-pro) argE*(Am) *rif malA recA56* λ*pir*	43
S17-1	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	61
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-pro*) [F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]	Stratagene
DH5αPRO	*deoR endA1 gyrA96 hsdR17*($r_K^-m_K^+$) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80Δ*lacZ*Δ*M15* F$^-$ λ$^-$ P_{N25}/*tetR* P_{lacI^q}/*lacI* Sp^r	Clonetech
C. violaceum CV026	Km^r; strain ATCC 31532, defective in C6-HSL production due to a mini-Tn*5* insertion in the *cviI* gene	29, 34, 69
V. anguillarum		
NB10	Wild type, serotype O1, clinical isolate from the Gulf of Bothnia	49
AC10	In-frame deletion of *vanT*	This study
DM70	Cm^r; polar mutation in *sat* gene	This study
Plasmids		
pBluescript	Ap^r; ColE1 origin	Stratagene
pBSVanT-175	Ap^r; pBluescript containing 175-bp PCR fragment from *vanT* (bp 463–640)	This study
pBSVanT3-2	Ap^r; pBluescript containing cloned fragment with the *vanT* gene	This study
pDM4	Cm^r; suicide vector with an R6K origin (*pir* requiring) and *sacBR* genes from *Bacillus subtilis*	45
pDMVanT1	Cm^r; pDM4 derivative containing *vanT* bp 146–418 fused in-frame to bp 1028–1303	This study
pNQ705-1	Cm^r; suicide vector that contains an R6K origin (*pir* requiring)	37
pNQSat1	Cm^r; pNQ705-1 derivative containing a 220-bp internal 5′ fragment of *sat* gene	This study
pSup202	Cm^r Tc^r Ap^r; pBR325 derivative which carries mobilization genes (RP4 *mob*)	61
pMC1871	Tc^r; contains a promoterless *lacZ* gene that lacks a ribosome-binding site and first 8 amino acid codons	Pharmacia Biotech
pTL61T	Ap^r; contains a promoterless *lacZ* gene and the *lacZ* ribosome-binding site	32
pDM5	Cm^r Tc^r; pSup202 derivative containing the promoterless *lacZ* gene cassette from pMC1871 used for translational gene fusion studies	This study
pDM8	Cm^r Tc^r; pSup202 derivative containing the promoterless *lacZ* gene cassette from pTL61T used for transcriptional gene fusion studies	This study
pDM8-Vps73	Cm^r Tc^r; pDM8 derivative containing a *vps73*::*lacZ* transcriptional gene fusion	This study
pDM8-EmpA	Cm^r Tc^r; pDM8 derivative containing an *empA*::*lacZ* transcriptional gene fusion	This study
pDM8-SerA	Cm^r Tc^r; pDM8 derivative containing a *serA*::*lacZ* transcriptional gene fusion	This study
pDM8-HpdA	Cm^r Tc^r; pDM8 derivative containing an *hpdA*::*lacZ* transcriptional gene fusion	This study
pDM5-S5	Cm^r Tc^r; pDM5 derivative containing a *vps73*::*lacZ* translational gene fusion	This study
pDM5-S13	Cm^r Tc^r; pDM5 derivative containing an *hpdA*::*lacZ* translational gene fusion	This study
pDM5-Ssp4	Cm^r Tc^r; pDM5 derivative containing a *serA*::*lacZ* translational gene fusion	This study
pPROLar.A131	Kn^r; p15A origin, protein expression vector using a $P_{lac/ara-1}$ promoter	Clontech
pLar-VanT	Kn^r; derivative of pPROLar.A131 containing the *vanT* gene fused to the $P_{lac/ara-1}$ promoter	This study
pAC-EmpA	Cm^r; pACYC184 derivative containing the *empA* gene and its promoter	This study
pSB1075	Ap^r; contains a fusion of *lasRI′*::*luxCDABE* in pUC18	69

recombinant plasmids, the chromosomal DNA inserts were unstable, as deletions occurred during excision from the bacteriophage (data not shown). To acquire a stable DNA fragment and an active *vanT* gene for sequencing and functional analyses, we used preliminary data suggesting that *E. coli* does not express the *empA* gene without VanT present in the cell. Thus, pAC-EmpA, which carries *empA* and its promoter, was transformed into XL1-Blue *E. coli*. pAC-EmpA was created by ligating an *Xba*I- and *Sal*I-digested *empA*-containing fragment from pBS80 (44) to *Xba*I- and *Xho*I-digested pACYC184. Excised plasmids from the positive plaques were transformed into XL1-Blue carrying pAC-EmpA and plated onto TSA medium containing 2% skim milk. A colony that gave a reproducible zone of clearing was picked. The pBluescript plasmid pBSVanT3-2, containing the cloned DNA fragment, was purified and used for sequencing the *vanT* gene.

Construction of *vanT* mutant strain. For functional analyses, an in-frame deletion was made in *vanT* by allelic exchange as previously described (45). To create the new deletion allele, pBSVanT3-2 was used as the template with PCR primers VanT-A (5′-GGAAGATCTGCCAATACGCGAACC-3′) and VanT-B (5′-AAGTTAAGCCTAGTGCATGAGTTGTTAATC-3′) to create a fragment from bp 0 to 65 and with primers VanT-C (5′-CACTAGGCTTAACTTCCTA GTCTC-3′) and VanT-D (5′-CTCGAGCTCGGTTTGGCTATCGAG-3′) to create a fragment from bp 675 to 950. These two fragments contained an overlap of similar sequence and were used as templates in a second PCR using primers VanT-A and VanT-D. Using *Bgl*II and *Sac*I, the fragment was cloned into the *Bgl*II and *Sac*I sites of pDM4, creating pDMVanT1. After allelic exchange using pDMVanT1, the start codon of *vanT* was fused to the last amino acid codon of the gene, and the strain containing this deletion was called AC10. The in-frame deletion in AC10 was confirmed by PCR amplification of this locus using primers VanT-10B (5′-GGACTAGTAATTCAACAGTCATTGGCA-3′) and VanT-16B (5′-CTCGAGCTCCTTTGACGATTCATGCTCA-3′) and subsequent DNA sequencing.

Construction of a polar mutation within the *sat-vps73* locus. A mutant carrying a polar mutation in the *sat-vps73* locus (DM70) was made by the integration of a suicide plasmid, pNQSat1, into the *sat* gene as described previously (44). A 220-bp PCR fragment complementary to the 5′ end of the *sat* gene was amplified from the *V. anguillarum* chromosome using primers Sat1-A (5′-GGAAGATCT CATTGATCCCAAATGTTA-3′) and Sat1-B (5′-GGGACTAGTATACATCA TTTCCTATCT-3′). Using *Bgl*II and *Spe*I, the PCR fragment was cloned into similar sites of pNQ705-1, creating pNQSat1. The entire pNQSat1 was inserted 54 bp downstream of the *sat* start site. Since the PCR fragment did not contain the *sat* promoter or start codon, the plasmid insertion created a null mutation of *sat* and will have a polar effect on *vps73* and other possible uncharacterized downstream genes. The insertion of the plasmid was checked by PCR analysis as previously described (44). Stability of the insertion mutation was tested by growth for 30 generations in the absence of chloramphenicol.

Two-plasmid screening for genes regulated by VanT. A method was designed that used two plasmids in an *E. coli* background to identify *V. anguillarum* genes

that are either positively or negatively regulated by VanT. The first plasmid, pLar-VanT, is a pPROLar.A131 (Clontech Laboratories) derivative that contains the *vanT* gene fused to a hybrid regulatory unit ($P_{lac/ara-1}$) between the *lac* promoter and the *araBAD* promoter. To create pLar-VanT, a PCR fragment that contains a promoterless *vanT* gene (bp 63 to 770) was amplified using primers VanT-LarA (5′-CGGGGTACCCATGGAAACATCGATAGAA-3′) and VanT-LarB (5′-CGCGGATCCGTTCATTAAATTGCTGGT-3′). The fragment was digested with *Kpn*I and *Bam*HI and ligated into pPROLar.A131 similarly digested. Due to its fusion to the hybrid promoter, $P_{lac/ara-1}$, the expression of *vanT* was highly inducible by L-arabinose and IPTG (isopropylthiogalactopyranoside) and tightly repressed by AraC and LacI in *E. coli* DH5αPRO.

The second plasmid, pDM5, is a pSUP202 (61) derivative that contains a promoterless *lacZ* gene for translational gene fusion studies. The promoterless *lacZ* gene for pDM5 lacks a ribosome-binding site and the first eight nonessential amino-terminal amino acid codons. This 3.1-kb promoterless *lacZ* gene cartridge was removed from pMC1871 (Pharmacia Biotech) using *Pst*I and ligated into the *Pst*I site within the ampicillin gene of pSUP202. A unique *Sma*I site is located just upstream of *lacZ* that allows promoter/gene fusions to the amino-terminal part of the promoterless *lacZ* gene. For randomization of gene fusions, *V. anguillarum* chromosomal DNA was digested with *Dra*I, *Ssp*I, or *Eco*RV, and the resulting chromosomal fragments were ligated to *Sma*I-digested pDM5. The ligation was transformed into DH5αPRO containing pLar-VanT and plated on LB medium containing 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml but no inducers. After incubation at 37°C, the colonies were replica plated onto LB medium containing 50 μg of X-Gal per ml, 1 mM IPTG, and 0.2% L-arabinose. Any colonies that appeared blue on one plate but white or lighter blue on the other within 48 h were picked for further analysis. As pDM5 contains mobilization genes, plasmids carrying the gene fusions were mobilized into *V. anguillarum* strains for further gene regulation studies using β-galactosidase assays.

Construction of transcriptional β-galactosidase fusions to *empA, vps73, serA,* and *hpdA*. Transcriptional gene fusions were constructed between the reporter gene *lacZ* from *E. coli* and four *V. anguillarum* promoters, *serA, vps73, hpdA-hgdA,* and *empA*. For these studies, the vector, pDM8, was designed to contain the entire *lacZ* gene and its ribosome-binding site but to lack the *lacZ* promoter. pDM8 is a derivative of pDM5 and pTL61T which carries a promoterless *lacZ* gene designed for transcriptional gene fusion studies (32). pDM5 was digested with *Sma*I and *Sac*I, and the 5′ region of the promoterless *lacZ* gene was removed and replaced with a 2,200-bp *Sma*I-*Sac*I fragment from the 5′ region of the promoterless *lacZ* gene from pTL61T. A unique *Sma*I site just upstream of the *lacZ* gene is used for the fusion of promoter regions from other genes.

For the *empA* transcriptional fusion, a PCR fragment containing the *empA* promoter (276 bp upstream) but lacking the possible ribosome-binding site was obtained using primers EmpA-βgal-1 (5′-TCCCCCGGGTTATATTGATAGTTATGT-3′) and EmpA-βgal-3 (5′-TCCCCCGGGGAGAGTTATTATTAGCAT-3′). For the *serA* transcriptional fusion, a PCR fragment containing the *serA* promoter (230 bp upstream) was obtained using primers SerA-βgal-A (5′-TCCCCCGGGTTTCATTACAAAGCGCAC-3′) and SerA-βgal-C (5′-TCCCCCGGGAAAATGGGAAGGTGGGCA-3′). For the *vps73* transcriptional fusion, a PCR fragment containing the possible *vps73* promoter found in the 3′ end of the *sat* gene (295 bp upstream) was obtained using primers Vps73-βgal-1 (5′-TCCCCCGGGGAGGGATAGGATGTGTCA-3′) and Vps73-βgal-3 (5′-TCCCCCGGGCTTGCCAAATAGAATGT-3′). For the *hpdA-hgdA* transcriptional fusion, a PCR fragment containing the *hpdA-hgdA* promoter (318 bp upstream) was obtained using primers VllY-βgal-1 (5′-TCCCCCGGGTGTCGGTTTTTAATG-3′) and VllY-βgal-2 (5′-TCCCCCGGGGTTAACGCTTGTATT-3′). All PCR fragments were digested with *Sma*I and ligated to pDM8 that was similarly digested, creating pDM8-EmpA, pDM8-SerA, pDM8-Vps73, and pDM8-HpdA. These plasmids were then conjugated into the *V. anguillarum* wild type and the *vanT* mutant.

β-Galactosidase assays. *V. anguillarum* cultures were grown overnight at 24°C in TSB containing tetracycline (2 μg/ml). Cell cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in the same medium and further incubated at 24°C with shaking. Samples were taken at the various time points, and β-galactosidase assays were performed in triplicate according to Miller (42).

Measurement of protease activity. Culture supernatants were assayed for protease activity as described by Denkin and Nelson (15) and Windle and Kelleher (68). Cells were grown overnight at 24°C in TSB with shaking, then diluted to an OD_{600} of 0.05 in TSB, and incubated further at 24°C with shaking. For comparative analysis between strains, a 2-h time period for incubation of the protease reaction mixture at 30°C was chosen.

Measurement of pigment production. *V. anguillarum* strains were grown overnight at 24°C in TSB. Bacterial cell cultures were then diluted to an OD_{600} of 0.05 in either TSB or TSB containing 5 mM L-tyrosine and incubated at 24°C with shaking. At various time points, 1-ml samples were taken from the culture, and the supernatants were collected by centrifugation (12,000 × *g*, 2 min). Pigment production was estimated in the supernatant by measuring the absorbance at 400 nm. Cell densities were determined by counting CFU on Trypticase soy agar or by measuring the OD_{600}.

Northern analysis. The *V. anguillarum* wild type and *vanT* mutant were grown for 30 h at 24°C in TSB. Total RNA was then isolated using the RNeasy Minikit from Qiagen GmbH (Germany), and 10 μg of each sample was applied to a 2.2 M formaldehyde1% agarose gel and electrophoresed according to Sambrook et al. (57). Transfer of the RNA to ZetaProbe GT membranes (Bio-Rad), prehybridization, hybridization, and washing of the filter were performed as described in the ZetaProbe GT Blotting Membranes instruction manual. DNA fragments used as probes were generated by PCR amplification using primers within each of the genes: *empA* (bp 641 to 1374), *serA* (bp 200 to 372), *vps73* (bp 959 to 1237), and *hpdA* (bp 289 to 1556). The *hpdA* DNA fragment was labeled using [α-^{32}P]dCTP and random oligonucleotide priming (57), and the *empA, serA,* and *vps73* DNA fragments were labeled using similar reaction conditions for random oligonucleotide priming, but instead of random hexamers, a specific primer within the DNA fragment was used. The radioactive bands were visualized using a Phosphorimager and the ImageQuant version 3.2 software from Molecular Dynamics.

Biofilm formation assays. Two types of biofilm formation assays were performed, one on glass and the other on untreated polyvinylchloride (PVC) plastic. For biofilm formation on PVC plastic, an assay similar to that described previously for *P. aeruginosa*, *E. coli*, and *Staphylococcus* (50, 53, 63) was used with a few modifications. *V. anguillarum* strains were grown overnight in biofilm growth medium and then diluted to an OD_{600} of 1. Five microliters of the diluted bacteria was transferred to wells of a PVC microtiter dish (Falcon 35117) containing 200 μl of biofilm growth medium. Bacteria were allowed to grow without agitation for 6, 8, 10, 12, and 14 h at 24°C. Biofilm formation was quantified as described previously (63). Each strain was tested in three separate experiments, and for each experiment five wells were used for each strain. The values recorded are averages of these experiments.

For biofilm formation on glass, microscope glass slides were cleaned by soaking in a solution of 10% HCl and 90% methanol for 1 h at room temperature, rinsed in distilled water, and dried. Four sterile glass slides were suspended from the top of a 400-ml beaker into 300 ml of sterile biofilm growth medium. An overnight bacterial culture, grown in biofilm growth medium, was diluted in the same medium to an OD_{600} of 1. Three milliliters was used to inoculate the beaker. The culture was allowed to grow with stirring at "moderate speed" at room temperature. After 4 h, one slide was removed, bacteria were wiped away from one side, and the opposite side was stained immediately with 1 ml of 0.1% acridine orange in potassium phosphate buffer (pH 7.4) (48) for 5 min at room temperature. After 5 min, the stain was removed and the slide was washed twice in distilled water before air-drying. To prevent nutrient depletion of the growth medium, the remaining slides were removed, dipped carefully into 200 ml of fresh biofilm growth medium, suspended into another beaker containing 300 ml of fresh biofilm growth medium, and incubated as above for an additional 12 h. One slide was removed every 4 h and stained with acridine orange. Biofilm samples were taken at 4, 8, 12, and 16 h, and all experiments were done in triplicate.

The slides were analyzed for biofilm formation by viewing under a Zeiss Axioplan fluorescent microscope coupled to a charge-coupled device camera. Biofilm formation was quantified by determining the percent area coverage. Five representative images were taken for each biofilm and stored as computer images using the Adobe Photoshop 5.0 program. The images were converted into a grey scale using the Adobe Photoshop program and then converted to a binary mode using the Image Tool Software, version 2, from the University of Texas Health Science Center at San Antonio. This software makes all pixels in the image either black (background) or white (biofilm). The area coverage was then given as the percentage of white pixels. This was done for all five images taken and averaged.

Computer analysis. Database searches were done using the sequence analysis software (17) of the Genetics Computer Group, Inc. (University of Wisconsin).

Fish infections. Rainbow trout (*Oncorhynchus mykiss*) weighing approximately 10 to 15 g were infected with *V. anguillarum* either by intraperitoneal injections or by immersion in seawater containing *V. anguillarum* as previously described (45). The immersion and intraperitoneal infections were done at least twice. Five fish were infected for each bacterial dilution used. The 50% lethal doses (LD_{50}s) were calculated as described by Reed and Muench (54). The LD_{50}s recorded are an averaged value for all infections for each strain.

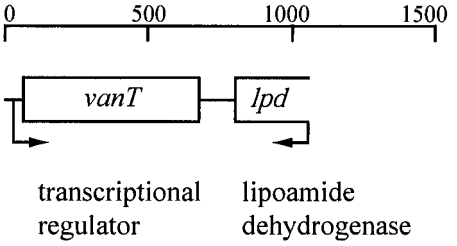

FIG. 1. Genetic organization of the *vanT* DNA locus. Arrows indicate the direction of transcription.

Nucleotide sequence accession numbers. The complete *vanT* DNA sequence and the partial DNA sequences of the *serA*, *sat-vps73*, and *hpdA-hgdA* loci have been submitted to GenBank. Accession numbers were assigned to *vanT* (AF457643), *serA* (AF457644), *hpdA-hgdA* (AF457645), and *sat-vps73* (AF457646).

RESULTS

Characterization and mutagenesis of the *vanT* gene. To determine whether *V. anguillarum* carries a gene homologous to the *luxR* gene of *V. harveyi*, degenerate primers based on the *luxR* coding sequence were designed. Using these primers, a 175-bp PCR fragment was generated. DNA sequence analysis of this fragment revealed the presence of an open reading frame (ORF), the deduced protein product of which showed 92% identity to LuxR. Using this PCR fragment as a probe, a recombinant clone containing the *V. anguillarum* gene was obtained from a *V. anguillarum* genomic library. The *V. anguillarum* DNA fragment was sequenced, and its genetic organization is shown in Fig. 1.

One complete ORF was identified and named *vanT*. The deduced VanT protein sequence was 81% identical to LuxR of *V. harveyi* (59), 74% identical to HapR of *V. cholerae* (27), 82% identical to SmcR of *Vibrio vulnificus* (35, 58), and 82% identical to OpaR of *V. parahaemolyticus* (33). The amino terminus (residues 4 to 72) of VanT was also 30% identical to the amino terminus of the TetR family of repressor proteins, which contains a helix-turn-helix motif characteristic of DNA-binding proteins (51). A putative helix-turn-helix motif was identified in VanT at amino acid residues 39 to 58 and was similarly located in all LuxR homologues. Using the TopPred-2 search program (65), a putative transmembrane region was identified near the carboxy terminus (amino acid residues 160 to 180).

In addition, one partial ORF was also found (Fig. 1). The deduced protein-coding sequence of this partial ORF is very similar to the *lpd* gene of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*. This gene encodes a putative dihydrolipoamide dehydrogenase and is also located downstream of the *luxR* homologues characterized in these *Vibrio* spp.

To determine the function of VanT, an in-frame mutation was made that deleted the entire *vanT* gene, leaving only the first and last amino acid codons. This was done by allelic exchange with the altered gene carried on a suicide vector as described previously (45). The mutant strain was called AC10.

VanT positively regulates expression of the metalloprotease gene *empA*. Since the EmpA metalloprotease of *V. anguillarum* is similar to the hemagglutinin protease of *V. cholerae* and since HapR regulates *hap* expression, it was thought that VanT may regulate *empA* expression. To test this idea, the promoter of *empA* was fused to a promoterless *E. coli lacZ* gene to generate a transcriptional fusion carried on plasmid pDM8-EmpA. This plasmid was conjugated into the wild-type *V. anguillarum* and the *vanT* mutant strain, and LacZ assays were done over the growth curve (Fig. 2A). For the wild type, LacZ activity was delayed until the cell density was high and then increased steadily throughout stationary-phase growth. In the *vanT* mutant, the *empA* promoter was virtually inactive compared to the wild type, suggesting that VanT is essential for expression of *empA* under these growth conditions.

For further confirmation, a quantitative protease assay (Fig. 2B) previously employed to assay EmpA (15) was used. The protease activity of the *vanT* mutant was negligible compared with that of the wild type. In addition, Northern analysis was done using total RNA from the wild-type and the *vanT* mutant strains. Figure 2C shows that the amount of RNA transcript hybridizing to a probe from the *empA* gene was significantly reduced in the *vanT* mutant compared to the wild type. Taken together, these results suggest a requirement for VanT in the expression of the *empA* gene under the conditions of growth used in this study, although we cannot rule out the possibility that VanT may also regulate additional proteases previously detected in an *empA* mutant (44).

AHL analysis in the *vanT* mutant. Previously, a mutation in the AHL synthase gene *vanM* resulted in the complete abolition of AHL production, including 3-oxo-C10-HSL, which is synthesized via VanI (47). One likely explanation for this observation is that *V. anguillarum* may contain a quorum-sensing circuit similar to that of *V. harveyi*, regulating the production of 3-oxo-C10-HSL via VanI. This possibility suggests that VanT may regulate 3-oxo-C10-HSL production. To test this hypothesis, AHL production was assayed in the wild type and the *vanT* mutant. TLC analysis of spent cell-free supernatants combined with AHL biosensor overlays were carried out to determine whether there were any changes in the AHL profile with respect to C6-HSL, 3-hydroxy-C6-HSL, and 3-oxo-C10-HSL in the *vanT* mutant compared with the wild type. No differences were observed, suggesting that regulation of 3-oxo-C10-HSL production in *V. anguillarum* is not likely to occur via VanT (data not shown).

Virulence analysis. For several bacterial pathogens, quorum sensing has been shown to regulate the production of virulence determinants (for reviews, see references 67 and 71). We have previously shown that mutations in *vanM* and *vanN* did not alter the virulence of *V. anguillarum* (47). However, no conclusion could be made from these results because a parallel quorum-sensing system, similar to the *luxSPQ* system of *V. harveyi,* may also exist in *V. anguillarum*, activating or inactivating the same repressor as the *vanMN* circuit. Hence, a mutation in *vanT*, located downstream of the parallel circuits, would aid in the interpretation of additional virulence studies. The LD_{50}s were therefore determined in rainbow trout for the wild type and the *vanT* mutant strain. Both immersion in infected seawater and intraperitoneal infection were used. For the immersion route, there was only a sixfold difference between the LD_{50}s for the wild type (3×10^3 bacteria per ml of seawater) and the *vanT* mutant (5×10^2 bacteria per ml of seawater). For the intraperitoneal route, again the LD_{50}s were similar for the wild type and the *vanT* mutant, 21 and 24

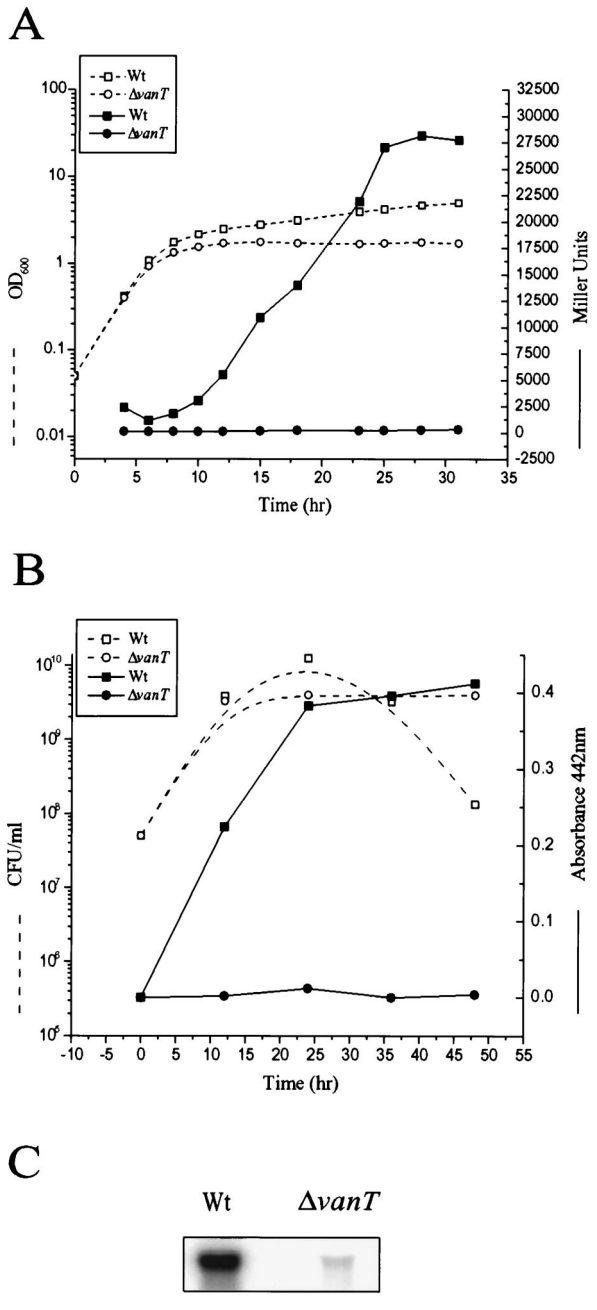

FIG. 2. Analyses of metalloprotease activity in the wild type (Wt) and the *vanT* mutant. (A) Growth curve and expression of *empA*::*lacZ* transcriptional gene fusion. Overnight cultures were diluted to an $OD_{600\ nm}$ of 0.05 and then incubated with shaking at 24°C. Samples were taken at various times and analyzed for growth (OD_{600}, open symbols) and β-galactosidase expression (Miller units, solid symbols). Miller units for the vector control were between 100 and 200 U. (B) Protease activity. Overnight cultures were diluted to an OD_{600} of 0.05 and then incubated with shaking at 24°C. Samples were taken at various times and analyzed for growth (open symbols) and for azocasein degradation (OD_{442}, solid symbols). For protease activity, 100 μl of filtered-sterilized culture supernatant was mixed with 100 μl of azocasein solution and incubated at 30°C for 2 h. The reaction was stopped by the addition of trichloroacetic acid, the unreacted azocasein was removed by centrifugation, and the absorbance at 442 nm was determined. (C) Northern analysis. Total RNA was isolated from 30-h cultures of the wild type and *vanT* mutant grown at 24°C and hybridized to a DNA fragment complementary to the *empA* gene.

bacterial cells, respectively. These data suggest that VanT is not essential for virulence.

Screening for genes regulated by VanT. Since *V. anguillarum* contains β-galactosidase activity that utilizes X-Gal but not ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as a substrate, we could not use transposons that contained promoterless *lacZ* genes for promoter characterization in *V. anguillarum*. Thus, to identify additional genes regulated by VanT, we designed a two-plasmid system for the random screening in *E. coli* of *V. anguillarum* genes regulated by VanT. Both plasmids were maintained within the same *E. coli* strain, DH5αPRO. The first plasmid, pLar-VanT, contains a hybrid arabinose and lactose promoter, $P_{lac/ara-1}$, that was fused to the *vanT* gene. This promoter tightly regulates the expression of the *vanT* gene by allowing gene expression only when arabinose and IPTG are present. The second plasmid is a derivative of pDM5 (Table 1) which carries a promoterless *lacZ* gene used in translational gene fusion studies.

To create random *V. anguillarum* promoter-gene fusions, we inserted chromosomal DNA fragments digested with either *Dra*I, *Ssp*I, or *Eco*RV into a unique *Sma*I restriction site located just upstream of the *lacZ* gene. For some DNA fragments, this ligation resulted in in-frame promoter-gene fusions to the promoterless *lacZ* gene of pDM5. To screen for promoters regulated by VanT, the ligated products were transformed into *E. coli* DH5αPRO(pLar-VanT), and transformants were spread onto plates containing only X-Gal. After overnight growth, the colonies were replica plated onto X-Gal plates containing arabinose and IPTG to induce $P_{lac/ara-1}$-*vanT*. Bacterial colonies containing promoter-*lacZ* fusions that were positively activated by VanT were darker blue in the presence of arabinose and IPTG and white or lighter blue in the absence of these inducers. Three colonies with the highest expression of LacZ were chosen for further analysis (pDM5-Ssp4, pDM5-S5, and pDM5-S13). To confirm similar regulatory effects in *V. anguillarum*, the three plasmids were mobilized, utilizing mobilization genes carried on pDM5 derivatives, into the *V. anguillarum* wild type and the *vanT* mutant. Since the β-galactosidase of *V. anguillarum* does not respond to ONPG as a substrate, LacZ activity was measured (data not shown). All three constructs showed a two- to fivefold lower LacZ expression in the *vanT* mutant compared to the wild-type strain.

The *V. anguillarum* DNA inserts from pDM5-Ssp4, pDM5-S5, and pDM5-S13 were sequenced. The DNA sequence of pDM5-Ssp4 indicated that a small 34-codon ORF was fused in-frame to the *lacZ* gene. The 34 amino acids encoded by the DNA sequence showed 85% identity to 3-phosphoglycerate dehydrogenase from *V. cholerae*, which is encoded by the VC2481 locus on chromosome I, which is similar to the *serA* gene of *E. coli* (26). In *E. coli*, 3-phosphoglycerate dehydrogenase oxidizes 3-phosphoglycerate to 3-phosphohydroxypyruvate, the first step in the biosynthesis of serine, glycine, and one-carbon-unit molecules. No additional ORF was seen in the DNA sequence 300 bp upstream of the *serA* peptide. Thus, it is likely that this 300 bp contains a promoter.

The 1,377-bp insert from pDM5-S5 (Fig. 3A) contained a partial ORF encoding 275 amino acids that was fused in-frame with the *lacZ* gene. These 275 amino acids showed 50% identity to the protein encoded by the VCO926 locus (also called *vps73*) on chromosome I of *V. cholerae* (26). Vps73 is involved

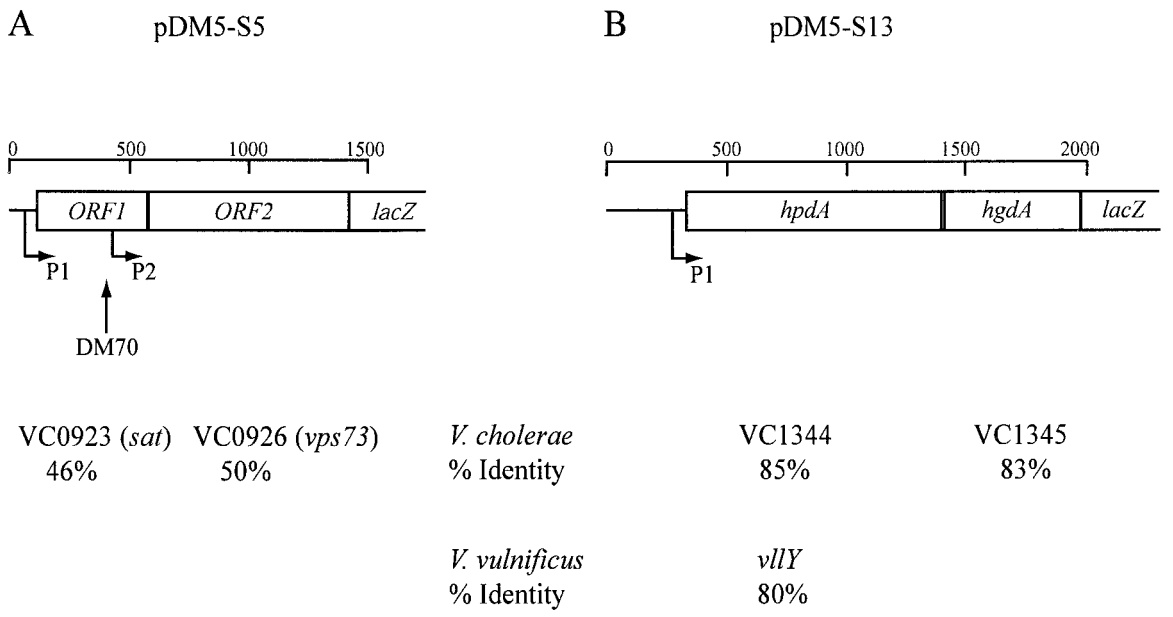

FIG. 3. Genetic organization of the cloned DNA fragments fused to the *E. coli lacZ* gene carried on pDM5-S5 and pDM5-S13. The horizontal arrows indicate the putative promoters and direction of transcription. The vertical arrow indicates the site of the plasmid insertion within the *sat* gene that created the polar mutation in the *sat-vps73* DNA locus (strain DM70).

in the production of extracellular polysaccharides (EPS) for biofilm formation (73). Just upstream of ORF2 (*vps73*), a complete ORF encoding a 150-amino-acid protein homologous to numerous bacterial serine acetyltransferases was found. When the *V. cholerae* genome database was searched, these 150 amino acids showed 46% identity with the serine acetyltransferase-related protein encoded by region VCO923 of chromosome I (26).

Thus, ORF1 (*sat*) and ORF2 (*vps73*) of *V. anguillarum* are located in a similar DNA locus as the coding regions VCO923 and VCO926 in *V. cholerae*. However, the genetic organization appears to be different between these two vibrios, since there are two additional genes between these two coding regions in *V. cholerae*. In addition, a very highly conserved consensus σ^{70} promoter sequence was found within the 3′ end of ORF1 just upstream of ORF2 (*vps73*), and a less conserved σ^{70} promoter sequence was found upstream of ORF1 (*sat*) (Fig. 3A).

For pDM5-S13, 2 kb upstream of the *lacZ* gene were sequenced, and the genetic organization is shown in Fig. 3B. Within this 2 kb, one complete ORF and a partial ORF were found. The partial ORF, encoding 194 amino acids, was fused in-frame to the *lacZ* gene. A GenBank database search revealed similarity between these 194 amino acids and numerous homogentisic acid oxidases. Hence, this ORF was designated *hgdA*. A *V. cholerae* genome search with these 194 amino acids showed 82.5% identity to the coding region VC1345, encoding a putative oxidoreductase. The complete ORF upstream of *hgdA* encodes a 357-amino-acid protein that showed similarity to numerous prokaryotic and eukaryotic HPPDs and was thus designated *hpdA*. The HPPDs that gave the highest identity (80%) were VllY from *V. vulnificus* (11) and the encoded protein from VC1344 of *V. cholerae* (26). VllY has been shown to confer hemolytic and pigment-producing activities when expressed in *E. coli*.

Interestingly, *hpdA* from *V. anguillarum* also appears to induce pigment production. Figure 4A shows a spectrophotometric profile of colored supernatant from the wild-type and the *vanT* mutant strains containing pDM5-S13 at various times during growth. The *V. anguillarum* strains were grown in the presence or absence of L-tyrosine, which has been shown to enhance pigment production in *V. cholerae* (55). As shown in Fig. 4A, at 48 h of growth, pigment production decreased fourfold for the *vanT* mutant in the absence of L-tyrosine and twofold in the presence of L-tyrosine, compared to the wild type. Moreover, pigment production was enhanced two- to threefold in the presence of L-tyrosine. There was no obvious ORF in the 300 bp upstream of the *hpdA* gene, indicating that a possible promoter upstream of *hpdA* is likely driving the expression of *lacZ*.

To confirm that VanT regulates the putative promoters upstream of the *vps73*, *serA*, and *hpdA* genes, transcriptional gene fusions were made between these promoters and a promoterless *E. coli lacZ* gene and carried on plasmids pDM8-Vps73, pDM8-SerA, and pDM8-HpdA, respectively. These constructs were mobilized into the *V. anguillarum* wild type and *vanT* mutant, and LacZ assays were done. The results are shown in Fig. 4B and Fig. 5A and 5C. As the bacteria entered stationary phase, a two- to threefold decrease in LacZ activity was seen for each of the three promoters. To confirm the *lacZ* data, Northern analyses were done using total RNA from the wild type and the *vanT* mutant. The amount of RNA transcript hybridizing to a DNA probe from the *hpdA* gene (Fig. 4C), the *vps73* gene (Fig. 5B), and the *serA* gene (Fig. 5D) was significantly decreased in the *vanT* mutant compared to the wild type. These data suggest that VanT positively regulates pigment production, serine biosynthesis, and genes possibly involved in EPS production.

VanT and the *sat-vps73*-containing DNA locus are involved

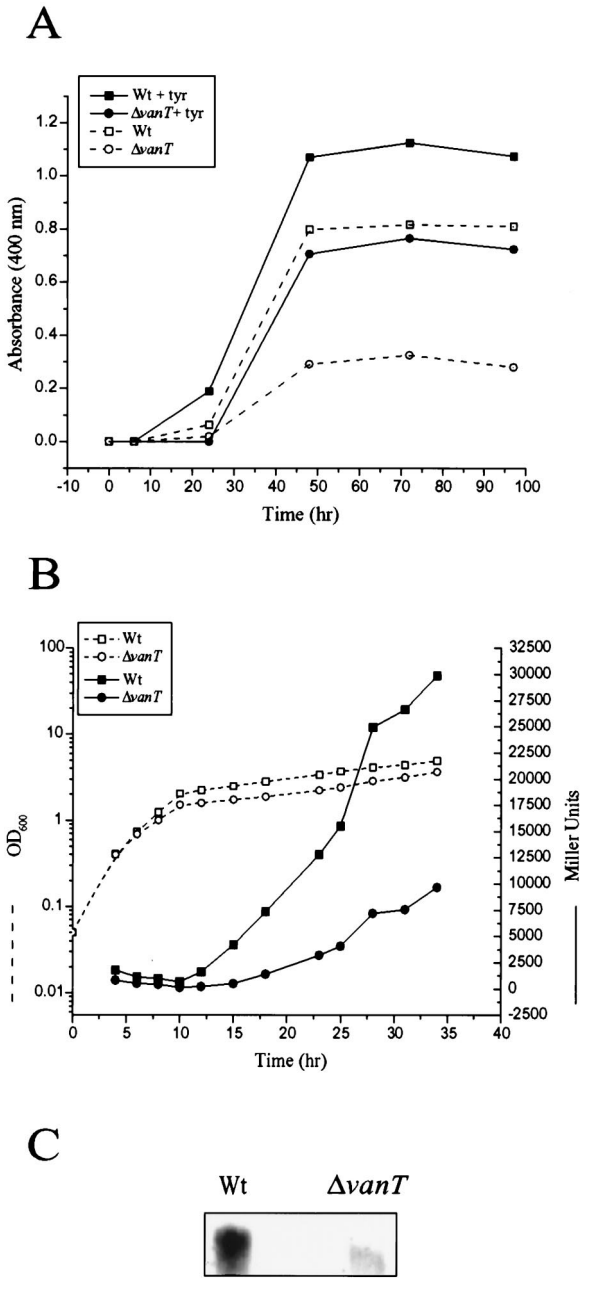

FIG. 4. Measurement of pigment production and promoter activity of the *hpdA* gene. (A) For pigment production, overnight cultures of the wild type (Wt) and the *vanT* mutant containing pDM5-S13, which carries the *hpdA* gene and its promoter, were diluted to an OD_{600} of 0.05 into TSB medium with (solid symbols) and without 5 mM L-tyrosine (open symbols) and incubated with shaking at 24°C. Samples were taken from the culture at various times, and the supernatant was filter sterilized to remove bacteria. Pigment production in the supernatant was estimated by measuring the absorbance at 400 nm. (B) For *hpdA* promoter activity, an *hpdA-lacZ* transcriptional gene fusion was made and contained on pDM8-HpdA in the wild type and the *vanT* mutant. Overnight cultures were diluted into fresh TSB medium to an OD_{600} of 0.05 and then incubated with shaking at 24°C. Samples were taken at various times and analyzed for growth (OD_{600}, open symbols) and β-galactosidase expression (Miller units, solid symbols). Miller units for the vector control were between 100 and 200 U. (C) Northern analysis. Total RNA was isolated from 30-h cultures of the wild type and *vanT* mutant grown at 24°C and hybridized to a DNA fragment complementary to the *hpdA* gene.

in biofilm formation. Yildiz and Schoolnik (73) have shown that the *vps73*-containing region of *V. cholerae* is involved in EPS production and biofilm formation. To determine if the *sat-vps73*-containing DNA locus in *V. anguillarum* is involved in biofilm formation, a polar mutation was made by inserting a suicide plasmid into ORF1 (*sat*) of the wild-type strain (Fig. 3), resulting in mutant strain DM70. This insertion resulted in a null mutation of ORF1 (*sat*) and should exert a polar effect on the downstream ORF2 (*vps73*) and any other genes that may be downstream of ORF2 on the chromosome. This mutant, DM70, was then tested for its ability to form a biofilm on a glass surface compared to the wild type.

Biofilms were formed by suspending glass slides into biofilm growth medium, inoculated with a bacterial strain, and allowing cells to grow on the glass at room temperature with gentle stirring. Spent medium was replaced with fresh medium after 4 h, and the biofilm was allowed to continue growing for 16 h. A glass slide was withdrawn from the medium at 4 h, 8 h, 12 h, and 16 h and stained with acridine orange, and the percent area coverage was determined. These data are shown in Fig. 6. Both the *vanT* mutant and the mutant containing a polar mutation in the *sat-vps73* DNA locus attached to the glass surface. By 16 h, the *vanT* mutant was unable to develop any biofilm structure, whereas the mutant carrying a polar mutation in the *sat-vps73* DNA locus appeared to begin forming a biofilm, although it was not as extensive as the wild-type biofilm.

To further confirm the biofilm data, the same *V. anguillarum* strains were also tested for biofilm formation on a PVC plastic surface. A previously described method using a 96-well PVC microtiter dish was used (50, 53, 63). The wild type, the *vanT* mutant, and the mutant carrying a polar mutation in the *sat-vps73* DNA locus were grown for 6 h, 8 h, 10 h, 12 h, and 14 h in biofilm growth medium in a 96-well PVC microtiter plate at 24°C without shaking. The wells were rinsed, fixed, and stained with crystal violet. To quantitate the bacteria present in the biofilms, the crystal violet was removed using 33% acetic acid, and the OD_{570} was determined. Figure 7 shows that the in-frame *vanT* mutant produced very little biomass that adhered to the plastic after 14 h, whereas the mutant carrying a polar mutation in the *sat-vps73* DNA locus produced a reduced level of biomass compared to the wild type. Taken together, these data suggest that the *V. anguillarum sat-vps73* DNA locus is involved in biofilm formation and that VanT is a positive regulator of genes found at this DNA locus. Moreover, VanT may also regulate additional genes involved in biofilm formation.

DISCUSSION

V. anguillarum has been shown to contain multiple quorum-sensing circuits (46, 47), incorporating VanIR, which is similar to the LuxIR system from *V. fischeri*, and VanMN, which is similar to the LuxMN system of *V. harveyi*. However, the role of these quorum-sensing circuits in *V. anguillarum* remains unclear. To investigate the possibility of a dual quorum-sensing channel system in this organism similar to that of *V. harveyi*, we cloned and characterized the *vanT* gene, encoding a homologue of the LuxR transcriptional activator of bioluminescence in *V. harveyi*.

During studies characterizing the LuxR homologues from *V.*

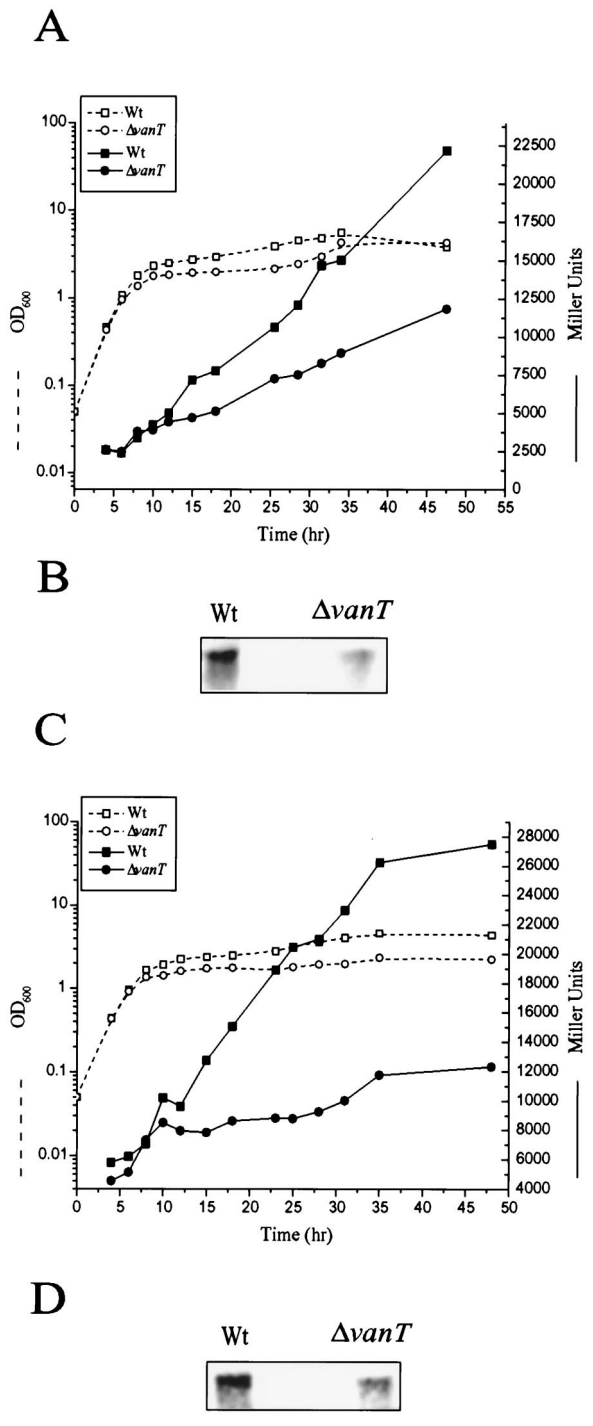

FIG. 5. β-Galactosidase activity of *vps73*::*lacZ* and *serA*::*lacZ* transcriptional gene fusions. (A) *vps73*::*lacZ* was carried on pDM8-Vps73 and (C) *serA*::*lacZ* was carried on pDM8-SerA in the wild type (Wt) and *vanT* mutant. Overnight cultures were diluted into fresh TSB medium to an OD_{600} of 0.05 and then incubated with shaking at 24°C. Samples were taken at various times and analyzed for growth (OD_{600}, open symbols) and β-galactosidase expression (Miller units, solid symbols). Miller units for the vector control were between 100 and 200 U. (B and D) Northern analysis. Total RNA was isolated from 30-h cultures of the wild type and *vanT* mutant grown at 24°C and hybridized to a DNA fragment complementary to either the *vps73* gene (B) or the *serA* gene (D).

cholerae (HapR) and *V. vulnificus* (SmcR), a *hapR* DNA probe was shown to hybridize weakly to chromosomal DNA from *V. anguillarum*, suggesting that *V. anguillarum* may also contain a LuxR homologue (27, 35). Both HapR and SmcR were shown to positively regulate homologous genes, *hap* and *vvp*, respectively, which encode metalloproteases (27, 58). EmpA of *V. anguillarum* is a homologue to the Hap and Vvp metalloproteases (12, 44), and in the present study, we have shown that this organism possesses a LuxR homologue, VanT, which positively regulates *empA* expression. These results suggest that this family of regulatory proteins, thus far only found in *Vibrio* spp. (27, 33, 35, 58, 59), may have very similar regulatory functions.

In a previous study (47), a mutation in *vanM* was shown to abolish production of C6-HSL and 3-hydroxy-C6-HSL as well as 3-oxo-C10-HSL, which is synthesized via VanI (46). This suggests that the VanMN circuit in some way regulates the expression of VanIR. Interestingly, a *vanN* mutant produced wild-type levels of AHLs, suggesting that *V. anguillarum* possesses an alternative mechanism for the regulation of 3-oxo-C10-HSL production, different from the dual sensory channel model of *V. harveyi.* To further support this idea, we show in this study that VanT, the possible regulator of the dual sensory channel model, produces wild-type levels of AHLs. This suggests that the *vanM* mutation may affect the production of 3-oxo-C10-HSL via a sensory circuit different from that used by *V. harveyi*.

To find genes in addition to *empA* that are regulated by VanT, a random approach was taken. A *lacZ* reporter gene fusion screening system was designed to allow the identification of *V. anguillarum* genes activated or repressed by VanT. In this study, three DNA loci were characterized that were positively regulated by VanT. The first *V. anguillarum* DNA locus contained genes encoding homologues of homogentisate 1,2-dioxygenase (HGD) and an HPPD. Both of these enzymes are involved in tyrosine catabolism. L-Tyrosine undergoes transamination to 4-hydroxypyruvate, which is converted to homogentisate by HPPD. HGD then converts homogentisate to maleylacetoacetate, which, after additional enzymatic steps, is finally converted to fumarate and acetoacetate. Both continuous expression of HPPD and inactivation of HGD results in accumulation of homogentisate, which can be oxidized, polymerizing into the red-brown pigment pyomelanin (40).

The homogentistate pathway for the production of melanin-like pigments has been shown for several bacteria (16, 41, 55), and homogentistate is the primary precursor of melanin-like pigments for marine bacteria, including *V. cholerae, Shewanella colwelliana*, and a *Hyphomonas* strain (28). The *V. anguillarum* HPPD enzyme was shown to be highly homologous to that of *V. cholerae* (VC1344) (26) and *S. colwelliana* (23). In addition, the *V. anguillarum* HPPD appears to be positively regulated by VanT and to be responsible for the production of a red-brown pigment in stationary phase when expressed from a plasmid-borne copy of *hpdA*. Moreover, the presence of L-tyrosine enhanced the production of this pigment, as has been shown for *V. cholerae* (55). This suggests that, in *V. anguillarum*, pigment production likely occurs via the homogentistate pathway and that the pigment is likely of the pyomelanin type.

Interestingly, the HPPD enzymes of *V. vulnificus* (11) and of *Legionella pneumophila* (70) have both hemolytic and pigment

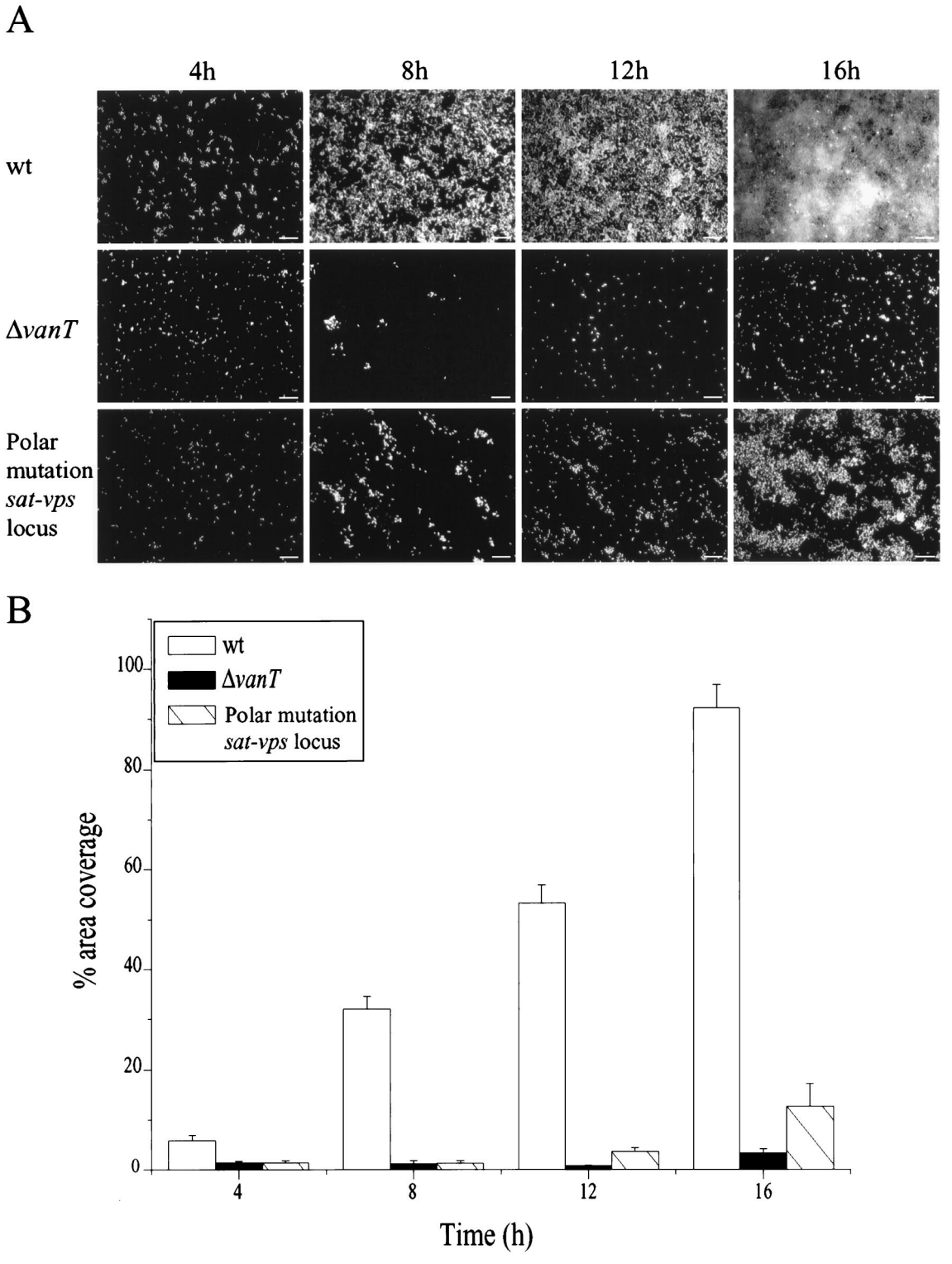

FIG. 6. Biofilm attachment to a glass surface for the wild type (wt), the *vanT* mutant, and the mutant carrying a polar mutation in the *sat-vps73* DNA locus. (A) Typical view of the progression of biofilm formation for each strain at 4 h, 8 h, 12 h, and 16 h. Slides were stained with acridine orange and then viewed with a fluorescent microscope. Bar, 20 μm. (B) Biofilm formation was quantified by determining the percent area coverage on a glass slide. For each strain, five images were taken at each time point and saved as a computer image. These images were converted into black (background) and white (biofilm) pixels using the Image Tool Software, version 2, and the percentage of white pixels is given as the percentage of biofilm covering the glass surface.

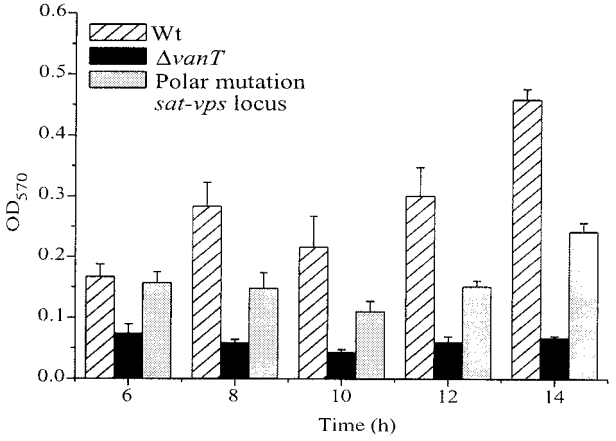

FIG. 7. Quantification of bacteria in biofilms formed on PVC plastic by the wild-type (Wt) NB10, the *vanT* mutant, and the mutant carrying a polar mutation in the *sat-vps73* DNA locus. Biofilms were allowed to form in a 96-well PVC microtiter dish. The microtiter dish was incubated for 6 h, 8 h, 10 h, 12 h, and 14 h at 24°C. The unattached bacteria were removed, and the biofilms were stained with crystal violet. The crystal violet was solubilized, and the absorbance, which correlated to the biofilm mass, was determined.

production activities. However, bacteria defective in this hemolytic activity have not yet been tested for virulence in animal models. Melanin pigments have previously been shown to be free-radical traps (2), and in one bacterial pathogen, *Burkholderia cepacia*, melanin pigment is thought to protect it from the bactericidal effects of the respiratory burst via free-radical-scavenging properties (74). *V. cholerae* has been shown to induce melanin production in response to stressful physiological conditions encountered in the host as well as in the environment. This suggests that melanin production may play a role in survival of this bacterium during stress (14). In *Sinorhizobium meliloti*, the tyrosine catabolism pathway has also been suggested to play a role in the starvation response (41). Whether the *V. anguillarum* HPPD enzyme has hemolytic activity, free-radical-scavenging properties, or stress-related properties is not known, but it is interesting to speculate that the role of melanin could be similar to that in other bacteria.

The second DNA locus regulated by VanT carried genes similar to VCO926 (*vps73*) and VCO923 (serine acetyltransferase) on chromosome I of *V. cholerae* (26). Yildiz and Schoolnik (73) have implicated this region of the *V. cholerae* genome in the production of EPS for biofilm formation. In addition, two transposon insertion sites in the *V. cholerae* genome that lead to a defect in rugose polysaccharide production have been mapped to the *vps* region (3). These similarities suggested that the *sat-vps73* DNA locus in *V. anguillarum* may be involved in biofilm production. In support of this hypothesis, a mutant carrying a polar mutation within the *sat-vps73* locus produced a very reduced biofilm.

These observations prompted us to determine whether VanT was also involved in the formation of biofilms. Under the growth conditions used in this study, a *vanT* isogenic mutant was unable to form a biofilm. Thus, *V. anguillarum* may have a set of *vps*-like genes similar to those of *V. cholerae* that are required for EPS production during biofilm formation. VanT, and possibly quorum sensing, may regulate the expression of these genes. Characterization of the possible *vps*-like locus in *V. anguillarum* is in progress.

Interestingly, the *V. harveyi*-like LuxR transcriptional regulators HapR and OpaR have been suggested to regulate the rugose colony morphology of *V. cholerae* (27) and the opaque colony morphology of *V. parahaemolyticus* (33), respectively. In addition, EPS is necessary for the rugose phenotype, which plays a role in biofilm formation (3, 67, 73), and for the opaque phenotype (20). Furthermore, LuxR from *V. harveyi* has been speculated to be involved in regulation of rugose colony morphology (31), and SmcR, the LuxR homologue of *V. vulnificus*, was shown to negatively regulate biofilm formation, as determined by microtiter plate assays (36). Hence, it is likely that all five characterized LuxR homologues regulate colony morphology or biofilm production by controlling EPS production.

Regulation of EPS production is likely to be a common function for this family of transcriptional regulators among the vibrios. However, these LuxR homologues probably differ in the way in which they regulate EPS production. VanT and OpaR appear to be activators of EPS production, whereas HapR, SmcR, and possibly LuxR negatively regulate either biofilm formation or a rugose phenotype and hence appear to repress EPS production.

The third DNA locus positively regulated by VanT contained the *serA* gene, which, in *E. coli,* catalyzes the first step in biosynthesis of serine, glycine, and one-carbon-unit molecules. Furthermore, the gene for lipoamide dehydrogenase (*lpd*), located downstream of all *luxR* homologues characterized thus far, is part of the glycine cleavage (GCV) enzyme system of *E. coli* (62), which is used in the final step of this biosynthesis pathway. The GCV enzyme complex in *E. coli* is composed of four protein complexes, one of which is lipoamide dehydrogenase. The GCV enzyme complex is suggested to maintain proper levels of glycine and C_1-unit-molecule concentrations by converting excess glycine to C_1-unit molecules. The GCV system is suggested to be highly regulated, as is probably the biosynthesis of serine and glycine. The global regulator, the leucine-responsive regulatory protein, regulates both the *serA* gene and the GCV system. It is likely that other global regulators are also involved in the regulation of this pathway.

Serine, glycine, and C_1-unit-molecule biosynthesis, tyrosine catabolism, and EPS production are all regulated by environmental conditions, which lead to a physiological response by the bacterium. A bacterium's response to its environment can lead to numerous adaptive physiological changes that are tightly controlled by a number of regulatory proteins or signal molecules. Recently, quorum sensing has been suggested to be an integral component of gene regulatory networks that control bacterial responses such as entry into stationary phase, nutrient limitation, and stress response (30, 72). Results presented in this study suggest that the role of quorum sensing in *V. anguillarum* may be that of a global regulator.

For many pathogenic bacteria, the production of proteases and pigments as well as the capacity to form biofilms contributes to virulence. Thus, it is surprising that *vanT* is not essential for the pathogenesis of *V. anguillarum* infections. Several factors are known to contribute to the virulence of *V. anguillarum* (for reviews, see references 1 and 5). The well-characterized siderophore-based iron uptake system carried on the plasmid pJM1 is essential for promoting growth in vivo and therefore

virulence. While lipopolysaccharides contribute to serum resistance, motility is important for entry into the fish host. In addition, although not genetically proven, extracellular products such as hemolysins, lipases, proteases, and a neurotoxic acetylcholinesterase have all been suggested to play a role in the pathology of vibriosis.

Although it is not clear why mutation of *vanT* does not reduce the virulence of *V. anguillarum*, it is possible that VanT may only be required for regulation of metalloprotease, pigment production, and biofilm formation outside of the fish host.

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REFERENCES

1. **Actis, L. A., M. E. Tomalsky, and J. H. Crosa.** 1999. Vibriosis, p. 523–557. *In* P. T. K. Woo and E. W. Bruno (ed.), Fish diseases and disorders, vol. 3: viral, bacterial and fungal infections. Cab International Publishing, Wallingford, United Kingdom.
2. **Agodi, A., S. Stefani, C. Corsaro, F. Campanile, S. Gribaldo, and G. Sichel.** 1996. Study of a melanic pigment of *Proteus mirabilis*. Res. Microbiol. **147:** 167–174.
3. **Ali, A., Z. H. Mahmud, J. G. Morris, Jr., S. Sozhamannan, and J. A. Johnson.** 2000. Sequence analysis of Tn *phoA* insertion sites in *Vibrio cholerae* mutants defective in rugose polysaccharide production. Infect. Immun. **68:**6857–6864.
4. **Alsina, M., J. Mart&ıacute;nez-Picado, J. Jofre, and A. R. Blanch.** 1994. A medium for presumptive identification of *Vibrio anguillarum*. Appl. Environ. Microbiol. **60:**1681–1683.
5. **Austin, B., and D. A. Austin.** 1999. Bacterial fish pathogens: disease of farmed and wild fish, p. 272–275. Springer and Praxis Publishing Ltd., Chichester, United Kingdom..
6. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol. Microbiol. **9:**773–786.
7. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. Mol. Microbiol. **12:**403–412.
8. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273–286.
9. **Cámara, M., M. Daykin, and S. R. Chhabra.** 1998. Detection, purification and synthesis of *N*-acylhomoserine lactone quorum sensing molecules. Methods Microbiol. Bacterial Pathogenesis **27:**319–330.
10. **Cao, J.-G., and E. A. Meighen.** 1989. Purification and structural identification of an autoinducer for the luminescence system of *V. harveyi*. J. Biol. Chem. **264:**21670–21676.
11. **Chang, T. M., Y. C. Chuang, J. H. Su, and M. C. Chang.** 1997. Cloning and sequence analysis of a novel hemolysin gene (*vllY*) from *Vibrio vulnificus*. Appl. Environ. Microbiol. **63:**3851–3857.
12. **Cheng, J. C., C. P. Shao, and L. I. Hor.** 1996. Cloning and nucleotide sequencing of the protease gene of *Vibrio vulnificus*. Gene **183:**255–257.
13. **Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, G. S. A. B., Stewart, P. Williams, and B. W. Bycroft.** 1993. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J. Antibiot. **46:**441–449.
14. **Coyne, V. E., and L. al-Harthi.** 1992. Induction of melanin biosynthesis in *Vibrio cholerae*. Appl. Environ. Microbiol. **58:**2861–2865.
15. **Denkin, S. M., and D. R. Nelson.** 1999. Induction of protease activity in *Vibrio anguillarum* by gastrointestinal mucus. Appl. Environ. Microbiol. **65:** 3555–3560.
16. **Denoya, C. D., D. D. Skinner, and M. R. Morgenstern.** 1994. A *Streptomyces avermitilis* gene encoding a 4-hydroxyphenylpyruvic acid dioxygenase-like protein that directs the production of homogentisic acid and an ochronotic pigment in *Escherichia coli*. J. Bacteriol. **176:**5312–5319.
17. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
18. **Dunny, G. M., and S. C. Winans.** 1999. Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
19. **Eberhard, A., A. L. Burlingame, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444–2449.
20. **Enos-Berlage, J. L., and L. L. McCarter.** 2000. Relation of capsular polysaccharide production and colonial cell organization to colony morphology in *Vibrio parahaemolyticus*. J. Bacteriol. **182:**5513–5520.
21. **Freeman, J. A., and B. L. Bassler.** 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibiro harveyi*. Mol. Microbiol. **31:**665–677.
22. **Freeman, J. A., and B. L. Bassler.** 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. J. Bacteriol. **181:**899–906.
23. **Fuqua, W. C., V. E. Coyne, D. C. Stein, C.-M. Lin, and R. M. Weiner.** 1991. Characterization of *melA*, a gene encoding melanin biosynthesis from the marine bacterium *Shewanella colwelliana*. Gene **109:**131–136.
24. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
25. **Hardman, A. M., G. S. A. B. Stewart, and P. Williams.** 1998. Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and nonpathogenic bacteria. Antonie van Leeuwenhoek J. Microbiol. **74:**199–210.
26. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–484.
27. **Jobling, M. G., and R. K. Holmes.** 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harvey luxR* gene. Mol. Microbiol. **26:**1023–1034.
28. **Kotob, S. I., S. L. Coon, E. J. Quintero, and R. M. Weiner.** 1995. Homogentisic acid is the primary precursor of melanin synthesis in *Vibrio cholerae,* a *Hyphomonas* strain, and *Shewanella colwelliana*. Appl. Environ. Microbiol. **61:**1620–1622.
29. **Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. A. B. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PA01. Mol. Microbiol. **17:**333–343.
30. **Lazazzera, B. A.** 2000. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. **3:**177–182.
31. **Lilly, B. N., and B. L. Bassler.** 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol. Microbiol. **36:**940–954.
32. **Linn, T., and R. St. Pierre.** 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. J. Bacteriol. **172:**1077–1084.
33. **McCarter, L.** 1998. OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. J. Bacteriol. **180:**3166–3173.
34. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition of the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.
35. **McDougald, D., S. A. Rice, and S. Kjelleberg.** 2000. The marine pathogen *Vibrio vulnificus* encodes a putative homologue of the *Vibrio harveyi* regulatory gene, *luxR*: a genetic and phylogenetic comparison. Gene **248:**213–221.
36. **McDougald, D., S. A. Rice, and S. Kjelleberg.** 2001. SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus*. J. Bacteriol. **183:**758–762.
37. **McGee, K., P. Hörstedt, and D. Milton.** 1996. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. J. Bacteriol. **178:**5188–5198.
38. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
39. **Meighen, E. A.** 1994. Genetics of bacterial bioluminescence. Annu. Rev. Genet. **28:**117–139.
40. **Menon, I. A., S. D. Persad, H. F. Haberman, P. K. Basu, J. F. Norfray, C. C. Felix, and B. Kalyanaraman.** 1991. Characterization of the pigment from homogentisic acid and urine and tissue from alkaptonuria patient. Biochem. Cell Biol. **69:**260–273.
41. **Milcamps, A., and F. J. de Bruijn.** 1999. Identification of a novel nutrient-deprivation-induced *Sinorhizobium meliloti* gene (*hmgA*) involved in the degradation of tyrosine. Microbiology **145:**935–947.
42. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

43. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
44. **Milton, D. L., A. Norqvist, and H. Wolf-Watz.** 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. J. Bacteriol. **174:**7235–7244.
45. **Milton, D. L., R. O'Toole, P. Hörstedt, and H. Wolf-Watz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178:**1310–1319.
46. **Milton, D. L., A. Hardman, M. Camara, S. R. Chhabra, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI*/*vanR* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. J. Bacteriol. **179:**3004–3012.
47. **Milton, D. L., V. J. Chalker, D. Kirke, A. Hardman, M. Cámara, and P. Williams.** 2001. The LuxM homologue, VanM, from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone. J. Bacteriol. **183:**3537–3547.
48. **Mittelman, M.** 1999. Recovery and characterization of biofilm bacteria associated with medical devices. Methods Enzymol. **310:**534–551.
49. **Norqvist, A., Å. Hagström, and H. Wolf-Watz.** 1989. Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. Appl. Environ. Microbiol. **55:**1400–1405.
50. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
51. **Pan, W., and B. G. Spratt.** 1994. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. Mol. Microbiol. **11:**769–775.
52. **Ponce, M. R., and J. L. Micol.** 1992. PCR amplification of long DNA fragments. Nucleic Acids Res. **20:**623.
53. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis, and type I pili. Mol. Microbiol. **30:**285–293.
54. **Reed, L. J., and J. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
55. **Ruzafa, C., A. Sanchez-Amat, and F. Solano.** 1995. Characterization of the melanogenic system in *Vibrio cholerae* ATCC 14035. Pigment Cell Res. **8:**147–152.
56. **Salmond, G. P. C., B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Mol. Microbiol. **16:**615–624.
57. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **Shao, C.-P., and L.-I. Hor.** 2001. Regulation of metalloprotease gene expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR homologue. J. Bacteriol. **183:**1369–1375.
59. **Showalter, R. E., M. O. Martin, and M. R. Silverman.** 1990. Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. J. Bacteriol. **172:**2946–2954.
60. **Silhavy, T., M. Berman, and L. Enquist.** 1984. Experiments with gene fusions . Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
61. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**787–796.
62. **Stauffer, G. V.** 1996. Biosynthesis of serine, glycine, and one-carbon units, p. 506–513. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
63. **Stepanovic, S., D. Vukovic, I. Dakic, B. Savic, and M. Svabic-Vlahovic.** 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods **40:**175–179.
64. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Excherichia coli, Salmonella typhimurium,* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA **96:**1639–1644.
65. **von Heijne, G.** 1992. Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:**487–494.
66. **Wai, S. N., Y. Mizunoe, A. Takade, S-I. Kawabata, and S-I Yoshida.** 1998. *Vibrio cholerae* 01 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. Appl. Environ. Microbiol. **64:**3648–3655.
67. **Williams, P., M. Camara, A. Hardman, S. Swift, D. Milton, V. J. Hope, K. Winzer, B. Middleton, D. I. Pritchard, and B. W. Bycroft.** 2000. Quorum sensing and the population dependent control of virulence. Phil. Trans. R. Soc. Lond. Sect. B **355:**667–680.
68. **Windle, H. J. P., and D. Kelleher.** 1997. Identification and characterization of a metalloprotease activity from *Helicobacter pylori*. Infect. Immun. **65:**3132–3137.
69. **Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jorgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. N. B. Stewart.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acylhomoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185–192.
70. **Wintermeyer, E., U. Rdest, B. Ludwig, A. Debes, and J. Hacker.** 1991. Characterization of legiolysin (*lly*), responsible for haemolytic activity, colour production and fluorescence of *Legionella pneumophila*. Mol. Microbiol. **5:**1135–1143.
71. **Winzer, K., and P. Williams.** 2001. Quorum sensing and the regulation of gene expression in pathogenic bacteria. Int. J. Med. Microbiol. **291:**131–143.
72. **Withers, H., S. Swift, and P. Williams.** 2001. Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. Curr. Opin. Microbiol. **4:**186–193.
73. **Yildiz, F. H., and G. K. Schoolnik.** 1999. *Vibrio cholerae* 01 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc. Natl. Acad. Sci. USA **96:**4028–4033.
74. **Zughaier, S. M., H. C. Ryley, and S. K. Jackson.** 1999. A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocyte respiratory burst activity by scavenging superoxide anion. Infect. Immun. **67:**908–913.