2006

Identification and Characterization of a Hemolysin Gene Cluster in *Vibrio anguillarum*

Jessica L. Rock

David R. Nelson
University of Rhode Island

Citation/Publisher Attribution

Rock, J. L., & Nelson, D. R. (2006). Identification and Characterization of a Hemolysin Gene Cluster in *Vibrio anguillarum*. *Infection and Immunity, 74*(5), 2777-2786. doi: 10.1128/IAI.74.5.2777-2786.2006
Available at: http://dx.doi.org/10.1128/IAI.74.5.2777-2786.2006

Identification and Characterization of a Hemolysin Gene Cluster in *Vibrio anguillarum*

INFECTION AND IMMUNITY, May 2006, p. 2777–2786 Vol. 74, No. 5
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.5.2777–2786.2006

Identification and Characterization of a Hemolysin Gene Cluster in *Vibrio anguillarum*

Jessica L. Rock† and David R. Nelson*

Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island 02881

Received 1 July 2005/Returned for modification 29 August 2005/Accepted 6 February 2006

***Vibrio anguillarum* is a causative agent of vibriosis in fish. Hemolytic activity has been suggested as a virulence factor by contributing to hemorrhagic septicemia and diarrhea. In order to identify and characterize the hemolysin genes and examine the role of hemolytic activity in virulence, a mini-Tn*10*Kan mutagenesis clone bank of *V. anguillarum* was screened. While no hemolysin-negative strains were observed, several mutants with two- to threefold-increased hemolytic activity were found. The region containing the insertion mutation was cloned, sequenced, and found to contain the *V. anguillarum* hemolysin (*vah1*) and two other open reading frames, coding for a putative lactonizing lipase (*llpA*) and a putative phospholipase (*plp*). The mini-Tn*10*Kan was inserted into *plp*. Site-directed mutagenesis of each gene revealed that mutations in *vah1* and *llpA* did not affect hemolytic activity, but insertions into *plp* caused a two- to threefold increase in hemolysis. Double mutations in *plp* and either *vah1* or *llpA* resulted in wild-type hemolytic activity. Complementation of *plp* restored hemolytic activity to wild-type levels. Spectrophotometric determination of hemolysin specific activity revealed that activity on a per cell basis peaked during the first 2 h of growth in LB20. Real-time quantitative reverse transcriptase PCR used to quantitate transcription of the hemolysin genes *plp* and *vah1* in *V. anguillarum* wild-type strains M93Sm and NB10 revealed that transcription of *plp* and *vah1* peaked at 2 h of growth in LB20. Additionally, expression of *vah1* measured in the *plp* mutant strain, JL01, during the first 2 h of growth was >8 times higher than that in M93Sm. Mutations in *plp* and *llpA* did not affect virulence of *V. anguillarum*. The mutation in *vah1* attenuated *V. anguillarum* virulence in fish. These data show that several genes are responsible for hemolytic activity in *V. anguillarum*. At least three genes (*plp*, *llpA*, and *vah1*) are responsible for one hemolytic activity. The data also suggest that *plp* acts as a negative regulator of *vah1* and *llpA*.**

Vibrio anguillarum, one of the causative agents of vibriosis in finfish, crustaceans, and bivalves, is a gram-negative, motile marine bacterium (2, 7). Vibriosis is a highly significant disease of cultured and wild marine fish, but outbreaks have also been recorded in freshwater, usually associated with feeding of marine fish (2, 23, 24). Vibriosis causes a systemic disease of fish characterized by hemorrhagic septicemia resulting in high mortalities among infected fish. The main sources of *V. anguillarum* infection are thought to be carrier fish and benthic organisms in the marine environment (2). Vibriosis is often the major limiting factor in the successful rearing of salmonids (12, 19).

Factors demonstrated to contribute to the virulence of *V. anguillarum* include the iron acquisition and transport system (6), the EmpA metalloprotease (4, 18), and several genes affecting chemotaxis and motility (15, 17, 21, 22). Hemolytic activity by *V. anguillarum* cells has also been suggested to be a virulence factor during infection of fish by contributing to hemorrhagic septicemia and diarrhea (11). Hirono et al. (11) cloned and sequenced the putative *V. anguillarum* hemolysin gene *vah1* with over 57% amino acid sequence homology to the *Vibrio cholerae* El Tor hemolysin HlyA. *Escherichia coli* cells containing the cloned *vah1* exhibited hemolytic activity, suggesting that *vah1* contains the gene responsible for hemolytic activity in *V. anguillarum*. Additionally, Hirono et al. (11) demonstrated that 25 of 28 strains of *V. anguillarum* contain DNA sequences that hybridize to a *vah1* probe.

In this study, we sought to further characterize the role and expression of the *V. anguillarum* hemolysin. Mini-transposon (mini-Tn*10*Kan) mutagenesis was used to create and screen for hemolysin mutants. Only mutants that exhibited a two- to threefold increase in hemolytic activity above wild-type hemolytic activity were detected. The region surrounding this mutation in *V. anguillarum* was cloned and sequenced. Three separate open reading frames (ORFs) were identified, and each was mutated by site-directed mutagenesis. Each mutant strain was tested for virulence in juvenile Atlantic salmon and compared to the parental wild-type, M93Sm. Hemolysin assays and real-time quantitative reverse transcription-PCR (qRT-PCR) were performed to determine changes in hemolytic activity and expression of hemolysin genes in the wild-type strains M93Sm and NB10, as well as in the original mini-Tn*10*Kan mutant strain, JL01.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. All *V. anguillarum* strains were routinely grown in Luria-Bertani broth (LB) plus 2% NaCl (LB20) (9), supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Other growth media included LB20, nine salts solution (NSS; a carbon-, nitrogen-, and phosphorus-free salt solution) (9, 14), marine minimal medium (3M) (9, 19), and NSS plus salmon gastrointestinal mucus (200 μg protein ml^{-1}) (NSSM) (9). Cell densities were determined by serial dilution and plating on LB20 agar plates or by measuring the optical density at 600 nm (OD_{600}). Antibiotics were used at the following concentrations: kanamycin, 85 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1};

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, 117 Morrill Science Building, University of Rhode Island, Kingston, RI 02881. Phone: (401) 874-5902. Fax: (401) 874-2202. E-mail: dnelson@uri.edu.

† Present address: Cubist Pharmaceuticals, 65 Hayden Ave., Lexington, MA 02421.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
V. anguillarum		
M93Sm	Spontaneous Sm^r mutant of M93 (serotype J-O-1)	Denkin and Nelson (5)
NB10	Wild type (serotype O1)	Milton et al. (18)
JL01	Sm^r Km^r *plp* mutant; mini-Tn*10*Km, insertion into *plp*	This study
JR1	Sm^r Cm^r *vah1* mutant; pNQ-*vah1* insertion into *vah1*	This study
JR2	Sm^r Cm^r *plp* mutant; pNQ-*plp* insertion into *plp*	This study
SC1	Sm^r Cm^r *llpA* mutant; pNQ-*llpA* insertion into *llpA*	This study
JR3	Sm^r Km^r Cm^r *plp* mutant; mini-Tn*10*Km insertion into *plp vah1* mutant; pNQ-*vah1* insertion into *vah1*	This study
JR8	JL01 complemented with pSUP202-*plp*	This study
JR10	Sm^r Km^r Cm^r *plp* mutant; mini-Tn*10*Km *llpA* mutant; pNQ-*llpA* insertion into *llpA*	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
XL1MRF′	*recA1 endA1 gryA96 thi-1 hsdR17 supE44 relA1* (*lac-pro*) [F′ *proABlacI lacZ* M15 Tn*10* (Tet^r)]	Stratagene
SM10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ*pir*)	Milton et al. (18)
CC118	λ*pir* pLOFKm	Herrero (10)
Plasmids		
pBluescript SKII$^+$	Ap^r *lacZ*; pUC ORI	Stratagene
pCR2.1	Kn^r Ap^r; pUC origin, LacZα	Invitrogen
pNQ-705.1	Suicide vector, requires *pir* Cm^r	Milton et al. (18)
pSUP202	Tc^r Ap^r Cm^r	Milton et al. (18)
pSUP202-*plp*	*plp* inserted into Cm^r cassette of pSUP202	This study
pNQ-705 *plp*	pNQ705 + *plp* fragment in SacI/XbaI site	This study
pNQ-705 *vah1*	pNQ705 + *vah1* fragment in SacI/XbaI site	This study
pNQ-705 *llpA*	pNQ705 + *llpA* fragment in SacI/XbaI site	This study
pCR2.1-*plp*	pCR2.1 + *plp* (amplified with plpCF/plpCR)	This study

and streptomycin, 200 μg ml^{-1}. All *E. coli* strains were routinely grown in LB supplemented with the appropriate antibiotic(s) in a shaking water bath at 37°C.

Mini-Tn*10*Kan mutagenesis. Mini-Tn*10*Kan mutagenesis was carried out using a modification of the method developed by Herrero et al. (10). Briefly, *V. anguillarum* was mated with *E. coli* CC118(λ*pir*)(pLOFKm) containing the mini-Tn*10*Kan. Aliquots (100 μl) from overnight cultures of each organism were mixed in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$. The cells were vacuum filtered onto a 0.45-μm filter and placed cell side up on LB15 agar plates (Luria-Bertani agar plus 1.5% NaCl) and incubated for 16 h at 27°C. After incubation, the filter was removed from the plate and suspended in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$. The suspension was vortexed vigorously to remove bacteria from the filter, and aliquots (100 μl) of the cell suspension were spread plated onto LB20 Sm^{200} Kan^{85} (200 μg ml^{-1} streptomycin and 85 μg ml^{-1} kanamycin) plates to select for *V. anguillarum* mutants containing a mini-Tn*10*Kan insertion (5, 10). *V. anguillarum* colonies able to grow on LB20 Sm^{200} Kan^{85} were transferred onto blood agar plates, and hemolytic activity was determined by measuring β-hemolysis after 24 h at 27°C.

Selection for hemolysin mutants and hemolysin assays. *V. anguillarum* colonies resulting from either mutagenesis procedure (mini-Tn*10*Kan or site directed) were transferred onto blood agar plates, and hemolytic activity was determined by measuring β-hemolysis after 24 h at 27°C. The level of hemolytic activity was also quantitated using a modification of the method described by Hirono et al. (11). The assay was done in 96-well microtiter plates. Twofold dilutions of 500 μl cell supernatant from *V. anguillarum* strains were added to 1 ml 5% sheep erythrocytes in 10 mM Tris-Cl (pH 7.5)–0.9% NaCl buffer and incubated at 27°C for 24 h, and the optical density of the sample was measured at 428 nm using an Ultraspec 4000 spectrophotometer (Pharmacia) and compared to a negative control consisting of buffer plus sheep erythrocytes. A third method to quantitate hemolytic activity of mutants was done in microcentrifuge tubes. The tubes contained 1 ml 5% sheep erythrocytes and twofold dilutions of cell supernatant taken at various time points of growth (0, 1, 2, 4, 6, 12, 24, and 48 h) and added to 10 mM Tris-Cl (pH 7.5)–0.9% NaCl buffer and incubated for 24 h. The samples were centrifuged at 1,000 × *g* for 2 min, and the optical density of the resulting supernatant was read at 428 nm. Hemolysis units were calculated as OD_{428}/dilution. In most cases, a 2^{-1} dilution was used. The hemolytic specific activities were calculated as (hemolysis units/CFU ml^{-1}) × 10^9.

DNA isolation. Genomic DNA was isolated from *V. anguillarum* strains using the QIAGEN DNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The isolated DNA was further purified and concentrated by ethanol precipitation (3). The purified genomic DNA was quantitated spectrophotometrically by measuring absorption at 260 nm and 280 nm using an Ultraspec 4000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ).

Southern analysis. Total genomic DNA was extracted from *V. anguillarum* M93Sm, JL01, and JL03 using a DNeasy tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA from each bacterial strain (4 μg) was digested to completion with SacI and XbaI (Promega, Madison, WI) according to the instructions of the manufacturer, and the fragments were separated by agarose gel electrophoresis (0.8% agarose gel, 100 V) in Tris-acetate-EDTA buffer (3). The samples were prepared for Southern analysis as described by Ausubel et al. (3), with a modification to the transfer process to allow the usage of the Turboblotter Rapid Downward Transfer system (Schleicher & Schuell Inc., Keene, NH). The DNA was transferred onto a nylon membrane using the Turboblotter system according to the instructions of the manufacturer. The transfer was allowed to run for 3 h. The DNA was bound to the membrane by cross-linking using a UV cross-linker (FB-UVXC-1000; Fisher Scientific). Blots were probed with a digoxigenin (DIG)-dUTP-labeled probe (Boehringer Mannheim) for the mini-Tn*10*Kan. The kanamycin resistance gene probe was created by PCR amplification of a 200-bp region of the kanamycin resistance gene using primers KanF and KanR (Table 2) and the Boehringer Mannheim DIG-PCR probe synthesis kit (DIG labeling kit; Roche). Blots were hybridized at 51°C for 16 h in a Roller Blot Hybridizer HB-30 (Techne, Cambridge, England).

Detection of the hybridized fragments was carried out using the kanamycin resistance gene probe with the enhanced chemiluminescence kit (DIG High

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Target
KanD-S1	GTTTCATTTGATGCTCGATGAG	Km^r gene
KanD-S2	GATGTTGGACGAGTCGGAATCG	Km^r gene
KanD-S3	GCGTACCTTTGCCATGTTTCAG	Km^r gene
KanD-S4	CGAGCAAGACGTTTCCCGTTG	Km^r gene
MT3	GCGCAATTAACCCTCACTAAAGGG	pBluescript
MT7	GCGTAATACGACTCACTATAGGGC	pBluescript
SD vah1-R	GCTAG**TCTAGAT**TTGCGCGTTATTAG	*vah1*
SD vah1-F	GCTAG**GAGCTC**TACGCGAGTGTTTTG	*vah1*
pNQ705-R	TTTGCGTAACGGCAAAAGCACCGC	pNQ705
SD Lip/Heme R1	GCTAG**TCTAGA**ACGGATACCACCTCAGA	*plp*
SD Lip/Heme F1	GCTAG**GAGCTC**AGTGTCTCTTCACACC	*plp*
SD Lip/Heme F2	GCTAG**GAGCTC**TATTCTGACCTTGCCAT	*plp*
SD Lip/Heme R2	GCTAG**TCTAGA**CGCTGATGAATCCCCTA	*plp*
SD Lip/Heme F3	GCTAG**GAGCTC**AATCTGTTGCTGGGT	*plp*
SD LacLip F	GCTAG**GAGCTCT**CTAAGTGGTTAC	*llpA*
SD LacLip R	GCTAG**TCTAGA**GGGCACATTAAAGAGGG	*llpA*
RT llp-F	GCCAAGCCCGTTGAATTTCATC	*llpA*
RT llp-R	GCTGGCCGGAGTCGATTATTTCT	*llpA*
RT vah1-R1	GACCGCCGAATCGATGATGAATC	*vah1*
RT vah1-R2	CGCTATTGCCATTATGTCAGG	*vah1*
RT plp-R1	GAGAACCTATTGTCTGCTCGAA	*plp*
RT plp-R2	GAGGGTATTTTCTGGCTGGTAG	*plp*
llpF RT	GGTGGCGTTAAGTAAGACAGGCTA	*llpA*
llpR RT	TAGAAATAATCGACTCCGGCCAGC	*llpA*
plpF RT	CAGACGACCACCAGTAACCACTAA	*plp*
vah1F RT	TAGATGATGATACAACGGGTGCGG	*vah1*
vah1R RT	GTCGACCAGTCTCGGAAATAAGCA	*vah1*
plpR RT	GCAATCATGATGACCCAGCAACAG	*plp*
vah1 CR	GCTAGCTGCAGTCGCATAGTTTTGGT	*vah1*
vah1 CF	GTCAGCTGCAGATAAGCGGTAACTGGTT	*vah1*
plp CF	GCTAGCTGCAGTTTCAGGTGCGTAT	*plp*
plp CR	GCTAGCTGCAGTTTGGAACGCCGACT	*plp*
llp iPCR	GCAGTACTCACTTGGGTAAGGTGATC	*llpA*
plp iPCR	GGTGACAGCCTTTCGGATACAGGAA	*plp*
F vah1 RT (AFT)	ACCGTTACTTCCGGTGAGTTCAAG	*vah1*
R vah1 RT (AFT)	CATCGTGGGTACTGATTGCGTAGT	*vah1*

[a] Restriction sites within primers are in boldface.

Prime DNA labeling and detection starter kit; Boehringer, Mannheim, Germany).

Cloning of the mini-Tn*10*Kan insertion mutation. The region surrounding the gene interrupted in the mini-Tn*10*Kan mutagenesis was cloned into pBluescript SKII$^+$. Briefly, genomic DNA from *V. anguillarum* JL01 was digested with SacI, treated with calf intestinal alkaline phosphatase (Promega, Madison, WI), and then ligated using T4 DNA ligase (Promega, Madison, WI) into the SacI-digested site of pBluescript SKII$^+$. The ligated DNA was used to transform *E. coli* XL1MRF′ by electroporation using a Bio-Rad electroporation system. Transformants were selected on LB agar plates supplemented with kanamycin and ampicillin (13). Plasmid DNA was purified from the clone using a QIAGEN Mini-Prep kit (QIAGEN). The plasmids (pJL01.1 to -1.7) were checked for the presence of inserted *V. anguillarum* DNA containing mini-Tn*10*kan by restriction digestion and agarose gel electrophoresis. Clones of interest were saved for future study.

PCR amplification. All PCRs were done using *Taq* DNA polymerase (QIAGEN) under the following conditions: 93°C for 3 min, 93°C for 30 s, 58 to 60°C for 1 min, and 68°C for 30 s, repeated for 40 cycles with a final extension at 68°C for 7 to 10 min. All PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The modifications to the PCR cycle were dependent on the melting temperature of the primers used and the length of the desired amplicon.

All real-time qRT-PCRs were carried out using Mx4000 Multiplex QPCR system (Stratagene, La Jolla, CA). Total RNA from the wild-type strain M93Sm and all mutant strains created in this study were isolated using an RNeasy tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The RNA was treated with DNase, quantitated, and then diluted to 10 ng RNA in 15 μl of reaction buffer. The RNA was added to Brilliant SYBR Green qRT-PCR Master Mix kit (Stratagene, La Jolla, CA). The following temperatures and times for the qRT-PCR: 1 cycle for 30 min at 50°C, 1 cycle for 10 min at 95°C, and 40 cycles of PCR with activation at 95°C for 15 min, denaturation at 95°C for 30 s, annealing at 58°C, and extension for 1 min at 72°C. Calculation of transcript number was per 10 ng of total *V. anguillarum* DNA. Each time point presented is the average of duplicate samples. Each experiment was repeated at least twice.

Construction of pNQ705-*vah1*, pNQ705-*plp*, and pNQ705-*llpA*. Total genomic DNA from M93Sm was isolated using a DNeasy tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Restriction sites (SacI and XbaI) were added to the PCR primer set that amplifies a 200- to 400-base sequence in the center of the gene of interest. Briefly, primers from the *vah1* sequence (*vah1*F and *vah1*R) (Table 2) were used to amplify a 400-bp fragment of *vah1* from *V. anguillarum* M93Sm genomic DNA located starting at 400 bp from the 5′ terminus of the *vah1* gene. Primers for *plp* (*plp*F and *plp*R) (Table 2) were used to amplify a 250-bp fragment from *V. anguillarum* genomic DNA located 400 bp from the 5′ terminus of the *plp* gene. Primers for *llpA* (*llp*F and *llp*R) (Table 2) were used to amplify a 250-bp fragment of *llpA* from *V. anguillarum* genomic DNA located 400 bp from the 5′ terminus of the *llpA* gene. The amplified PCR product for each gene of interest (*vah1*, *plp*, and *llpA*) was digested with the restriction enzymes SacI and XbaI (Promega, Madison, WI) and ligated using T4 DNA ligase (Promega, Madison, WI) into the suicide vector pNQ705, previously digested with SacI and XbaI to yield pNQ705-*vah1*, pNQ705-*plp*, or pNQ705-*llpA*, respectively. The resulting plasmids were then introduced into *E. coli* SM10 by electroporation transformation using a Gene Pulser (Bio-Rad, Richmond, CA). Transformants were incubated for 1 h at 37°C in a shaking water bath and plated onto LB agar plates containing 20 μg/μl chloramphenicol (Cm^{20}). Plasmid DNA was harvested from overnight *E. coli* cultures using QIAGEN Miniprep Spin kit according to the manufacturer's instructions. To confirm that the insert was successfully ligated into pNQ705, the purified plasmid DNA was digested with SacI and XbaI and the resulting DNA fragments were separated by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA buffer run at 80 V for 1.5 h (3).

Site-directed mutagenesis. Site-directed mutagenesis was carried out using a modification of the procedure described by Milton and Wolf-Watz (18). Briefly, overnight cultures of *V. anguillarum* M93Sm and *E. coli* SM10 containing the pNQ705-based plasmids (pNQ705-*vah1*, -*plp*, and -*llpA*) were prepared and mixed at recipient/donor ratios of 1:1 or 3:1 in NSS plus 10 mM $MgSO_4$. The cell suspension was vacuum filtered onto a 0.22-μm-pore nylon membrane, which was placed on an LB15 agar plate and allowed to incubate overnight at 27°C. Following incubation, the cells were removed from the filter by vigorous mixing in NSS plus 10 mM $MgSO_4$. The cell suspension (100 μl) was plated on LB20 Sm^{200} Cm^5 and allowed to incubate at 27°C until *V. anguillarum* colonies were observed (16 to 24 h).

Each site-directed mutation was confirmed by PCR amplification of the novel junction between pNQ705 and the cloned gene fragment for each gene of interest (*plp*, *vah1*, and *llpA*) using primer sets containing pNQ705-R and *plp*F, *vah1*F, or *llp*F, respectively.

Complementation of mutants. The cloned *V. anguillarum plp* gene was tested for its ability to complement JL01. The mutant was complemented by cloning the wild-type gene into the shuttle vector pSUP202 (accession no. AY428809). Briefly, total genomic DNA from M93Sm was isolated using a DNeasy tissue kit (QIAGEN, Valencia, CA). Restriction sites (PstI) were added to the PCR primer set that amplifies the gene of interest plus flanking DNA that may include promoter regions. The amplified PCR product was digested with PstI (Promega, Madison, WI). The fragment was ligated into pSUP202 (also digested with PstI) using T4 DNA ligase (Promega, Madison, WI) to yield pSUP202-*plp*. Briefly, primers amplifying the entire *plp* gene plus 250 bases flanking both the 5′ and 3′ends were amplified using primers plpCF and plpCR (Table 2), resulting in a 2-kbp amplicon. The 2-kbp amplicon was ligated into pCR2.1 using the TOPO TA cloning system, resulting in the plasmid pCR2.1-*plp*. Insertion of *plp* into pCR2.1 was confirmed using PCR (primers plpCF and plpCR) and restriction analysis with EcoRI. pCR2.1-*plp* was digested with EcoRI, and the *plp*-containing fragment was ligated into the unique EcoRI site of the shuttle vector pSUP202, resulting in the plasmid pSUP202-*plp*. Insertion was confirmed by PCR amplification (using primers plpCF and plpCR) and restriction analysis with EcoRI. pSUP202-*plp* was introduced into *E. coli* DH5α by electroporation and then transferred into *V. anguillarum* JL01 by conjugation to complement the *plp* mutation.

Aliquots (100 μl) from overnight cultures of *E. coli* DH5α (pSUP202-*plp*) and JL01 were mixed in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$. The cells were vacuum filtered onto a 0.45-μm-pore filter, placed cell side up on LB15 agar plates (Luria-Bertani agar plus 1.5% NaCl), and incubated for 16 h at 27°C. After incubation, the filter was removed from the plate and suspended in 2.5 ml NSS plus 2.5 ml 10 mM $MgSO_4$. The suspension was vortexed vigorously to remove bacteria from the filter, and aliquots (100 μl) of the cell suspension were spread plated onto LB20 Sm^{200} Kan^{85} Ap^{200} (200 μg ml^{-1} streptomycin, 85 μg ml^{-1}

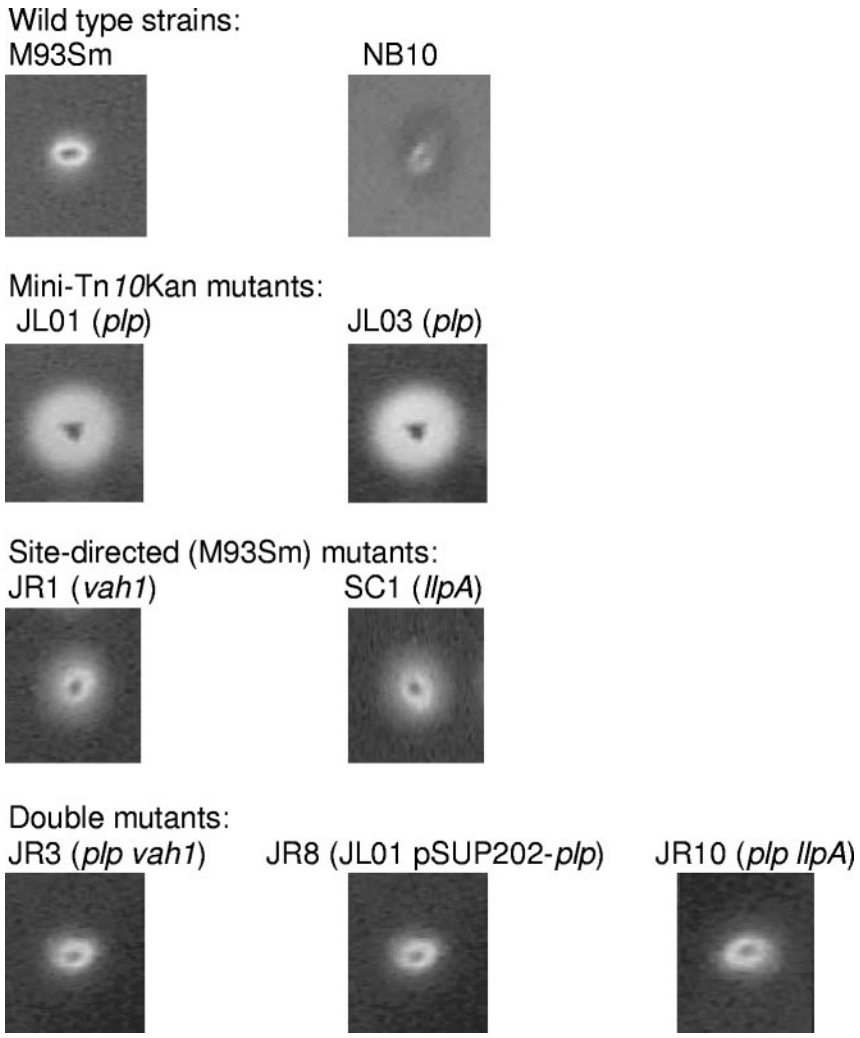

FIG. 1. Hemolytic activity of *V. anguillarum* wild-type strains (M93Sm and NB10) and mutant strains (JL01, JL03, JR1, SC1, JR3, JR8, and JR10) on TSA plus sheep blood agar. Colonies were transferred onto a TSA-sheep blood agar plate and incubated at 27°C for 18 h.

kanamycin, and 200 μg ml^{-1} ampicillin) plates to select for *V. anguillarum* mutants containing pSUP202-*plp* (5).

RNA isolation. Exponential-phase cells (2×10^6 to 3×10^6 CFU ml^{-1} and 2×10^7 to 3×10^7 CFU ml^{-1}) and stationary-phase cells (2×10^9 to 3×10^9 CFU ml^{-1}) of various *V. anguillarum* strains were harvested by centrifugation (11,000 × *g*, 10 min at 4°C). Total RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions after 0, 2, 4, and 24 h of growth in LB20. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm using an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ).

DNA sequencing. All DNA sequencing was done at the URI Genomics and Sequencing Center (University of Rhode Island, Kingston, RI) using a CEQ8000 genetic analysis system (Beckman Coulter). A Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) was used in the sequencing reactions. Homology searches and alignments were performed with the Basic Local Alignment Search Tool (BLAST) Network Service at the National Center for Biotechnology Information (www.ncbi.nih.gov), National Institutes of Health, Bethesda, MD (1).

Fish infections. All hemolysin mutants were tested for virulence in juvenile Atlantic salmon (*Salmo salar* L.) (observed to be free from any clinical signs of infection or injury) by intraperitoneal (i.p.) and anal intubations (AIB) as described previously by Denkin and Nelson (4). Briefly, *V. anguillarum* cells grown in LB20, supplemented with appropriate antibiotics, in a shaking water bath for 18 h at 27°C were harvested by centrifugation (9,000 × *g*, 10 min, 4°C), washed twice in NSS, and suspended in NSS to ~2×10^9 CFU ml^{-1}. Fifteen fish (20 to 30 g) were used to test the virulence of each bacterial strain used in each study. Fifteen fish were sham inoculated with NSS as a negative control. To prevent possible cross-contamination, fish inoculated with different bacterial strains (and the NSS negative control) were maintained in separate tanks. Five fish were inoculated per dose, and three different doses per strain were used. Fish were inoculated i.p. or AIB with equal volumes (100 μl) of cells (ranging from ~10^5 to 10^7 CFU ml^{-1}) in NSS or NSS alone (control fish). The fish were anesthetized in water supplemented with tricaine methane sulfonate (100 mg ml^{-1}), prior to inoculation and allowed to recover before returning to the tank. Death due to vibriosis was determined by the observation of gross clinical signs and confirmed by the recovery and isolation of *V. anguillarum* cells that were resistant to the appropriate antibiotics from infected organs of dead fish. Observations for clinical signs of vibriosis continued for 21 days. All fish used in this research project were obtained from the URI East Farm Aquaculture Center.

RESULTS

Mini-Tn*10* mutagenesis. Mini-Tn*10* mutagenesis (10) was used to create mutants of *V. anguillarum* M93Sm that exhibited altered hemolytic activity. Over 5,000 mini-Tn*10*Kan-containing colonies created by three rounds of mutagenesis were screened for altered hemolytic activity on tryptic soy agar (TSA)-blood agar plates by measuring the zones of hemolysis around the colonies after 24 h at 27°C. Two clones (JL01 and

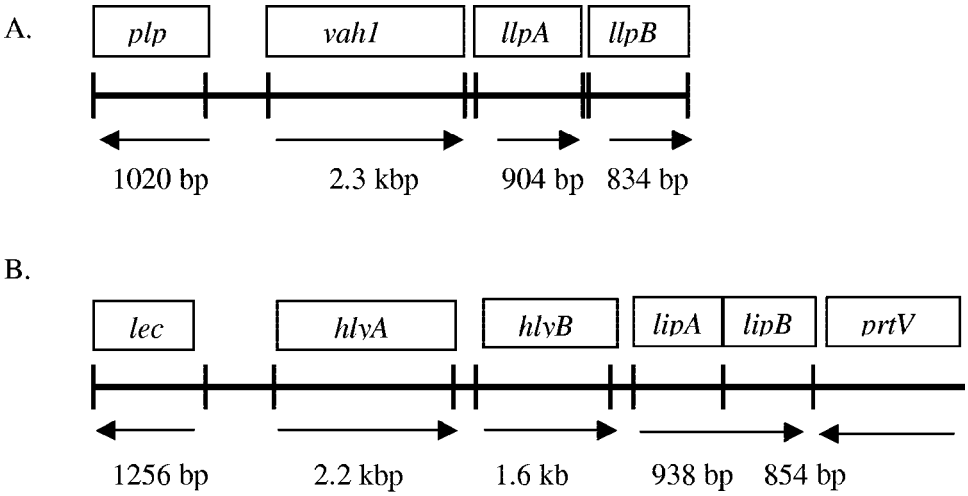

FIG. 2. Map of hemolysin gene region from (A) *V. anguillarum* and (B) *V. cholerae*. For the hemolysin region of *V. anguillarum*, genes coding for the following products are represented: phospholipase/lecithinase (*plp*), hemolysin (*vah1*), lactonizing lipase (*llpA*), and lactonizing lipase activator (*llpB*). For the hemolysin region in *V. cholerae*, genes coding for the following products are represented: lecithinase (*lec*), hemolysin (*hlyA*), chemotaxis transducer (*hlyB*), lipase accessory (*lipBC*), and putative metalloprotease (*prtV*). Arrows indicate the direction of transcription. Figure sizes are approximate.

JL03) that exhibited two- to threefold-higher hemolysin activity compared to the wild-type strain M93Sm were identified (Fig. 1). No hemolysin-negative mutants were observed during the mini-Tn*10*Kan mutagenesis procedure and screening.

Southern blot analysis. Southern hybridization analysis of *V. anguillarum* JL01 and JL03 genomic DNA digested with both SacI and XbaI and probed with a DIG-labeled Kan^r DNA probe was carried out to compare the approximate sizes of the DNA fragments from each mutant containing the mini-Tn*10*Kan insertions. The SacI-XbaI double digestions resulted in a single hybridizable band at 11 kbp for both strains (data not shown). These results, confirmed by DNA restriction mapping and sequence analysis, demonstrated that JL01 and JL03 contain the mini-Tn*10*Kan insertion in the same location; therefore, *V. anguillarum* JL01 was chosen for further analysis and study.

Cloning and identification of *V. anguillarum* putative hemolysin genes, *vah1*, *plp*, and *llpA*. In order to identify and characterize the gene interrupted by the mini-Tn*10*Kan insertion, the region surrounding the mini-Tn*10*Kan insertion was cloned into the SacI site of pBluescript SKII$^+$. The resulting plasmid was designated pJL01.3. Restriction digestion of pJL01.3 using SacI yielded a 17-kbp insertion.

Forward and reverse primers from the mini-Tn*10*Kan (KanDS1 and KanDS4), and modified T7 and T3 pBluescript-specific primers (Table 2) were used to initiate sequencing of pJL01.3. The sequence of the inserted DNA was determined by primer walking. DNA sequence analysis by BLASTn and BLASTx (1) resulted in the identification of the previously described *V. anguillarum* hemolysin gene (*vah1*) (11), a putative phospholipase/lecithinase gene (*plp*) containing the mini-Tn*10*Kan insertion, a putative lactonizing lipase gene (*llpA*), as well as several other previously unidentified genes in *V. anguillarum* M93Sm, including those coding for trehalose-6-phosphate hydrolase, response regulator, hypothetical protein, and transcriptional regulator (Fig. 2). Additionally, BLASTx analysis of the cloned region revealed that both the amino acid sequences of the individual ORFs and the gene order are highly conserved in *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio mimicus* (Table 3 and Fig. 2). The *plp* amino acid sequence was found to have 69% identity and 84% similarity to a lecithinase of *V. mimicus*. The *vah1* amino acid sequence exhibited ≥ 96% identity to the Vah1 sequence previously reported by Hirono et al. (11). Additionally, the predicted amino acid sequence of *llpA* was 87% identical and 94% similar to the lactonizing lipase (VCA0221) sequence found in *V. cholerae*.

Identification of putative hemolysin genes in *V. anguillarum* NB10. Since the wild-type strain NB10 exhibited very weak hemolytic activity (Fig. 1), we sought to determine whether this strain had the same complement of hemolysin-like genes as the other wild-type strain, M93Sm. Putative hemolysin genes in *V. anguillarum* NB10 were identified using both PCR analysis and Southern blot analysis. Primer sets for *vah1* (RT *vah1*F/RT *vah1*R), *plp* (RT *plp*F/RT *plp*R), and *llpA* (RT *llp*F/RT *llp*R) (Table 2) were used to amplify fragments of these genes from both M93sm and NB10 DNA (Fig. 3). Fragments of identical sizes were amplified for *vah1*, *plp*, and *llpA* from both M93Sm and NB10. Additionally, Southern blot analysis of BamHI-HindIII double digests of genomic DNA from M93Sm and NB10 probed with DIG-labeled *vah1*, *plp*, and *llpA* probes revealed bands of identical sizes from each strain (Fig. 3). These results strongly suggest that the same genes are present in both strains.

Growth of *V. anguillarum vah1*, *plp*, and *llpA* mutant strains. Growth experiments were performed to determine whether the mutations created in *vah1*, *plp*, and *llpA* affect the growth rate of the *V. anguillarum* strains grown in LB20, NSSM, and 3M. All mutant strains grew at about the same rate and to the same cell density as the wild-type strain, M93Sm (see Fig. 4 for growth in LB20). As expected, the highest cell densities and fastest growth rates were observed in LB20-grown cells (3×10^9 to 4×10^9 CFU ml^{-1}; 32 to 37 min per generation); slightly reduced cell densities and growth rates were seen in NSSM-

TABLE 3. Hemolysin gene sequence similarity compared to other *Vibrio* species

ORF	Size (bp)	Predicted protein size (aa)[a]	Accession no.	Homology to predicted encoded protein	% Amino acid: Identity	% Amino acid: Similarity	Homologue accession no.
plp	918	305	DQ008059	*V. mimicus* lecithinase	69	84	AAC63951
				V. vulnificus CMCP6 phospholipase/lecithinase/hemolysin	69	80	NP_763362
vah1	2,349	782	DQ008059	*L.* (*Vibrio*) *anguillarum* Vah1	>96	>96	AAB50894
				V. cholerae hemolysin HlyA	56	72	AAA27528
llpA	885	294	DQ008059	*V. cholerae* lactonizing lipase	87	94	NP_232620
				V. vulnificus CKM-1 lipase	85	89	AAQ04476
llpB	834	278	DQ008059	*V. cholerae* O1 biovar El Tor N16961 lipase activator (foldase) protein	42	62	AE004362
				V. parahaemolyticus RIMD 2210633 lipase activator protein	43	57	NP_797559

[a] aa, amino acids.

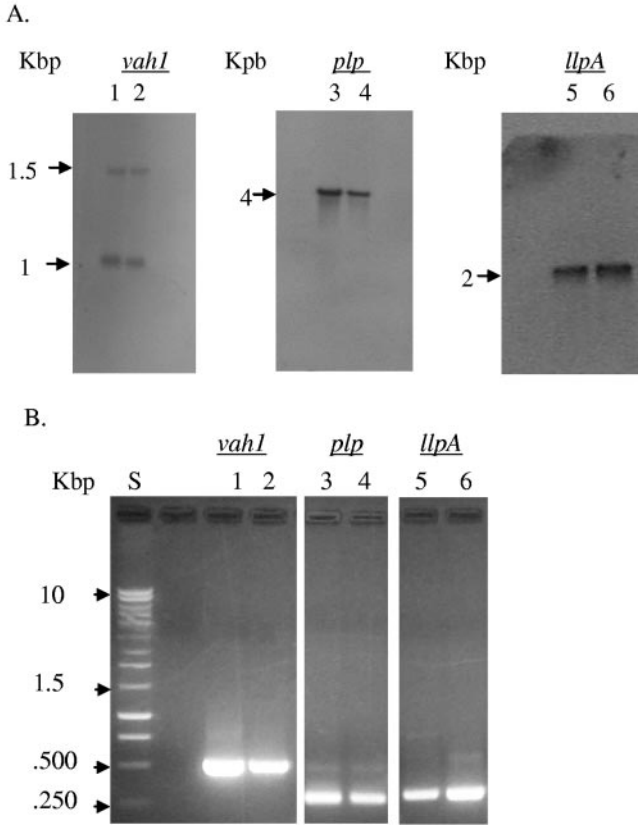

FIG. 3. Detection of hemolysin genes in *V. anguillarum* strains M93Sm and NB10 by Southern blot analysis and PCR amplification. (A) Southern blot analysis of hemolysin genes *vah1* (lanes 1 and 2), *plp* (lanes 3 and 4), and *llpA* (lanes 5 and 6) in *V. anguillarum* strains M93Sm (lanes 1, 3, and 5) and NB10 (lanes 2, 4, and 6). Genomic DNA digested with BamHI and HindIII was separated on a 1% agarose gel and transferred to a nylon membrane, and the blot was probed with a PCR-amplified digoxigenin-labeled probe specific for each gene. (B) PCR amplification of *vah1* (lanes 1 and 2), *plp* (lanes 3 and 4), and *llpA* (lanes 5 and 6) from *V. anguillarum* strains M93 (lanes 1, 3, and 5) and NB10 (lanes 2, 4, and 6) using gene-specific primers (Table 2).

grown cells (2.4×10^9 to 3.2×10^9 CFU ml^{-1}; 40 to 46 min per generation); and the lowest cell densities and growth rates were found in 3M-grown cells (5.3×10^8 to 7.3×10^8 CFU ml^{-1}; 43 to 55 min per generation). These experiments showed that the mutations in the genes (*vah1*, *plp*, and *llpA*) examined in this study did not affect growth in 3M, NSSM, or LB20.

Hemolytic activity of putative hemolysin mutants. Hemolytic activities of the wild type and the putative hemolysin mutants were determined by two methods: (i) diameter of hemolytic zones on blood agar plates and (ii) spectrophotometric determination of erythrocyte lysis (11). Strains JL01 and JR2 with insertions in *plp* had two- to threefold-larger zones of β-hemolysis than M93Sm, as well as two- to threefold-greater hemolytic values than M93Sm by spectrophotometric determinations at 480 nm (Fig. 1 and 4). Strains JR1 and SC1, with mutations in *vah1* and *llpA*, respectively, each exhibited hemolytic activity identical to that of the wild-type strain M93Sm (Fig. 1 and 4). Further, when a *plp* mutant strain acquired a second mutation in either *vah1* (strain JR3) or *llpA* (strain JR10), the zones of hemolysis declined from two- to threefold above that of the wild-type strain M93Sm to wild-type levels (Fig. 1). The second wild-type strain, NB10, exhibited only about one-half of the beta-hemolytic activity of M93Sm (Fig. 1).

In order to determine whether the amounts of hemolytic activity for each strain (Fig. 4) were proportional to the cell density, the level of hemolytic activity for each strain was normalized to CFU. When the hemolytic specific activity was plotted against time of growth in LB20, it was found that activity peaked at about 2 h (Fig. 5). These data suggested that the expression of the hemolysin genes occurs during the initiation of exponential growth.

Complementation of the *plp* mutant JL01. In order to determine whether the mutation in *plp* was directly responsible for increased hemolytic activity, the wild-type *plp* gene was cloned into pSUP202 and the resulting plasmid (pSUP202-*plp*) was introduced into JL01 by conjugation. The complemented strain, JR8, grew at approximately the same rate and to the same cell density as the wild-type strain M93Sm. Hemolytic activity was restored to levels observed in the M93Sm wild-type strain (Fig. 1 and 4).

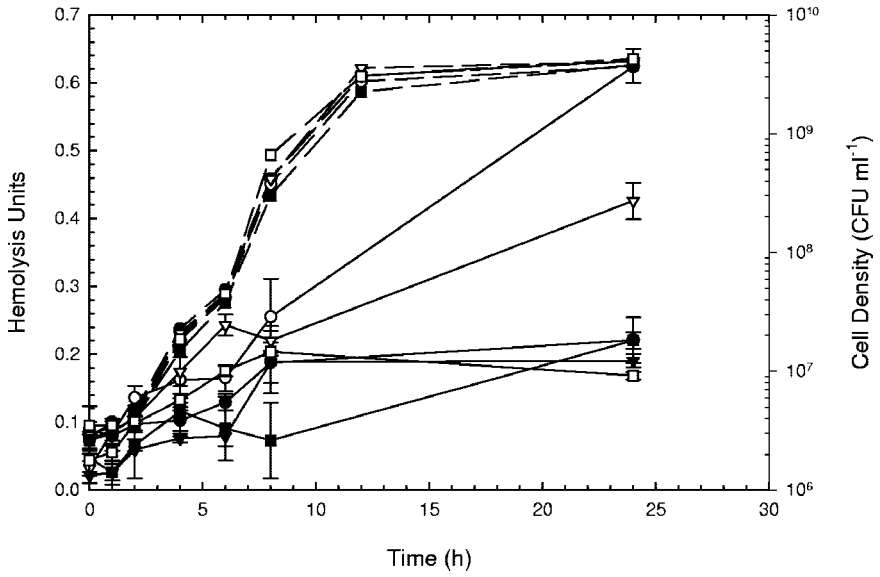

FIG. 4. Spectrophotometric assay of hemolysin activity in the wild-type strain (M93Sm [●]) and mutant strains (JL01 [○], JR1 [▼], JR2 [▽], SC1 [■], and JR8 [□]) of *V. anguillarum*. *V. anguillarum* cells grown overnight in LB20 (27°C with shaking) were diluted 1:1,000 in fresh LB20, samples (0.5 μl) were taken at the times indicated, and hemolytic activity was determined using a twofold dilution of culture supernatant added to 5% sheep erythorcytes. Samples were added to a 96-well microtiter plate, and optical density was determined at 428 nm. The hemolytic activity was calculated as OD_{428}/dilution, and the values were plotted as solid lines. Additionally, the cell density for each strain was determined and plotted (dashed lines). The data presented are from a representative experiment that was repeated three times. Error bars indicate standard deviation.

Determination of *vah1* and *plp* expression by qRT-PCR. Since hemolysin specific activity peaked early in exponential growth (Fig. 5), qRT-PCR was used to determine whether transcription of the hemolysin genes *plp* and *vah1* corresponded to the hemolysin specific activity. Transcription of both *plp* and *vah1* peaked at 2 h of growth in LB20 (Fig. 6). Specifically, in M93Sm transcript levels of *plp* and *vah1* increased by 62-fold and 1.8-fold, respectively, during the first 2 h of growth in LB20 (Fig. 6). When transcription of *plp* and *vah1* was measured in the weakly hemolytic wild-type strain NB10, *plp* increased by 2.9-fold, while *vah1* showed no increase during the first 2 h of growth in LB20. These increases were 4.7% and 56%, respectively, of those in M93Sm. As in M93Sm, levels of *plp* and *vah1* transcripts declined after 2 h of growth in LB20.

Expression of *plp* and *vah1* was also measured in the *plp* mutant strain, JL01 (Fig. 6). As expected, no *plp* transcripts were detected. However, accumulation of *vah1* transcripts in JL01 increased 12.6-fold during the first 2 h of growth in LB20

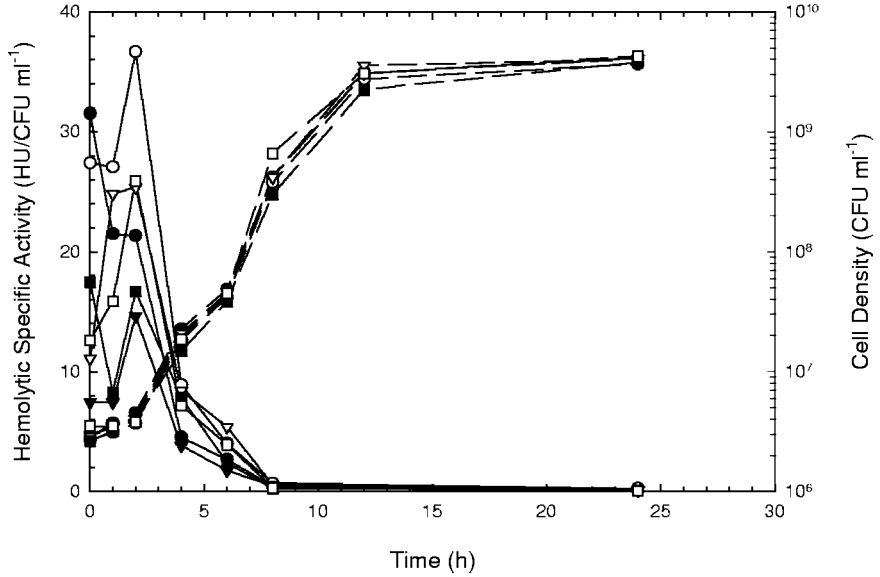

FIG. 5. Hemolytic specific activity of *V. anguillarum* strains M93Sm (●), JL01 (○), JR1 (▼), JR2 (▽), SC1 (■), and JR8 (□). Hemolytic activity (data from Fig. 4) was normalized to cell density (CFU ml^{-1}) of each culture and is plotted as solid lines. Cell density for each strain is plotted as dashed lines.

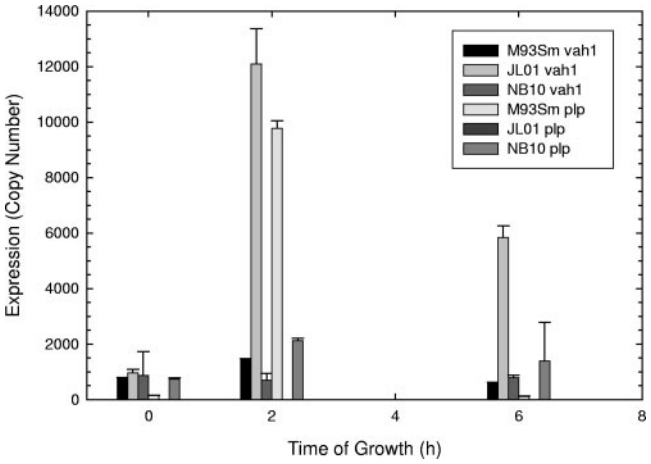

FIG. 6. Change in transcription of the hemolysin genes *vah1* and *plp* in *V. anguillarum* strains M93Sm, JL01, and NB10 determined by qRT-PCR. Cells were grown overnight in LB20, diluted to 3×10^6 in fresh LB20, and allowed to incubate at 27°C with shaking. Cell samples were taken at 0, 2, and 6 h of growth, total RNA was extracted, and the copy number of *vah1* and *plp* was determined for each strain by qRT-PCR. Copy number is shown per 10 ng of total RNA. The data are from one representative experiment of three replicates. Each data point is an average of two determinations, and the error bars indicate the standard deviation. The first three data bars at each time point represent *vah1* transcripts, and the second three data bars represent *plp* transcripts. In each group of three, the *V. anguillarum* strains are M93Sm, JL01, and NB10, respectively.

or was 7 times higher than that in the wild-type strain M93Sm (Fig. 6). These data suggest that *plp* acts to decrease hemolytic activity at the transcriptional level either by repressing *vah1* transcription or possibly by destabilizing *vah1* transcripts.

Virulence study of JL01 in Atlantic salmon. Juvenile Atlantic salmon were infected with *V. anguillarum* M93Sm, JL01, JR1, SC1, and JR8 by i.p. injection (Table 4). Fish inoculated with $\sim 3 \times 10^6$ CFU of the wild-type strain M93Sm suffered 100% mortality by 2 days, while fish inoculated with $\sim 3 \times 10^5$ CFU exhibited 60% mortality by 6 days. Similar levels of killing were observed in fish inoculated with JL01 (*plp*) or SC1 (*llpA*). In contrast, salmon inoculated with JR1 (*vah1*) suffered only 80% mortality over 6 days at the highest dose (1.3×10^6 CFU) and no deaths when inoculated with lower doses ($\sim 1 \times 10^5$ CFU). Fish inoculated with JR8 (JL01 complemented with pSUP202-*plp*) were killed at the same levels and rates as fish inoculated with the wild-type strain M93Sm (Table 4).

Juvenile Atlantic salmon were also inoculated with lethal doses of *V. anguillarum* M93Sm and JL01 by anal intubation (Table 4). Mortalities due to vibriosis occurred at almost the same rates in fish inoculated with the wild-type M93Sm strain as with the *plp* mutant, JL01.

DISCUSSION

Hemolytic activity by *V. anguillarum* cells has been suggested to be a virulence factor during infection of fish by contributing to hemorrhagic septicemia and diarrhea (11). In this study, three hemolysin-related genes of *V. anguillarum* (*plp*, *vah1*, and *llpA*) were identified, mutated, and characterized with regard to hemolysin activity, expression, and virulence. Initial mini-Tn*10*Kan mutagenesis created a mutation in *plp* (JL01) that caused a two- to threefold increase in hemolytic activity over that of the wild type. The regions surrounding the insertion mutation were cloned and sequenced, revealing a hemolysin region containing *plp*, *vah1*, *llpA*, and *llpB*. A second *plp* mutant, JR2, created by site-directed mutagenesis exhibited an identical phenotype to JL01. Complementation of JL01 with wild-type *plp* inserted into the shuttle vector pSUP202 restored hemolytic activity to wild-type levels. Additionally, the *plp*-null mutant JL01 produces seven-fold-higher levels of *vah1* transcript than does the wild-type strain M93Sm (Fig. 6). Taken together, these data strongly suggest that *plp* acts to repress hemolytic activity. The inferred *plp* amino acid sequence shows 69% identity and 80% similarity to the phospholipase/lecithinase/hemolysin of *V. vulnificus* CMCP6 with similar scores for relatedness to the lecithinase (encoded by *phl*) of *V. mimicus* and the thermolabile hemolysin/cytolysin/lecithinase (encoded by *lec*) of *V. cholerae*.

The deduced amino acid sequences of the *vah1* gene and the previously reported *Listonella* (*Vibrio*) *anguillarum vah1* (11), *Vibrio cholerae* EI Tor hemolysin (*hlyA*), and *V. fluvialis* hemolysin showed high degrees of amino acid sequence identity,

TABLE 4. Virulence of *V. anguillarum* strains in juvenile Atlantic salmon

Strain	Dose/fish (CFU)	Total % mortality	Time of death in days (no. of deaths/total fish)
i.p. inoculation			
M93Sm	3.69×10^6	100	1 (1/5), 2 (5/5)
	3.34×10^5	60	4 (2/5), 6 (3/5)
	2.89×10^4	20	6 (1/5)
JL01 (*plp*)	3.85×10^6	100	2 (4/5), 3 (5/5)
	3.00×10^5	40	5 (1/5), 7 (2/5)
	3.03×10^4	0	NA[a]
JR1 (*vah1*)	1.30×10^6	80	4 (1/5), 5 (2/5), 6 (4/5)
	1.10×10^5	0	NA
	1.10×10^4	0	NA
SC1 (*llpA*)	3.60×10^6	100	1 (1/5), 2 (3/5), 4 (5/5)
	3.20×10^5	60	4 (2/5), 5 (3/5)
	3.34×10^4	0	NA
JR8 (JL01 pSUP202-*plp*)	1.00×10^6	100	1 (2/5), 2 (3/5), 3 (5/5)
	1.00×10^5	40	4 (1/5), 6 (2/5)
	1.00×10^4	0	NA
Control (NSS)		0	NA
AIB[c] inoculation			
M93Sm	3.69×10^6	40	2 (1/5), 4 (2/5)
	3.34×10^5	20	3 (1/5)
	2.89×10^4	20	6 (1/5)
JL01	3.85×10^6	40	2 (1/5), 7 (2/5)
	3.00×10^5	40	3 (1/5), 4 (2/5)
	3.03×10^4	0	NA
Control (NSS)		6.67	4 (1/15)[b]

[a] NA, not applicable; no fish deaths occurred during the 21-day experiment.
[b] The dead fish did not show clinical symptoms of vibriosis, and no *V. anguillarum* cells could be isolated from the fish.
[c] AIB, anal intubation.

with identities of >96%, 56%, and 59%, respectively. Additionally, the amino acid similarities between *V. anguillarum vah1* and the hemolysins of *V. cholerae* and *V. fluvialis* were 72% and 71%, respectively. The HlyA, VmhA, and *V. fluvialis* hemolysins act as pore formers to create anion-permeable channels in membranes that cause ion leakage and, ultimately, cell lysis and cell death (16). The pore-forming properties of these cytolytic toxins occur when single protein subunits oligomerize to form anion-selective channels in either biological or artificial membranes (16).

The gene order of the hemolysin region was found to be highly conserved in various *Vibrio* species (8, 20). As illustrated in Fig. 2, the hemolysin region in *V. cholerae* contains genes (*lec*, *hlyA*, *hlyB*, *lipA*, and *lipB*) that, with the exception of *hlyB*, are homologous to *plp*, *vah1*, *llpA*, and *llpB* of *V. anguillarum*, respectively. The *hlyB* gene of *V. cholerae* apparently encodes a methyl-accepting chemotactic protein (20). No homologue to *hlyB* is found in the hemolysin region of *V. anguillarum*. It has been proposed that the genetic organization of this region of *V. cholerae* is part of a pathogenicity island, encoding products capable of damaging host cells and/or involved in nutrient acquisition (20). The same may be the case in *V. anguillarum* as well.

While phospholipases are associated with virulence in bacterial diseases (8), the role of phospholipases in the colonization of the gastrointestinal tract or infectious disease pathogenesis is unknown. Sequence analysis of M93Sm revealed the 918-bp open reading frame *plp* (*p*hospho*l*ipase), which is homologous to *lec*, encoding a 305-amino-acid protein. The predicted sequence exhibits strong amino acid sequence homology to phospholipases in other *Vibrio* species.

While mutations in *plp* consistently resulted in two- to threefold-increased hemolytic activity, mutations in either *vah1* or *llpA* had no effect on hemolytic activity. However, double mutations in *plp* and *vah1* or in *plp* and *llpA* restored hemolytic activity to wild-type levels. These data support the idea that *plp* negatively regulates *vah1* and *llpA*. The data also suggest that there may be more than one gene responsible for hemolytic activity in *V. anguillarum* M93Sm since no single or double mutation in the gene cluster containing *plp*, *vah1*, and *llpA* results in the loss of hemolytic activity. It is possible that a mutation in *vah1* could have a polar effect on *llpA*. Double mutations in *plp* and in *vah1* or *llpA* result in a restoration of hemolytic activity to wild-type levels from the two- to threefold increase in hemolytic activity exhibited by *plp* mutants. Hirono et al. (11) showed that in *E. coli*, *vah1* functions as a hemolysin; however, it is not clear whether *llpA* encodes a hemolysin. A mutation in the *vah1* gene attenuates virulence of *V. anguillarum* in juvenile Atlantic salmon, while an *llpA* mutant (SC1) is not attenuated and is as virulent as the wild-type strain M93Sm (Table 4). These data suggest that *vah1* is a virulence factor, while *llpA* appears to assist in hemolytic activity but is not required for virulence.

In fish infection studies (Table 4), single mutations in *plp* and *llpA* had no effect on virulence. The *plp*-complemented strain, JR8, also exhibited no change in virulence. As noted above, virulence in Atlantic salmon is attenuated in strain JR1 (*vah1*). Specifically, 60% of fish inoculated i.p. with doses of $\sim 10^5$ CFU of M93Sm die of vibriosis within 6 days. However, no fish die when inoculated i.p. with $\sim 10^5$ CFU of JR1. These data strongly suggest that *vah1* is a virulence gene for *V. anguillarum*.

While M93Sm and NB10 both contain the hemolysin genes characterized in this study (*vah1*, *plp*, and *llpA*), the hemolytic activities differ between the two wild-type strains (Fig. 1 and 4). The data presented in Fig. 6 suggest that the differences in hemolytic activity between the two wild type strains are the result of different levels of expression of the hemolysin genes (Fig. 6). Strain NB10 expresses lower levels of *vah1* and *plp* than does M93Sm. In both NB10 and M93Sm, expression data obtained by RT-qPCR show that expression of the hemolysin genes is turned on during the early exponential phase. Denkin and Nelson (4, 5) describe other differences in the pathogenicity of M93Sm and NB10. They examined the expression and role of the EmpA metalloprotease in pathogenesis in M93Sm and NB10 (4). Since *empA* is expressed only during the stationary phase and the hemolysin genes are expressed most strongly during the exponential phase, these two virulence factors (hemolysins and metalloprotease) may have different relative values in promoting pathogenesis by each wild-type strain. We show here that M93Sm exhibits about twofold more hemolytic activity than does NB10. In contrast, Denkin and Nelson (4) showed that NB10 exhibits greater EmpA activity. Further, the *empA* mutant of NB10, NB12, is avirulent in juvenile Atlantic salmon, while M99, the *empA* mutant of M93Sm, shows from no reduction to only mild reduction in virulence in juvenile Atlantic salmon, depending upon the route of infection (4).

We also analyzed the *empA* mutant strain M99 to determine whether the knockout of *empA* effects hemolytic activity. It has been reported by Song et al. (25) that aerolysin is activated by metalloprotease in *Aeromonas veronii* biovar *sobria*. Our results show that inactivation of the EmpA protease in *V. anguillarum* M93Sm does not inhibit hemolytic activity (data not shown). We also note that genes involved in hemolysin activity are induced in early exponential phase, while Denkin and Nelson (4, 5) show that the *empA* metalloprotease is induced in stationary phase.

Despite screening over 5,000 mini-Tn*10*Kan transponson mutants, no hemolysin-negative mutants were detected; only the up-regulated mutants Jl01 and Jl03 were observed. These data strongly suggest that a second gene cluster acts with *vah1* and *plp* in the regulation and production of hemolysin activity in *V. anguillarum* M93Sm. Identification of a second hemolysin gene or gene cluster and the creation of a null mutant in hemolytic activity will further elucidate the role of hemolysins in promoting pathogenesis by this organism.

ACKNOWLEDGMENTS

This work was supported by the National Research Initiative of the USDA Cooperative State Research, Education, and Extension Service, grant no. 2002-35204-12252 and 2005-35204-16294, awarded to D.R.N.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Austin, B., and D. A. Austin.** 1999. Characteristics of the pathogens. Bacterial fish pathogens: disease of farmed and wild fish, 3rd ed. Praxis Publishing Co., London, United Kingdom.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A.**

Smith, and K. Struhl. 1987. Current protocols in molecular biology. Green Publishing Associates and Wiley Interscience, New York, N.Y.
4. **Denkin, S. M., and D. R. Nelson.** 2004. Regulation of *Vibrio anguillarum empA* metalloprotease expression and its role in virulence. Appl. Environ. Microbiol. **70:**4193–4204.
5. **Denkin, S. M., and D. R. Nelson.** 1999. Induction of protease activity in *Vibrio anguillarum*. Appl. Environ. Microbiol. **65:**3555–3560.
6. **Di Lorenzo, M., M. Stork, M. E. Tolmasky, L. A. Actis, D. Farrell, T. J. Welch, L. M. Crosa, A. M. Wertheimer, Q. Chen, P. Salinas, L. Waldbeser, and J. H. Crosa.** 2003. Complete sequence of virulence plasmid pJM1 from the marine fish pathogen *Vibrio anguillarum* strain 775. J. Bacteriol. **185:** 5822–5830.
7. **Evelyn, T. P. T.** 1996. The fish immune system: organism, pathogen, and environment. Academic Press, San Diego, Calif.
8. **Fiore, A. E., J. M. Michalski, R. G. Russell, C. L. Sears, and J. B. Kaper.** 1997. Cloning, characterization, and chromosomal mapping of a phospholipase (lecithinase) produced by *Vibrio cholerae*. Infect. Immun. **65:**3112–3117.
9. **Garcia, T., K. Otto, S. Kjelleberg, and D. R. Nelson.** 1997. Growth of *Vibrio anguillarum* in salmon intestinal mucus. Appl. Environ. Microbiol. **63:**1034–1039.
10. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
11. **Hirono, I., T. Masuda, and T. Aoki.** 1996. Cloning and detection of the hemolysin gene of *Vibrio anguillarum*. Microb. Pathog. **21:**173–182.
12. **Mahnken, C. V. W.** 1975. Status report on commercial salmon culture in Puget Sound. Commer. Fish Farmer Aquacult. News **2:**8–11.
13. **Maniatis, T., J. Sambrook, and E. F. Fritsch.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Marden, P., A. Tunlid, K. Malmcrona-Friberg, G. Oldham, and S. Kjelleberg.** 1985. Physiological and morphological changes during short term starvation of marine bacterial isolates. Arch. Microbiol. **142:**326–332.
15. **McGee, K., P. Hörstedt, and D. L. Milton.** 1996. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. J. Bacteriol. **178:**5188–5198.
16. **Menzl, K., E. Maier, T. Chakraborty, and R. Benz.** 1996. HlyA hemolysin of *Vibrio cholerae* O1 biotype E1 Tor. Identification of the hemolytic complex and evidence for the formation of anion-selective ion-permeable channels. Eur. J. Biochem. **3:**646–654.
17. **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.
18. **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.
19. **Neidhardt, F. C., P. L. Block, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
20. **Ogierman, M. A., A. Fallarino, T. Riess, S. G. Williams, S. R. Attridge, and P. A. Manning.** 1997. Characterization of the *Vibrio cholerae* El Tor lipase operon *lipAB* and a protease gene downstream of the *hly* region. J. Bacteriol. **179:**7072–7080.
21. **O'Toole, R., D. Milton, P. Horstedt, and H. Wolf-Watz.** 1997. RpoN of the fish pathogen *Vibrio* (*Listonella*) *anguillarum* is essential for flagellum production and virulence by the water-borne but not intraperitoneal route of inoculation. Microbiology **143:**3849–3859.
22. **O'Toole, R., D. L. Milton, and H. Wolf-Watz.** 1996. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. Mol. Microbiol. **19:**625–637.
23. **Ross, A. J., J. E. Martin, and V. Bressler.** 1968. *Vibrio anguillarum* from an epizootic in rainbow trout (*Salmo gairdneri*) in the USA. Bull. Off. Int. Epizoot. **69:**1139–1148.
24. **Rucker, R. R.** 1959. Vibrio infections among marine and fresh water fish. Prog. Fish-Cult. **21:**22–25.
25. **Song, T., C. Toma, N. Nakasone, and M. Iwanaga.** 2004. Aerolysin is activated by metalloprotease in *Aeromonas veronii* biovar *sobria*. J. Med. Microbiol. **53:**477–482.

Editor: V. J. DiRita