HlyU Is a Positive Regulator of Hemolysin Expression in *Vibrio anguillarum*

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HlyU Is a Positive Regulator of Hemolysin Expression in *Vibrio anguillarum*

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The two hemolysin gene clusters previously identified in *Vibrio anguillarum*, the vah1 cluster and the rtxACHBDE cluster, are responsible for the hemolytic and cytotoxic activities of *V. anguillarum* in fish. In this study, we used degenerate PCR to identify a positive hemolysin regulatory gene, hlyU, from the unsequenced *V. anguillarum* genome. The *hlyU* gene of *V. anguillarum* encodes a 92-amino-acid protein and is highly homologous to other bacterial HlyU proteins. An *hlyU* mutant was constructed, which exhibited an ~5-fold decrease in hemolytic activity on sheep blood agar with no statistically significant decrease in cytotoxicity of the wild-type strain. Complementation of the *hlyU* mutation restored both hemolytic activity and cytotoxic activity. Both semiquantitative reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) were used to examine expression of the hemolysin genes under exponential and stationary-phase conditions in wild-type, *hlyU* mutant, and *hlyU* complemented strains. Compared to the wild-type strain, expression of *rtx* genes decreased in the *hlyU* mutant, while expression of *vah1* and *plp* was not affected in the *hlyU* mutant. Complementation of the *hlyU* mutation restored expression of the *rtx* genes and increased *vah1* and *plp* expression to levels higher than those in the wild type. The transcriptional start sites in both the *vah1-plp* and *rtxH-rtxB* genes' intergenic regions were determined using 5' random amplification of cDNA ends (5'-RACE), and the binding sites for purified HlyU were discovered using DNA gel mobility shift experiments and DNase protection assays.

*Vibrio anguillarum* is a marine member of the class *Gamma-proteobacteria*. This highly motile Gram-negative bacterium is the causative agent of warm-water vibriosis, a fatal hemorrhagic septicemic disease in fish, crustaceans, and bivalves (1). The mortality rate from *V. anguillarum* infections ranges from 30% to 100% (1). Infections by these bacteria have resulted in severe economic losses to aquaculture worldwide (1, 21) and affect many farm-raised fish, including Pacific salmon, Atlantic salmon, sea bass, cod, and eel (1, 4, 5, 21).

Hemolytic activity has been considered to be a virulence factor for *V. anguillarum* and is thought to contribute to the hemorrhagic septicemia characteristic of vibriosis (7, 16). Rock and Nelson (16) reported that the *vah1* hemolysin gene cluster contains at least two genes, *vah1* and *plp*, that affect hemolytic activity. Vah1 is a putative pore-forming hemolysin that causes vacuolization of target cells (10). It was suggested that pore-forming hemolysins, like HlyA in *Escherichia coli*, cause direct lysis of blood cells by disrupting the membrane integrity (13). Mutations in the divergently transcribed *plp* gene result in both increased expression of *vah1* and increased hemolysis, suggesting that *plp* is a putative repressor of *vah1* transcription (16). Additionally, restoration of *plp* by complementation restores the wild-type level of *vah1* transcription and hemolysis (16). Plp is a phosphatidylcholine (PC)-specific phospholipase A2 (PLA2), which causes lysis of PC-rich fish erythrocytes (9).

Besides the *vah1* cluster, a second hemolysin gene cluster, *rtxACHBDE*, was identified in the *V. anguillarum* (10). This gene cluster contains *rtxA*, which encodes a potent MARTX toxin and the specialized type I secretion system (T1SS) genes (*rtxDBE*) responsible for the secretion of the RtxA hemolysin/cytotoxin. A mutant containing mutations in both *vah1* and *rtxA* completely lost hemolytic activity on sheep blood agar (10). Additionally, RtxA exhibits cytotoxic activity and causes Atlantic salmon kidney (ASK) cells to round and die (10).

HlyU, a member of the SmtB/ArsR family, is a metal-regulated transcriptional regulatory protein (17). It has been reported that HlyU is a positive regulator of hemolysin and toxin genes in *Vibrio* species. In *Vibrio cholerae*, the HlyU protein positively regulates expression of hemolysin gene *hlyA*, as well as the *hlyA*-coregulated gene *hcp* (22, 23). Williams et al. (22) reported that a mutation in *hlyU* attenuates *V. cholerae* O17 in the infant mouse cholera infection model. Recently, HlyU was also identified in *Vibrio vulnificus*, and it appears to be a positive regulator of virulence genes (8, 11). Kim et al. (8) reported that HlyU of *V. vulnificus* may be one of the master regulators of *in vivo* virulence gene expression. Specifically, in a *V. vulnificus* hlyU mutant, cytotoxic activity against HeLa cells was nearly abolished, and the 50% lethal dose (LD₅₀) of *V. vulnificus* in mice by intraperitoneal infection was increased by 10- to 50-fold (8). Liu et al. (11) also demonstrated that HlyU was required for virulence of RtxA1, a homologue of RtxA of *V. anguillarum* in *V. vulnificus* CMCP6. In *V. vulnificus*, HlyU acted as a competitor that antagonized the binding of H-NS, a repressor of *rtxA1*, in the upstream region of the *rtxA1* operon so that the presence of HlyU resulted in derepression of *rtxA1* (12).

In this report, we identified the *hlyU* homologue in *V. anguillarum* by degenerate PCR and constructed an *hlyU* mutant strain and its complement. The hemolytic activity and cytotox-
icity of the mutant were determined and compared to those of the wild-type and complemented strains. We also identified the transcriptional start site genes in both the vah1 cluster and rtxA operon and localized the HlyU binding sites to the upstream region of the two hemolysins by gel mobility shift and DNase protection assays. Additionally, the amounts of transcriptional start site of genes in both the wild-type and complemented strains. We also identified the complemented strains. We also identified the

### MATERIALS AND METHODS

**Fish cell line, bacterial strains, plasmids, and growth conditions.** Atlantic salmon kidney (ASK) cells (ATCC CRL-2747) were cultured at 20°C in Leibovitz-15 medium containing 100 μg/ml ampicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum (FBS) (Invitrogen). All bacterial strains and plasmids used in this report are listed in Table 1. V. anguillarum strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10) (18). Specific conditions for each experiment are described in the text. E. coli strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10) (18). Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm200); ampicillin, 100 μg/ml (Ap100); chloramphenicol, 20 μg/ml (Cm20) for E. coli and 5 μg/ml (Cm5) for V. anguillarum; kanamycin, 50 μg/ml (Km50) for E. coli and 80 μg/ml (Km80) for V. anguillarum; and tetracycline, 15 μg/ml (Tc15) for E. coli and 2 μg/ml (Tc2) for V. anguillarum.

**Degenerate PCR.** Degenerate PCR was used to identify the hlyU gene in V. anguillarum. Previously sequenced hlyU genes, including their flanking genes from various *Vibrio* species, were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov) and aligned using the ClustalW program (20). Degenerate primers (Table 2) were designed from the conserved regions (Fig. 1) and used to amplify the possible hlyU gene from V. anguillarum M93Sm genomic DNA. The PCR products were separated and purified from a 1% agarose gel and then subcloned into pCR2.1 vector (Invitrogen). Colonies containing the cloned hlyU gene in pCR2.1 were selected on LB10 plates plus Ap100 and Km80, and the presence of hlyU was confirmed by plasmid purification and DNA sequencing.

### Insertional mutagenesis of hlyU.** Insertional mutagenesis by homologous recombination was used to create a gene interruption within the hlyU gene by using a modification of the procedure described by Milton and Wold-Watz (14). Briefly, primers (Table 2) were designed based on the hlyU gene sequence of M93Sm (GenBank accession no. HQ149334). Then a 161-bp hlyU DNA fragment was PCR amplified by using primer pair Pm297 and Pm298 (Table 2) and cloned into the suicide vector pNQ705 by using SacI and XbaI restriction sites to yield the suicide vector pNQ705 derivative plasmid, which was confirmed by both PCR amplification and restriction analysis. The mobilizable suicide vector was transferred from *E. coli* Sm10 (λ pir) into V. anguillarum M93Sm by conjugation (14). Transconjugants were selected on LB10 plates plus Ap100 and Km80, and the presence of hlyU was confirmed by plasmid purification and DNA sequencing.

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and features</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. anguillarum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M93Sm</td>
<td>Spontaneous Sm’ mutant of M93 (serotype J-O-1)</td>
<td>3, 4</td>
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<tr>
<td>S305</td>
<td>Sm’ Cm’; M93Sm hlyU mutant</td>
<td>This study</td>
</tr>
<tr>
<td>S307</td>
<td>Sm’ Cm’ Tet’; M93Sm hlyU complement</td>
<td>This study</td>
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<tr>
<td>S183</td>
<td>Sm’ Cm’ Kan’; M93Sm rtxA vah1 double mutant</td>
<td>10</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sm10</td>
<td>thi thr leu tonA lacY supE recA RP4-2-Tc::Muc:Km (λ pir)</td>
<td>14</td>
</tr>
<tr>
<td>M15</td>
<td>NaN’ Str’ Rif’ thi lac ara’ gal’ mil’ F’ recA’ uvr’ lon’ (pREP4; Km’)</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pNQ705-1</td>
<td>Cm’; suicide vector with R6K origin</td>
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<tr>
<td>pSUP202</td>
<td><em>E. coli</em>-V. anguillarum shuttle vector</td>
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<tr>
<td>PCR2.1</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
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<tr>
<td>pQF30UA</td>
<td>Expression vector with N-terminal His6 tag</td>
<td>Qiagen</td>
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### TABLE 2. Primers used in this study

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<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)*</th>
<th>Description</th>
</tr>
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<td>Pm301</td>
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<td>Degenerate hlyU F</td>
</tr>
<tr>
<td>Pm302</td>
<td>CGTGCYCGACAYAAYGCCTAGCCGTTCGYTC</td>
<td>Degenerate hlyU R</td>
</tr>
<tr>
<td>Pm297</td>
<td>ACTGAGA GTCCGGTTGTTGTAAGGCCTAGG GCC</td>
<td>hlyU insertion mutation F</td>
</tr>
<tr>
<td>Pm298</td>
<td>ATCGATCTAGAGTATCCACTAACCCC CATCT TT</td>
<td>hlyU insertion mutation R</td>
</tr>
<tr>
<td>R vah1 RT (BF)</td>
<td>GGCTCAACCTCCTCTTGTTAACCAA</td>
<td>5’-RACE vah1</td>
</tr>
<tr>
<td>plp RT</td>
<td>CAGACGGACACACCAT AAAACCACAT AA</td>
<td>5’-RACE plp</td>
</tr>
<tr>
<td>Pm112</td>
<td>TGGTTGTAAGCCGGACGAC</td>
<td>5’-RACE rtxB</td>
</tr>
<tr>
<td>Pm163</td>
<td>GGGTATGCTGACATGCTGACATGCT GAT</td>
<td>5’-RACE anchor primer</td>
</tr>
<tr>
<td>Primer AP</td>
<td>GACCACCGTGATGCAGTCAATGCTAGT</td>
<td>Entire HlyU protein F</td>
</tr>
<tr>
<td>Pm303</td>
<td>ATGGAAAAAATTTCCGTA AAAAGCA</td>
<td>Entire HlyU protein F</td>
</tr>
<tr>
<td>Pm304</td>
<td>CTAGCGGCGATTA AAAACCCTGTTAA</td>
<td>Entire HlyU protein F</td>
</tr>
<tr>
<td>Pm305</td>
<td>CCCGGATCCGACACTTATTCCTGCA TTGATG</td>
<td>hlyU complementation F</td>
</tr>
<tr>
<td>Pm306</td>
<td>CCCCTGATCCGACACTTATTCCTGCA TTGATG</td>
<td>hlyU complementation R</td>
</tr>
</tbody>
</table>

* Restriction sites for SacI (GAGCTC), XbaI (TCTAGA), and EcoRI (GGATCC) are underlined. V = A, C, or G; N = A, C, T, or G; R = A or G; and Y = C or T.
taining Trypticase soy agar (TSA) plus sheep blood agar after 24 h at 27°C, as were determined by measuring the diameter of beta-hemolysis on plates containing the indicator.

Complementation of the hlyU mutant. The mutant was complemented by cloning the appropriate hlyU gene fragment into the shuttle vector pSUP202 (GenBank accession no. AY428809), as described previously by Rock and Nelson (16). Briefly, primers (Table 2) were designed and EcoRI sites were introduced at the 5′ end of the primers. The primer pair was then used to amplify the entire hlyU gene plus ~500 bp of the 5′ and 3′ flanking regions from genomic DNA of V. anguillarum M93Sm. The PCR product was cloned into the pCR2.1 vector (Invitrogen) and digested with EcoRI restriction enzyme, and the DNA fragments were separated on a 1% agarose gel. Subsequently, the gel-purified PCR fragment was ligated into pSUP202 after digestion with EcoRI and the ligation mixture was introduced into E. coli Sm10 (pir) by electroporation with Bio-Rad Gene Pulser II. Transformants were selected on LB-Ap 100 agar plates. The complementing plasmid, pSUP202-hlyU, was transferred from E. coli Sm10 into the V. anguillarum hlyU mutant (S305) by conjugation using the procedures described previously (16). The transconjugants were confirmed by PCR amplification and restriction digestion.

Hemolytic activity assay. Hemolytic activities of various V. anguillarum strains were determined by measuring the diameter of beta-hemolysis on plates containing Trypticase soy agar (TSA) plus sheep blood agar after 24 h at 27°C, as previously described (16).

Cytotoxicity assay. Cytotoxic activity of V. anguillarum strains was determined by changes to cell morphology or by measurement of released lactate dehydrogenase (LDH). ASK cells were seeded into a six-well microtiter plate (Costar) in Leibovitz’s L-15 medium supplemented with 20% fetal bovine serum and grown at 20°C to a cell density of 5 × 10^9 cells ml⁻¹. The cells were harvested, washed twice in NSS, and resuspended in NSS (at a cell density of ~2 × 10^6 cells ml⁻¹). The complementing plasmid, pSUP202-hlyU, was transferred from E. coli Sm10 into the V. anguillarum hlyU mutant (S305) by conjugation using the procedures described previously (16). The transconjugants were confirmed by PCR amplification and restriction digestion.

RNA isolation. Exponential-phase cells (~0.5 × 10^9 CFU ml⁻¹) and stationary-phase cells (~2 × 10^9 CFU ml⁻¹) of various V. anguillarum strains were harvested by centrifugation. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 and 280 nm using a NanoDrop spectrophotometer and stored at −75°C for future use.

Semiquantitative RT-PCR and real-time qRT-PCR. Total RNA was isolated from exponential- and stationary-growth-phase V. anguillarum cells as described above. All RNA samples were treated with DNase, and 100 μg of RNA was used as the template for reverse transcription-PCR (RT-PCR). RT-PCR was performed using Brilliant II SYBR green single-step quantitative RT-PCR (qRT-PCR) master mix (Stratagene). Briefly, gene-specific primers (Table 2) were used to reverse transcribe the specific cDNA from RNA templates, and the resulting cDNA was used as the template with which to amplify the specific DNA product, using 25-cycle regular PCR to give a semiquantitative determination of the original RNA amount. Genomic DNA (100 μg) extracted from wild-type strain M93Sm was used as the positive control. The thermal profile was 30°C for 30 min and 95°C for 15 min and then 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR product was visualized in a 1% agarose gel using a 100-bp DNA molecular weight ladder (Promega) as a standard. All real-time quantitative RT-PCRs (qRT-PCRs) were performed using an Mx3005 or Mx4000 multiplex quantitative PCR system (Stratagene). The primers used were the same as the semiquantitative RT-PCR (Table 2). Quantitation of various mRNAs was performed using Brilliant II SYBR green single-step qRT-PCR master mix (Stratagene) with 10 ng of total RNA in 25-μl reaction mixtures. The thermal profile was 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s and 55°C for 30 s. Fluorescence was measured at the end of the 55°C step during every cycle. Samples were run in triplicate plus a no-RT control and no-template control.

5′-RACE assay. Total RNA was isolated from exponential-phase V. anguillarum cells grown in LB20 using the RNeasy kit (Qiagen). To identify the transcriptional start site, RNA was subjected to 5′ rapid amplification of cDNA ends (5′-RACE) using the 2nd generation 5′-RACE kit (2). Primers used in RT-PCR are listed in Table 2. Briefly, 5 μg of RNA was used to generate specific first-strand cDNA from target mRNA (valh1, ppi, rtxH, or rtxB) in a reverse transcriptase reaction with a gene-specific primer. Poly(A) tails were added to the 3′ cDNA end using dATP and terminal deoxynucleotidyl transferase [or in

**FIG. 1.** Protein alignments of the hlyU flanking genes, encoding ribosomal protein S20P (A) and the transcriptional regulator NahR (B), in five Vibrio-related bacterial species. The bars above the alignment indicate the relative amount of conservation of amino acid residues. The regions enclosed in black boxes were used to design the degenerate primers according to their original DNA sequences. The black arrows show the orientation of primers.
some cases, poly(G) tails were added with dGTP and terminal deoxynucleotidyl transferase. A PCR product was amplified from the tailed cDNA by using a 3′-RACE anchor primer (AP) (Table 2) and the primer specific for that sequence. The PCR product was cloned into PCR2.1 cloning vector (Invitrogen), and plasmids from appropriate transformants were purified and sequenced.

DNA sequence and analysis. All DNA sequencing was done at the RI Genomics and Sequencing Center (University of Rhode Island, Kingston), using an ABI 3170xl genetic analyzer unit (Applied Biosystems). Multiple alignments and phylogenetic trees were analyzed using the ClustalW method in the DNASTAR Lasergene 7 program.

Overexpression and purification of the V. anguillarum HlyU protein. The DNA fragment encoding HlyU was PCR amplified using primers Pm303 and Pm304 (Table 2) and cloned into the 6×His (His6) tag expression plasmid pQE30-UA (Qiagen, Inc.), generating the plasmid pQE30-UA/HlyU, which encodes HlyU with an N-terminal fusion tag. The correct recombinant clone confirmed by sequencing was used for expression of His-tagged HlyU protein in E. coli M15 (S301). Ten milliliters of overnight bacterial culture growing at 37°C in a Luria broth supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin was inoculated into 250 ml of the same fresh medium. When the optical density at 600 nm (OD600) reached 0.6, 1 mM IPTG (isopropyl-β-D-thiogalactospyranoside) was added to induce the expression of HlyU protein. After bacteria were grown for an additional 5 h at 37°C, the cells were collected by centrifugation (8,000 × g, 10 min) and the cell pellets were resuspended in 5 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole [pH 8.0]). The cell suspension was incubated with lysozyme (0.5 mg/ml) on ice for 30 min and then sonicated (six bursts at 20 s per burst with 30-s intervals on ice). The resulting cell lysate was centrifuged (10,000 × g, 20 min), and the soluble supernatant containing HlyU-His6 was collected. The recombinant protein was then purified from this fraction by affinity chromatography using Ni-nitrilotriacetic acid resin (Qiagen, Inc.) according to the manufacturer’s instructions. The concentration of the purified HlyU protein was determined by measuring the absorbance at 280 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

Gel mobility shift assay. The gel mobility shift assay was performed using a 2nd generation digoxigenin (DIG) gel shift kit (Roche, Indianapolis, IN). Three fragments (a, b, and c) from the rtxH-rtxB intergenic region and five fragments (d, e, f, g, and h) from the plp-vah1 intergenic region were amplified by PCR and then 3′ end labeled with digoxigenin-11-dUTP using terminal deoxynucleotidyl transferase. After the labeling efficiency was determined, each of the labeled probes (0.4 ng for fragments a, b, and c, and 0.2 ng for fragments d, e, f, g, and h) was incubated with 350 ng purified HlyU protein in 20 μl binding buffer [100 mM HEPES (pH 7.6), 5 mM EDTA, 50 mM (NH4)2SO4, 5 mM dithiothreitol, 1% (w/v) Tween 20, 150 mM KCl]. For competition analysis, labeled probe (0.4 ng for fragments a, b, and c, and 0.2 ng for fragments d, e, f, g, and h) and 350 ng HlyU protein were incubated with 100 ng/ml unlabeled specific probe. The binding reactions were carried out at room temperature for 15 min, and then samples were separated by 6% polyacrylamide DNA retardation gel (Invitrogen, Carlsbad, CA). The DNA-protein complex was transferred to positively charged nylon membrane by electroblotting, and then immunological detection and chemiluminescent signal detection were carried out according to the instructions of the manufacturer (Roche, Indianapolis, IN).

DNaI protection assay. The DNA probes for the DNaI protection assay were amplified from V. anguillarum genomic DNA by PCR using primers (Integrated DNA Technologies, Inc.) shown in Table 3. Thus, two rtxH-rtxB intergenic region probes (4 and 5) were labeled with 6-carboxyfluorescein (6-FAM) at the 5′-end on the upper strand and the lower strand, respectively. The two plp-vah1 intergenic probes (2 and 3) were also labeled with 6-FAM at the 5′ end on the upper strand and lower strand, respectively. The assay was carried out using a method modified from Zianni et al. (25). Briefly, 40 ng of a DNA probe and various amounts of recombinant HlyU (rHlyU) (up to 1.88 μg) were incubated at 37°C in a total volume of 20 μl, containing binding buffer (4 μl, 5× concentration) from the 2nd generation DIG gel shift kit (Roche Applied Science), for 1 h. The DNA-protein complex was then digested by adding 0.001 U RQ1 RNase-free DNase (Promega Corporation) in a total volume of 23 μl containing reaction buffer (2.3 μl, 10X concentration) at 37°C for 1 min. The reaction was stopped by adding 2.6 μl stop solution (10X concentration) followed by heating (95°C, 10 min). The DNA was purified with a QIAquick PCR purification kit (Qiagen, Inc.), using a QIAcube and its standard protocol, except that the elution volume was adjusted to 30 μl. The DNA in the eluant (5 μl) was added to 10 μl Hi-Di formamide containing 0.1 μl GeneScan 600 LIZ size standard (Applied Biosystems), and the mixture was submitted to capillary electrophoresis fragment analysis (Rhode Island Genomics and Sequencing Center).

## RESULTS

Identification of the hlyU gene in V. anguillarum. Previous studies indicated that the hlyU gene is a conserved transcriptional regulator in many Vibrio species (11, 12, 22, 23). We hypothesized that HlyU could be a putative regulator of the two hemolysin gene clusters in V. anguillarum. In order to identify the unknown hlyU gene in V. anguillarum, several hlyU genes from Vibrio species, including V. cholerae, V. vulnificus, V. fischeri, and V. para-haemolyticus, were compared using freely available software and database from the Integrated Microbial Genomes (IMG) website (http://img.jgi.doe.gov). The comparison revealed that the flanking genes of hlyU were identical among these Vibrio species and encoded a transcriptional activator protein, NhaR, and a ribosomal protein, S20P (Fig. 1). Conserved regions (sequence data obtained from http://www.ncbi.nlm.nih.gov) from both flanking regions were aligned (Fig. 1), and degenerate PCR primers (Table 2) were designed and used to perform degenerate PCR to amplify the putative hlyU gene and flanking DNA from V. anguillarum. A single PCR product was obtained by degenerate PCR (Fig. 2) and was purified, cleaned, cloned, and sequenced. As expected, DNA sequence data revealed that the PCR product included the intact 294-bp hlyU gene homolog (GenBank accession no. HQ149334), which encodes a predicted protein with 97 amino acids, a molecular mass of 11,095 Da, and strong homology to HlyU protein found in a variety of Vibrio species.

<table>
<thead>
<tr>
<th>Intergenic region</th>
<th>Probe no. (bp)</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Primer 5′ label</th>
<th>Primer strand</th>
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<td>vah1 sense</td>
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FIG. 2. The degenerate PCR product was amplified from \textit{V. anguillarum} M93Sm genomic DNA using primer pair Pm301/Pm302. The PCR product was separated and visualized in a 1% agarose gel using a Promega 1-kb DNA ladder as the size standard and was 1.6 kbp long. The PCR product was purified, cleaned, and cloned into pCR2.1 vector and then transformed into the \textit{E. coli} DH5\textalpha{} strain. The plasmid, purified from the appropriate colony, was sequenced.

including \textit{Vibrio fumissii} (97\% similarity and 92\% identity), \textit{Vibrio coralliilyticus} (95\% similarity and 91\% identity), \textit{V. cholerae} (93\% similarity and 86\% identity), \textit{V. parahaemolyticus} (92\% similarity and 88\% identity), and \textit{V. vulnificus} (94\% similarity and 87\% identity).

\textbf{Mutation in hlyU decreases hemolytic activity.} An insertional mutation by single-crossover homologous recombination in the \textit{hlyU} gene was obtained. The hemolytic activity of the \textit{hlyU} mutant was determined and found to decrease about 5-fold compared with wild-type strain M93Sm on sheep blood agar (Fig. 3). Complementation of the \textit{hlyU} mutant restored the hemolytic activity, which was even higher than wild type (Fig. 3), indicating that HlyU is a positive regulator of hemolysis in \textit{V. anguillarum}.

\textbf{Mutation in hlyU has no significant effect on cytotoxicity.} One hemolysin gene, \textit{rtxA}, has been shown to be a major virulence factor for \textit{V. anguillarum} (10). Previous studies revealed that RtxA has strong cytotoxic activity against Atlantic salmon kidney (ASK) cells and causes cells to round-up, detach, and die (10). However, experiments showed that ASK cells still rounded up and died when incubated with S305, M93Sm, or S307 cells (Fig. 4A) at an MOI of 100 for 4 h, indicating the mutation in \textit{hlyU} did not completely knock out the cytotoxicity of \textit{V. anguillarum}. Indeed, the LDH release assay revealed that S305 retained \textasciitilde{}75 to 80\% (\textit{P} $> 0.1$) of cytotoxicity at all MOI values compared to the wild-type strain M93Sm (Fig. 4B), confirming that the mutation of \textit{hlyU} had only a small, statistically insignificant, effect on cytotoxicity. As a negative control, the \textit{rtxA vah1} double mutant strain S183 exhibited no cytotoxicity compared to the wild-type strain M93Sm (Fig. 4B), confirming that \textit{rtxA} and \textit{vah1} are the major cytotoxins in \textit{V. anguillarum} (10). When strain S307 was assayed for cytotoxicity activity by the LDH release assay, the activity was restored to the same levels seen in M93Sm (Fig. 4B).

\textbf{HlyU positively regulates hemolysin genes at the transcriptional level.} Semiquantitative RT-PCR and real-time qRT-PCR were performed to determine the expression levels of hemolysin genes, including \textit{vah1}, \textit{plp}, \textit{rtxA}, \textit{rtxB}, and \textit{rtxA}, in the wild-type strain (M93Sm), \textit{hlyU} mutant (S305), and the \textit{hlyU} complement (S307) during both the exponential and stationary growth phases. Previously, we demonstrated that the \textit{rtx} gene cluster contains two divergently cotranscribed sets of genes: \textit{rtxA1} with the \textit{rtxH} promoter proximal, and \textit{rtxBDE}, with the \textit{rtxB} promoter proximal (10). As shown in Fig. 5, RNA expression of \textit{rtxH}, \textit{rtxA}, and \textit{rtxB} decreased in the \textit{hlyU} mutant compared to wild-type expression levels during both exponential and stationary phases, indicating that the transcriptional levels of \textit{rtx} genes were downregulated in the absence of HlyU. Indeed, complementation of the \textit{hlyU} mutation upregulated the expression of \textit{rtx} genes back to wild-type levels (or higher), indicating that HlyU positively regulates the expression of \textit{rtx} genes. Real-time qRT-PCR data also revealed that in the \textit{hlyU} mutant during the exponential and stationary phases, respectively, expression of \textit{rtxA} decreased by 7.94- and 20-fold; expression of \textit{rtxB} decreased by 3.56- and 8.07-fold; and expression of \textit{rtxH} decreased by 5.9- and 15.1-fold (Table 4). The data strongly suggest that HlyU is a positive regulator of \textit{rtx} gene expression, playing an important role in the expression of \textit{rtx} genes during both exponential and stationary phases. In fact, the data show that the mutation in \textit{hlyU} has a larger effect on stationary-phase expression of \textit{rtx} genes than on exponential-phase expression. Additionally, expression of the same \textit{rtx} genes increased to levels higher than wild type in the \textit{hlyU} complement (Table 4), indicating the overexpression of \textit{hlyU} positively regulates the expression of \textit{rtx} genes.

In contrast to \textit{rtx} genes, expression of genes in the \textit{vah1} cluster, including \textit{vah1} and \textit{plp}, exhibited little or no decrease in the \textit{hlyU} mutant by the semiquantitative RT-PCR experiments (Fig. 5). Measurements of expression of \textit{vah1} and \textit{plp} by real-time qRT-PCR were consistent with data from the semiquantitative experiments showing no significant changes in expression in the \textit{hlyU} mutant (Table 4), indicating that the absence of HlyU does not affect either \textit{vah1} or \textit{plp} expression. However, when the expression of \textit{vah1} and \textit{plp} was examined by both semiquantitative RT-PCR (Fig. 6) and qRT-PCR (Table 4) in the \textit{hlyU} complement (S307), we observed that expression of both genes increased. Specifically, expression of \textit{vah1} in S307 increased over wild-type (M93Sm) levels by 11.6- and 26.3-fold during the exponential and stationary phases, respectively, and expression of \textit{plp} increased over levels in M93Sm by 8.32- and 86.2-fold during the exponential and stationary phases, respectively. These data indicate that overexpression of HlyU can positively regulate expression of \textit{vah1} and \textit{plp}.

\textbf{Mapping transcriptional start sites of hemolysin genes.} Since it had been reported that HlyU is a DNA binding protein (17) that positively regulates \textit{hlyA} (homologue of \textit{vah1}) in \textit{V. cholerae} (23) and \textit{rtxA1} (homologue of \textit{rtxA}) in \textit{V. vulnificus} (11), we wanted to determine possible HlyU binding sites in...
the vah1 gene cluster and rtxACHBDE cluster in V. anguillarum. The transcriptional start sites of both hemolysin clusters were identified using 5'-RACE. In the vah1 gene cluster, there is a 508-bp intergenic region between the divergent plp and vah1 genes. The 5'-RACE results demonstrated that the region between the transcriptional start sites of plp and vah1 was 318 bases long (Fig. 6B). The transcriptional start site (A) of plp is 73 bases prior to its start codon, with a predicted 35-10 promoter sequence of TTGATT-N13-ATAAAT (Fig. 6B). The divergent hemolysin gene, vah1, had a transcriptional start site (G) 119 bases before the vah1 start codon, with a predicted -35 and -10 promoter sequence of TTGTGT-N16-TATTAA (Fig. 6B).

For the rtx gene cluster, the intergenic space between the divergent rtxH and rtxB genes is 325 bp. 5'-RACE results show that the region between the transcriptional start sites of rtxH and rtxB is 187 bp (Fig. 6A). The +1 transcriptional start site (G) of rtxH is 103 bases prior to its start codon, with a predicted -35 and -10 promoter sequence of TTTCGT-N15-TATTAA (Fig. 6A). The divergent rtxA transporter gene, rtxB, was found to have a transcriptional start site (C) 34 bases before the rtxB start codon, with a predicted -35 and -10 promoter sequence of TTGAGC-N18-TATTAA (Fig. 6A).

Analysis of the predicted promoter regions of these two hemolysin clusters revealed strong similarities to a σ70 consensus promoter, TTGACA-N17-TATAAT. Additionally, the putative ribosomal binding site (RBS) for all genes was also located upstream of the ATG start codons (Fig. 6A and B).

HlyU binds to the intergenic promoter regions of the hemolysin gene clusters. Previously, Liu et al. (11) demonstrated that HlyU binds to the promoter region of the rtxA1 operon of V. vulnificus. In an effort to determine whether HlyU acted in a similar fashion to help regulate expression of the hemolysin gene clusters in V. anguillarum, we carried out gel mobility shift experiments using purified HlyU-His6 protein. Briefly, the purified protein (350 ng) was reacted with each of the three DIG-labeled DNA subfragments amplified from the intergenic region between rtxH and rtxB (Fig. 7A) and with each of the five DIG-labeled DNA subfragments amplified from the intergenic region between plp and vah1 (Fig. 7C). DNA mobility shift experiments were performed on the mixtures containing HlyU plus DIG-labeled DNA. The results revealed that HlyU bound to fragment b of the rtxH-rtxB intergenic region (Fig. 7B) and to fragment f of the plp-vah1 intergenic region (Fig. 7D). When unlabeled competitor DNA was added to each of these reactions, binding was decreased or abolished.

In an effort to more closely characterize the binding sites of HlyU for each hemolysin gene cluster, each of the two DNA subfragments that bound HlyU was examined by a DNase I protection assay, as described in Materials and Methods. The
results of these experiments revealed that HlyU protected an 18-bp region (5'-TAATAAAAATCTTAAAAA-3') in fragment b (Fig. 8A) with two 5-bp direct repeats of TAAAA. This region starts 103 bp upstream of the rtxH site of rtxH and 67 bp upstream from rtxB. Similarly, HlyU protected a 22-bp region (5'-AATAAAAATATCAATAAAATTA 3') in fragment f (Fig. 8B) with the same two 5-bp direct repeats of TAAAA. The binding region in subfragment f starts 192 bp upstream of plp and 106 bp upstream of the rtxH site for vah1. DISCUSSION

Hemolytic activity of V. anguillarum has been considered the virulence factor responsible for hemorrhagic septicemia during infection (1, 5). We previously reported that there are two major hemolysin gene clusters in V. anguillarum M93Sm (10, 16). The vah1 cluster consists of four genes, plp, vah1, llpA, and llpB. Vah1 is a putative pore-forming hemolysin, which shows strong homology to HlyA of V. cholerae. HlyA integrates into the erythrocyte membrane to cause lysis (13). The plp gene, divergent from the vah1 gene, encodes a hemolysin with phospholipase A2 activity specific for phosphatidylcholine and is highly conserved among members of the Vibrionaceae as a lecithinase/thermolabile hemolysin. Plp has the ability to lyse fish erythrocytes because of the abundance of phosphatidylcholine in their membranes (9). Additionally, mutations in plp result in increased expression of vah1 (16). The second hemolysin gene cluster in V. anguillarum is the rtxACHBDE cluster (10), in which rtxHAC is divergently transcribed from rtxBDE.

The V. anguillarum RtxA is a major virulence factor for V. anguillarum with both hemolytic and cytotoxic activities (10). While mutations of both vah1 and rtxA are required for the complete loss of hemolytic activity on sheep blood agar (10), mutations in plp, vah1, and rtxA are required for a 90% loss of hemolytic activity against fish erythrocytes. Thus, all three genes encode proteins that are major hemolysins in the fish host (9). However, prior to this study, little was known about the regulation of these hemolysins in V. anguillarum. It has been reported that HlyU regulates the expression of hemolysins in Vibrio species. In V. cholerae, HlyU positively regulates the expression of hemolysin HlyA (23) and the HlyA-coregulated gene hcp (24). It was also suggested that mutation of hlyU attenuates the virulence of V. cholerae O17 in the infant mouse cholera infection model (22). Recent evidence suggests that HlyU is a master regulator of virulence in V. vulnificus, as several virulence factors, including vvh1 and rtxA1, a homologue of rtxA of V. anguillarum, appear to be regulated by HlyU (8, 11). Therefore, we hypothesized that the hlyU gene in V. anguillarum might encode a regulator for both hemolysin clusters in V. anguillarum. In this study, we used degenerate PCR to discover the unknown hlyU gene from the V. anguillarum genome. The experiment successfully identified an hlyU gene (Fig. 2) from V. anguillarum with strong homology to other hlyU genes in Vibrio species. HlyU is a member of SmtB-ArsR protein family. Some members of this transcriptional regulator family, such as NolR of Rhizobium meliloti, SmtB of Synechococcus sp. strain PCC.
Vibrio HlyU, as well as other using the ClustalW program reveals that the metal binding site. Analysis of the HlyU amino acid sequence recently solved by Nishi et al. (15), confirmed that HlyU has no absence of the metal binding site on HlyU (17). Furthermore, HlyU in DNA binding site (19). However, recent studies suggested that located on the repressor protein to enhance binding to the gene expression by binding metal ions to a metal binding site

<table>
<thead>
<tr>
<th>Parameter and gene expressed</th>
<th>V. anguillarum strain</th>
<th>Expression at:</th>
<th>Log phase</th>
<th>Stationary phase</th>
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<td>3.45 x 10^2 ± 800</td>
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<td></td>
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<td></td>
<td>1.18 x 10^2 ± 424</td>
<td>1.48 x 10^2 ± 1,237</td>
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<td>Relative change (fold) in expression</td>
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<tr>
<td></td>
<td>S305</td>
<td></td>
<td>-7.94 ± 0.4</td>
<td>-20.00 ± 0.6</td>
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<tr>
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<td>S305</td>
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<td>1.06 ± 0.05</td>
<td>1.24 ± 0.1</td>
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<td>1.00</td>
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<tr>
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<td></td>
<td>1.56 ± 0.05</td>
<td>-2.08 ± 0.01</td>
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<tr>
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<td>8.32 ± 1.1</td>
<td>86.2 ± 7.8</td>
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</table>

a The data presented are from a representative experiment of two independent experiments. Each sample is the average of three replicates.
b M93Sm is the wild type, S305 is the hlyU mutant, and S307 is the hlyU complement.
c Gene expression is shown as either upregulated (positive number) or downregulated (negative number) compared to expression in M93Sm.

7942, and ArsR of Staphylococcus aureus, act to repress target gene expression by binding metal ions to a metal binding site located on the repressor protein to enhance binding to the DNA binding site (19). However, recent studies suggested that HlyU in V. cholerae acts as a positive regulator because of the absence of the metal binding site on HlyU (17). Furthermore, the crystal structure of HlyU from V. vulnificus strain CMCP6, recently solved by Nishi et al. (15), confirmed that HlyU has no metal binding site. Analysis of the HlyU amino acid sequence using the ClustalW program reveals that the V. anguillarum HlyU, as well as other Vibrio species (V. cholerae, V. vulnificus, V. fischeri, and V. parahemolyticus), does not contain a metal binding site and probably has similar binding characteristics to the homologues found in V. cholerae and V. vulnificus.

Evidence suggests that mutation of hlyU has a strong effect on virulence. For example, a mutation in hlyU attenuates V. cholerae O17 virulence in the infant mouse cholera infection model (22). In V. vulnificus, the LD_{50} increased about 10^2-fold in an hlyU mutant using the iron-overloaded mouse infection model (11) or the iron-normal mouse infection model (8). Additionally, cytotoxic activity was lost in an hlyU mutant of V. vulnificus (11). However, we found that in V. anguillarum,
cytotoxicity of the hlyU mutant remained relatively high according to both the LDH release assay and observations of morphological changes in ASK cells exposed to the hlyU mutant (Fig. 4). These observations indicate that rtxA was still expressed in the hlyU mutant, even though rtxA expression was significantly decreased in the mutant (Fig. 5 and Table 4). While our data indicate that HlyU is a positive regulator of rtxA, rtxH, and rtxB, these genes are still expressed in the absence of HlyU in V. anguillarum. It is interesting to note that levels of transcription of rtxA, rtxH, and rtxB in the wild-type strain and hlyU mutant all decrease during stationary phase (Table 4). This may suggest that either greater amounts of HlyU are required during stationary phase or hlyU expression may be repressed during stationary phase.

Additionally, cytotoxicity data were consistent with the hemolytic activity assay, in which the hlyU mutant did not completely eliminate the hemolysis on the sheep blood agar (Fig. 3), indicating that the hemolysins were expressed in the mutant. Interestingly, real-time RT-PCR data showed that the hlyU mutant did not affect the expression of vah1 and plp compared to the wild-type strain (Table 4). However, the observation that overexpression of HlyU in the hlyU complement dramatically increased expression of both vah1 and plp suggests that vah1 and plp are regulated by HlyU in a different manner than the rtx gene cluster. This is supported by our previous observation that plp null mutations increase hemolysin activity and vah1 transcription (16). Thus, the results presented here suggest that in addition to HlyU, one or more other factors may regulate the hemolysins/cytotoxins of V. anguillarum.

While HlyU is a positive regulator of V. cholerae hlyA (22, 23), there is no experimental evidence to demonstrate that HlyU binds to the hlyA promoter region. Therefore, it is still unclear if HlyU is a direct transcriptional activator binding to the hlyA promoter region or if it interferes with an unknown repressor of hlyA to cause derepression of hlyA by HlyU (17). The latter assumption was recently supported by the study of V. vulnificus, where HlyU was found to bind to the upstream region of rtxH, which competes with the binding site of rtxA1 repressor H-NS (12). Similar to V. anguillarum, expression of rtxH and rtxA1 in V. vulnificus is regulated by the same rtxH proximal promoter. Therefore, it was suggested that the absence of HlyU would increase the H-NS binding, which repressed the expression of rtxA1 in V. vulnificus (12). It is reasonable to think that a similar situation might exist in the both hemolysin clusters of V. anguillarum.

In this study, transcriptional start sites of both hemolysin clusters were identified, and promoter regions for the potential
HlyU binding were targeted (Fig. 6). We found that the central regions of the intergenic sequence for each hemolysin gene cluster contain a conserved binding site for HlyU, as determined by both DNA mobility shift experiments (Fig. 7) and DNase I protection assays (Fig. 8). The two binding sites are quite similar (Fig. 8): the intergenic rtxH-rtxB protected binding region is 18 bp long, while the intergenic plp-vah1 region is 22 bp long, and both have identical 5-bp direct repeats of

![DNA gel mobility shift demonstrating binding of purified HlyU to intergenic regions of the rtxACHBDE operon (A and B) and the vah1-plp gene cluster (C and D). DIG-labeled DNA fragments of the intergenic region between rtxH and rtxB (A) and between plp and vah1 (C) were obtained by PCR amplification. Individual DIG-labeled fragments (0.4 ng) were reacted with no additions (lane 1), 350 ng HlyU (lane 2), and 350 ng HlyU plus 100 ng unlabeled DNA fragment (lane 3). (B) Fragments a, b, and c from the intergenic regions of the rtxACHBDE operon. (D) Fragments d, e, f, g, and h from the intergenic regions of the vah1-plp gene cluster.](image1)

![Capillary electrophoresis of 6-FAM-labeled DNA fragments b (A) and f (B) from DNase protection assays in the presence (gray traces) and absence (black traces) of HlyU, demonstrating that HlyU binds to specific sequences in fragments b and f of the rtxACHBDE and plp-vah1 intergenic regions, respectively, and protects against DNase I digestion. DNA fragments b and f were prepared and labeled with 6-FAM, reacted with HlyU (0 or 1.88 μg) followed by DNase I, and then analyzed by DNA fragment analysis as described in Materials and Methods. The double black lines show the binding regions. The binding region sequences are shown below the double black line. Underlined bases indicate those that are higher in the presence of HlyU (gray trace) than in its absence (black trace).](image2)
and 68 bp upstream of the rtxH

We thank Maureen Varina Driscoll for her help with this study.

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USDA Cooperative State Research, Education, and Extension Ser-

are nearly identical with 10-bp inverted repeats at each end

intergenic region has nearly identical 10-bp inverted re-

nucleotide sequence of ATAATAAAAA. Similarly, the

10-bp inverted repeats at each end of its 25-bp region with a

intergenic regions contain 25- to 26-bp regions that

repeat may be a bit longer than 5 bp. If one uses an imperfect

suggested by Saha and Chakrabarti (17). In fact, the direct

VOL. 193, 2011 REGULATION OF HEMOLYSIN EXPRESSION IN

V. anguillarum and the plp-vah1 intergenic region contains identi-

plp-rtxB and the plp-vah1 intergenic regions contain 25- to 26-bp regions that

5′-TTAATAAAAA-3′. The nucleotide sequence of the vah1 proximal repeat is 3′-ATAAT-

AAAT-5′. Furthermore, comparison between the HlyU binding sites identified here and the site identified by Liu et al. (12) reveals that in both cases HlyU binds to AT-rich regions up-

of the transcriptional start sites of the regulated hemo-

lysin genes. However, Liu et al. (10, 12) found that HlyU

bound far upstream (bp −376 to −417) of the transcriptional start site of the

rtxAI operon. In contrast, we have located HlyU binding somewhat closer to the start transcription sites of rtxH-rtxB and plp-vah1. The binding sites of HlyU are 104 bp and 68 bp upstream of the rtxH and rtxB +1 sites, respectively, and 150 bp and 145 bp upstream of the plp and vah1 +1 sites, respectively. While we have not yet demonstrated H-NS re-

pressor binding to these regulatory regions in V. anguillarum, the shorter distance between HlyU binding sites and the transcrip-

tional start sites in V. anguillarum compared to V. vulnifi-

cus may indicate that V. anguillarum has fewer H-NS binding sites than the five sites found for the V. vulnificus rtxA1 regulatory region (12).

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dom.


1591.


3555–3560.


1039.


2620–2632.


1319.


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