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H-NS Is a Negative Regulator of the Two Hemolysin/Cytotoxin Gene Clusters in *Vibrio anguillarum*

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Hemolysins produced by *Vibrio anguillarum* have been implicated in the development of hemorrhagic septicemia during vibriosis, a fatal fish disease. Previously, two hemolysin gene clusters responsible for the hemolysis and cytotoxicity of *V. anguillarum* were identified: the *vah1-plp* gene cluster and the *rtxACHBDE* gene cluster. In this study, we identified the *hns* gene, which encodes the H-NS protein and acts as a negative regulator of both gene clusters. The *V. anguillarum* H-NS protein shares strong homology with other bacterial H-NS proteins. An *hns* mutant exhibited increased hemolytic activity and cytotoxicity compared to the wild-type strain. Complementation of the *hns* mutation restored hemolytic activity and cytotoxicity levels to nearly wild-type levels. Furthermore, expression of *rtxA*, *rtxH*, *rtxB*, *vah1*, and *plp* increased in the *hns* mutant and decreased in the *hns*-complemented mutant strain compared to expression in the wild-type strain. Additionally, experiments using DNase I showed that purified recombinant H-NS protected multiple sites in the promoter regions of both gene clusters. The *hns* mutant also exhibited significantly attenuated virulence against rainbow trout. Complementation of the *hns* mutation restored virulence to wild-type levels, suggesting that H-NS regulates many genes that affect fitness and virulence. Previously, we showed that HlyU is a positive regulator of expression for both gene clusters. In this study, we demonstrate that upregulation by *hlyU* is *hns* dependent, suggesting that H-NS acts to repress or silence both gene clusters and HlyU acts to relieve that repression or silencing.

Vibrio anguillarum is the causative agent of vibriosis, a fatal hemorrhagic septicemic disease. *V. anguillarum* infects more than 50 fresh- and saltwater fish species, including various species of economic importance to the larviculture and aquaculture industries, such as salmon, rainbow trout, turbot, sea bass, sea bream, cod, eel, and ayu (1). Infections by this bacterium have mortality rates of 30% to 100%, resulting in severe economic losses to aquaculture worldwide (2).

The ability of *V. anguillarum* to infect and cause disease in fish is dependent upon several virulence factors and their proper regulation (3). One of these virulence factors is hemolytic activity. In *V. anguillarum* M93Sm, there are two known gene clusters that encode at least three hemolysins (4, 5). Rock and Nelson (4) reported that the *vah1-plp* hemolysin gene cluster (Fig. 1A) contains at least two genes, *vah1* and *plp*, that affect hemolytic activity. Vah1 (encoded by *vah1*) is a putative pore-forming hemolysin causing vacuolization of target cells that has strong amino acid sequence identity to *Vibrio cholerae* El Tor hemolysin (*hlyA*) and *V. fluvialis* hemolysin (5). Mutations in the divergently transcribed *plp* gene result in both increased expression of *vah1* and increased hemolysis of sheep's blood, suggesting that Plp (encoded by *plp*) is a putative repressor of *vah1* transcription (4). Restoration of *plp* by complementation restores the wild-type levels of *vah1* transcription and hemolysis (4). Plp is a phosphatidylcholine (PC)-specific phospholipase A2 (PLA2) which causes lysis of PC-rich fish erythrocytes (L. Li et al., unpublished data). These observations suggest that Plp plays a dual role as both a repressor and a phospholipase. A second hemolysin gene cluster, *rtxACHBDE* (Fig. 1B), was identified in *V. anguillarum* (5). This gene cluster contains *rtxA*, which encodes a multifunctional autoprocessing repeat-in-toxin (MARTX) toxin, and specialized type 1 secretion system (T1SS) genes (*rtxDBE*) responsible for the secretion of RtxA. RtxA exhibits cytotoxic activity that causes Atlantic salmon kidney (ASK) cells to round and die (5). Loss of *rtxA* function results in avirulence (5), while mutation of *vah1* causes a slight attenuation of *V. anguillarum* virulence (4). Strains with mutations in both *vah1* and *rtxA* lost 98% cytotoxicity in ASK cells, suggesting that Vah1 and RtxA are the two major cytotoxins when ASK cells are treated with *V. anguillarum* (6). These observations strongly suggest that the RtxA hemolysin is a major virulence factor of *V. anguillarum*, while Vah1 plays a more minor role in virulence.

The histone-like nucleoid structuring protein (H-NS) is a conserved global regulator that belongs to a family of small nucleoid-associated proteins including the factor for inversion stimulation (FIS), the heat-unstable protein (HU), and the integration host factor (IHF) (7). It has been reported that H-NS function is based on self-oligomerization and binding to DNA motifs to create DNA-protein-DNA bridges that can impede the movement of RNA polymerase (8). H-NS has been shown to repress expression of several virulence genes, including the cholera toxin (*ctx*) (9, 10) and exopolysaccharide biosynthesis (*vps*) genes in *V. cholerae* (10, 11), the RTX toxin gene (*rtxA1*) in *V. vulnificus* (12), and T3SS1 genes in *V. parahaemolyticus* (13). In many bacterial species, repression by H-NS can be relieved by other regulators, and each bacterial system has developed specific approaches to attenuate

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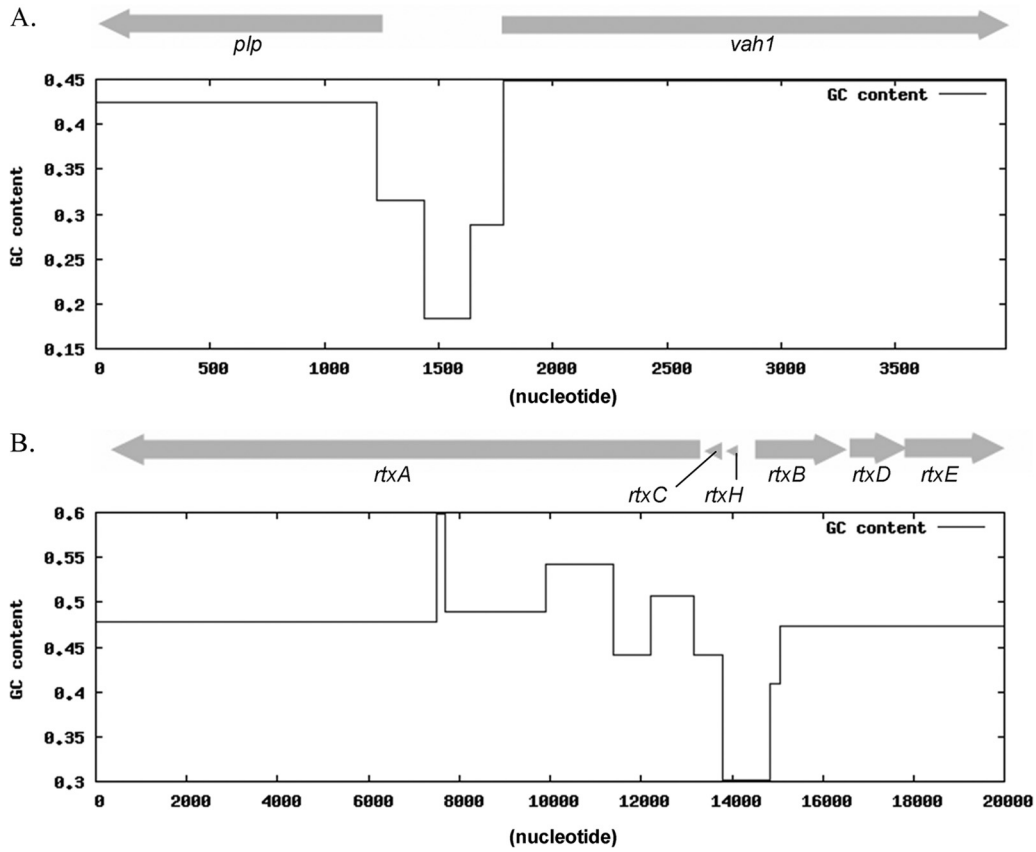

FIG 1 *V. anguillarum* hemolysin genes are arranged in two gene clusters: the *vah1-plp* gene cluster (A) and the *rtxACHBDE* gene cluster (B). The GC content of each gene cluster is shown.

the repressive action of H-NS (8). In *V. vulnificus*, HlyU acts as a competitor that antagonizes the binding of H-NS, resulting in derepression of *rtxA1* (12). Transcriptional silencing of the *V. cholerae tcpA* and *ctx* promoters by H-NS is antagonized by the AraC-like transcriptional regulator ToxT and by IHF (10, 14, 15). While there is no report regarding H-NS in *V. anguillarum*, we hypothesize that H-NS is a regulator of the two hemolysin gene clusters in *V. anguillarum*.

In this study, we identified the sequence of an *hns* homologue in *V. anguillarum* by using the *V. anguillarum* M93Sm draft genome and subsequently constructed several *hns* mutant strains, including an *hns* mutant, an *hns hlyU* double mutant, an *hns hlyU* double mutant complemented with *hns*, and an *hns hlyU* double mutant complemented with *hlyU*. The hemolytic activity and cytotoxicity of these strains were determined. The expression levels of various hemolysin genes, including *vah1*, *plp*, *rtxA*, *rtxH*, and *rtxB*, were also quantified for these strains. Additionally, the H-NS binding sites in the intergenic regions in both hemolysin gene clusters were localized. Finally, the virulence of the *hns* mutant and *hns*-complemented strains was tested in rainbow trout (*Oncorhynchus mykiss*) and compared to the virulence of the wild-type strain.

MATERIALS AND METHODS

Identification of genes in *V. anguillarum*. The *V. anguillarum* M93Sm draft genome (unpublished data) was annotated by the RAST (Rapid Annotation using Subsystem Technology) service (http://rast.nmpdr.org/rast.cgi), using the default settings (16).

Fish cell line, bacterial strains, plasmids, and growth conditions. ASK cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured at 20°C in Leibovitz-15 medium containing 100 μg/ml streptomycin and 17% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY). 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 2% NaCl (LB20) (17), supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. *Escherichia coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10). Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml (Sm^{200}); ampicillin, 100 μg/ml (Ap^{100}); chloramphenicol, 20 μg/ml (Cm^{20}) for *E. coli* and 5 μg/ml (Cm^{5}) for *V. anguillarum*; kanamycin, 50 μg/ml (Km^{50}) for *E. coli* and 80 μg/ml (Km^{80}) for *V. anguillarum*; tetracycline, 15 μg/ml (Tc^{15}) for *E. coli*, 1 μg/ml (Tc^{1}) for *V. anguillarum* grown in liquid medium, and 2 μg/ml (Tc^{2}) for *V. anguillarum* grown in solid medium.

Insertional mutagenesis. Insertional mutations were made by using a modification of the procedure described by Milton et al. (18). Briefly, primers SD_hns(F) and SD_hns(R) (Table 2) were designed based on the target gene sequence of M93Sm. A 200- to 300-bp DNA fragment of *hns* was then PCR amplified and ligated into the suicide vector pNQ705 (GenBank accession no. KC795685) after digestion with SacI and XbaI. The ligation mixture was introduced into *E. coli* Sm10 by electroporation using a Bio-Rad Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transformants were selected on LB10 Cm^{20} agar plates. The construction of pNQ705-*hns* was confirmed by both PCR amplification and restriction

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or feature(s)	Reference or source
Strains		
V. anguillarum strains		
M93Sm	Spontaneous Sm^r mutant of M93 (serotype J-O-1); virulent	31
S305	*hlyU* mutant; Sm^r Cm^r	6
M114	*hns* mutant; Sm^r Cm^r	This study
M116	*hns*-complemented strain; Sm^r Cm^r Tet^r	This study
ES114	*hns hlyU* double mutant; Sm^r Cm^r Km^r	This study
ES115	*hns hlyU* double mutant complemented with *hlyU*; Sm^r Cm^r Tet^r Km^r	This study
ES116	*hns hlyU* double mutant complemented with *hns*; Sm^r Cm^r Tet^r Km^r	This study
E. coli strains		
Sm10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ *pir*) Km^r	39
M15	Nal^s Str^s Rif^s *thi lac ara*$^+$ *gal*$^+$ *mtl* F^- *recA*$^+$ *uvr*$^+$ *lon*$^+$ (pREP4; Km^r)	Qiagen
D112	*E. coli* Sm10 with pDM4-hns5′-Kan-hns3′; Cm^r Km^r	This study
Plasmids		
pNQ705-1	Cm^r; suicide vector with R6K origin	40
pNQ705-hns	Used for *hns* insertional mutation	This study
pSUP202	*E. coli-V. anguillarum* shuttle vector	39
pSUP202-hlyU	Used for complementation of *hlyU*	6
pSUP202-hns	Used for complementation of *hns*	This study
pDM4	Cm^r Kan^r $SacBC^r$; suicide vector	18
pDM4-hns5′-Kan-hns3′	Used for *hns* deletion mutation	This study
pQE-30 UA	Expression vector with N-terminal His_6 tag	Qiagen
pQE-30 UA/H-NS	Used for expression of rH-NS	This study

analysis. The mobilizable suicide vector was transferred from *E. coli* Sm10 into *V. anguillarum* M93Sm by conjugation (18). Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of pNQ705-*hns* was confirmed by PCR amplification.

Construction of *hns hlyU* double mutant. The *hns hlyU* double mutant was constructed by allelic exchange of *hns* followed by insertional mutation of *hlyU*. The allelic exchange mutation was made by using a modification of the procedure described by Milton et al. (18). Briefly, the plasmid pDM4 (GenBank accession no. KC795686) was used to construct an *hns*::*Km* allelic exchange mutant as described previously (18). The 5′ region of *hns* was amplified using the primer pair pr40 and pr41 (Table 2), digested with XhoI and XbaI, and then cloned into the region between the XhoI and XbaI sites on pDM4. The 3′ region of *hns* was amplified using the primer pair pr42 and pr37 (Table 2), digested with XbaI and SacI, and then cloned into the region between the XbaI and SacI sites on the derivative pDM4 plasmid containing the 5′ region of *hns*. Finally, the kanamycin resistance gene was amplified from the TOPO2.1 vector (Life Technologies) with the primer pair pr38 and pm173 (Table 2), digested with XbaI, and inserted into the XbaI site between the 5′ and 3′ *hns* regions on the derivative pDM4 plasmid. The resulting plasmid, pDM4-*hns*::*Km*, was transformed into *E. coli* Sm10 to produce the transformant strain D112, which was mated with *V. anguillarum* M93Sm. Single-crossover transconjugants were selected with LB20 Kan^{80} Sm^{200} Cm^5 plates, and subsequently, double-crossover transconjugants were selected with LB20 Kan^{80} Sm^{200} plates containing 5% sucrose. The resulting *V. anguillarum* mutants were checked for the desired allelic exchange by PCR amplification and then were subjected to insertional mutation of *hlyU* as described above.

Complementation of the mutants. The various mutants were complemented by cloning the appropriate target gene fragment into the shuttle vector pSUP202 (GenBank accession no. AY428809) as described by Rock and Nelson (4). Briefly, primers hns_comp(F) and hns_comp(R) (Table 2) were designed with a PstI site added at the 5′ end of each primer. The primer pair was then used to amplify the entire target gene plus ~500 bp of the 5′-flanking region and ~200 bp of the 3′-flanking region from genomic DNA of *V. anguillarum* M93Sm. The DNA fragment was then ligated into pSUP202 after digestion with EcoRI and AgeI, and the ligation mixture was introduced into *E. coli* Sm10 by electroporation using a Bio-Rad Gene Pulser II apparatus. Transformants were selected on LB10 $Tc^{15}Ap^{100}$ agar plates. The complementing plasmid was transferred from *E. coli* Sm10 into the *V. anguillarum* mutant by conjugation (18). Transconjugants were selected by utilizing the tetracycline resistance gene located on the plasmid. The transconjugants were then confirmed by PCR amplification and restriction digestion.

Hemolytic activity assay. The blood agar hemolysis assay was carried out using the method described by Rock and Nelson (4). Briefly, *V. anguillarum* colonies were transferred onto blood agar plates, and hemolytic activity was determined by measuring the diameter of beta-hemolysis on plates containing Trypticase soy agar (TSA) plus either 5% sheep blood (Northeast Laboratories Service, Waterville, ME) or 5% trout blood after 24 h at 27°C. Trout blood was taken from live, healthy, farm-raised rainbow trout (*Oncorhynchus mykiss*) by use of a 3-ml sterile syringe supplemented with 10 μl 0.5 M disodium EDTA (Sigma-Aldrich). The blood was then stored on ice and used in casting plates within 6 h.

Cytotoxicity assay. The cytotoxicity assay was carried out using a modification of the method described by Li et al. (6). Cytotoxic activity of *V. anguillarum* strains was determined by measurement of released lactate dehydrogenase (LDH). ASK cells (20,000 cells/well for assays using *V. anguillarum* supernatants and 10,000 cells/well for assays using washed *V. anguillarum* cells) were seeded into a 96-well tissue culture plate and incubated in Leibovitz-15 medium supplemented with 17% FBS at 20°C for 24 h to allow cells to attach. *V. anguillarum* cultures grown for 18 h were centrifuged (9,000 × *g*, 5 min, 4°C). The resulting culture supernatant was harvested and filter sterilized using 22-μm filters (Millipore Corp., Billerica, MA). The bacterial pellet was washed twice in nine-salts solution (NSS) (19) and resuspended in fresh NSS (at ~2×10^9 cells ml^{-1}). *V. anguillarum* culture supernatant (50 μl) was added to wells containing ASK cells plus 50 μl phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) and incubated at 20°C for 6 h. Washed bacterial cells were added to ASK cells at a multiplicity of infection (MOI) of 200 and incubated at 20°C for 4 h. To determine the release of LDH, a CytoTox-ONE homogeneous membrane integrity assay kit (Promega, Madison, WI) was used following the manufacturer's instructions. The assay measures the generation of the fluorescent resorufin product, which is proportional to the amount of LDH, using an excitation wavelength of 560 nm and an emission wave-

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Use and description	Reference
SD_hns(F)	GCTAGGAGCTCCAGCTTGAAGAAGCACTAGA	*hns* insertional mutation, forward primer, SacI site	This study
SD_hns(R)	GCTAGTCTAGACCAGAAAGTGCAGAAATTAA	*hns* insertional mutation, reverse primer, XbaI site	This study
hns_comp(F)	GCTAGCTGCAGTCGGCGATAAAACCTTTCAC	*hns* complementation, forward primer, PstI site	This study
hns_comp(R)	GCTAGCTGCAGGTTTACCTGAACGTGACGAC	*hns* complementation, reverse primer, PstI site	This study
Pm416	TTAAATCTCGAATTCTTCTAGAGATTTACC	*hns* open reading frame, forward primer	This study
Pm417	ATGTCTGAATTAACAAAAACTCTACTTAAT	*hns* open reading frame, reverse primer	This study
pr40	CGGCTACTCGAGAGATTTACCTGCATCAAGTTG	*hns* 5′ region, forward primer, XhoI site	This study
pr41	CGGCTATCTAGAGCACTTTCTGGTGAAACTAAG	*hns* 5′ region, reverse primer, XbaI site	This study
pr42	CGGCTATCTAGAATTAATGCGCTTACATCAATA	*hns* 3′ region, forward primer, XbaI site	This study
pr37	CGGCTAGAGCTCAGAAGCACTAGATAAATTAAC	*hns* 3′ region, reverse primer, SacI site	This study
pr38	CGGCTATCTAGAGAAAAGCTTGAACACGTAGAA	Kanamycin resistance gene, forward primer, XbaI site	This study
Pm173	ACTGATCTAGATCAGAAGAACTCGTCAAGAAG	Kanamycin resistance gene, reverse primer, XbaI site	This study
RT vah1-R1	GACCGCCGAATCGATGATGAATC	*vah1* qRT-PCR, forward primer	4
pvah1JR	GGTAGGACTGATGCCCACCTACAA	*vah1* qRT-PCR, reverse primer	This study
plpF RT	CAGACGACCACCAGTAACCACTAA	*plp* qRT-PCR, forward primer	4
plpR RT	GCAATCATGATGACCCAGCAACAG	*plp* qRT-PCR, reverse primer	4
Pm111	GGAAATTATTCCGCCGACGATGGA	*rtxA* qRT-PCR, forward primer	5
Pm112	GCCGATACCGTATCGTTACCTGAA	*rtxA* qRT-PCR, reverse primer	5
Pm285	GTGATGGTAGAAAACCTGCGG	*rtxH* qRT-PCR, forward primer	This study
Pm286	ATGTCTGAGAAATTTGTCCAAACA	*rtxH* qRT-PCR, reverse primer	This study
iPCR rtxB R	CCGCTAACCGCATTGATATTAAGCTTGGC	*rtxB* qRT-PCR, forward primer	This study
Pm104	TCACAATCGCCCCAACTTGCCTTG	*rtxB* qRT-PCR, reverse primer	This study
Pm297	ACTGAGAGCTCGGTGTTGTTAAAGGCTATGGC	*hlyU* qRT-PCR, forward primer	6
Pm298	ATCGATCTAGAGTATCCACTAACCCATCTCTT	*hlyU* qRT-PCR, reverse primer	6
Pm412	CCGTATTTTCTGCAATCGCCATGG	*vah1* promoter region (probe 1), forward primer, 5′ labeled with FAM	6
Pm324	CACATATTGACTGATTATAATTTTATTGATATT	*vah1* promoter region (probe 1), reverse primer	6
pr323a	AGGGTTTTTATAAATCCTAATTTAGATA	*plp* promoter region (probe 2), forward primer, 5′ labeled with FAM	This study
Pm320	GAATACCCATTTTTTATTTTTTCAGACC	*plp* promoter region (probe 2), reverse primer	6
Pm327	GTATTTTCTGCAATCGCCATG	*vah1-plp* (probe 3) intergenic region, forward primer	6
Pm413	CACCTTTGTGGCGAATTATTAATAGATCTT	*vah1-plp* (probe 3) intergenic region, reverse primer, 5′ labeled with FAM	6
Pm414	CAGTGGCTCATAAAAGCAGTTGC	*rtxB-rtxH* intergenic region (probe 4), forward primer, 5′ labeled with FAM	6
Pm318	CAGCGGTAAGTAGACTGATA	*rtxB-rtxH* intergenic region (probe 4), reverse primer	6
Pm315	CTCAGACATAAATAAATCACC	*rtxB-rtxH* intergenic region (probe 5), forward primer	6
Pm415	CAGCGGTAAGTAGACTGATAAGCAATG	*rtxB-rtxH* intergenic region (probe 5), reverse primer, 5′ labeled with FAM	6

[a] Underlined sequences are engineered restriction sites.

length of 590 nm. Fluorescence was read by a Stratagene MX3005P QPCR system at an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

RNA isolation. Exponential-phase cells (~0.5×10^8 CFU ml^{-1}) and stationary-phase cells (2×10^9 CFU ml^{-1}) of various *V. anguillarum* strains were treated with RNAprotect bacterial reagent (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was isolated using an RNeasy kit and QIAcube (Qiagen) following the manufacturer's instructions. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm, using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA), and were stored at −75°C for future use.

Real-time qRT-PCR. Quantitative reverse transcriptase PCR (qRT-PCR) was used to quantify various mRNAs by use of an Mx3005 multiplex quantitative PCR system and Brilliant II SYBR green single-step qRT-PCR master mix (Agilent Technologies, Wilmington, DE) 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 along with no-RT and no-template controls. All experiments were repeated at least twice.

Overexpression and purification of the *V. anguillarum* H-NS protein. The DNA fragment encoding H-NS was PCR amplified by using primers Pm416 and Pm417 (Table 2) and then cloned into a six-His-tag expression plasmid, pQE30-UA (Qiagen), generating the plasmid pQE-30 UA/H-NS (Table 1), which encodes H-NS with an N-terminal fusion tag. The correct recombinant clone (rH-NS; confirmed by sequencing) was used for expression of the His-tagged H-NS protein in *E. coli* M15. Expression and purification of rH-NS were carried out using a modification of the procedures described by Li et al. (6). Briefly, 10 ml of an overnight bacterial culture growing at 37°C in Luria broth supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin was added to 250 ml of the same fresh medium. When the optical density at 600 nm (OD_{600}) reached 0.6, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce the expression of the H-NS protein. After bacteria were grown for an additional 5 h at 37°C, the cells were collected and lysed by sonication under nondenaturing conditions. The soluble supernatant containing rH-NS was then purified from this fraction by affinity chromatography using Ni-nitrilotriacetic acid resin columns (Qiagen) according to the manufacturer's instructions. The concentration of the purified rH-NS protein was determined by measuring the absorbance at 280 nm, using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The purity

of rH-NS was assessed by SDS-polyacrylamide gel electrophoresis, with only a single band visible following staining with Coomassie blue.

DNase I protection assay. DNA probes for the intergenic region of each of the hemolysin gene clusters were amplified from *V. anguillarum* genomic DNA by PCR (Table 2). Probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end (see Fig. S1 in the supplemental material). The assay was carried out using a method modified from the work of Li et al. (6). Briefly, 7.5×10^{11} copies of DNA probe and various amounts of rH-NS (up to 3 μM) were incubated for 1 h at 27°C in a total volume of 20 μl containing 4 μl of 5× binding buffer, 1 μg poly-L-lysine, and 1 μg poly(dI-dC) (DIG gel shift kit, 2nd generation; Roche Applied Science, Indianapolis, IN). The DNA-protein complex was then digested by adding 0.005 U RQ1 RNase-free DNase (New England BioLabs, Ipswich, MA) in a total volume of 25 μl containing 2.5 μl of 10× concentrated reaction buffer at 37°C for 15 min. The reaction was stopped by heating at 95°C for 10 min. The DNA was purified using a QIAquick PCR purification kit (Qiagen) and a QIAcube according to the standard protocol, except that the elution volume was adjusted to 30 μl. The DNA in the eluate (3 μl) was added to 9 μl Hi-Di formamide containing 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).

GC content plotting. The GC contents of the two gene clusters (Fig. 1) were plotted using the GC-Profile program (http://tubic.tju.edu.cn/GC-Profile) (20) with the following settings: halting parameter of 1 and minimum length to segment of 100 bp.

Fish infection studies. Various *V. anguillarum* strains were tested for virulence in rainbow trout (*Oncorhynchus mykiss*) by intraperitoneal (i.p.) injection. Briefly, *V. anguillarum* cells grown in LB20 supplemented with appropriate antibiotics for 22 h at 27°C were harvested by centrifugation (9,000 × *g*, 5 min, 4°C), washed twice in NSS, and resuspended in NSS ($\sim 2 \times 10^9$ cells ml^{-1}). Initial cell density was estimated by measurement of the OD_{600}. The actual cell density of NSS suspensions was determined by serial dilution and spot plating. All fish were examined prior to the start of each experiment to determine that they were free of disease or injury. It should be noted that all the negative-control fish survived. Fish were anesthetized with tricaine methanesulfonate (Western Chemical, Ferndale, WA) at 100 mg/liter for induction and 52.5 mg/liter for maintenance. *V. anguillarum* strains were injected i.p. into fish in 100 μl NSS vehicle. Fish that were between 15 and 25 cm long were injected with bacteria diluted with NSS at a dose of $\sim 4 \times 10^5$ CFU/fish, or with NSS only as a negative control. Ten fish were used for each experimental group. Fish inoculated with different bacterial strains were maintained in separate 10-gallon tanks with constant water flow (200 ml/min) at 19 ± 1°C. The tanks were separated to prevent possible cross-contamination. Death due to vibriosis was determined by the observation of gross clinical signs and was confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the head kidneys of dead fish. Observations were made for 14 days. All fish used in this research project were obtained from the URI East Farm Aquaculture Center. All fish infection protocols were approved by the URI IACUC.

Statistical analysis. Two-tailed Student's *t* tests assuming unequal variances were used for statistical analyses for all experiments except the fish infection experiment (*P* values of <0.05 were considered statistically significant). For fish infection experiments, a Kaplan-Meier survival analysis with the log rank significance test was performed on fish survival percentages (*P* values of <0.05 were considered statistically significant).

RESULTS

Identification of *hns* in *V. anguillarum*. The *V. anguillarum hns* gene (GenBank accession number KC795684) was found in the RAST annotation of the *V. anguillarum* M93Sm draft genome (unpublished data). It encodes a predicted 137-amino-acid protein with a molecular mass of 15,299 Da and has strong homology

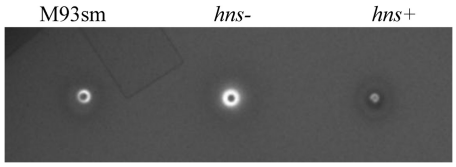

FIG 2 Hemolytic activity of *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−) and *hns*-complemented mutant (*hns*+) strains on TSA-5% sheep blood agar after 24 h at 27°C.

to H-NS proteins found in a variety of *Vibrio* species, including *Vibrio harveyi* (92% similarity and 84% identity), *Vibrio coralliilyticus* (84% similarity and 76% identity), *V. cholerae* (90% similarity and 82% identity), *V. parahaemolyticus* (90% similarity and 85% identity), and *V. vulnificus* (93% similarity and 87% identity).

Mutation of *hns* increases hemolytic activity. It was previously shown that *V. anguillarum* wild-type cells exhibit beta-hemolysis on 5% TSA-sheep blood agar (5). When the hemolytic activity of the *hns* mutant (M114) was tested on 5% TSA-sheep blood agar, it was found that mutation of *hns* resulted in increased hemolysis compared to that of the wild type (M93Sm) (Fig. 2). Furthermore, when the *hns* mutation was complemented (M116; hns^+), hemolysis was reduced to levels below those of the wild type (Fig. 2), suggesting that H-NS is a negative regulator of at least one of the two hemolysins (RtxA and Vah1). The lower hemolytic activity was probably due to the overexpression of H-NS, since pSUP202 is a multicopy plasmid.

Mutation of *hns* increases cytotoxicity against ASK cells. It was previously demonstrated that both *vah1* and *rtxA* contribute to the cytotoxicity of *V. anguillarum* cells against ASK cells (5). In order to determine whether *hns* acts to regulate cytotoxic activity, we tested the cytotoxic activities of both culture supernatants and washed cells of the *hns* mutant and the *hns*-complemented mutant (hns^+) and compared them against those of culture supernatant and washed cells of the wild type (M93Sm). The results showed that mutation of *hns* significantly increased the cytotoxicity of both *V. anguillarum* culture supernatant (>40%) and cells (MOI of 200) (~80%) against ASK cells compared to the cytotoxicity of wild-type *V. anguillarum* M93Sm. Complementation of the *hns* mutation significantly decreased cytotoxicity of both culture supernatant and cells against ASK cells compared to the cytotoxic activity of the *hns* mutant (Fig. 3A and B). These data support the suggestion that *hns* negatively regulates at least one of the two hemolytic/cytotoxic activities encoded by *rtxA* and *vah1*.

H-NS negatively regulates hemolysin genes at the transcriptional level. Since the two hemolysin/cytotoxin gene clusters are each organized into two divergent transcriptional units (Fig. 1), with intergenic regions shown to bind HlyU (6), we wanted to investigate the effects of H-NS upon the expression of the various genes within the gene clusters. Real-time qRT-PCR was performed to quantify expression of members of the hemolysin gene clusters, including *vah1*, *plp*, *rtxA*, *rtxH*, and *rtxB*, in the wild-type strain (M93Sm), the *hns* mutant, and the *hns*-complemented mutant (hns^+) during both the exponential and stationary growth phases. Real-time qRT-PCR data revealed that for the *hns* mutant compared to the wild type during the exponential and stationary growth phases, expression of *rtxA* increased 2.91- and 2.14-fold, respectively; expression of *rtxB* increased 1.28- and 1.43-fold, re-

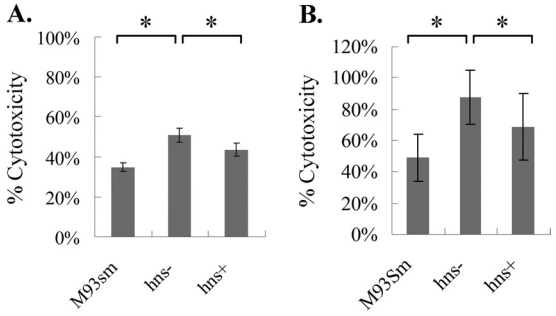

FIG 3 (A) Cytotoxicity of culture supernatants from *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−), and *hns*-complemented mutant (*hns*+) strains against ASK cells after 6 h at 20°C. (B) Cytotoxicity of washed cells of *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−), and *hns*-complemented mutant (*hns*+) strains against ASK cells after 4 h at 20°C (MOI = 200). Asterisks represent *P* values of <0.05 between two bracketed strains. Error bars represent 1 standard deviation.

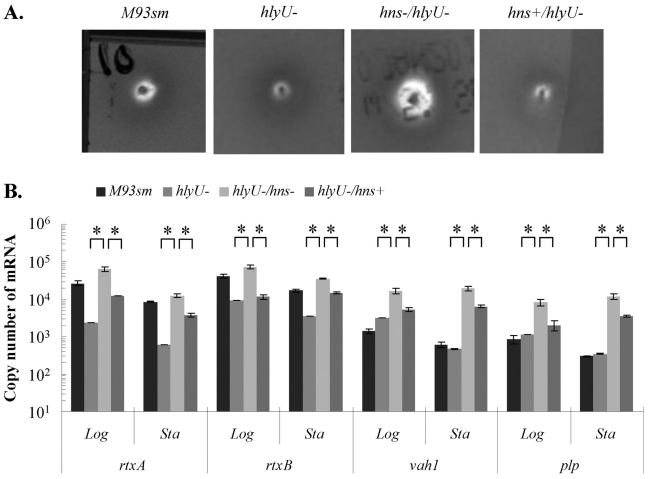

FIG 5 (A) Hemolytic activity of *V. anguillarum* wild-type (M93Sm), *hlyU* mutant (*hlyU*−), *hns hlyU* double mutant (*hns*−/*hlyU*−), and *hns*-complemented *hns hlyU* double mutant (*hns*+/*hlyU*−) strains on TSA-5% sheep blood agar after 24 h at 27°C. (B) Expression of *rtxA*, *rtxB*, *plp*, and *vah1* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm), *hlyU* mutant (*hlyU*−), *hns hlyU* double mutant (*hlyU*−/*hns*−), and *hns*-complemented *hns hlyU* double mutant (*hlyU*−/*hns*+) strains during logarithmic (Log)- and stationary (Sta)-phase growth. The data presented are representative of two independent experiments. Each value is the average for three replicates. Asterisks represent *P* values of <0.05 between two bracketed strains. Error bars represent 1 standard deviation.

spectively; expression of *rtxH* increased 4.56- and 2.39-fold, respectively; expression of *vah1* increased 16.21- and 20.01-fold, respectively; and expression of *plp* increased 31.27- and 36.88-fold, respectively (Fig. 4; see Table S1 in the supplemental material). Furthermore, complementation of the *hns* mutation down-regulated the expression of these genes back to or below wild-type levels. The data strongly suggest that H-NS is a negative regulator of gene expression from both the *rtxACHBDE* and *vah1-plp* gene clusters.

Mutation of *hns* does not affect the expression of *hlyU*. Since HlyU was previously shown to bind to the intergenic regions of both hemolysin gene clusters to increase their transcription (6), we wanted to determine whether mutation of *hns* would affect *hlyU* transcription. Real-time qRT-PCR was performed to measure the expression of *hlyU* in the wild-type strain (M93Sm), the *hns* mutant, and the *hns*-complemented mutant (hns^+) during the exponential and stationary growth phases (see Fig. S2A in the supplemental material). No statistically significant difference in expression of *hlyU* was found between M93Sm and both the *hns* mutant and the *hns*-complemented mutant (hns^+) in either the log phase or stationary phase (see Fig. S2A). These results rule out the possibility that H-NS regulates hemolysin gene expression by regulating the expression of *hlyU*.

Upregulation of hemolysin genes by *hlyU* is *hns* dependent. As noted above, Li et al. (6) showed that an *hlyU* mutant (S305) had decreased hemolytic activity on sheep blood agar compared to that of the wild type and that complementation of *hlyU* (S307) resulted in increased activity compared to that of the wild type. In an effort to determine the roles of *hns* and *hlyU* in the regulation of hemolysin gene transcription, we examined the hemolytic activity and measured the transcription of hemolysin genes (*vah1*, *plp*, *rtxA*, and *rtxB*) in each hemolysin transcriptional unit. The first set of these determinations was carried out with cells lacking a functional *hlyU* gene: the *hlyU* mutant, the *hns hlyU* double mutant (ES114), and the *hns hlyU* double mutant complemented with *hns* (ES116; hns^+) (Fig. 5). Determinations of hemolytic activity and hemolysin gene expression were also done for wild-type M93Sm.

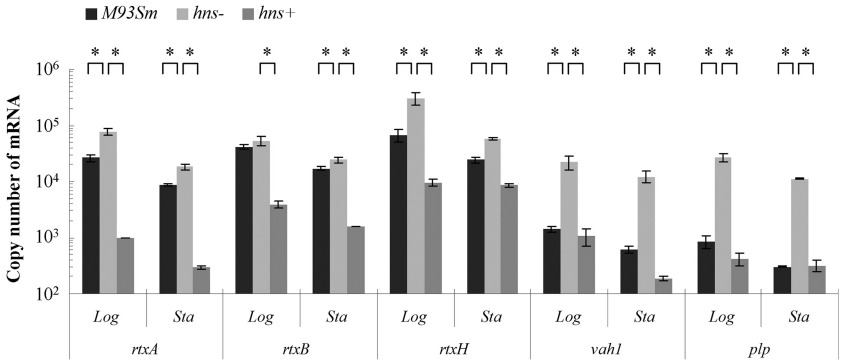

FIG 4 Expression of *rtxA*, *rtxB*, *rtxH*, *plp*, and *vah1* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−), and *hns*-complemented mutant (*hns*+) strains during logarithmic (Log)- and stationary (Sta)-phase growth. The data presented are representative of two independent experiments. Each value is the average for three replicates. Asterisks represent *P* values of <0.05 between two bracketed strains. Error bars represent 1 standard deviation.

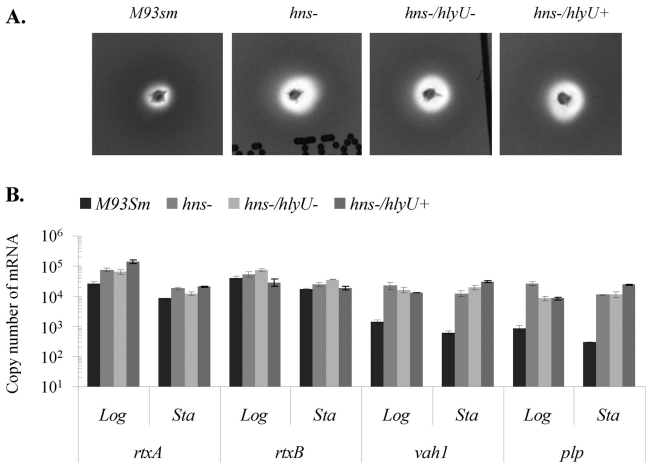

FIG 6 (A) Hemolytic activity of *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−), *hns hlyU* double mutant (*hns*−/*hlyU*−), and *hlyU*-complemented *hns hlyU* double mutant (*hns*−/*hlyU*+) strains on TSA-5% sheep blood agar after 24 h at 27°C. (B) Expression of *rtxA*, *rtxB*, *plp*, and *vah1* determined by qRT-PCR analysis of *V. anguillarum* wild-type (M93Sm), *hns* mutant (*hns*−), *hns hlyU* double mutant (*hns*−/*hlyU*−), and *hlyU*-complemented *hns hlyU* double mutant (*hns*−/*hlyU*+) strains during logarithmic (Log)- and stationary (Sta)-phase growth. The data presented are representative of two independent experiments. Each value is the average for three replicates. Error bars represent 1 standard deviation.

Hemolytic activity in the *hlyU* mutant decreased compared to that of wild-type M93Sm, as previously reported by Li et al. (6). In contrast, hemolytic activity in the *hlyU hns* double mutant increased over that in M93Sm, and when *hns* was used to complement the double mutant, hemolysis decreased to the levels seen in the *hlyU* mutant (compare Fig. 5A with Fig. 2). Changes in transcription of *rtxA*, *rtxB*, *vah1*, and *plp* corresponded with the changes in hemolysis (Fig. 5B; see Table S1 in the supplemental material). Specifically, transcription of each gene (*rtxA*, *rtxB*, *vah1*, and *plp*) increased in the absence of a functional *hns* gene and decreased in the presence of a functional *hns* gene.

The second set of determinations was carried out with cells lacking a functional *hns* gene: the *hns* mutant, the *hns hlyU* double mutant, and the *hns hlyU* double mutant complemented with *hlyU* (ES115; *hlyU*$^+$) (Fig. 6). Determinations of hemolytic activity and hemolysin gene expression were also done for wild-type M93Sm. In the absence of a functional *hns* gene, hemolytic activity increased regardless of the presence or absence of *hlyU* (Fig. 2 and 6A). Determinations of hemolysin transcription by qRT-PCR corresponded with the results of the hemolysis assay (Fig. 6B). In the absence of *hns*, *rtxA* and *rtxB* expression increased over the levels in wild-type cells. For *rtxA*, all increases were >2-fold and were significant ($P < 0.05$). For *rtxB*, increases were small (generally <2-fold) and generally not significant. The presence or absence of *hlyU* had little or no effect (<2-fold) on *rtxA* and *rtxB* gene expression (Fig. 6B; see Table S1 in the supplemental material). Similarly, in the absence of a functional *hns* gene, expression of both *vah1* and *plp* increased >9-fold in both exponential- and stationary-phase cells, regardless of the presence or absence of a functional *hlyU* gene (Fig. 6B; see Table S1). As with the *rtxACHBDE* gene cluster, our data show that in the absence of *hns*, complementation with *hlyU* gave only minimal changes in expression of both *vah1* and *plp* (around 2-fold) over that in the *hns* mutant strain, with almost no change in *vah1* and *plp* expression between the two strains (Fig. 6B; see Table S1). These data indicate that upregulation of hemolysin genes by *hlyU* is *hns* dependent.

H-NS binds to the intergenic regions of both hemolysin gene clusters. Previously, Li et al. (6) demonstrated that HlyU binds to the intergenic regions between the divergently transcribed genes of each of the two hemolysin gene clusters to upregulate gene expression. In an effort to determine whether H-NS acted in a similar fashion to help regulate expression of the hemolysin gene clusters in *V. anguillarum*, we carried out DNase I protection assays as described in Materials and Methods. The results of these experiments revealed that rH-NS protected multiple regions in both the *rtxB-rtxH* and *vah1-plp* intergenic regions (Fig. 7). These regions are AT-rich (72 to 74% AT) and correspond to other H-NS binding sites described for other bacteria (21–27) (Fig. 8). The H-NS binding sites cover the promoter regions of all four genes (*rtxB*, *rtxH*, *plp*, and *vah1*), with little or no overlap with the HlyU binding site in each of the intergenic regions (Fig. 8) (6). In the *vah1-plp* intergenic region, rH-NS bound to five sites, covering the −10 and −35 regions of both the *plp* and *vah1* promoters, but did not cover the HlyU binding site. In the *rtxB-rtxH* intergenic region, rH-NS bound to six sites, covering the −35 regions of both the *rtxB* and *rtxH* promoters. In addition, rH-NS also bound to a seventh site, just within the *rtxB* coding sequence. rH-NS was also found to protect the three *rtxB*-proximal bases of the HlyU binding site (Fig. 8).

The *vah1-plp* and *rtxACHBDE* gene clusters are unlikely to have been acquired horizontally. Recently, it was reported that a major role of H-NS proteins is to silence horizontally acquired genetic elements distinguished by AT-rich sequences (25–27). This raised the question of whether either or both of the hemolysin gene clusters (*rtxACHBDE* and *vah1-plp*) might be xenogeneic in origin. We examined the GC contents of the two gene clusters and compared them to the average GC content of the whole genome of *V. anguillarum*. The results of this examination revealed that the GC content of the *rtxACHBDE* gene cluster is 47.3% and the GC content of the *vah1-plp* gene cluster is 42.5%. Both values are very similar to the average GC content of the whole genome (44.51%) of *V. anguillarum* (28). The intergenic regions do have low GC/elevated AT percentages, with the *plp-vah1* intergenic region having 26% GC and the *rtxB-rtxH* region having 28% GC (Fig. 1). Additionally, examination of the published *V. anguillarum* 775 genome (GenBank assembly ID GCA_000217675.1) revealed that the placement of the hemolysin genes in relation to the surrounding genes, within 7.5 kbp to 10 kbp of DNA flanking each gene cluster, is the same as that found in strain M93Sm. Furthermore, we saw no evidence of any tRNA genes, transposase genes, interrupted genes, or pseudogenes in these surrounding regions. Finally, when we examined the codon usage patterns of the hemolysin genes and compared them to those for the chromosomes in which they are found (chromosome I for the *rtx* genes and chromosome II for the *plp* and *vah1* genes), no significant differences were observed. These observations suggest that while the two hemolysin gene clusters are negatively regulated by H-NS, they were not acquired horizontally.

The *hns* mutant has attenuated virulence against rainbow trout. Since the expression of both hemolysin gene clusters is affected by H-NS, we tested the virulence of M93Sm, the *hns* mutant, and the *hns*-complemented mutant (*hns*$^+$). Groups of 10 rainbow trout were infected by i.p. injection as described in Ma-

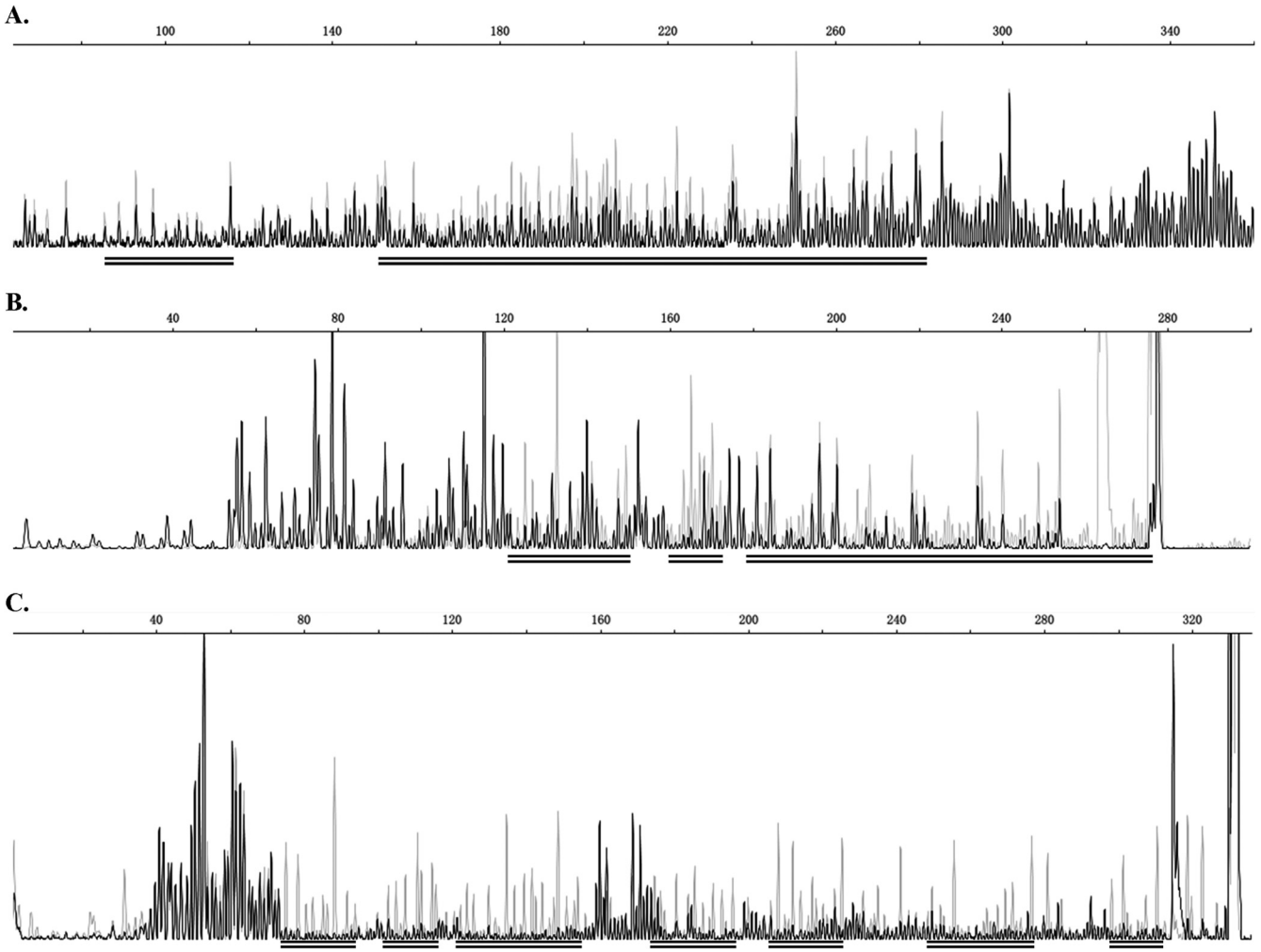

FIG 7 Capillary electrophoresis of FAM-labeled DNA probe 1 (A) and probe 2 (B), specific for the *vah1-plp* intergenic region, and probe 4 (C), specific for the *rtxB-rtxH* intergenic region, from DNase protection assays in the presence (black lines) and absence (gray lines) of rH-NS, demonstrating that H-NS binds to specific sequences in the *vah1-plp* and *rtxB-rtxH* intergenic regions and protects against DNase I digestion. DNA probes were prepared and labeled with FAM, incubated with rH-NS (0 or 3 μM) followed by DNase I, and then analyzed by DNA fragment analysis as described in Materials and Methods. The binding region sequences are indicated by double black underlining. The underlined DNA fragments indicate those that are more common in the presence of rH-NS (black lines) than in its absence (gray lines). The location of each probe is shown in Fig. S1 in the supplemental material.

terials and Methods, using wild-type M93Sm, the *hns* mutant, or the *hns*-complemented mutant of *V. anguillarum* in NSS at a dose of ~4×10^5 CFU/fish, or using NSS only as a negative control. All M93Sm-infected trout died by day 4, while 60% of *hns* mutant-infected trout died by day 14. These results (Fig. 9) show that there is a significant difference ($P = 0.005$) in virulence between the M93Sm wild type and the *hns* mutant. Complementation of *hns* restored virulence back to wild-type levels, with 90% mortality by day 4. Thus, there was a significant difference ($P = 0.029$) in the virulence of the *hns*$^+$ and *hns* mutant strains and no significant difference ($P = 0.413$) between the wild-type and *hns*$^+$ strains.

At first glance, the decline in virulence for the *hns* mutant appears to be counterintuitive, since both hemolysin gene clusters are upregulated in the *hns* mutant. However, *hns* is considered important for bacterial fitness by properly regulating virulence and other genes during growth (26, 27). To determine whether the loss of *hns* affected the growth of *V. anguillarum*, we tested the growth of M93Sm, the *hns* mutant, and the *hns*-complemented mutant (*hns*$^+$) in LB20 (see Fig. S3 in the supplemental material). While the three strains grew to nearly identical cell densities ($OD_{600} = 1.04$ for M93Sm, 0.97 for the *hns* mutant, and 0.97 for the *hns*-complemented mutant) at stationary phase, the *hns* mutant had a longer generation time than the wild type (58 min versus 48 min; $P < 0.05$). However, complementing the *hns* mutation did not result in a shorter generation time than that of the *hns* mutant (60 min versus 58 min), suggesting that there is no correlation between virulence in fish and fitness in LB20 for these three strains.

DISCUSSION

Vibriosis caused by *V. anguillarum* has been recognized as a major problem for salmonid culture due to the significant economic losses it causes (29). While this bacterium uses a variety of virulence factors, including iron transport/siderophore systems (30), the EmpA metalloprotease (31, 32), motility (33, 34), lipopolysaccharides (LPS) (33, 34), and exopolysaccharides (EPS) (35), it is

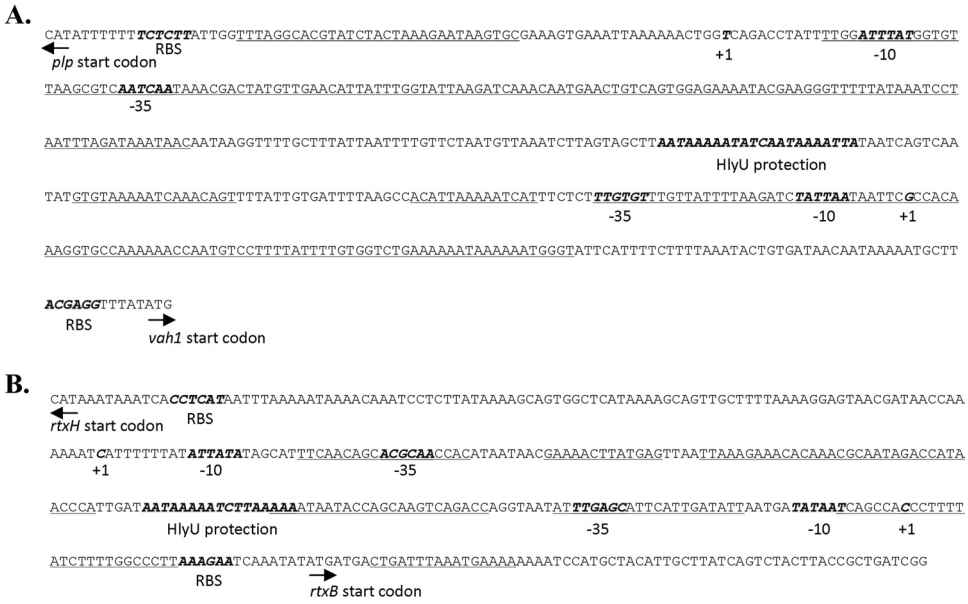

FIG 8 Intergenic regions of the *vah1-plp* (A) and *rtxACHBDE* (B) gene clusters. The transcriptional start sites are shown as bold, italicized sequences and labeled "+1." The −10 and −35 promoter sequences are shown as bold, italicized sequences and labeled "−10" and "−35." Ribosomal binding sites are shown as bold, italicized sequences and labeled "RBS." Sequences protected by HlyU are shown in bold italics and labeled "HlyU protection." Sequences protected by H-NS are underlined.

the hemolysins/cytotoxins that directly kill host cells (4, 5) and are thought to be the major contributors to the hemorrhagic septicemia that is characteristic of vibriosis (2). Previously, we identified and described three hemolysin/cytotoxin genes of *V. anguillarum* M93Sm: *vah1* (4, 36), *rtxA* (5), and *plp* (4; L. Li, X. Mou, and D. R. Nelson, unpublished data). The three hemolysin genes (and associated transport genes) are organized into two gene clusters (Fig. 1). Additionally, both hemolytic activity and expression of the three hemolysin genes (*vah1*, *plp*, and *rtxA*) are higher in log phase than in stationary phase (6). Recently, we reported that HlyU positively regulates the expression of both hemolysin gene clusters by specifically binding to the *vah1-plp* and *rtxB-rtxH* intergenic regions (6).

In this study, we examined the role of H-NS in the regulation of hemolysin activity and gene expression in *V. anguillarum* M93Sm. Initially, the *hns* homologue in *V. anguillarum* was identified using the *V. anguillarum* M93Sm draft genome, and an *hns* mutant and *hns*-complemented strain were constructed. Mutation of *hns* resulted in increased hemolytic activity on 5% TSA-sheep blood agar, while complementation of the *hns* mutation reduced hemolysis to levels below those of the wild type (Fig. 2). Mutation of *hns* also increased the cytotoxicity of both *V. anguillarum* culture supernatant (diluted 1:1 with PBS) and *V. anguillarum* cells (at an MOI of 200) against ASK cells, while complementation of the *hns* mutation reduced cytotoxic activity (Fig. 3). Transcription of the three hemolysin genes (and related *rtx* genes) in the presence and absence of *hns* corresponded with hemolysin and cytotoxin activity, with increased transcription in the *hns* mutant and decreased transcription in the *hns*-complemented mutant (Fig. 4; see Table S1 in the supplemental material). These data show that H-NS is a negative regulator of hemolytic and cytotoxic activity by acting as a repressor of hemolysin gene expression.

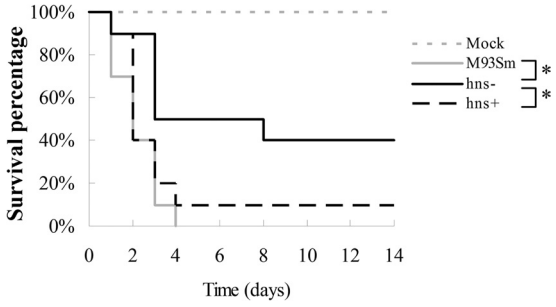

FIG 9 Survival percentages for rainbow trout injected i.p. with NSS only (mock; gray dashed line) or with ~4 × 10^5 CFU/fish of wild-type M93Sm (gray solid line), the *hns* mutant (*hns*−; black solid line), or the *hns*-complemented mutant (*hns*+; black dashed line). Each experimental group had 10 fish. *V. anguillarum* cells were suspended in NSS. Asterisks represent *P* values of <0.05 between two bracketed strains.

The results presented here correspond with the well-documented role of H-NS as a repressor and silencer of many genes in Gram-negative bacteria, especially in the repression of virulence genes (8). Recently, Liu et al. (12) demonstrated that expression of *rtxA1* in *Vibrio vulnificus* is repressed by H-NS and that HlyU acts as an antirepressor by interfering with H-NS binding to the upstream regulatory region of *rtxA1*. Specifically, data from competitive gel mobility shift assays between HlyU and H-NS showed that HlyU could displace H-NS from the promoter, with binding at a low concentration, and that H-NS needs a much higher concentration to displace the bound HlyU protein (12). Similarly, our data show that H-NS represses expression from both hemolysin gene clusters in *V. anguillarum*, regardless of the presence of *hlyU*, and that upregulation of hemolysin gene expression by HlyU is dependent upon the presence of *hns* (Fig. 5 and 6; see Table S1). These observations strongly suggest that in *V. anguillarum*, H-NS functions to repress transcription of both hemolysin gene clusters

and that HlyU acts as an antirepressor. Additionally, both gene clusters are arranged as divergently transcribed genes, with one HlyU binding site in the center of each intergenic region (Fig. 8) (also see Fig. 7 in the work of Li et al. [6]) flanked by 2 to 4 H-NS binding sites that extend toward the promoter sites (Fig. 8). The sites protected by rH-NS (Fig. 8) are AT-rich. The five H-NS binding sites in the *vah1-plp* intergenic region have A+T% values that range from 64.5% to 84.3%, while the seven H-NS-protected sites in the *rtxB-rtxH* intergenic region have A+T% values that range from 47.4% to 81.25%. In contrast, the flanking structural genes have much lower A+T% values. The A+T% for *plp* is 57%, that for *vah1* is 55.7%, that for *rtxACH* is 51.6%, and that for *rtxBDE* is 54.5% (Fig. 1). This reveals an interesting discontinuity between the structural genes and the intergenic regulatory regions. A similar discontinuity is also seen between the *rtx* structural genes and the intergenic regions in *V. vulnificus* and *V. cholerae*. Additionally, the fact that the structural genes have A+T% values nearly identical to that for the whole genome of *V. anguillarum* (55.49%) suggests that these virulence genes were not acquired horizontally. This is further supported by the observations detailed above showing that there is no evidence of any tRNA genes, transposase genes, interrupted genes, or pseudogenes in the 7.5 to 10 kbp of DNA flanking the hemolysin gene clusters. Furthermore, codon usage in the hemolysin genes is not significantly different from that in the chromosomes in which the gene clusters reside.

It has been suggested that self-oligomerization and binding of H-NS to AT-rich DNA to form DNA-protein-DNA bridges impede the movement of RNA polymerase, thus repressing gene expression (8). H-NS repression may be reversed by different mechanisms (8). In *V. cholerae*, the binding site of the transcriptional activator ToxT overlaps H-NS binding sites. ToxT displaces H-NS and directly activates transcription (15). In contrast, the activation of *pagC* and *ugtL* transcription from H-NS-mediated repression in *Salmonella enterica* requires both SlyA and PhoP, with SlyA displacing H-NS and PhoP acting as a transcriptional activator (37). There are several SlyA and H-NS binding sites in the *pagC* promoter region, with little or no overlap between the sites (37). In *V. vulnificus*, the binding site of the antirepressor HlyU is far upstream of the transcription start site (positions −376 to −417) for the *rtxA1* operon and overlaps two H-NS binding sites (12). Binding of HlyU to DNA relieves the H-NS repression at all H-NS binding sites of the *rtxA1* promoter region (12). Our results demonstrate that in *V. anguillarum*, HlyU relieves H-NS repression but does not act to directly activate transcription. We have not yet identified a transcriptional activator of hemolytic activity. However, our data do indicate that transcription from both hemolysin gene clusters is higher during exponential-phase growth than during stationary-phase growth. Additionally, the binding sites for HlyU and H-NS in the *V. anguillarum rtxH-rtxB* intergenic region are much closer to the +1 transcription start sites than is the case in *V. vulnificus* CMCP6. In *V. vulnificus*, the five H-NS binding sites are at positions −289 to −459 relative to the transcription start site of *rtxH* (vv20481) (12), while the H-NS binding sites in *V. anguillarum* are at positions −23 to −100 for *rtxH*-proximal sites and positions +42 to −70 for *rtxB*-proximal sites (Fig. 8). These differences probably reflect the relative sizes of the intergenic regions for each organism. In *V. vulnificus* CMCP6, the intergenic region between *rtxH* and *rtxB* is 1,028 nucleotides (nt), while in *V. anguillarum* M93Sm, the intergenic region is only 325 nt. It would be interesting to examine the H-NS and HlyU binding sites in the *rtx* intergenic region of *V. vulnificus* YJ016, which is only 362 nt.

Although mutation of *hns* resulted in increased expression and activities of the three hemolysins (RtxA, Vah1, and Plp), the overall virulence of the *hns* mutant was slightly attenuated in rainbow trout. Similar results were observed in uropathogenic *Escherichia coli*. Mice injected intravenously with 10^8 CFU of the *hns* mutant had a higher survival rate than those injected with the wild type, although the mutant showed a higher level of alpha-hemolysin expression and activity (38). Our data suggest that there is no correlation between virulence in fish and fitness in LB20, but it should be noted that growth in LB20 is very different from growth in fish. It is likely that removal of H-NS-mediated repression/gene silencing results in an unfavorable alteration of virulence gene expression and a reduction in fitness in the host environment.

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