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Xiangyu Mou

University of Rhode Island, mouxiangyu@gmail.com

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REGULATION OF HEMOLYSIN GENE EXPRESSION AND THE EFFECTS OF
METABOLISM MUTATIONS ON THE VIRULENCE OF VIBRIO
ANGUILLARUM

BY
XIANGYU MOU

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN
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2013

DOCTOR OF PHILOSOPHY DISSERTATION
OF
XIANGYU MOU

APPROVED:

Dissertation Committee:

Major Professor David R. Nelson

Paul S. Cohen

David C. Rowley

Nasser H. Zawia

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2013

Abstract

Vibrio anguillarum is the causative agent of vibriosis, a fatal hemorrhagic septicemic disease. *V. anguillarum* infects more than 50 fresh and salt-water fish species including various species of economic importance to the larviculture and aquaculture industry. In vibriosis, *V. anguillarum* invades its host fish through the intestine and skin. Infected fish usually die with systemic infection of *V. anguillarum*. Bacterial hemolysins are exotoxins that cause lysis of erythrocytes in the host and thus the release of the intracellular heme, and are therefore identified as important virulence factors. Moreover, hemolysins are known to also cause lysis in other cell types, including mast cells, neutrophils and polymorphonuclear cells. Two hemolysin gene clusters, *vah1-plp* and *rtxACHBDE*, have been previously identified and described. The activities of the protein encoded by the *plp* gene were not known.

In the first manuscript, we describe the biochemical activities of the *plp*-encoded protein and its role in pathogenesis. The *plp* gene, one of the components in *vah1* cluster, encodes a 416-amino-acid protein (Plp), which has homology to lipolytic enzymes containing the catalytic site amino acid signature SGNH. Hemolytic activity of the *plp* mutant increased 2-3-fold on sheep blood agar indicating that *plp* represses *vah1*; however, hemolytic activity of the *plp* mutant decreased by 2-3-fold on fish blood agar suggesting that Plp has different effects against erythrocytes from different species. His₆-tagged recombinant Plp protein (rPlp) was over-expressed in *E. coli*. Purified and re-folded active rPlp exhibited phospholipase A2 activity against phosphatidylcholine and no activity against phosphatidylserine,

phosphatidylethanolamine, or sphingomyelin. Characterization of rPlp revealed broad optimal activities at pH 5–9 and at temperatures of 30–64°C. Divalent cations and metal chelators did not affect activity of rPlp. We also demonstrated that Plp was secreted using thin layer chromatography and immunoblot analysis. Additionally, rPlp had strong hemolytic activity towards rainbow trout erythrocytes, but not to sheep erythrocytes suggesting that rPlp is optimized for lysis of phosphatidylcholine-rich fish erythrocytes. Further, only the loss of the *plp* gene had a significant effect on hemolytic activity of culture supernatant on fish erythrocytes, while the loss of *rtxA* and/or *vahI* had little effect. However, *V. anguillarum* strains with mutations in *plp* or in *plp* and *vahI* exhibited no significant reduction in virulence compared to the wild type strain when used to infect rainbow trout.

In the next manuscript, 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 ~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 and cytotoxic activity. Both semi-quantitative RT-PCR and real time RT-qPCR 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 *vahI* and *plp* was not affected in the *hlyU* mutant. Complementation of the *hlyU*

mutation restored expression of the *rtx* genes and increased *vahI* and *plp* expression to levels higher than in the wild type. The transcriptional start sites in the intergenic regions of both *vahI/plp* and *rtxH/rtxB* genes were determined using 5'-RACE and the binding sites for purified HlyU was discovered using DNA gel mobility shift experiments and DNase protection assays.

In the third manuscript, 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 near wild type levels. Further, expression of *rtxA*, *rtxH*, *rtxB*, *vahI* and *plp* increased in the *hns* mutant, and decreased in the complemented *hns* mutant strain when compared to the wild type strain. Additionally, experiments using DNase I, showed that purified recombinant H-NS protected multiple sites in the promoter region 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 up-regulation 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.

In the last manuscript, we aimed to create avirulent and immunogenic *V.*

anguillarum strains that can be used as a live vaccine for fish, without knocking out any of the hemolysin genes. For this purpose, six genes (*mdh*, *icd*, *sucA*, *sucC*, *sdhC*, and *fumA*) encoding enzymes in tricarboxylic acid (TCA) cycle and one gene (*cra*) encoding a fructose metabolism repressor were identified and mutated in wild type *V. anguillarum* M93Sm (serotype O2a). Among all mutants, *icd* mutant showed high attenuation of virulence and lowest cell density limit in two forms of rich media. All mutants exhibited the same or higher levels of hemolysin gene expression compared to wild type during log phase. Further, fish that were pre-treated by immersion with *icd* mutant protected rainbow trout from the subsequent challenge of *V. anguillarum* M93Sm; and fish that were pre-treated with injection of the *icd* mutant elicited cross-serotype immunity against the subsequent challenge of *V. anguillarum* NB10 (serotype O1). The results suggest the TCA cycle mutation approach is likely to be an easy method to construct modified live vaccine for a wide variety of pathogenic bacteria.

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PREFACE

This dissertation has been prepared in the Manuscript Format according to the guidelines of the Graduate School of the University of Rhode Island. The first manuscript: “Characterization of Plp, a phosphatidylcholine-specific phospholipase and hemolysin of *Vibrio anguillarum*” was submitted to *BMC Microbiology* on August, 12th, 2013 and is in review. The second manuscript: “HlyU is a positive regulator of hemolysin expression in *Vibrio anguillarum*” was published in *Journal of Bacteriology* in 2011. The third manuscript: “H-NS is a Negative Regulator of the Two Hemolysin/Cytotoxin Gene Clusters in *Vibrio anguillarum*” was published in *Infection and Immunity* in 2013, and the fourth manuscript: “A *Vibrio anguillarum* isocitrate dehydrogenase mutant is highly attenuated and immunogenic in rainbow trout (*Oncorhynchus mykiss*).” has been written in the same form as the third manuscript and will be submitted for publication.

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Manuscript I

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Title: Characterization of Plp, a phosphatidylcholine-specific phospholipase and hemolysin of *Vibrio anguillarum*

Authors: Ling Li^{1†}, Xiangyu Mou[†], and David R. Nelson*

Author Affiliations: Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI 02881

Key Words: *Vibrio anguillarum*, vibriosis, phospholipase, hemolysis, virulence

***Corresponding author:** David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

[†]Both authors contributed equally to this investigation.

Abstract

Background

Vibrio anguillarum is the causative agent of vibriosis in fish. Several extracellular proteins secreted by *V. anguillarum* have been shown to contribute to virulence. While two hemolysin gene clusters, *vah1-plp* and *rtxACHBDE*, have been previously identified and described, the activities of the protein encoded by the *plp* gene were not known. Here we describe the biochemical activities of the *plp*-encoded protein and its role in pathogenesis.

Results

The *plp* gene, one of the components in *vah1* cluster, encodes a 416-amino-acid protein (Plp), which has homology to lipolytic enzymes containing the catalytic site amino acid signature SGNH. Hemolytic activity of the *plp* mutant increased 2-3-fold on sheep blood agar indicating that *plp* represses *vah1*; however, hemolytic activity of the *plp* mutant decreased by 2-3-fold on fish blood agar suggesting that Plp has different effects against erythrocytes from different species. His₆-tagged recombinant Plp protein (rPlp) was over-expressed in *E. coli*. Purified and re-folded active rPlp exhibited phospholipase A2 activity against phosphatidylcholine and no activity against phosphatidylserine, phosphatidylethanolamine, or sphingomyelin. Characterization of rPlp revealed broad optimal activities at pH 5–9 and at temperatures of 30–64°C. Divalent cations and metal chelators did not affect activity of rPlp. We also demonstrated that Plp was secreted using thin layer chromatography and immunoblot

analysis. Additionally, rPlp had strong hemolytic activity towards rainbow trout erythrocytes, but not to sheep erythrocytes suggesting that rPlp is optimized for lysis of phosphatidylcholine-rich fish erythrocytes. Further, only the loss of the *plp* gene had a significant effect on hemolytic activity of culture supernatant on fish erythrocytes, while the loss of *rtxA* and/or *vahI* had little effect. However, *V. anguillarum* strains with mutations in *plp* or in *plp* and *vahI* exhibited no significant reduction in virulence compared to the wild type strain when used to infect rainbow trout.

Conclusion

The *plp* gene of *V. anguillarum* encoding a phospholipase with A2 activity is specific for phosphatidylcholine and, therefore, able to lyse fish erythrocytes, but not sheep erythrocytes. Mutation of *plp* does not affect the virulence of *V. anguillarum* in rainbow trout.

Background

Vibrio anguillarum, a highly motile marine member of the γ -Proteobacteria, is one of the causative agents of vibriosis, a fatal hemorrhagic septicemic disease of both wild and cultured fish, crustaceans, and bivalves [1]. Fish infected with *V. anguillarum* display skin discoloration and erythema around the mouth, fins, and vent. Necrotic lesions are also observed in the abdominal muscle [2]. Mortality rates in infected fish populations range as high as 30-100% [1, 3]. Vibriosis has caused severe economic losses to aquaculture worldwide [1, 3] and affects many farm-raised fish including Pacific salmon, Atlantic salmon, sea bass, cod, and eel [3, 4]. *V. anguillarum* enters its fish host through the gastrointestinal tract (GI) and quickly colonizes this nutrient rich environment [2, 5]. Garcia *et al.* [6] have shown that *V. anguillarum* grows extremely well in salmon intestinal mucus and that mucus-grown cells specifically express a number of different proteins, including several outer membrane proteins [6] and the extracellular metalloprotease EmpA [2, 5].

Several genes have been reported to be correlated with virulence by *V. anguillarum*, including the *vahI* hemolysin cluster [7, 8], the *rtx* hemolysin cluster [9], the siderophore mediated iron transport system [10], the *empA* metalloprotease [2, 5], and the *flaA* gene [11]. Hemolytic activity of *V. anguillarum* has been considered to be the virulence factor responsible for hemorrhagic septicemia during infection [10]. We have identified two hemolysin gene clusters in *V. anguillarum* that contribute to the virulence of this pathogen [8, 9]. One gene cluster, *rtxACHBDE*, encodes a MARTX

toxin and its type I secretion system [9]. The second hemolysin gene cluster in *V. anguillarum* strain M93Sm contains the hemolysin gene *vah1* linked to two putative lipase-related genes (*llpA* and *llpB*) and a divergently transcribed hemolysin-like gene (*plp*) that appears to function as a repressor of hemolytic activity [8]. The *plp*-encoded protein has very high sequence similarity to phospholipases found in other pathogenic *Vibrio* species [8]. However, the enzymatic characteristics of Plp in *V. anguillarum* were not described.

Generally, phospholipases are divided into several subgroups depending on their specificity for hydrolysis of ester bonds at different locations in the phospholipid molecule. Phospholipases A (PLAs) cleave long chain fatty acids at sn-1 (PLA1) or sn-2 (PLA2) position from phospholipid to yield lysophospholipid and free fatty acid; phospholipases C (PLCs) cleave phospholipid into diacylglycerol and a phosphate-containing head group; and phospholipases D (PLDs) cleave phospholipid into phosphatidic acid and an alcohol. It is known that some phospholipid products are used as secondary messages, which play a central role in signal transduction [12].

In this study, we determined that *plp* encodes a phospholipase A2 in *V. anguillarum*, and then purified recombinant Plp protein (rPlp) from *E. coli* to investigate biochemical characteristics. We also described the contribution and specificity of rPlp for hydrolysis of phospholipids, and its ability to lyse fish erythrocytes.

Results

Identification of a putative phospholipase gene *plp*. Previously, a putative phospholipase gene, *plp*, was identified in the *vah1* hemolysin cluster of *V. anguillarum* strain M93Sm [8]. The 1251-bp *plp* gene (Genbank accession EU650390) was predicted to encode a protein of 416 amino acids. A BLASTx [13] search revealed that the deduced Plp amino acid sequence exhibited homology with many lipolytic enzymes including the phospholipase/lecithinase/hemolysin of *Vibrio vulnificus* (identity, 69%; similarity, 82%); the lecithin-dependent hemolysin (LDH)/thermolabile hemolysin (TLH) of *Vibrio parahaemolyticus* (identity, 64%; similarity, 80%); the lipolytic enzyme/hemolysin VHH of *Vibrio harveyi* (identity, 63%; similarity, 78%); and the thermolabile hemolysin of *Vibrio cholerae* (identity, 62%; similarity, 78%); The phylogenetic tree created by the Clustal-W program from 17 Plp homologous proteins revealed that while the most closely related Plp proteins were all from pathogenic members of the genus *Vibrio*, the Plp of *V. anguillarum* was an outlier among the *Vibrio* species (Fig. 1). According to Flieger's classification [14, 15], the alignment of Plp with other homologous proteins indicated that Plp could be classified into subgroup B of this lipolytic family with its long N-terminal tail prior to the block I [14]. Additionally, close examination of the amino acid sequences of these enzymes revealed that the typical GDSL motif for lipolytic enzymes is not totally conserved in all of these 17 proteins, in which leucines (L) are replaced with isoleucines (I) in *Photobacterium*, *Marinomonas*, and *Shewanella* (Fig. 1). In this case, *V. anguillarum* Plp should be considered as a member of the SGNH hydrolase

family, based on the Molgaard's suggestion that only four amino acids (S, G, N, and H) are completely conserved among the GDSL proteins [16].

Plp affects hemolysis of fish erythrocytes. The hemolysin gene *vah1* is divergently transcribed from *plp* [17]. Mutation of *plp* increased hemolytic activity by 2-3-fold on Trypticase soy agar plus 5% sheep blood (TSA-sheep blood) plate compared with wild type strain (M93Sm) (Fig. 2A) [8]. Rock and Nelson [8] also demonstrated that the *plp* mutant had increased *vah1* transcription (by 2-4-fold), indicating that Plp is a putative repressor of *vah1*. Previously, we demonstrated that a double mutant in *vah1* and *rtxA* resulted in a hemolysis negative mutant when plated on TSA-sheep blood agar [9]. Similar results were observed when using Luria-Bertani broth plus 2% NaCl plus 5% sheep blood (LB20-sheep blood) agar (data not shown). However, on LB20 plus 5% rainbow trout blood (LB20-rainbow trout blood) agar, the *plp* mutant exhibited a smaller zone of hemolysis compared to wild type strain M93Sm (Fig. 2B); complementation of *plp* restored the hemolytic activity of the mutant strain (Fig. 2B). Similar results were observed when using LB20 plus 5% Atlantic salmon blood agar (data not shown), suggesting that the ability of Plp to lyse erythrocytes is dependent upon the source of erythrocytes and, therefore, their lipid composition.

Plp has phospholipase A2 activity. Thin layer chromatography (TLC) was used to examine the pattern of phospholipid cleavage by Plp. BODIPY-labeled phosphatidylcholine (BPC) was incubated with various enzyme standards, including

phospholipase A2 (PLA2), phospholipase C (PLC), or phospholipase D (PLD). TLC analysis revealed distinct cleavage patterns (Fig. 3A) by these standard enzymes indicating that BPC was an appropriate substrate to examine Plp activity. Cell lysate prepared from *E. coli* strain S299, which contains the shuttle plasmid pSUP202-*plp* that was able to complement the *plp* mutation in *V. anguillarum* [8], cleaved BPC to yield BODIPY-lysophosphatidylcholine (BLPC) (Fig. 3B Lane 5) plus unlabeled free fatty acid (FFA) that is not detected. The cleavage products were identical to those generated by PLA2 (Fig. 3A) and demonstrate that Plp has phospholipase A2 activity. Additionally, the culture supernatant from S299 had only ~5% of the activity of that in cell lysate, indicating that Plp accumulated in the cell lysate instead of being secreted by the *E. coli* strain. No phospholipase activity was detected in PBS buffer or in *E. coli* DH5 α containing only the pSUP202 vector (Fig. 3B). Further, phospholipase A2 activity was examined in various subcellular fractions prepared from *E. coli* strain S299, including cytoplasmic, cytoplasmic membrane, and outer membrane fractions. Most Plp activity was detected in Tween-20 soluble membrane fraction, indicating that Plp was mainly localized in the cytoplasmic membrane of *E. coli* S299 (data not shown). No BODIPY-labeled free fatty acid (FFA) (at sn-1 position) was detected in the TLC analysis when an apolar solvent was used (data not shown), and BODIPY-labeled LPC was not further degraded by Plp in the reaction, indicating that Plp had no lysophospholipase or phospholipase B activity.

Enzymatic characteristics of rPlp protein. To examine the enzymatic characteristics of Plp, the entire coding sequence of *plp* was cloned and inserted into the expression

vector pQE60, which adds a His₆ (His-6×) tag to the carboxyl end of Plp. The over-expressed recombinant Plp (rPlp) formed inclusion bodies in *E. coli*. To recover active rPlp, purification of the inclusion bodies followed by solubilization under mild conditions and re-folding was performed (16) as described in the Materials and Methods. Purity of refolded rPlp protein was confirmed by SDS-PAGE and silver staining (data not shown). The final concentration of purified rPlp protein was 8 µg/ml with a recovery of <10%.

Subsequently, the enzymatic characteristics of refolded rPlp were examined under various chemical and physical conditions. The enzymatic activity of rPlp was positively correlated to its concentration from 1 µg/ml to 8 µg/ml (Fig. 4A); therefore, 4 µg/ml rPlp protein was routinely used in other activity assays. The enzymatic activity unit of refolded rPlp (1 unit = amount of protein that cleaves 1 µmole of BODIPY-PC per minute) was about 2,500-fold higher than standard PLA2 enzyme extracted from porcine pancreas (Sigma# P8910), which indicated that Plp had a high activity against the BPC phospholipid substrate. Plp enzyme activity exhibited a broad temperature optimum from 37°C to 64°C (Fig. 4B) with 75% activity retained at 27°C and 50% activity at 20°C. While rPlp activity rapidly decreased at temperatures above 70°C, the enzyme retained full activity at 64°C for at least 1 h. The data demonstrate that rPlp is a relatively thermostable phospholipase.

The effect of pH on enzyme activity was determined for pH values ranging from 2 to 12. The data showed that rPlp had a broad pH optimum from pH 5.3 to pH 8.7 with activity dropping off rapidly at pH values above and below the optimum (Fig. 4C). rPlp activity was not affected by treatment with the chelating reagents EGTA (Fig. 4D) or EDTA (data not shown) at concentrations up to 100 mM. Additionally, treatment with divalent metal ions, such as calcium or magnesium had no effect on activity (data not shown).

Plp is a secreted protein in *V. anguillarum*. Subcellular fractions from *V. anguillarum* strains M93Sm and S262 (*plp*) were prepared and phospholipase A2 activity examined using BPC and TLC. Initial studies revealed that at 37°C phospholipase A2 activity was detected in all cell fractions, including the culture supernatant, periplasm, cytoplasm, cytoplasmic membrane, and outer membrane, from both M93Sm and S262 (Fig. 5A). However, when the assay was performed at 64°C (to inactivate heat labile phospholipases), phospholipase A2 activity in S262 was significantly decreased in all fractions including the supernatant (Fig. 5B). Additionally, when the assay was performed at 64°C for M93Sm subcellular fractions, only the culture supernatant exhibited phospholipase activity against BPC (about 100-fold higher activity compared to the phospholipase activity of the S262 supernatant). The data demonstrated that Plp was secreted into the culture supernatant of *V. anguillarum*. TLC results also revealed that there was at least one other protein in *V. anguillarum* M93Sm exhibiting phospholipase A2 activity besides

the secreted, heat stable Plp protein. This was a thermolabile PLA2 activity inactivated at 64°C.

In order to confirm that Plp was localized in the supernatant of *V. anguillarum*, protein samples prepared from various subcellular fractions were separated by SDS-PAGE and analyzed by western blot analysis using polyclonal rabbit anti-Plp antiserum. An immuno-reactive band of ~45 kDa was detected only in the supernatant of M93Sm, but was absent in the supernatant of *plp* mutant (Fig. 5C). Taken together with the phospholipase A2 activity data, these data indicate that Plp is a secreted protein in *V. anguillarum*.

rPlp has a specific activity against phosphatidylcholine. Various fluorescently-labeled phospholipid substrates (described in Materials and Methods) were used to determine the specificity of the rPlp protein. rPlp exhibited high activity against phosphatidylcholine, cleaving BPC to yield BLPC and free fatty acid (Fig. 3 and 6A). However, rPlp had almost no activity against both NBD-phosphatidylethanolamine (NBD-PE) (Fig. 6B) and NBD-phosphatidylserine (NBD-PS) (Fig. 6C), showing only 2% and 5%, respectively, of the activity of the standard PLA2 protein against each of the substrates. The data indicated that the rPlp protein does not efficiently cleave either phosphatidylethanolamine or phosphatidylserine. Additionally, unlike the standard sphingomyelinase (Sigma), rPlp was not able to cleave the NBD-sphingomyelin into the NBD-ceramide and

phosphocholine (Fig. 6D), indicating that rPlp had no sphingomyelinase activity. Taken together, the data demonstrated that Plp is a phosphatidylcholine-specific PLA2 enzyme.

rPlp is able to lyse the fish erythrocytes directly. Membrane phospholipid compositions are quite varied among the animal species, especially for phosphatidylcholine. It is known that phosphatidylcholine makes up 58% of the total phospholipid in fish erythrocytes [18]; however, no phosphatidylcholine is found in sheep erythrocytes [19]. In order to determine whether the differential hemolysis observed for *plp* mutants of *V. anguillarum* (Fig. 2) is due to the activity of Plp against PC, we tested the ability of purified rPlp to lyse Atlantic salmon erythrocytes. Addition of recombinant Plp resulted in the lysis of Atlantic salmon erythrocytes, with the amount of lysis directly related to the amount of rPlp added to the blood suspension (Fig. 7). In contrast, addition of rPlp to a suspension of sheep erythrocytes resulted in no lysis of those cells (Fig. 7). These data show that Plp has phosphatidylcholine-specific phospholipase A2 activity and can directly lyse fish erythrocytes.

Plp is one of the hemolysins of *V. anguillarum*. Previously, we demonstrated that there are two major hemolysin gene clusters in the M93Sm, the *vah1* cluster [8] and the *rtxA* cluster [9]. Mutation of both *vah1* and *rtxA* completely eliminated the hemolytic activity of M93Sm on TSA-sheep blood agar [9]. While, mutation of the

plp gene resulted in 2-3-fold increased hemolytic activity on TSA-sheep blood agar, with *vah1* expression increased both transcriptionally and translationally in the *plp* mutant, indicating that Plp is a putative repressor of *vah1* [9], Plp also has hemolytic activity against fish erythrocytes due to its phosphatidylcholine-specific activity (Figs. 6 and 7). To investigate the relationship of the three hemolysins, culture supernatants obtained from various *V. anguillarum* strains (Table 1) were used to examine the hemolytic activity against the fish blood (Table 2).

In contrast to the strong hemolytic activity against 5% rainbow trout blood mixed with culture supernatant from the wild type strain M93Sm, hemolytic activity of culture supernatant from strain S262 (*plp*) declined by >70% (Table 2). Additionally, all mutants containing a knockout of *plp* exhibited significant declines ($P < 0.05$) in hemolytic activity. The triple hemolysin mutant, XM90 (*plp vah1 rtxA*) had no ability to lyse fish erythrocytes (Table 2). However, mutations in either *vah1* or *rtxA*, but not *plp*, resulted in little or no decline in hemolytic activity against fish erythrocytes compared to supernatants from wild type cells (Table 2). Further, complementation of *plp* restored the hemolytic activity of supernatants from both the *plp*-complemented strains (XM31, *plp*⁺ and XM93, *vah1 rtxA plp*⁺) (Table 2). Taken together, these data clearly demonstrate that Plp is the major hemolytic enzyme responsible for the lysis of fish erythrocytes by culture supernatants of *V. anguillarum*.

Plp is not a major virulence factor for *V. anguillarum* during fish infection. In order to determine whether the *plp* gene affects virulence in fish, an infection study was performed by inoculating rainbow trout by IP injection with either the wild type strain M93Sm or mutant strains S262 (*plp*) or JR03 (*vah1plp*). The results of this experiment (Fig. 8) indicated that there were no statistical differences in mortality between the three strains. This suggested that mutation of either or both *plp* or *vah1* did not decrease the virulence of M93Sm. These results are consistent with our previous observations that *rtxA* is a major virulence factor of M93sm and that mutation of *vah1* does not affect virulence [8], and demonstrate that Plp is not a major virulence factor in the *V. anguillarum* M93Sm.

Discussion

In this report, we describe the characteristics of the *V. anguillarum* phospholipase protein (Plp) encoded by *plp*, and its contribution to the hemolytic activity of *V. anguillarum*. Specifically, we show that Plp is a secreted phospholipase with A2 activity with specificity for phosphatidylcholine. The enzyme has a broad temperature optimum (37 – 64°C) and a broad pH optimum (pH5.5 – 8.7). Phospholipases are broadly distributed among the *Vibrionaceae* and often contribute to the virulence of the pathogenic members of this family. For example, the TLH (synonym: lecithin-dependent hemolysin, LDH) of *V. parahaemolyticus* [20-22] was the first well-studied lecithin-dependent PLA / lysophospholipase [23]. A lecithinase (encoded by *lec*) was also identified in *V. cholerae* [24]. Fiore *et al*[24] found that a *lec* mutant strain was unable to degrade lecithin and the culture supernatant exhibited decreased cytotoxicity. However, the mutant did not exhibit decreased fluid accumulation in a rabbit ileal loop assay, suggesting that fluid accumulation in animals is not affected by lecithinase activity. Additionally, the phospholipase A (PhlA) in *V. mimicus* was found to exhibit hemolytic activity against trout and tilapia erythrocytes and was cytotoxic to the fish cell line CHSE-214 [25]. Recently, the *V. harveyi* hemolysin (VHH) was shown to be a virulence factor during flounder infection and also had phospholipase activity on egg yolk agar [26]. Rock and Nelson [8] reported that the putative phospholipase gene (*plp*) from *V. anguillarum* exhibits 69% amino acid identity with the *V. cholerae* *lec* gene. Both *plp* and *lec* are located divergently adjacent to a hemolysin gene (*vah1* and *hlyA*, respectively) [8, 24]. Additionally, data strongly suggested that functional *plp* repressed transcription of its adjacent

hemolysin gene, *vah1*, in *V. anguillarum*[8]. However, the enzymatic characteristics of Plp in *V. anguillarum* were not described.

Usually, phospholipases are divided into phospholipases A (A1 and A2), C, and D according to the cleavage position on target phospholipids. Most of lipolytic enzymes contain a putative lipid catalytic motif (GDSL) that was previously demonstrated in other bacterial and eukaryotic phospholipases [27]. However, Molgaard [16] proposed that four amino acid residues (SGNH) form a catalytic site, and are conserved in all members of the phospholipase family; therefore, phospholipases were re-named as a SGNH family. Multiple alignment analysis of 17 phospholipase homologues (Fig. 1) demonstrates that *V. anguillarum* Plp belongs to SGNH hydrolase family, instead of GDSL family, since the GSDL motif was not fully conserved in these proteins (Fig. 1). Recently, it was reported that mutation of the serine residue in the SGNH motif resulted in the complete loss of the phospholipase and hemolytic activities of VHH in *V. harveyi* [28] demonstrating the importance of this motif on the activity of phospholipase.

In contrast to the similarities of their catalytic motifs, the biochemical characteristics of bacterial phospholipases appear to be variable. For example, *V. mimicus* PhlA has a phospholipase A activity, which cleaves the fatty acid at either sn-1 or sn-2 position, but no lysophospholipase activity [25]. Two phospholipases identified from mesophilic *Aeromonas* sp. serogroup O:34 show phospholipase A1 and C activity [29].

In addition, TLH of *V. parahaemolyticus* has PLA2 and lysophospholipase activity, and demonstrates a loss of activity at 55°C for 10 min [20]. In this report, we show that *V. anguillarum* Plp has PLA2 activity, and is able to maintain activity at 64°C for 1 h (Figs. 6 and 7). Therefore, the enzymatic characteristics of specific phospholipases are distinct even when they all belong to the SGNH hydrolase family (Fig. 1).

Phospholipases have been implicated in the pathogenic activities of a number of bacteria [30, 31]. It is known that phospholipase activities often lead to cell destruction by degrading the phospholipids of cell membranes [30, 32]. However, the relationships between phospholipases and virulence are not always clear. While the purified rPlp exhibits strong hemolytic activity against Atlantic salmon erythrocytes (Fig. 7), Rock and Nelson [8] showed that a knock-out mutation of either the *plp* gene or the *vah1* gene in *V. anguillarum* did not affect virulence of *V. anguillarum* during an infection study on juvenile Atlantic salmon. In this report, we show that when groups of rainbow trout are infected with either a *plp* mutant or a *plp vah1* double mutant there is no significant difference in mortalities compared to fish infected with the wild type strain. Our data suggest that neither *plp* nor *vah1* are major virulence factors during pathogenesis of salmonids. It was also reported that the deletion of lecithinase (Lec) activity in *V. cholerae* did not significantly diminish fluid accumulation in the rabbit ileal loop assay, indicating the lecithinase activity does not contribute significantly to enterotoxin activity [24]. In contrast, the direct IP injection of purified *V. harveyi* VHH protein caused the death of flounder with an LD₅₀ of about 18.4 µg protein/fish [26]. The rPhlA of *V. mimicus* also has a direct

cytotoxic effect on the fish cell line CHSE-214 [25] suggesting that this phospholipase is a virulence factor during fish infection. In addition, the lecithinase purified from *A. hydrophila* (serogroup O:34) has been shown to be an important virulence factor to rainbow trout and mouse [29].

Generally, the hemolytic activity of phospholipases is dependent upon the hydrolysis of the phospholipids that reside in the erythrocyte membrane. Erythrocytes contain various phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM). PC makes up 58% of the total erythrocyte phospholipids in the Atlantic salmon [33], but only 34% and 1% in rabbit and sheep erythrocytes, respectively [19]. Taken together with the high specificity of rPlp for PC (Fig. 6), it was not surprising that rPlp was able to lyse the fish erythrocytes, but not sheep erythrocytes (Fig. 7), and that the *plp* mutant had decreased hemolytic activity on LB20-fish blood agar (Fig. 2). Our results are consistent with those reported for *V. mimicus* PhlA[25] and *V. harveyi* VHH [26], in which PhlA and VVH specifically lyse the fish erythrocytes.

We have previously reported that there are two hemolysin gene clusters in *V. anguillarum* M93Sm, the *vahI-plp* cluster and *rtxACHBDE* cluster [9] and have described their regulation by H-NS and HlyU [17, 34]. Mutation of both *vahI* and *rtxA* results in the loss of all hemolytic activity on TSA-sheep blood agar [9], which is consistent with the data reported here that Plp has no activity on sheep erythrocytes.

We have also previously demonstrated that Plp is a putative repressor of Vah1, since mutation of *plp* increases *vah1* expression by 2-3 fold [8]. In this report, we examined the hemolytic activity of various hemolysin mutants using freshly collected Rainbow trout blood (Table 2) to investigate the relationships among three hemolysins of *V. anguillarum*. While culture supernatants from two of the three single mutants (JR1 and S123) and one of three double mutants (S183) exhibited $\geq 94\%$ of the hemolytic activity as supernatants from the wild type strain M93Sm (Table 2), the hemolytic activity of one single mutant (S262, *plp*) and two double mutants (JR03, *plp vah1* and S187, *plp rtxA*) were reduced to 28%, 14%, and 12% of that in M93Sm, respectively. Our data indicate that only the loss of the *plp* gene has a significant effect on hemolysis of fish erythrocytes by *V. anguillarum* culture supernatant, while the loss of *rtxA* and/or *vah1* has little effect. Further, supernatant from the hemolysin triple mutant XM90 (*vah1 rtxA plp*) exhibits no hemolytic activity on fish blood compared to M93Sm (Table 2), indicating that Vah1, RtxA, and Plp are responsible for all secreted hemolytic activity by *V. anguillarum*. Finally, complementation of any *plp* mutant with *plp* (*in trans*) restores hemolytic activity to *V. anguillarum* culture supernatant (Table 2).

Conclusion

V. anguillarum Plp is a secreted hemolysin with phosphatidylcholine-specific phospholipase A2 activity. The ability of Plp to digest the abundant phosphatidylcholine found in the membrane of fish erythrocytes causes their lysis. The three hemolysins, Plp, Vah1 and RtxA, account for all hemolytic activity in *V. anguillarum* culture supernatant under the experiment conditions described in this study. Finally, infection studies in rainbow trout demonstrate that the *plp* and *vah1* genes are not required for virulence.

Methods

Bacterial strains, plasmids, and growth conditions. 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) [35], supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. *E. coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10). Antibiotics were used at the following concentrations: streptomycin, 200 µg/ml; 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*; tetracycline, 15 µg/ml (Tc¹⁵) for *E. coli*, 1 µg/ml (Tc¹) for *V. anguillarum* grown in liquid medium, and 2 µg/ml (Tc²) for *V. anguillarum* grown on agar plates.

Insertional mutagenesis. Insertional mutations were made by using a modification of the procedure described by Milton *et al.*[25]. Briefly, primers (Table3) were designed based on the target gene sequence of M93Sm. Then a 200-300 bp DNA fragment of the target gene was PCR amplified and ligated into the suicide vector pNQ705-1 (GenBank accession no. KC795685) after digestion with *Sac*I and *Xba*I. The ligation mixture was introduced into *E. coli* Sm10 by electroporation using BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm²⁰ agar plates. The construction of the recombinant pNQ705 was confirmed by both PCR amplification and restriction analysis. The mobilizable suicide vector was transferred

from *E. coli* Sm10 into *V. anguillarum* by conjugation. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the recombinant pNQ705 was confirmed by PCR amplification.

Allelic exchange mutagenesis. The allelic exchange *rtxA* mutation in *V. anguillarum* S264 was made by using a modification of the procedure described by Milton *et al.*[25]. The 5' region of *rtxA* was amplified using the primer pair pm256 and pm257 (Table3), digested with *Xho*I and *Xba*I, and then cloned into the region between the *Xho*I and *Xba*I sites on pDM4 (GenBank accession no. KC795686), deriving pDM4-*rtxA*5'. The 3' region of *rtxA* was amplified using the primer pair pm258 and pm259 (Table3), digested with *Xba*I and *Sac*I, and then cloned into the region between the *Xba*I and *Sac*I sites on the pDM4-*rtxA*5'. The resulting pDM4-*rtxA*5'-*rtxA*3' was transformed into *E. coli* Sm10 to produce the transformant strain S252, which was mated with *V. anguillarum* S171 (*vahI*). Single-crossover transconjugants were selected with LB20 Kan⁸⁰ Sm²⁰⁰ Cm⁵ plates and, subsequently, double-crossover transconjugants were selected with LB20 Kan⁸⁰ Sm²⁰⁰ 5% sucrose plates. The resulting *V. anguillarum* colonies were transferred to TSA-sheep blood agar (Northeast Laboratories Service, Waterville, ME) and screened for non-hemolytic colonies (*vahI rtxA*). The resulting colonies were checked for the desired allelic exchange using PCR amplification.

Complementation of mutants. The various mutants were complemented by cloning the appropriate target gene fragment into the shuttle vector pSUP202 (GenBank

accession no. AY428809) as described previously by [8]. Briefly, primers (Table 3) were designed with *EcoRI* and *AgeI* sites and then used to amplify the entire target gene plus ~500 bp of the 5' and ~200 bp 3' flanking regions 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 BioRad Gene Pulser II. Transformants were selected on LB10 Tc¹⁵ Ap¹⁰⁰ agar plates. The complementing plasmid was transferred from *E. coli* Sm10 into the *V. anguillarum* mutant by conjugation. Transconjugants were selected by tetracycline resistance (Tc²). The transconjugants were then confirmed by PCR amplification and restriction digestion.

Bacterial conjugation. Bacterial conjugations were carried out using the procedure modified from by Varina *et al* [36]. Briefly, 100 µl *V. anguillarum* grown overnight was added into 2.5 ml nine salts solution (NSS) [37]; 100 µl *E. coli* culture overnight was added into 2.5 ml 10 mM MgSO₄. The resulting *V. anguillarum* and *E. coli* suspension was mixed, vacuum filtered onto an autoclaved 0.22-µm-pore-diameter nylon membrane (Millipore, USA.), placed on an LB15 agar plate (LB-plus-1.5% NaCl), and allowed to incubate overnight at 27°C. Following incubation, the cells were removed from the filter by vigorous vortex mixing in 1 ml NSS. Cell suspensions (70 µl) were spread on LB20 plated with appropriate antibiotics and the plates were incubated at 27°C until *V. anguillarum* colonies were observed (usually 24 to 48 h).

Cloning, over-expression, purification, and refolding of the Plp protein. The whole length of the *plp* gene (stop codon not included) was amplified by PCR with a sense primer introducing a BamHI site and an antisense primer introducing BglII site, respectively. Genomic DNA extracted from *V. anguillarum* M93Sm was used as template. The amplified PCR product was digested with BamHI and BglII, and ligated into a pQE60 (QIAGEN, USA) vector, which was also cut with BamHI and BglII. The ligation mix was transformed into *E. coli* M15 (pREP4) and clones with pQE60-plp were selected on LB10 agar containing kanamycin and ampicillin. A clone harboring plasmid pQE60-plp was selected and the plasmid DNA sequence isolated from the clone confirmed by sequencing. The clone was designated as S269. Subsequently, *E. coli* strain S269 was grown at 37°C in 500 ml LB10 broth to OD₆₀₀ = 0.5, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture (final concentration, 1 mM) to induce the expression of Plp. Then, the induced *E. coli* cells grown for 4 h at 37 °C were harvested at 8000 × g for 10 min. The cell pellet was stored at -20°C overnight to improve lysis. Inclusion bodies of Plp were crudely purified using Cellytic B reagent (Sigma, USA). Refolding of Plp protein from the inclusion body preparation was carried out using a modification of the method described by Santa *et al* [38]. Briefly, 500 µl of purified inclusion body (2 mg protein/ml) was completely solubilized in 1ml of 50 mM Tris buffer (pH 12) containing 2 M urea. The solubilized Plp was diluted into 20 ml dilution buffer (50 mM Tris-HCl, pH8.0; 0.2 M glycine; 10% glycerol; 2 M urea; 0.5 mM EDTA, and 0.2 mM DTT) at 4 °C. No aggregation was observed during the dilution. The diluted Plp protein was dialyzed with the addition of 500 ml 50 mM Tris-HCl (pH8.0)

until the total dialysis volume up to 3 L. The dialyzed Plp protein was concentrated with QIAGEN Ni-NTA Protein Purification Kit (QIAGEN) under native purification condition according to the instructions of the manufacturer.

Hemolytic assays. The hemolytic activity of *V. anguillarum* strains was measured by two methods. First, single *V. anguillarum* colonies were transferred onto TSA-sheep blood agar, LB20-sheep blood agar (LB20 agar plus 5% sheep blood with heparin, obtained from Hemostat Laboratories) or LB20-fish blood agar (LB20 agar plus 5% rainbow trout or Atlantic salmon blood with heparin). Hemolytic activity of each colony was determined by measuring hemolytic zone surrounding the colonies after 24 h at 27°C. Additionally, the level of hemolytic activity was also quantitated using a microcentrifuge tube assay. The tubes contained 500 µl 5% erythrocytes (fish or sheep, suspended in 10 mM Tris-Cl, pH 7.5 – 0.9% NaCl buffer) were mixed with 500 µl of bacterial supernatant or rPlp and incubated for 20 h at 27°C. The samples were centrifuged at 1500 × g for 2 min at 4°C, and the optical density of the resulting supernatant was read at 428 nm.

Phospholipase assay and thin-layer chromatography (TLC) analysis.

Phospholipase assays were performed *in vitro* with a BODIPY-phosphatidylcholine (BPC or 2-decanoyl-1-(O-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)undecyl)-sn-glycero-3-phosphocholine; Invitrogen),

NBD-phosphatidylethanolamine (NBD-PE, *N*-(NBD-Aminododecanoyl)L-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine; Sigma), NBD-phosphatidylserine (NBD-PS or 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-Glycero-3-Phospho-L-Serine; Avanti Polar Lipid), NBD-sphingomyelin (NBD-SM, N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-Sphingosine-1-Phosphocholine; Avanti Polar Lipid). 20 μ M phospholipid substrates (10 μ l) were reacted with an equal volume (10 μ l) of various samples, and incubated at different conditions, as described for each experiment. For some experiments, purified standard phospholipases were used: PLA2 (Sigma) from porcine pancreas, PLC (Sigma) from *Clostridium perfringens*, and PLD (Sigma) from cabbage. The reaction products were analyzed by thin-layer chromatography (TLC). Briefly, 20 μ l of 1-butanol was added to the above reaction mixes (20 μ l), followed by vigorous vortex mixing for 30 s and centrifugation (10,000 $\times$ g, 1 min). The upper lipid extract layer (5 μ l) was loaded onto a plastic-backed silica gel G60 plate without fluorescent indicator (Sigma) and air-dried for 20 min. TLC was performed either with chloroform-methanol-water-acetic acid (45/45/10/1 by vol.) when BODIPY-PC was used as the substrate, or with chloroform-methanol-acetic acid (60/20/1 by vol.) when NBD-PE, NBD-PS, or NBD-SM used as the substrates. For some experiments, an apolar solvent (n-hexane (70): diethyl ether (30): acetic acid (4)) was used. Fluorescence was detected and quantified using a Typhoon 9410 laser scanner.

Subcellular fractionation. *V. anguillarum* cells were fractionated as described previously [6] and the subcellular location of Plp determined. Briefly, 100 ml NSS-washed overnight grown bacteria cells were resuspended in 10 ml of ultrapure water for 20 min to cause osmotic shock and centrifuged ($10,000 \times g$, 5°C , 10 min) to collect the periplasmic fraction (the supernatant). The remaining pellets were washed twice with ultrapure water and lysed by sonication. The sonicated cells were centrifuged ($10,000 \times g$, 5°C , 20 min) to remove cell debris and any unlysed cells, and the supernatant cell lysate was separated by ultracentrifugation ($200,000 \times g$, 1 h, 4°C) to yield the cytosolic (supernatant) and membrane (pellet) fractions. The membrane fraction was treated with 1% Sarkosyl to obtain Sarkosyl-soluble (inner membrane) and -insoluble (outer membrane) fractions. Protein concentration in various fractions was measured using BCA protein determination kit (Pierce).

Preparation of polyclonal antibody. Truncated Plp protein was over-expressed and purified to serve as the antigen to create polyclonal antibody against Plp. Briefly, primer Pm212 and Pm213 (listed in Table 3) were used to amplify central portion of the *plp* gene, which encodes the truncated Plp protein (amino acid 93 to 293). PCR product was ligated into pQE30UA vector (QIAGEN), and transformed into *E. coli* M15 and transformants were selected on LB10 agar containing kanamycin and ampicillin. Plasmid DNA was purified and the sequence confirmed by DNA sequencing. Protein purification was performed under denaturing conditions according to the instructions of the manufacturer (QIAGEN, USA) and protein purity was determined by SDS-PAGE and Coomassie blue staining. Subsequently, the

purified truncated Plp was used as antigen to prepare polyclonal antibody in two New Zealand White rabbits (Charles River Lab, MA). Briefly, 1 ml purified antigen (concentration = 100 µg/ml) was vigorously mixed with 1 ml TiterMax Gold adjuvant (Sigma) into a homogeneous suspension. About 10 ml of blood was withdrawn from the rabbits before immunization as a control. For the first injection, antigen-adjuvant mix was subcutaneously injected at 4 sites (over each shoulder and thigh; 100 µl/site). The rabbits were boosted with single injections of antigen-adjuvant (100 µl) at day 28, 42, and 56. Blood was withdrawn 7-10 days after the 2nd and 3rd boosts to test the titer of antiserum using the western blot analysis. Antiserum with a high titer (> 1:10,000) was aliquoted and stored at -70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Purified proteins or other protein samples were separated in 10% SDS-polyacrylamide gels. Prestained protein standards (Bio-Rad) and Laemmli sample buffer (Sigma) were used in all gels. Electrophoresis was performed at 100 V for 60-90 min. Gels were stained with either Coomassie blue G-250 or silver stain (Pierce, USA) to visualize the protein bands. Alternatively, proteins were transferred to nitrocellulose membranes for western blot analysis using the mini-Protean II system (Bio-Rad). Protein transfers were performed as described by Towbin *et al* [39] at 100 V for 1 h. Nitrocellulose membranes were blocked with the addition of 5% skim milk. Detection of specific protein bands was accomplished by reacting the blot with the 1:5000 diluted anti-Plp antibody, followed by the addition of the secondary

antibody goat anti-rabbit IgG conjugated with peroxidase, and then developed by TMB Development Liquid (Sigma, USA).

DNA sequence and analysis. All DNA sequencing was done at the URI Genomics and Sequencing Center (University of Rhode Island, Kingston, RI), using an ABI 3170xl Genetic Analyzer unit (Applied Biosystems). Multiple alignment and phylogenetic tree were analyzed using the Clustal-W method in DNA-STAR Lasergene7 program.

Fish infection studies. Various *V. anguillarum* strains were tested for virulence with rainbow trout (*Oncorhynchus mykiss*) by intraperitoneal (IP) injection as described by Mou *et al* [29]. Briefly, *V. anguillarum* cells grown in LB20 supplemented with appropriate antibiotics for 22 h at 27°C were harvested by centrifugation ($9,000 \times g$, 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 optical density at 600 nm. 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. Fish were anesthetized with tricaine methanesulfonate (Western Chemical, Ferndale, WA), with 100 mg/L for induction and 52.5 mg/L for maintenance. *V. anguillarum* strains were IP-injected into fish in 100 μL NSS vehicle. Fish that were between 15 and 25 cm long were injected with bacteria diluted with NSS at various doses or NSS only as negative control. Five 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 confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the head kidney 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 reviewed and approved by the University of Rhode Island Institutional Animal Care and Use Committee (URI IACUC reference number AN06-008-002; protocols renewed 14 January 2013).

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

LL, XM and DRN designed the study. XM and LL created the strains used in this study. LL and XM performed all the assays. LL, XM and DRN wrote the paper. Formatting of the paper was done by XM and DRN. All authors have read and approved the final version of manuscript.

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Figure 1. The phylogenetic tree (A) and amino acid sequence alignment (B) of *V. anguillarum* Plp with members of the SGNH family. Sequences of the 16 closest matches to Plp are aligned along the five conserved blocks of the SGNH family (Block IV not shown). The rectangle bars above the alignment indicate the amount of conservation of amino acid residues. The four residues conserved in all SGNH family members are boxed.

A

B

Figure 2. Hemolytic activity of M93Sm and S262 (*plp*) on TSA-sheep blood agar (A) and LB20 + 5% rainbow trout blood agar (B). A single colony of M93Sm and S262 was transferred onto each of the blood agars and incubated at 27°C for 24 h. The zones of hemolysis were measured and the diameters were given in the figure. This is a representative experiment from 3 replicate trials.

Figure 3. Thin-layer chromatography (TLC) demonstrates phospholipase A2 activity of Plp. BODIPY-labeled phosphatidylcholine (BPC) was incubated with various standard enzymes or sample preparations for 1 h at 37°C. Subsequently, the lipids were extracted and separated by TLC. (A) The cleavage patterns of BPC by standard proteins PLA2, PLC, and PLD were able to distinguish the different phospholipase activities. (B) Cleavage patterns of BPC by supernatants (lanes 2 and 3) and cell lysates (lanes 4 and 5) from *E. coli* DH5 α containing cloned *plp* (lanes 3 and 5) or just the cloning vector pSUP202 (lanes 2 and 4). Lane 1 contains only BPC incubated in the presence of PBS buffer. BLPC, BODIPY-labeled lysophosphatidylcholine; PA, phosphatic alcohol; PBt, phosphaticbutanol; DAG, di-acylglycerol.

A**PLA2 PLC PLD**

← DAG

← PA
PBt

← BPC

← BLPC

B**1 2 3 4 5**

Figure 4. Effects of chemical and physical conditions on rPlp activity. (A) The enzymatic activity was proportional to rPlp concentration between 1 µg/ml to 8 µg/ml. (B) The effect of temperature on rPlp activity indicated that rPlp activity was optimal at 37°C and stable up to 64°C. (C) The pH effect on rPlp protein indicated that the optimal pH for rPlp activity ranged from 5 to 9. (D) EGTA did not have any effect on rPlp activity.

Figure 5. Plp is a secreted protein. The phospholipase activity assays of various cell fractions prepared from wild type (wt) strain M93sm and *plp* mutant strain S262 (*plp*-) were performed at 37°C (A) and 64°C (B). PBS buffer, LB20, and PBS buffer + 1% sarcosylate were served as negative controls. The refolded rPlp protein (PLP +) served as positive control. The top spots on each chromatogram are the BPC substrate and the bottom spots are the BLPC reaction product. The proteins from the same cell fractionation preparations were analyzed by SDS-PAGE and Western blot analysis (C) as described in the Materials and Methods. The refolded rPlp protein was served as positive control.

Figure 6. rPlp has specific activity against phosphatidylcholine. BPC, NBD-PE, NBD-PS, and NBD-Sm were used as phospholipid substrates to examine the specificity of rPlp. Phosphate-buffered saline (PBS) was used as a negative control, and PLA2 enzyme from porcine pancreas as a positive control. TLC analysis showed that rPlp only cleaved BPC into BLPC and FFA (A), but could not cleave other phospholipids (B, C, and D). BPC: BODIPY-labeled phosphatidylcholine; BLPC: BODIPY-labeled lysophosphatidylcholine; NBD-PE: NBD-labeled phosphatidylethanolamine; NBD-LPE: NBD-labeled lysophosphatidylethanolamine; NBD-PS: NBD-labeled phosphatidylserine; NBD-FFA: NBD-labeled free fatty acid; NBD-SM: NBD-labeled sphingomyelin; NBD-CE: NBD-labeled ceramide.

Figure 7. Lysis of Atlantic salmon erythrocytes by recombinant Plp protein (rPlp). 500 µl 5% fish (triangle) and sheep (square) erythrocytes were incubated with various concentration rPlp at 27°C for 20 h. The lysis of erythrocytes was measured at 428 nm. Erythrocyte resuspension buffer (10mM Tris-HCl, 0.9% NaCl, pH7.2) was used as negative control. All values were calculated from three independent experiments. Error bars show one standard deviation.

Figure 8. Fish infection experiment. Survival rate of rainbow trout injected IP with wild type and mutant strains of *V. anguillarum* strains at doses of **A)** 3×10^6 , **B)** 3×10^5 or **C)** 3×10^4 CFU/fish. No statistically significant difference was observed between the strains.

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

Strain or plasmid	Description	Reference
<i>V. anguillarum</i> strains		
M93sm	Spontaneous Sm ^r mutant of M93 (serotype O2a)	[2]
JR1	Sm ^r Cm ^r <i>vahI</i> ; insertional <i>vahI</i> mutant of M93Sm	[8]
XM21	Sm ^r Cm ^r Tc ^r <i>vahI</i> +; <i>vahI</i> complement strain of JR1	This study
S262	Sm ^r Cm ^r <i>plp</i> ; insertional <i>plp</i> mutant of M93Sm	This study
XM31	Sm ^r Cm ^r Tc ^r <i>plp</i> +; <i>plp</i> complement strain of S262	This study
S123	Sm ^r Cm ^r <i>rtxA</i> ; insertional <i>rtxA</i> mutant of M93Sm	[9]
JR3	Sm ^r Cm ^r Km ^r <i>vahI plp</i> ; insertional <i>vahI</i> mutant of JL01	[8]
S183	Sm ^r Cm ^r Km ^r <i>vahI rtxA</i> ; insertional <i>rtxA</i> mutant of S171	[9]
XM62	Sm ^r Cm ^r Km ^r Tc ^r <i>vahI</i> + <i>rtxA</i> ; <i>vahI</i> complement strain of S183	This study
S187	Sm ^r Cm ^r Km ^r <i>plp rtxA</i> ; insertional <i>rtxA</i> mutant of JL01	This study
XM90	Sm ^r Cm ^r Km ^r <i>vahI plp rtxA</i> ; insertional <i>plp</i> mutant of S264	This study
XM93	Sm ^r Cm ^r Km ^r Tc ^r <i>vahI plp</i> + <i>rtxA</i> ; <i>plp</i> complement strain of XM90	This study
JL01	Sm ^r Km ^r <i>plp</i> ; mini-Tn10Km insertion into <i>plp</i>	[8]
S171	Sm ^r Km ^r <i>vahI</i> ; allelic exchange <i>vahI</i> mutant	[9]
S264	Sm ^r Km ^r <i>vahI rtxA</i> ; allelic exchange <i>vahI</i> and <i>rtxA</i> mutant	This study
<i>E. coli</i> strains		
Sm10	<i>thi thr leu tonA lacY supE recA</i> RP4- γ (λ <i>pir</i>) u::Km	[40]

S253	Km ^r Cm ^r ; Sm10 containing plasmid pNQ705- <i>plp</i>	This study
S118	Km ^r Cm ^r ; Sm10 containing plasmid pNQ705- <i>rtxA</i>	[9]
S250	Km ^r Cm ^r ; Sm10 containing plasmid pDM4- <i>rtxA5'</i>	This study
S252	Km ^r Cm ^r ; Sm10 containing plasmid pDM4- <i>rtxA5'-rtxA3'</i>	This study
U21	Km ^r Ap ^r Tc ^r ; Sm10 containing plasmid pSUP202- <i>vah1</i>	This study
U31	Km ^r Ap ^r Tc ^r ; Sm10 containing plasmid pSUP202- <i>plp</i>	This study
M15	Nal ^S Str ^S Rif ^S <i>thi⁻ lac⁻ ara⁺ gal⁺ mtl⁻ F⁻ recA⁺ uvr⁺ lon⁺</i> (pREP4)	QIAGEN, USA
S238	Km ^r Ap ^r ; M15 containing plasmid pQE30UA- <i>plp</i>	This study
S269	Km ^r Ap ^r ; M15 containing plasmid pQE60- <i>plp</i>	This study
Plasmid		
PCR2.1	Km ^r Ap ^r ; Cloning vector	Invitrogen, USA
pNQ705-1	Cm ^r ; suicide vector with R6K origin	[41]
pNQ705- <i>vah1</i>	Cm ^r ; for insertional <i>vah1</i> mutation	[8]
pNQ705- <i>plp</i>	Cm ^r ; for insertional <i>plp</i> mutation	This study
pNQ705- <i>rtxA</i>	Cm ^r ; for insertional <i>rtxA</i> mutation	[9]
pDM4	Cm ^r SacBC ^r ; suicide vector with R6K origin	[11]
pDM4- <i>rtxA5'-rtxA3'</i>	Cm ^r SacBC ^r ; for allelic exchange <i>rtxA</i> mutation	This study
pSUP202	Cm ^r Ap ^r Tc ^r ; <i>E. coli</i> – <i>V. anguillarum</i> shuttle vector	[40]
pSUP202- <i>vah1</i>	Ap ^r Tc ^r ; for complementation of <i>vah1</i>	This study
pSUP202- <i>plp</i>	Ap ^r Tc ^r ; for complementation of <i>plp</i>	This study
pQE-30 UA	Ap ^r ; expression vector with N-terminal His ₆ -tag	QIAGEN, USA

pQE30UA- <i>plp</i>	Ap ^r ; for expression of rPlp that is used to make anti-Plp	This study
pQE60	Ap ^r ; expression vector with C-terminal His ₆ -tag	QIAGEN, USA
pQE-60- <i>plp</i>	Ap ^r ; for expression of rPlp for enzymatic activity analysis	This study

Table 2. Hemolytic activity of culture supernatant from *V. anguillarum* wild-type and various *V. anguillarum* mutant strains against rainbow trout blood cells

<i>V. anguillarum</i> strain or treatment	Hemolytic Activity (% of Wild-Type control $\pm$ SD) ^a
M93Sm	1.00 ($\pm$ 0.12)
JR1 (<i>vahI</i>)	0.98 ($\pm$ 0.16)
XM21 (<i>vahI</i> +)	1.20 ($\pm$ 0.28)
S262 (<i>plp</i>)	0.28 ($\pm$ 0.09) ^b
XM31 (<i>plp</i> +)	0.99 ($\pm$ 0.04)
S123 (<i>rtxA</i>)	0.94 ($\pm$ 0.22)
JR03 (<i>plp vahI</i>)	0.14 ($\pm$ 0.09) ^b
S183 (<i>vahI rtxA</i>)	1.51 ($\pm$ 0.29)
XM62 (<i>vahI</i> + <i>rtxA</i>)	0.73 ($\pm$ 0.03)
S187 (<i>plp rtxA</i>)	0.12 ($\pm$ 0.09) ^b
XM90 (<i>vahI rtxA plp</i>)	-0.04 ($\pm$ 0.09) ^b
XM93 (<i>vahI rtxA plp</i> +)	1.33 ($\pm$ 0.01)
Water (positive control)	1.15 ($\pm$ 0.16)

^aHemolytic activity assays carried out using the tube assay method as described in the Materials and Methods. Hemolysis by M93Sm was given the value of 1.00. The data are representative of two independent experiments, each with three replicates, $\pm$ one standard deviation (SD).

^bStatistically different from hemolytic activity for M93Sm ($P < 0.05$).

TABLE 3. Primers used in this study

Primers	Sequence (5' to 3', underlined sequences are designed restriction sites)	Purpose and description	Reference
Pm262	ATCGAG <u>GGATCC</u> ATGAA ACTAATGACGTTATTG	For whole Plp protein, forward	This study
Pm263	ATCGA <u>AGATCT</u> TTTGAA ATTGAAATGACGCGAG	For whole Plp protein, reverse	This study
Pm212	GACACCTCACAATATG AAATAAAA	For truncated Plp protein, forward	This study
Pm213	TTTGAGCTGCGGGGCTT TGGTTGC	For truncated Plp protein, reverse	This study
Pm261	ATCGAGAGCTCGCAGA ATCGTGA <u>CTGACG</u> CCG	For insertional <i>plp</i> mutation, forward, with <i>SacI</i> site	This study
SD Lip/Heme R1	GCTAGTCTAGAACGGA TACCACCTCAGA	For insertional <i>plp</i> mutation, reverse, with <i>XbaI</i> site	[8]
pr1	GGGGAATTCTTATTCAA ATTGAAATGACGCGAG	For <i>plp</i> complement, forward, with <i>EcoRI</i> site	This study
pr2	GGGACCGGTGAATACC CATTTTTTATTTTTTC	For <i>plp</i> complement, reverse, with <i>AgeI</i> site	This study
pr3	GTTGAATTCGTATTTTC TGCAATCGCCATG	For <i>vah1</i> complement, forward, with <i>EcoRI</i> site	This study
pr4	GGGACCGGTCTATTTTA TAATAAATTGAATACC AT	For <i>vah1</i> complement, reverse, with <i>AgeI</i> site	This study
Pm256	ATCGA <u>CTCGAGCT</u> GGA GAAGATGTACTCTGCG	For allelic exchange <i>rtxA</i> mutation, flanking the 5' region, forward, with <i>XhoI</i> site	This study
Pm257	ATCGATCTAGACGTATC ATCTACAGCTTTTGC	For allelic exchange <i>rtxA</i> mutation, flanking the 5' region, reverse, with <i>XbaI</i> site	This study

Pm258	ATCGATCTAGATTATAT TAATCATGTCTTTTATG GG	For allelic exchange <i>rtxA</i> mutation, flanking the 3' region, forward, with <i>XbaI</i> site	This study
Pm259	ATCGAGAGCTCCTGATT GCCTAGCAGTAGCCC	For allelic exchange <i>rtxA</i> mutation, flanking the 3' region, reverse, with <i>SacI</i> site	This study
pr7	CAGGAAACAGCTATGA CCATGATTACG	For sequencing of the DNA fragment inserted in pCR2.1 TA-ligation site	This study
pr8	CTACGGGCTTGAGCGT GACAATC	For sequencing of the DNA fragment inserted in pSUP202 <i>AgeI</i> site	This study
pr25ex	GCTGTCCCTCCTGTTCA GCTACTGACGGGGTGG TGCG	For sequencing of the DNA fragment inserted in pNQ705-1 Multi-cloning site	This study

Manuscript II

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Title: HlyU is a positive regulator of hemolysin expression in *Vibrio anguillarum*

Authors: Ling Li, Xiangyu Mou, and David R. Nelson*

Affiliation: Department of Cell and Molecular Biology
University of Rhode Island

Key Words: *Vibrio anguillarum*, hemolysin, regulation, HlyU, RTX

***Corresponding author:** David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

Abstract

The two hemolysin gene clusters previously identified in *V. 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 ~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 and cytotoxic activity. Both semi-quantitative RT-PCR and real time RT-qPCR 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 in the wild type. The transcriptional start sites in the intergenic regions of both *vah1/plp* and *rtxH/rtxB* genes were determined using 5'-RACE and the binding sites for purified HlyU was discovered using DNA gel mobility shift experiments and DNase protection assays.

Introduction

Vibrio anguillarum is a marine member of the class *Gammaproteobacteria*. 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, which causes vacuolization of target cells (10). It was suggested that pore-forming hemolysins, like HlyA in *E. coli*, cause direct lysis of blood cells by disrupting the membrane integrity (13). Mutations in the divergently transcribed *plp* 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 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 *vahI* and *rtxA* completely lost hemolytic activity on sheep blood agar (10). Additionally, RtxA also exhibits cytotoxic activity and causes Atlantic salmon kidney (ASK) cells to round and die (10).

HlyU, a member of 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 *V. cholerae*, the HlyU protein positively regulates expression of hemolysin gene *hlyA*, as well as the HlyA-co-regulated 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 *V. vulnificus*, and 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 LD₅₀ dose 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 *rtxAI*, in the upstream region of the *rtxAI* operon so that the presence of HlyU resulted in derepression of *rtxAI* (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 cytotoxicity of the mutant were determined and compared to the wild type

and complemented strains. We also identified the transcriptional start site of genes in both the *vahI* 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 transcription from various hemolysin genes, including *vahI*, *plp*, *rtxA*, *rtxH* and *rtxB* were determined in the *hlyU* mutant and its isogenic wild-type parent and complement by real-time RT-PCR.

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, USA). 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) (6), supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Overnight cultures of *V. anguillarum*, grown in LB20, were harvested by centrifugation (8,000 × g, 10 min), and the pelleted cells washed twice with Nine Salt Solution (NSS) (6). Washed cells were resuspended to appropriate cell densities in experimental media. Specific conditions are described in the text for each experiment. *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*; 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 CLUSTW program (20). Degenerate primers (Table II) 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, USA). Colonies containing the cloned *hlyU* in PCR2.1 were selected on LB10 plates plus Ap¹⁰⁰ and Km⁵⁰ and the presence of *hlyU* 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 Wolf-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 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 by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the suicide vector into the *hlyU* gene was confirmed by PCR analysis, as described previously (14). The resulting *V. anguillarum hlyU* mutant was designated S305 (Table 1) for future use.

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 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, USA) and digested with EcoRI restriction enzyme and the DNA fragments separated on a 1% agarose gel. Subsequently, the gel-purified PCR fragment was ligated into pSUP202 after digestion with EcoRI and the ligation mixture introduced into *E. coli* Sm10(λ pir) by electroporation with BioRad Gene Pulser II. Transformants were selected on LB10-Ap¹⁰⁰ agar plates. The complementing plasmid, pSUP202-*hlyU* plasmid, 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 β -hemolysis on TSA plus sheep blood agar plates 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 $\sim 2 \times 10^5$ cells ml⁻¹. *V. anguillarum* cultures grown overnight

were harvested, washed twice in NSS, and resuspended in NSS (at a cell density of $\sim 2 \times 10^9$ cells ml⁻¹). Washed bacterial cells were added to ASK cells at multiplicities of infection (moi) = 100, and incubated at 20°C for 4 h. Changes in cell morphology were assessed and photographed by viewing live cells with an inverted microscope (Nikon TE2000 model). To determine the released LDH, a CytoTox-ONE™ Homogeneous Membrane Integrity Assay Kit (Promega, USA) was used. Briefly, ASK cells were seeded into a 96-well white wall microtiter plate (Costar) as described above at a cell number of 20,000 cells /well. NSS-washed bacterial cells were added to each well at moi=10, 20, 50, and 100, and then incubated at 20°C for 4 h. The assay measures the generation of the fluorescent resorufin product, which is proportional to the amount of LDH at 560 nm_{Excitation}/590 nm_{Emission}.

RNA isolation. Exponential-phase cells ($\sim 0.5 \times 10^8$ 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, USA) according to the manufacturer's instructions. All purified RNA samples were quantified spectrophotometrically by measuring absorption at 260 nm and 280 nm using a NanoDrop® spectrophotometer and stored at -75°C for future use.

Semi-quantitative RT-PCR and Real-time RT-qPCR. 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 RNA used as the template for reverse transcription (RT)-PCR. RT-PCR was performed using Brilliant 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 semi-quantitative 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 50°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. PCR product was visualized in a 1% agarose gel use a 100-bp DNA molecular weight ladder (Promega) as a standard. All Real-time quantitative RT-PCR (RT-qPCR) were performed using an Mx3005 or Mx4000 Multiplex Quantitative PCR System (Stratagene, USA). Primers used were the same as the semi-quantitative RT-PCR (Table 2). Quantitation of various mRNAs was performed using Brilliant 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 15min, 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 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 (*vahI*, *plp*, *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 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 5' RACE anchor primer (AP) (Table 2) and the primer specific for that sequence. The PCR product was cloned into PCR2.1 cloning vector (Invitrogen, USA), 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, RI), using an ABI 3170xl Genetic Analyzer unit (Applied Biosystems). Multiple alignment and phylogenetic tree were analyzed using the Clustal-W method in DNA-STAR Lasergene7 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 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 bacteria culture growing at 37°C in Luria broth supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin was inoculated in 250 ml of the same fresh medium. When the OD₆₀₀ reached 0.6, 1 mM IPTG 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 (8000 ×g, 10 min) and the cell pellets resuspended in 5 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH8.0). The cell suspension was incubated with lysozyme (0.5 mg/ml) on ice for 30 min, and then sonicated (six bursts, 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 digoxigenin gel shift kit (second generation; 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/vahI* intergenic region were amplified by PCR and then 3' end labeled with digoxigenin-11-ddUTP using terminal deoxynucleotidyl transferase. After the labeling efficiency was determined, each of the labeled probes (0.4 ng for fragment a, b and c, 0.2 ng for fragment 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% (wt/vol) Tween 20, 150 mM KCl). For competition analysis, labeled probe (0.4 ng for fragment a, b and c, 0.2 ng for fragment d, e, f, g and h) and 350 ng HlyU protein were incubated with 100 ng/µl

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).

DNase I protection assay. The DNA probes for the DNase I protection assay were amplified from *V. anguillarum* genomic DNA using 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-FAM at 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 5' end on the upper strand and the 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 rHlyU (up to 1.88 µg) were incubated at 37°C in a total volume of 20 µl, containing Binding Buffer (4 µl, 5x conc.) from the DIG Gel Shift Kit, (2nd Generation, 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 conc.) 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 using 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

elutant (5 μ l) was added to 10 μ l Hi-Di Formamide containing 0.1 μ l 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. parahaemolyticus*, 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 reaction 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 homologue (GenBank accession number HQ149334), which encodes a predicted protein with 97 amino acids, a molecular mass of 11,095 Da, and strong homology to HlyU proteins found in a variety of *Vibrio* species including *Vibrio furnissii* (97% similarity and 92% identity), *Vibrio coralliilyticus* (95% similarity and 91% identity), *V. cholerae* (93% similarity and 86% identity), *V. parahaemolyticus* (92% similarity and 88% identity), and *V. vulnificus* (94% similarity and 87% identity).

Mutation in *hlyU* decreases hemolytic activity. An insertional mutation by single crossover homologous recombination in the *hlyU* gene was obtained. The hemolytic activity of the *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 *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 *V. anguillarum*.

Mutation in *hlyU* has no significant effect on cytotoxicity. One hemolysin gene, *rtxA*, has been shown to be a major virulence factor for *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 a moi = 100 for 4 h, indicating the mutation in *hlyU* did not completely knock out the cytotoxicity of *V. anguillarum*. Indeed, the LDH release assay revealed that S305 retained ~75-80% ($P > 0.1$) of cytotoxicity at all moi values compared to the wild type strain M93Sm (Fig. 4B) confirming that the mutation of *hlyU* had only a small, but statistically insignificant effect on cytotoxicity. As a negative control, the *rtxA vahI* double mutant strain S183 exhibited no cytotoxicity compared to wild type strain M93Sm (Fig. 4B), confirming that *rtxA* and *vahI* are the major cytotoxins in *V. anguillarum* (10). When strain S307 was assayed for cytotoxic activity by the LDH release assay, the activity was restored to same levels seen in M93Sm (Fig. 4B).

HlyU positively regulates hemolysin genes at the transcriptional level.

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

In contrast to *rtx* genes, expression of genes in the *vah1* cluster, including *vah1*

and *plp*, exhibited little or no decrease in the *hlyU* mutant by the semi-quantitative RT-PCR experiments (Fig. 5). Measurement of expression for *vahI* and *plp* by real-time qRT-PCR were consistent with data from the semi-quantitative experiments showing no significant changes in expression in the *hlyU* mutant (Table 4), indicating that the absence of HlyU does not affect either *vahI* or *plp* expression. However, when the expression of *vahI* and *plp* were examined by both semi-quantitative RT-PCR (Fig. 6) and qRT-PCR (Table 4) in the *hlyU* complement (S307), we observed that expression of both genes increased. Specifically, expression of *vahI* in S307 increased over wild type (M93Sm) levels by 11.6- and 26.3-fold during exponential and stationary phase, respectively; and expression of *plp* increased over levels in M93Sm by 8.32- and 86.2-fold during exponential and stationary phase, respectively. These data indicate that over-expression of HlyU can positively regulate expression of *vahI* and *plp*.

Mapping transcriptional start sites of hemolysin genes. Since it had been reported that HlyU is a DNA binding protein (17) that positively regulates *hlyA* (homologue of *vahI*) in *V. cholerae* (23) and *rtxAI* (homologue of *rtxA*) in *V. vulnificus* (11), we wanted to determine possible HlyU binding sites in the *vahI* gene cluster and *rtxACHBDE* cluster in *V. anguillarum*. The transcriptional start sites of both hemolysin clusters were identified using 5'RACE. In the *vahI* gene cluster there is a 508-bp intergenic region between the divergent *plp* and *vahI* genes. The 5'-RACE results demonstrated that the region between the +1 sites of *plp* and *vahI* was 318 bases long (Fig. 6A). The +1 transcriptional start site (A) of *plp* is 73 bases prior to its start codon, with a predicted -35 and -10 promoter sequence of:

TTGATT-N₁₃-ATAAAT (Fig. 6A). The divergent hemolysin gene, *vahI*, had a transcriptional start site (G) 119 bases before the *vahI* start codon, with a predicted -35 and -10 promoter sequence of TTGTGT-N₁₆-TATTAA (Fig. 6A).

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. 6B). 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: TTGCGT-N₁₅-TATAAT (Fig. 7B). 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-TATAAT (Fig. 6B). Analysis of the predicted promoter regions of these two hemolysin clusters revealed strong similarities to a σ^{70} 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 *rtxAI* 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 sub-fragments amplified from the intergenic region between *rtxH* and *rtxB* (Fig. 7A) and with each of the five DIG-labeled DNA sub-fragments amplified from the intergenic region between *plp* and *vahI* (Fig. 7C).

DNA mobility shift experiments were performed on the HlyU + DIG-labeled DNA mixtures. The results revealed that HlyU bound to fragment b of the *rtxH-rtxB* intergenic region (Fig. 7B) and to fragment f of the *plp-vahI* 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 sub-fragments that bound HlyU was examined by a DNase I protection assay as described in the Materials and Methods. The results of these experiments revealed that HlyU protected an 18-bp region (5'TAATAAAAAATCTTAAAAA3') in fragment b (Fig. 8A) with two 5-bp direct repeats of TAAAA. This region starts 103 bp upstream of the +1 site of *rtxH* and 67 bp upstream from *rtxB*. Similarly, HlyU protected a 22-bp region (5'AATAAAAAATATCAATAAAATTA3') in fragment f (Fig. 8B) with the same two 5-bp direct repeats of TAAAA. The binding region in sub-fragment f starts 192 bp upstream of *plp* and 106 bp upstream of +1 site for *vahI*.

Discussion

Hemolytic activity of *V. anguillarum* has been considered to be 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 4 genes, *plp*, *vah1*, *llpA*, and *llpB* gene. *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 to 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 activity (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 co-regulated 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 *vahl* 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 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). Further, 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. parahaemolyticus*), 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 LD₅₀ increased about 10⁴-fold in a *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 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 that *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 effect the expression of *vahI* and *plp* compared to the wild type strain (Table 4). However, the observation that over-expression of HlyU in the *hlyU* complement dramatically increased expression of both *vahI* and *plp*, suggests that *vahI* 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 *vahI* 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 de-repression of *hlyA* by HlyU (17). The later assumption was recently supported by the study in *V. vulnificus* where HlyU was found to bind to the upstream region of *rtxH*, which competes with the binding site of *rtxAI* repressor H-NS (12). Similar to *V. anguillarum*, expression of *rtxH* and *rtxAI* in *V. vulnificus* are 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 *rtxAI* 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 contains 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-vahI* region is 22 bp long and both have

identical 5-bp direct repeats of TAAAA, strongly suggesting that HlyU binds as a dimer as suggested by Saha and Chakrabarti (17). In fact, the direct repeat may be a bit longer than 5 bp. If one uses an imperfect match, the direct repeat is 7-bp: (A/T)TAAAA(A/T). Additionally, examination of the sequences immediately adjacent to the protected regions reveals that both the *rtxH-rtxB* and the *plp-vahI* intergenic regions contain 25-to 26-bp regions that are nearly identical with 10-bp inverted repeats at each end (Fig. 6). The *rtxH-rtxB* intergenic region contains identical 10-bp inverted repeats at each end of its 25-bp region with a nucleotide sequence of: ATAATAAAAA. Similarly, the *plp-vahI* intergenic region has nearly identical 10-bp inverted repeats at each end of the 26-bp region. The nucleotide sequence of the *plp* proximal repeat is: 5'-TTAATAAAAA-3'. The nucleotide sequence of the *vahI* proximal repeat is: 3'-ATATTAAAAT-5'. Further, 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 upstream of the transcriptional start sites of the regulated hemolysin genes. However, Liu et al (10, 12) found that HlyU bound far upstream (-376 to -417 bp) of the transcriptional start site of the *rtxA1* operon. In contrast, we have located HlyU binding somewhat closer to the start transcription sites of *rtxH-rtxB* and *plp-vahI*. The binding site of HlyU is 104 bp and 68 bp upstream of the *rtxH* and *rtxB* +1 sites, respectively, and 150 bp and 145 bp upstream of *plp* and *vahI* +1 sites, respectively. While we have not yet demonstrated H-NS repressor binding to these regulatory regions in *V. anguillarum*, the shorter distance between HlyU binding sites and the transcriptional start sites in *V. anguillarum* compared to *V. vulnificus* may indicate that *V. anguillarum* has fewer

H-NS binding sites than the five sites found for the *V. vulnificus rtxAI* regulatory region (12).

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Table 1. Bacterial strains and plasmids used in this study.

Strains	Genotype and features	References
<i>V. anguillarum</i>		
M93Sm	Spontaneous Sm ^r mutant of M93 (serotype J-O-1)	(3, 4)
S305	Sm ^r Cm ^r ; M93Sm <i>hlyU</i> mutant	This study
S307	Sm ^r Cm ^r Tet ^r ; M93Sm <i>hlyU</i> complement	This study
S183	Sm ^r Cm ^r Kan ^r ; M93Sm <i>rtxA vahI</i> double mutant	(10)
<i>E. coli</i>		
Sm10	<i>thi thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu::Km (λ <i>pir</i>)	(14)
M15	Nal ^S Str ^S Rif ^S <i>thi</i> ⁻ <i>lac</i> ⁻ <i>ara</i> ⁺ <i>gal</i> ⁺ <i>mtl</i> ⁻ F ⁻ <i>recA</i> ⁺ <i>uvr</i> ⁺ <i>lon</i> ⁺ (pREP4, Km ^R)	Qiagen, USA
Plasmid		
pNQ705-1	Cm ^r ; suicide vector with R6K origin	(14)
pSUP202	<i>E. coli</i> – <i>V. anguillarum</i> shuttle vector	(14)
PCR2.1	Cloning vector	Invitrogen, USA
pQE30UA	Expression vector with N-terminal His ₆ -tag	QIAGEN, USA

Table 2. Primers used in this study.

Primers	Sequence (5' to 3')	Description
Pm301	AGYTAYGARATGYTNGATA CGTNAAAYTA	Degenerate <i>hlyU</i> F
Pm302	CGTCGYCAGCAYAAYGCTA GCCGTCGYTC	Degenerate <i>hlyU</i> R
Pm297	ACTGAGAGCTCGGTGTTGT TAAAGGCTATGGC	<i>hlyU</i> insertional mutation F
Pm298	ATCGATCTAGAGTATCCAC TAACCCATCTCTT	<i>hlyU</i> insertional mutation R
R vah1 RT (BF)	GGCTCAACCTCTCCTTGTA ACCAA	5' RACE <i>vah1</i>
plpF RT	CAGACGACCACCAGTAACC ACTAA	5' RACE <i>plp</i>
Pm112	TGGTTGTAAGCCGCAGCAC	5' RACE <i>rtxH</i>
Pm163	GGGGTATCTGAGTCACATG GATGAATT	5' RACE <i>rtxB</i>
Primer AP	GACCACGCGTATCGATGTC GACTTTTTTTTTTTTTTTT	5' RACE anchor primer
Pm303	ATGGAAAAAATTCCGCTA AAGCA	Entire HlyU protein F
Pm304	CTAGCGGCAGTATAAACCG TGTA	Entire HlyU protein R
Pm305	CCC <u>GGATCC</u> GCAACTTATC	<i>hlyU</i> complementation F

GGTCAGATTGATG

Pm306	<u>CCCGGATCCGATGCGCACT</u> TACATGAAGAAAAC	<i>hlyU</i> complementation R
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^a Restriction sites for SacI (GAGCTC), XbaI (TCTAGA), EcoRI (GGATCC) are underlined. V=A, C, or G; N=A, C, T, or G; R= A or G; Y=C or T

Table 3. List of primers used to amplify DNA probes in DNase I protection assay.

Intergenic Region	Probe Name	Probe Length	Primer Name	Primer Sequence	Primer 5' Label	Primer Strand
<i>rtxH/rtxB</i>	Probe #4	336 bp	Pm414	CAGTGGCT CATAAAAG CAGTTGC	6-FAM	<i>rtxH</i> sense strand
			Pm318	CAGCGGTA AGTAGACT GATA	none	<i>rtxB</i> sense strand
	Probe #5	394 bp	Pm315	CTCAGACA TAAATAAA TCACC	none	<i>rtxH</i> sense strand
			Pm415	CAGCGGTA AGTAGACT GATAAGCA ATG	6-FAM	<i>rtxB</i> sense strand
<i>plp/vah1</i>	Probe #2	496 bp	Pm412	CCGTATTT TCTGCAAT CGCCATGG	6-FAM	<i>plp</i> sense strand
			Pm322	AAAATAAA AGGACATT GGTTTTTT GG	none	<i>vah1</i> sense strand
	Probe #3	468 bp	Pm327	GTATTTTC TGCAATC GCCATG	none	<i>plp</i> sense strand
			Pm413	CACCTTTG TGGCGAAT	6-FAM	<i>vah1</i> sense

				TATTAATA GATCTT		strand
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Table 4. Real time qRT-PCR analysis^a

		<i>V. anguillarum</i> strain ^b	Log phase	Stationary phase
<i>rtxA</i> expression	RNA copy number	M93Sm	$2.74 \times 10^4 \pm 1300$	$8.88 \times 10^3 \pm 252$
		S305	$3.45 \times 10^3 \pm 800$	$4.47 \times 10^2 \pm 15$
		S307	$1.18 \times 10^5 \pm 424$	$1.48 \times 10^4 \pm 123$ 7
	Relative change (n-fold) in expression	M93Sm	1	1
		S305	- 7.94±0.4	-20.00±0.6
		S307	4.30±0.01	1.68±0.14
	RNA copy number	M93Sm	$3.40 \times 10^4 \pm 2298$	$2.92 \times 10^4 \pm 736$
		S305	$9.54 \times 10^3 \pm 596$	$3.62 \times 10^3 \pm 141$
		S307	$9.83 \times 10^4 \pm 6045$	$3.60 \times 10^4 \pm 777$ 1
<i>rtxB</i> expression	Relative change (n-fold) in expression	M93Sm	1	1
		S305	-3.56±0.2	-8.07±0.3
		S307	2.89±0.18	1.23±0.3
	RNA copy number	M93Sm	$4.74 \times 10^4 \pm 0$	$1.93 \times 10^4 \pm 933$ 3
		S305	$7.43 \times 10^3 \pm 643$	$1.18 \times 10^3 \pm 410$
		S307	$1.46 \times 10^5 \pm 1060$ 6	$2.96 \times 10^5 \pm 707$
	Relative change (n-fold) in expression	M93Sm	1	1
		S305	-5.90±0.5	-15.1±6
		S307	3.08±0.2	14.6±0.03
<i>rtxH</i> expression	RNA copy number	M93Sm	$3.17 \times 10^2 \pm 56$	$5.57 \times 10^2 \pm 302$
		S305	$3.36 \times 10^2 \pm 17$	$6.92 \times 10^2 \pm 58$
		S307	$3.66 \times 10^3 \pm 556$	$1.47 \times 10^4 \pm 157$ 2
	Relative change (n-fold) in expression	M93Sm	1	1
		S305	1.06 ±0.05	1.24±0.1
		S307	11.6 ±1.7	26.3 ±2.7

<i>plp</i> expression	RNA copy number	M93Sm	$2.70 \times 10^2 \pm 34$	$6.83 \times 10^2 \pm 46$
		S305	$4.22 \times 10^2 \pm 62$	$3.27 \times 10^2 \pm 53$
		S307	$2.25 \times 10^3 \pm 445$	$5.92 \times 10^4 \pm 3467$
	Relative change (n-fold) in expression	M93Sm	1	1
		S305	1.56 ± 0.05	-2.08 ± 0.01
		S307	8.32 ± 1.1	86.2 ± 7.8

^a The data presented is a representative experiment of two independent experiments.

Each sample is the average of three replicates.

^b *V. anguillarum* strains: M93Sm (wild type), S305 (*hlyU* mutant), and S307 (*hlyU* complement).

Figure 1. The 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 rectangle bars above the alignment indicate the relative amount of conservation of amino acid residues. The black boxed regions were used to design the degenerate primers according to their original DNA sequences. The black arrows shows the orientation of primers.

(A). Ribosomal Protein S20P protein alignment

(B). NahR protein alignment

Figure 2. The degenerate PCR product was amplified from *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 *E. coli* DH5α strain. The plasmid, purified from the appropriate colony, was sequenced.

Figure 3. Hemolytic activity of *V. anguillarum* strains M93Sm (wild type), S305 (*hlyU* mutant) colonies, and S307 (*hlyU* complement) transferred onto 5% TSA-sheep blood agar, and incubated at 27°C for 24 h. Relative hemolytic activity was determined by measuring the β -hemolysis zone surrounding each colony.

M93Sm

S305

S307

Figure 4. The *hlyU* mutation and its complement did not significantly effect the cytotoxicity of *V. anguillarum* against ASK cells. (A) ASK cells were incubated with various strains of *V. anguillarum* at moi =100 for 4 h at 20°C. Data showed that strain S305 (*hlyU* mutant) still caused ASK cells to round-up, as did wild type strain M93Sm and strain S307 (*hlyU* complement). ASK cells treated with NSS buffer (mock) exhibited no rounding or detachment during the course of the experiment. The photograph magnification was 100×. (B) LDH release from ASK cells treated with wild type M93Sm (●), *hlyU* strain S305 (△), *hlyU* complement S307 (▲), and *rtxA/vah1* double mutant S183 (○) at various moi (20, 50, 100, and 200) for 4 h. LDH release was measured in Relative Fluorescence Units (RFU), and then calculated to yield % cytotoxicity according to the instructions of the manufacturer. The incubation time at 0 was the base level of LDH in ASK cells treated with NSS buffer. Data are the representative of three separate experiments done with three replicates. Error bars show standard deviation of average. *, The statistical difference of strain S305 from the wild type treated data (P>0.1).

Figure 5. HlyU regulates both hemolysin clusters positively at the transcriptional level. Semi-quantitative RT-PCR was used to determine the expression levels of *rtxA*, *rtxB*, *rtxH*, *vah1*, and *plp* with 100 µg of RNA extracted from *V. anguillarum* cells grown to exponential phase (A) and stationary phase (B). Data show that the expression of *rtxA*, *rtxB*, and *rtxH* decreased in the *hlyU* mutant (S305). However, expression of *vah1* and *plp* did not decrease in S305, but did increase in the *hlyU* complement (S307). The M93Sm genomic DNA was used as positive control (+). RT-PCR reactions with no added reverse transcriptase used as negative controls showed no amplified bands (data not shown). The DNA molecular size standards (Std) are a 100 bp Promega® DNA ladder.

(A). Log phase

(B). Stationary phase

Figure 6. Intergenic regions of the *rtxACHBDE* gene cluster (A) and *vah1/plp* gene cluster (B). The transcriptional start sites of hemolysin genes are indicated by solid triangle marked with a +1. The -10 and -35 promoter sequences of hemolysin genes are indicated by underlined nucleotides labeled as -10 and -35 and are predicted to be σ^{70} promoters. Black arrows indicate that start codons of genes. Lines indicating ribosomal binding sites of hemolysin genes are labeled RBS. The boxed sequences represent inverted repeats located with the HlyU-DNase I protected sequences, which are indicated as protected region.

A *rtxH* start codon

CATAAATAAATCACCTCATAATTTAAAAATAAAACAAATCCTCTTATAAAAGCAGTGGCTCATAAAGCAGTTGCTTTTAAAGGAGT
 ← RBS
 ACGATAACCAAAAAATCATTTTATATTATATAGCATTTCAACAGCACGCAACCCACATAATAACGAAAACCTTATGAGTTAATTAAA
 ▲ -10 -35
 GAAACACAAACGCAATAGACCATAACCCATTGATAATAAAAACTTTAAAAATAATCCAGCAAGTCAGACCAGGTAATATTGAGCA
 Protected Region -35
 TTCATTGATATTAATGATATAATCAGCCACCCTTTTATCTTTGGCCCTTAAAGAAATCAAATATATG
 -10 ▲ RBS *rtxB* start codon
 +1

B *plp* start codon

CATATTTTTTTCTCTTATTGGTTTAGGCACGTATCTACTAAAGAATAAGTGCAGAAAGTGAAATTAAAAAATGGTCAGACCTATTTTG
 ← RBS
 GATTTATGGTGTTAAGCGTCAATCAATAAACGACTATGTTGAACATTATTTGGTATTAAGATCAACAATGAAGTGTGAGAGAAA
 -10 -35
 ATACGAAGGGTTTTTATAAATCCTAATTTAGATAAATAACAATAAGGTTTGCCTTTATTAATTTGTTCTAATGTTAAATCTTAGTAG
 CTTAATAAAAAATCAATAAAAATTATATCAGTCAATATGTGTAAAAATCAAACAGTTTATTGTGATTTTAAGCCACATTAATAATC
 Protected Region
 ATTTCTCTTTGTGTTTGTATTTTAAGATCTATTAATAATTCGCCCAAAGGTGCCAAAAACCAATGTCCTTTTATTTGTGGTCTG
 -35 -10
 AAAAAATAAAAAATGGGTATTCATTTCTTTTAAATACGTGTGATAACAATAAAAAATGCTTACGAGGTTTATATG
 RBS *vah1* start codon
 +1

Figure 7. 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 + 100 ng unlabeled DNA fragment (lane 3); Panel B – fragments a, b, and c from the intergenic regions of the *rtxACHBDE* operon; and Panel D – fragments d, e, f, g, and h from the intergenic regions of the *vah1/plp* gene cluster.

A

C

B

D

Figure 8. Capillary electrophoresis of 6-FAM labeled DNA fragments b (A) and f (B) from DNase protection assays in the presence (grey traces) and absence (black traces) of HlyU demonstrating that HlyU binds to specific sequences in fragments b and f of the *rtxACHBDE* and *plp/vahI* 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 μ g) followed by DNase I, and then analyzed by DNA fragment analysis as described in the 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 (grey trace) than in its absence (black trace).

A. Probe 4, Fragment b region, *rtxH/B*

B. Probe 2, Fragment f region, *vah1/plp*

Manuscript III

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

Authors: Xiangyu Mou, Edward J. Spinard, Maureen V. Driscoll, Wenjing Zhao and David R. Nelson*

Affiliation: Department of Cell and Molecular Biology, University of Rhode Island

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***Corresponding author:** David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

Abstract

Hemolysins produced by *V. anguillarum* have been implicated in the development of hemorrhagic septicemia during vibriosis, a fatal fish disease. Previously, two hemolysin gene clusters were identified in *V. anguillarum*, the *vah1-plp* gene cluster and the *rtxACHBDE* gene cluster, which are responsible for the hemolysis and cytotoxicity of *V. anguillarum*. In this study, we identified the *hns* gene, which encodes the H-NS protein, and acts as a negative regulator of the 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 near wild type levels. Further, expression of *rtxA*, *rtxH*, *rtxB*, *vah1* and *plp* increased in the *hns* mutant, and decreased in the complemented *hns* mutant strain when compared to the wild type strain. Additionally, experiments using DNase I, showed that purified recombinant H-NS protected multiple sites in the promoter region 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 up-regulation 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.

Introduction

Vibrio anguillarum is the causative agent of vibriosis, a fatal hemorrhagic septicemic disease. *V. anguillarum* infects more than 50 fresh and salt-water fish species including various species of economic importance to the larviculture and aquaculture industry, such as salmon, rainbow trout, turbot, sea bass, sea bream, cod, eel, and ayu (1). Infections by this bacterium have a mortality rate 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 with strong amino acid sequence identity to *Vibrio cholerae* EI Tor hemolysin (*hlyA*) and *V. fluvialis* hemolysin (5). Mutations in the divergently transcribed *plp* 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 level of *vah1* transcription and hemolysis (4). Plp is a phosphatidylcholine (PC)-specific PLA₂, which causes lysis of PC-rich fish erythrocytes (L. Li et al., unpublished data). These observations suggest that Plp plays a dual role both as a repressor and as 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 T1SS genes (*rtxDBE*) responsible for the secretion of RtxA. RtxA exhibits cytotoxic activity causing Atlantic salmon kidney (ASK) cells to round and die (5). Loss of *rtxA* function results in avirulence (5), while mutation of *vah1* causes slight attenuation of *V. anguillarum* virulence (4). Mutations in both *vah1* and *rtxA* lost 98% cytotoxicity in ASK cells, suggesting Vah1 and RtxA are the major two cytotoxin 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 is reported that function of H-NS 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 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 the H-NS repressive action (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 *V. cholerae* *tcpA* and *ctx* promoters by H-NS is antagonized by the AraC-like transcriptional regulator ToxT and 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* 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 with *hns* complemented and an *hns/hlyU* double mutant with *hlyU* complemented. The hemolytic activity and cytotoxicity of these strains were determined. The expression levels of various hemolysin genes, including *vahI*, *plp*, *rtxA*, *rtxH* and *rtxB* in these strains were also quantified. 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*. *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. Atlantic salmon kidney (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. *E. coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10). 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*; tetracycline, 15 µg/ml (Tc¹⁵) for *E. coli*, 1 µg/ml (Tc¹) for *V. anguillarum* grown in liquid medium, and 2 µg/ml (Tc²) 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.

Then a 200-300 bp DNA fragment of *hns* was 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 BioRad Gene Pulser II (BioRad, Hercules, CA). Transformants were selected on LB10 Cm²⁰ agar plates. The construction of pNQ705-*hns* was confirmed by both PCR amplification and restriction 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 the *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 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 *Xba*I, and inserted into the *Xba*I site between the 5' and 3' *hns* regions on the derivative pDM4. The resulting 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 Kan⁸⁰ Sm²⁰⁰ Cm⁵ plates and, subsequently, double-crossover transconjugants were selected with LB20 Kan⁸⁰ Sm²⁰⁰ 5% sucrose plates. The resulting *V. anguillarum* mutants were checked for the desired allelic exchange using PCR amplification and then 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 previously by Rock and Nelson (4). Briefly, primers *hns_comp*(F) and *hns_comp*(R) (Table 2) were designed with a *Pst*I 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' and ~200 bp 3' flanking regions from genomic DNA of *V. anguillarum* M93Sm. The DNA fragment was then ligated into pSUP202 after digestion with *Eco*RI and *Age*I, and the ligation mixture was introduced into *E. coli* Sm10 by electroporation using a BioRad Gene Pulser II. Transformants were selected on LB10 Tc¹⁵Ap¹⁰⁰ 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 *et al.* (4). Briefly, *V. anguillarum* colonies were transferred onto blood agar plates and hemolytic activity was determined by measuring the diameter of β -hemolysis on plates containing Trypticase soy agar (TSA) plus either 5% sheep (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*) using a 3 ml sterile syringe supplemented with 10 μ l 0.5M Disodium EDTA (Sigma-Aldrich). The blood was then stored on ice and used in casting plates within 6 hours.

Cytotoxicity assay. This 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's L-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 ($9,000 \times g$, 5 min, 4°C). The resulting culture supernatant was harvested and filter-sterilized using 22 μ m filters (Millipore Corp., Billerica, MA). The bacterial pellet was washed twice in nine salts solution (NSS) (19) and resuspended in fresh NSS (at $\sim 2 \times 10^9$ cells ml⁻¹). *V. anguillarum* culture supernatant (50 μ l) was added to wells containing ASK cells plus 50 μ l pH =7.4 phosphate

buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) and incubated at 20°C for 6 h. Washed bacterial cells were added to ASK cells at multiplicities of infection (MOI) = 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 at 560 nm_{Excitation}/590 nm_{Emission}. Fluorescence was read by a Stratagene MX3005P QPCR System at 550 nm_{Excitation}/570 nm_{Emission}.

RNA isolation. Exponential phase cells ($\sim 0.5 \times 10^8$ CFU ml⁻¹) and stationary phase cells (2×10^9 CFU ml⁻¹) of various *V. anguillarum* strains were treated with RNAlater Bacteria Reagent (QIAGEN, Valencia, CA), following the manufacturer's instructions. Total RNA was isolated using the 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 stored at -75°C for future use.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was used to quantify various mRNAs using an Mx3005 Multiplex Quantitative PCR System and the 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.

Over-expression and purification of the *V. anguillarum* H-NS protein. The DNA fragment encoding H-NS was PCR amplified by using Pm416 and Pm417 (Table 2) and 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 (confirmed by sequencing) was used for expression of His-tagged H-NS protein in *E. coli* M15. Expression and purification of rH-NS was carried out using a modification of the procedures described in 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 OD₆₀₀ reached 0.6, 1 mM IPTG 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 non-denaturing 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 the 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 using PCR (Table 2). Probes were labeled with 6-FAM at the 5' end of a certain strand of each probe (Fig. S1). The assay was carried out using a method modified from Li *et al.* (6). Briefly, 7.5×10^{11} copies of DNA probe and various amounts of rH-NS (up to 3 μ M) were incubated for 1 h at 27°C in a total volume of 20 μ l, containing 4 μ l of 5 $\times$ binding buffer, 1 μ g poly-L-lysine, and 1 μ g poly [d(I-c1)] (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 μ l containing 2.5 μ l of 10 $\times$ concentrated reaction buffer at 37°C for 15 min. The reaction was stopped by heating (95°C, 10 min). The DNA was purified using a QIAquick PCR Purification Kit (QIAGEN) using a QIAcube and its standard protocol, except that the elution volume was adjusted to 30 μ l. The DNA in the eluate (3 μ l) was added to 9 μ l Hi-Di Formamide containing 1 μ l 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. GC content of the two gene clusters (shown in Fig. 1) were plotted using the GC-Profile program (<http://tubic.tju.edu.cn/GC-Profile>) (20) using the following settings (Halting parameter = 1; Minimum length to segment = 100 bp).

Fish infection studies. Various *V. anguillarum* strains were tested for virulence with rainbow trout (*Oncorhynchus mykiss*) by intraperitoneal (IP) injection. Briefly, *V. anguillarum* cells grown in LB20 supplemented with appropriate antibiotics for 22 h at 27°C were harvested by centrifugation ($9,000 \times g$, 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 optical density at 600 nm. 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), with 100 mg/L for induction and 52.5 mg/L for maintenance. *V. anguillarum* strains were IP-injected into fish in 100 μ l 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 NSS only as 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 confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the head kidney 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 for the fish infection experiment (P values of <0.05 were considered statistically significant. For fish infection experiments, a Kaplan-Meier survival analysis with log rank significance test was performed on fish survival percentage (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 *V. anguillarum* M93Sm draft genome (unpublished data). It encodes for a predicted 137 amino acid protein with a molecular mass of 15,299 Da and has strong homology 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 β -hemolysis on 5% TSA-sheep blood agar (5). When the hemolytic activity of the *hns* mutant (M114, *hns*-) was tested on 5% TSA-sheep blood agar, it was found that mutation of *hns* resulted in increased hemolysis when compared to the wild type (M93Sm) (Fig. 2). Further, when the *hns* mutation was complemented (M116, *hns*+), hemolysis was reduced to levels below 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* increased cytotoxicity against Atlantic salmon kidney (ASK) cells. It was previously demonstrated that both *vah1* or *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 activity of both culture supernatants and washed cells from the *hns* mutant (*hns*⁻) and the complemented *hns* mutant (*hns*⁺) and compared their activities against culture supernatant and washed cells from the wild type (M93Sm). The results show that mutation of *hns* significantly increased the cytotoxicity of both *V. anguillarum* culture supernatant by >40%, and cells (MOI 200) by ~80% against ASK cells compared to wild type *V. anguillarum* M93Sm. Complementation of the *hns* mutation decreased cytotoxicity significantly of both culture supernatant and cells against ASK cells compared to the cytotoxic activities 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 *vahI*.

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 *vahI*, *plp*, *rtxA*, *rtxH*, and *rtxB*, in the wild-type strain (M93Sm), *hns* mutant (*hns*⁻), and the *hns* complement (*hns*⁺) during both exponential and stationary growth phases. Real time qRT-PCR data revealed that in the *hns* mutant during exponential and stationary phase, respectively, when compared to the wild type, expression of *rtxA* increased by 2.91- and 2.14-fold; expression of *rtxB* increased by 1.28- and 1.43-fold; and expression of *rtxH* increased

by 4.56- and 2.39-fold; and expression of *vahI* increased by 16.21- and 20.01-fold; and expression of *plp* increased by 31.27 and 36.88-fold (Fig. 4 and Table S1). Further, 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 *vahI-plp* gene clusters.

Mutation of *hns* does not affect the expression of *hlyU*. Since HlyU had previously been shown to bind to the intergenic regions of both hemolysin gene clusters to increase their transcription (Li *et al.* 2011), 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 (*hns*-), and the *hns* complement (*hns*+) during exponential and stationary growth phases (Fig. S2A). No statistically significant difference in expression of *hlyU* was found between M93Sm and both the *hns* mutant (*hns*-) and the *hns* complement (*hns*+) in either log phase or stationary (Fig. S2A). These results rule out the possibility that H-NS regulates hemolysin gene expression by regulating the expression of *hlyU*.

Up-regulation of hemolysin genes by *hlyU* is *hns*-dependent. As noted above, Li *et al.* (6) showed that an *hlyU* mutant (S305, *hlyU*-) had decreased hemolytic activity on sheep blood agar compared to wild type, and that complementation of *hlyU* (S307) resulted in increased activity compared to 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 (*vahI*, *plp*, *rtxA*, and *rtxB*) in each hemolysin transcriptional unit. The first set of these determinations was carried out in cells lacking a functional *hlyU*: the *hlyU* mutant (*hlyU*-), the *hns/hlyU* double mutant (ES114, *hns-/hlyU*-), and the *hns/hlyU* double mutant with *hns* complemented (ES116, *hns+/hlyU*-); determinations of hemolytic activity and hemolysin gene expression were also done for wild type M93Sm (Fig. 5). 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 complemented in the double mutant, hemolysis decreased to levels seen in the *hlyU* mutant (compare Fig. 5A with Fig. 2). Changes in transcription of *rtxA*, *rtxB*, *vahI*, and *plp* corresponded with the changes in hemolysis (Fig. 5B and Table S1). Specifically, transcription of each gene (*rtxA*, *rtxB*, *vahI*, and *plp*) increased in the absence of a functional *hns* and decreased in the presence of a functional *hns*.

The second set of determinations was carried out in cells lacking a functional *hns*: the *hns* mutant (*hns*-), the *hns/hlyU* double mutant (*hns-/hlyU*-), and the *hns/hlyU* double mutant with *hlyU* complemented (ES115, *hns-/hlyU*+); determinations of hemolytic activity and hemolysin gene expression were also done for wild type M93Sm (Fig. 6). In absence of a functional *hns*, hemolytic activity increased regardless of the presence or absence of *hlyU* (Fig. 2 and Fig. 6A). Determination of hemolysin transcription by RT-qPCR corresponded with the hemolysis assay (Fig. 6B). In absence of *hns*, *rtxA* and *rtxB* expression increased over levels in wild type cells.

For *rtxA*, all increases were >2-fold and 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 and Table S1). Similarly, in the absence of a functional *hns*, 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* (Fig. 6B and Table S1). As with the *rtxACHBDE* gene cluster, our data show that in absence of *hns*, the complement of *hlyU* (*hns*-/ *hlyU*+) only exhibited minimal changes in expression of both *vah1* and *plp* (around 2-fold) over the *hns*- strain and almost no change in *vah1* and *plp* expression between the two strains (Fig. 6B and Table S1). These data indicate that up-regulation of hemolysin genes by *hlyU* is *hns*-dependent.

H-NS binds to the intergenic region 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 the Materials and Methods. The results of these experiments revealed that rH-NS protected multiple regions in both *rtxB/H* and *vah1/plp* intergenic regions (Fig. 7). These regions are AT-rich (72-74% AT) and correspond to other H-NS binding sites described in other bacteria (21-27) (Fig. 8). The H-NS binding sites cover the promoter regions of all four genes (*rtxB*, *rtxH*, *plp*, *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 *plp* and *vah1* promoters, but did not cover the HlyU binding site. In the *rtxB/H* intergenic region, rH-NS bound to six sites, covering the -35 region of both *rtxB* and *rtxH* promoters. In addition, rH-NS also bound to a seventh site just within the *rtxB* coding sequence. The rH-NS also was found to protect the three *rtxB*-proximal bases of the HlyU binding site (Fig. 8).

The *vah1-plp* and the *rtxACHBDE* gene clusters are unlikely to have been horizontally acquired. 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 xenogenic in origin. We examined the GC content of the two gene clusters and compared them to the average GC content of the whole genome of *V. anguillarum*. The result 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 at 26% GC and the *rtxB/H* region at 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. Further, we saw no evidence of any tRNA

genes, transposases, interrupted genes, or pseudogenes in these surrounding regions. Finally, when we examined the codon usage patterns for the hemolysin genes and compared them to the chromosome in which each is found (*rtx* genes in chromosome I and *plp* and *vahI* in chromosome II), no significant differences were found. These observations suggest that while the two hemolysin gene clusters are negatively regulated by H-NS, they were not horizontally acquired.

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, *hns*⁻, and *hns*⁺. Groups of ten rainbow trout were infected by IP-injection as described in the Materials and Methods with the wild type M93Sm, *hns*⁻, or *hns*⁺ strains of *V. anguillarum* in NSS at a dose of $\sim 4 \times 10^5$ CFU/fish, or with NSS only as a negative control. All M93Sm infected trout died by day 4, while 60% of *hns*⁻ infected trout died by day 14. These results (Fig. 9) show that there was a significant difference ($P = 0.005$) in the virulence of 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*⁻ 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 would appear to be counterintuitive, since both hemolysin gene clusters are up regulated 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, *hns*⁻, and *hns*⁺ in LB20 (Fig. S3). While the three strains grew to nearly identical cell densities (OD₆₀₀ = 1.04 (M93Sm), 0.97 (*hns*⁻) and 0.97 (*hns*⁺) at stationary phase, *hns*⁻ had a longer generation time compared to the wild type (58 min vs. 48 min, *P* < 0.05). However, complementing the *hns* mutation did not result in a shorter generation time than the *hns*⁻ (60 min vs. 58 min), suggesting 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 these bacteria use 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 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 in *V. anguillarum* M93Sm, *vah1* (4, 36), *rtxA* (5) and *plp* (4) (Li, Mou, and 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 all 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 the *rtxB/H* 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 an *hns* complement 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 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 MOI = 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* complement (Fig. 4 and Table S1). These data show that H-NS is a negative regulator of hemolytic and cytotoxic activity by acting as a repressor of hemolysin gene expression.

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 anti-repressor 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 HlyU could displace H-NS from the promoter, binding at a low concentration and that H-NS needs a much higher concentration to displace the bound HlyU (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 up-regulation of hemolysin gene expression by HlyU is dependent upon the presence of *hns* (Figs. 5 & 6, 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 anti-repressor. Additionally, both gene clusters are arranged as divergently transcribed genes with one HlyU binding site in the center of each

intergenic region (Fig. 8; see also Fig. 7 in Li *et al* (6)) flanked by 2-4 H-NS binding sites that extend towards the promoter sites (Fig. 8). The sites protected by rH-NS (Fig. 8) are AT-rich. The five H-NS binding sites in the *vahI/plp* intergenic region have A+T% that range from 64.5% to 84.3 %, while the seven H-NS protected sites in the *rtxB/H* intergenic region have A+T% that range from 47.4% to 81.25%. In contrast, the flanking structural genes have much lower A+T%. The A+T% for *plp* = 57% and for *vahI* = 55.7%; the A+T% for *rtxACH* = 51.6% and for *rtxBDE* = 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, that the structural genes have an A+T% nearly identical to the whole genome of *V. anguillarum* (55.49%) suggests that these virulence genes were not horizontally acquired. This is further supported by the observations detailed above that there is no evidence for any tRNA genes, transposases, interrupted genes or pseudogenes in the 7.5 to 10 kbp of DNA flanking the hemolysin gene clusters. Further, codon usage in the hemolysin genes is not significantly different from that of the chromosomes in which each gene cluster resides.

It has been suggested that self-oligomerization and binding of H-NS to AT-rich DNA to form DNA–protein–DNA bridges impedes the movement of RNA polymerase, thus repressing gene expression (8). H-NS repression may be reversed by different mechanisms (8). In *V. cholera*, the binding site of the transcriptional activator ToxT overlaps with 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 anti-repressor HlyU is far upstream of the start transcription site (-376 to -417) for the *rtxAI* operon and overlaps with two H-NS binding sites (12). Binding of HlyU to DNA relieves the H-NS repression at all H-NS binding sites of the *rtxAI* 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 growth than during stationary phase. Additionally, the binding sites for HlyU and H-NS for the *V. anguillarum* intergenic *rtxHB* region are much closer to the +1 start transcription sites than in *V. vulnificus* CMCP6. In *V. vulnificus* the five H-NS binding sites are at -289 to -459 nt from the start transcription site of *rtxH* (vv20481) (12), while the H-NS binding sites in *V. anguillarum* are between -23 to -100 for *rtxH* proximal sites and +42 to -70 from the +1 site 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 1028 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 is 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 compared to the wild type, although the mutant showed a higher level of alpha-hemolysin expression and activity (38). Our data suggest 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 the 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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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype and feature(s)						Reference
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 complement	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, hlyU complement	Sm ^R	Cm ^R	Tet ^R	Km ^R		This study
ES116	hns/hlyU double mutant, hns complement	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)						Km ^R QIAGEN

D112	<i>E. coli</i> Sm10 with pDM4-hns5'-Kan-hns3'	Cm ^R	Km ^R	This study
Plasmid				
pNQ705-1	Cm ^r ; suicide vector with R6K origin			(40)
pNQ705-hns	For <i>hns</i> insertional mutation			This study
pSUP202	<i>E. coli</i> – <i>V. anguillarum</i> shuttle vector			(39)
pSUP202-hly U	For complementation of <i>hlyU</i>			(6)
pSUP202-hns	For complementation of <i>hns</i>			This study
pDM4	Cm ^r Kan ^r SacBC ^r ; suicide vector			(18)
pDM4-hns5'- Kan-hns3'	For <i>hns</i> deletion mutation			This study
pQE-30 UA	Expression vector with N-terminal His ₆ -tag			QIAGEN
pQE-30 UA/H-NS	For expression of rH-NS			This study

Table 2. Primers used in this study.

Primers	Sequence (5' to 3', underlined sequences are engineered restriction sites)	Description	Reference
SD_hns(F)	GCTAG <u>GAGCTCC</u> AGCTT GAAGAAGCACTAGA	<i>hns</i> insertional mutation, forward, Sac I	This study
SD_hns(R)	GCTAGTCTAGACCAGAA AGTGCAGAAATTAA	<i>hns</i> insertional mutation, reverse, Xba I	This study
hns_com p(F)	GCTAG <u>CTGCAGT</u> CGGCG ATAAAACCTTTCAC	<i>hns</i> complement, forward, Pst I	This study
hns_com p(R)	GCTAG <u>CTGCAGT</u> TTTAC CTGAACGTGACGAC	<i>hns</i> complement, reverse, Pst I	This study
Pm416	TTAAATCTCGAATTCTTC TAGAGATTACC	<i>hns</i> ORF, forward	This study
Pm417	ATGTCTGAATTAACAAA AACTCTACTTAAT	<i>hns</i> ORF, reverse	This study
pr40	CGGCT <u>ACTCGAG</u> AGATT TACCTGCATCAAGTTG	<i>hns</i> 5' region, forward, Xho I	This study
pr41	CGGCTATCTAGAGCACT TTCTGGTGAAACTAAG	<i>hns</i> 5' region, reverse, Xba I	This study
pr42	CGGCTAT <u>CTAGA</u> ATTAA TGCGCTTACATCAATA	<i>hns</i> 3' region, forward, Xba I	This study
pr37	CGGCTAGAGCTCAGAAAG CACTAGATAAATTAAC	<i>hns</i> 3' region, reverse, Sac I	This study
pr38	CGGCTATCTAGAGAAAA GCTTGAACACGTAGAA	kanamycin resistant gene, forward, Xba I	This study
Pm173	ACTGAT <u>CTAGAT</u> CAGAA GAACTCGTCAAGAAG	kanamycin resistant gene, reverse, Xba I	This study
RT vah1-R1	GACCGCCGAATCGATGA TGAATC	vah1 qRT-PCR, forward	(4)
pvah1JR	GGTAGGACTGATGCCCCA CCTACAA	vah1 qRT-PCR, reverse	This study
plpF RT	CAGACGACCACCAGTAA CCACTAA	plp qRT-PCR, forward	(4)
plpR RT	GCAATCATGATGACCCA GCAACAG	plp qRT-PCR, reverse	(4)
Pm111	GGAAATTATTCCGCCGA CGATGGA	rtxA qRT-PCR, forward	(5)
Pm112	GCCGATACCGTATCGTT ACCTGAA	rtxA qRT-PCR, reverse	(5)
Pm285	GTGATGGTAGAAAACCT GCGG	rtxH qRT-PCR, forward	This study
Pm286	ATGTCTGAGAAATTTGT CCAAACA	rtxH qRT-PCR, reverse	This study
iPCR rtxB R	CCGCTAACCGCATTGAT ATTAAGCTTGGC	rtxB qRT-PCR, forward	This study

Pm104	TCACAATCGCCCCAACT TGCCTTG	rtxB qRT-PCR, reverse	This study
Pm297	ACTGAGAGCTCGGTGTT GTAAAGGCTATGGC	hlyU qRT-PCR, forward	(6)
Pm298	ATCGATCTAGAGTATCC ACTAACCCATCTCTT	hlyU qRT-PCR, reverse	(6)
Pm412	CCGTATTTTCTGCAATC GCCATGG	<i>vahI</i> promoter region (probe 1), forward, 5'-labeled with 6-FAM	(6)
Pm324	CACATATTGACTGATTA TAATTTTATTGATATT	<i>vahI</i> promoter region (probe 1), reverse	(6)
pr323a	AGGGTTTTTATAAATCC TAATTTAGATA	<i>plp</i> promoter region (probe 2), forward, 5'-labeled with 6-FAM	This study
Pm320	GAATACCCATTTTTTATT TTTTCAGACC	<i>plp</i> promoter region (probe 2), reverse	(6)
Pm327	GTATTTTCTGCAATCGC CATG	<i>vahI/plp</i> (probe 3) intergenic region, forward	(6)
Pm413	CACCTTTGTGGCGAATT ATTAATAGATCTT	<i>vahI/plp</i> (probe 3) intergenic region, reverse, 5'-labeled with 6-FAM	(6)
Pm414	CAGTGGCTCATAAAAGC AGTTGC	<i>rtxB/H</i> intergenic region (probe 4), forward, 5'-labeled with 6-FAM	(6)
Pm318	CAGCGGTAAGTAGACTG ATA	<i>rtxB/H</i> intergenic region (probe 4), reverse	(6)
Pm315	CTCAGACATAAATAAAT CACC	<i>rtxB/H</i> intergenic region (probe 5), forward	(6)
Pm415	CAGCGGTAAGTAGACTG ATAAGCAATG	<i>rtxB/H</i> intergenic region (probe 5), reverse, 5'-labeled with 6-FAM	(6)

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

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

M93sm

hns⁻

hns⁺

Figure 3. Cytotoxicity experiment. **A)** Cytotoxicity of culture supernatant from *V. anguillarum* wild type (M93Sm), *hns* mutant (*hns*⁻) and *hns* complement (*hns*⁺) against Atlantic salmon kidney (ASK) cells after 6 h at 20°C. **B)** Cytotoxicity of washed cells of *V. anguillarum* wild type (M93Sm), *hns* mutant (*hns*⁻) and *hns* complement (*hns*⁺) against Atlantic salmon kidney (ASK) cells after 4 h at 20°C at MOI 200. Asterisks (*) represent $P < 0.05$ between two bracketed strains. Error bars represent 1 standard deviation.

A.

B.

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

Figure 5. Repression of H-NS does not require *hlyU*. **A)** Hemolytic activity of *V. anguillarum* wild type (M93Sm), *hlyU* mutant (*hlyU*-), *hns/hlyU* double mutant (*hns-/hlyU*-) and *hns/hlyU* double mutant with *hns* complemented (*hns+/hlyU*-) 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 (*hns-/hlyU*-) and *hns/hlyU* double mutant with *hns* complemented (*hns-/hns*+) during logarithmic (Log) and stationary (Sta) phase. The data presented are a representative experiment from two independent experiments. Each sample is the average of three replicates. Asterisks (*) represent $P < 0.05$ between two bracketed strains. Error bars represent 1 standard deviation.

A.

B.

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

Figure 7. Dnase I protection assay. Capillary electrophoresis of 6-FAM labeled DNA (**A**) probe 1 and (**B**) probe 2 from the *vah1/plp* intergenic region, and (**C**) probe 4 from the *rtxB/H* intergenic region from DNase protection assays in the presence (black lines) and absence (grey line) of rH-NS demonstrating that H-NS binds to specific sequences in the *vah1/plp* and *rtxB/H* intergenic regions, respectively, and protects against DNase I digestion. DNA probes were prepared and labeled with 6-FAM, incubated with rH-NS (0 or 3 μ M) followed by DNase I, and then analyzed by DNA fragment analysis as described in the Materials and Methods. The binding region sequences are indicated by the double black underline. The underlined DNA fragments indicate those that are higher in the presence of rH-NS (black line) than in its absence (grey line). The location of each probe is shown in Fig. S1.

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

A.

CATATTTTTTCTCTTTTATTGGTTTAGGCACGTATCTACTAAAGAATAAGTGCGAAAGTGAAATTAAAAAACTGGTCAGACCTATTTGGATTATTTGGTGT
 ← RBS
 plp start codon +1 -10
 TAAGCGTCAATCAATAAACGACTATGTTGAACATTATTGGTATTAAGATCAAACAATGAAGTGCAGTGGAGAAAAACGAAGGGTTTTTATAAATCCT
 -35
 AATTAGATAAATAACAATAAGGTTTGTCTTATTAATTTGTTCTAATGTTAAATCTTAGTAGCTTAATAAAAAATATCAATAAAAAATTAATCAGTCAA
 HlyU protection
 TATGTGTAAAAATCAACAGTTTATTGTGATTTTAAGCCACATTAAAAATCATTCTCTTTGGTGTGTTTATTAAAGATCTATTAATAATTCGCCACA
 -35 -10 +1
 AAGGTGCCAAAAACCAATGTCCTTTTATTGTTGGTCTGAAAAATAAAAAATGGGTATTCATTTCTTTTAAATACTGTGATAACAATAAAAAATGCTT
 ACGAGCTTTATATC
 RBS → vah1 start codon

B.

CATAAATAATCACTCATTAATTTAAAAATAAAACAAATCCTCTTATAAAAGCAGTGGCTCATAAAAGCAGTTGCTTTTAAAGGAGTAACGATAACCAA
 ← RBS
 rtxH start codon
 AAAATCATTTTTTATATTATATAGCATTCAACAGCACGCAACCCACATAATAACGAAACTTATGAGTTAATTAAAGAAACACAAACGCAATAGACCATA
 +1 -10 -35
 ACCCATTTGATATAAAAAATCTTAAAAATAATACCAGCAAGTCAGACCAGGTAATATTTGAGCATTGATGATTAATGATATAATCATAGCCACCTTTT
 HlyU protection -35 -10 +1
 ATCTTTTGGCCCTTAAAGAAATCAAATATATGATGACTGATTTAAATGAAAAAAATCCATGCTACATTGCTTATCAGTCTACTTACCGCTGATCGG
 RBS → rtxB start codon

Figure 9. Fish infection experiment. Survival percentages of rainbow trout injected IP with NSS only (Mock, gray dashed line) or $\sim 4 \times 10^5$ CFU/fish of wild-type M93Sm (gray solid line), *hns* mutant (*hns*⁻, black solid line), or *hns* complement (*hns*⁺, black dashed line). Each experimental group had n = 10. *V. anguillarum* cells were suspended in NSS. Asterisks (*) represent $P < 0.05$ between two bracketed strains.

Table S1. Real time qRT-PCR analysis^a

Parameter and gene expressed	V. <i>anguillarum</i> strain ^b	Average (±SD ^c)	
		Log phase	Stationary phase
<i>rtxA</i>			
RNA Copy number	M93sm	$2.66 \times 10^4 (\pm 3.76 \times 10^3)$ 6	$8.57 \times 10^3 (\pm 3.60 \times 10^2)$
	<i>hns</i> -	$7.75 \times 10^4 (\pm 1.11 \times 10^4)$	$1.83 \times 10^4 (\pm 2.13 \times 10^3)$
	<i>hns</i> +	$9.72 \times 10^2 (\pm 10)$	$2.94 \times 10^2 (\pm 19)$
	<i>hlyU</i> -	$2.38 \times 10^2 (\pm 1.38 \times 10^2)$	$6.02 \times 10^2 (\pm 90)$
	<i>hns</i> -/ <i>hlyU</i> -	$6.47 \times 10^4 (\pm 9.92 \times 10^3)$	$1.23 \times 10^4 (\pm 1.47 \times 10^3)$
	<i>hns</i> -/ <i>hlyU</i> +	$1.43 \times 10^5 (\pm 2.00 \times 10^4)$	$2.13 \times 10^4 (\pm 4.91 \times 10^2)$
	<i>hns</i> +/ <i>hlyU</i> -	$1.24 \times 10^4 (\pm 1.75 \times 10^2)$	$3.74 \times 10^3 (\pm 4.65 \times 10^2)$
Relative change (fold) to M93Sm ^d	M93Sm	1.00 (±0.14)	1.00 (±0.04)
	<i>hns</i> -	2.91 (±0.42)	2.14 (±0.25)
	<i>hns</i> +	-27.37 (±0.00)	-29.15 (±0.00)
	<i>hlyU</i> -	-11.18 (±0.01)	-14.23 (±0.01)
Relative change (fold) to <i>hlyU</i> - ^d	<i>hlyU</i> -	1.00 (±0.06)	1.00 (±0.15)
	<i>hns</i> -/ <i>hlyU</i> -	27.21 (±4.17)	20.50 (±2.43)
	<i>hns</i> +/ <i>hlyU</i> -	5.22 (±0.07)	6.22 (±0.77)
Relative change (fold) to <i>hns</i> - ^d	<i>hns</i> -	1.00 (±0.14)	1.00 (±0.12)
	<i>hns</i> -/ <i>hlyU</i> -	-1.20 (±0.13)	-1.48 (±0.08)
	<i>hns</i> -/ <i>hlyU</i> +	1.84 (±0.26)	1.16 (±0.03)
<i>rtxH</i>			
RNA Copy number	M93Sm	$6.80 \times 10^4 (\pm 1.80 \times 10^4)$	$2.45 \times 10^4 (\pm 3.19 \times 10^3)$
	<i>hns</i> -	$3.10 \times 10^5 (\pm 8.58 \times 10^4)$	$5.85 \times 10^4 (\pm 3.56 \times 10^3)$
	<i>hns</i> +	$9.43 \times 10^3 (\pm 1.31 \times 10^3)$	$8.59 \times 10^3 (\pm 6.12 \times 10^2)$
Relative change (fold) to M93Sm ^d	M93Sm	1.00 (±0.26)	1.00 (±0.13)
	<i>hns</i> -	4.56 (±1.26)	2.39 (±0.15)
	<i>hns</i> +	-7.21 (±0.02)	-2.85 (±0.02)

<i>rtxB</i>			
RNA Copy number	M93sm	$4.17 \times 10^4 (\pm 4.01 \times 10^3)$	$1.71 \times 10^4 (\pm 1.23 \times 10^3)$
	<i>hns</i> -	$5.35 \times 10^4 (\pm 9.58 \times 10^3)$	$2.44 \times 10^4 (\pm 3.10 \times 10^3)$
	<i>hns</i> +	$3.89 \times 10^3 (\pm 5.19 \times 10^2)$	$1.59 \times 10^3 (\pm 18)$
	<i>hlyU</i> -	$9.54 \times 10^3 (\pm 5.96 \times 10^2)$	$3.62 \times 10^3 (\pm 1.41 \times 10^2)$
	<i>hns</i> -/ <i>hlyU</i> -	$7.31 \times 10^4 (\pm 7.23 \times 10^3)$	$3.56 \times 10^4 (\pm 7.66 \times 10^2)$
	<i>hns</i> -/ <i>hlyU</i> +	$2.92 \times 10^4 (\pm 8.00 \times 10^3)$	$1.85 \times 10^4 (\pm 2.48 \times 10^3)$
	<i>hns</i> +/ <i>hlyU</i> -	$1.14 \times 10^4 (\pm 1.42 \times 10^3)$	$1.47 \times 10^4 (\pm 5.74 \times 10^2)$
Relative change (fold) to M93Sm ^d	M93Sm	1.00 (± 0.10)	1.00 (± 0.07)
	<i>hns</i> -	1.28 (± 0.23)	1.43 (± 0.18)
	<i>hns</i> +	-10.72 (± 0.01)	-10.77 (± 0.00)
	<i>hlyU</i> -	-4.37 (± 0.01)	-4.73 (± 0.01)
Relative change (fold) to <i>hlyU</i> - ^d	<i>hlyU</i> -	1.00 (± 0.06)	1.00 (± 0.04)
	<i>hns</i> -/ <i>hlyU</i> -	7.67 (± 0.76)	9.84 (± 0.21)
	<i>hns</i> +/ <i>hlyU</i> -	1.20 (± 0.15)	4.06 (± 0.16)
Relative change (fold) to <i>hns</i> - ^d	<i>hns</i> -	1.00 (± 0.18)	1.00 (± 0.13)
	<i>hns</i> -/ <i>hlyU</i> -	1.37 (± 0.14)	1.46 (± 0.03)
	<i>hns</i> -/ <i>hlyU</i> +	-1.83 (± 0.15)	-1.32 (± 0.10)
<i>vahI</i>			
RNA Copy number	M93sm	$1.40 \times 10^3 (\pm 1.76 \times 10^2)$	$6.19 \times 10^2 (\pm 94)$
	<i>hns</i> -	$2.27 \times 10^4 (\pm 6.40 \times 10^3)$	$1.24 \times 10^4 (\pm 2.69 \times 10^3)$
	<i>hns</i> +	$1.06 \times 10^3 (\pm 3.62 \times 10^2)$	$1.88 \times 10^2 (\pm 17)$
	<i>hlyU</i> -	$3.24 \times 10^3 (\pm 1.94 \times 10^2)$	$4.74 \times 10^2 (\pm 88)$
	<i>hns</i> -/ <i>hlyU</i> -	$1.68 \times 10^4 (\pm 3.21 \times 10^3)$	$1.95 \times 10^4 (\pm 2.90 \times 10^3)$
	<i>hns</i> -/ <i>hlyU</i> +	$1.30 \times 10^4 (\pm 1.48 \times 10^2)$	$3.04 \times 10^4 (\pm 1.93 \times 10^3)$
	<i>hns</i> +/ <i>hlyU</i> -	$5.34 \times 10^3 (\pm 5.30 \times 10^2)$	$6.45 \times 10^3 (\pm 4.17 \times 10^2)$
Relative change (fold) to M93Sm ^d	M93Sm	1.00 (± 0.13)	1.00 (± 0.15)
	<i>hns</i> -	16.21 (± 4.57)	20.01 (± 4.35)
	<i>hns</i> +	-1.32 (± 0.26)	-3.30 (± 0.03)

	<i>hlyU</i> -	2.31 (± 0.14)	-1.30 (± 0.14)
Relative change (fold) to <i>hlyU</i> ^{-d}	<i>hlyU</i> -	1.00 (± 0.06)	1.00 (± 0.19)
	<i>hns</i> -/ <i>hlyU</i> -	5.20 (± 0.99)	41.02 (± 6.11)
	<i>hns</i> +/ <i>hlyU</i> -	1.65 (± 0.16)	13.60 (± 0.88)
Relative change (fold) to <i>hns</i> ^{-d}	<i>hns</i> -	1.00 (± 0.28)	1.00 (± 0.22)
	<i>hns</i> -/ <i>hlyU</i> -	-1.35 (± 0.14)	1.57 (± 0.23)
	<i>hns</i> -/ <i>hlyU</i> +	-1.75 (± 0.01)	2.46 (± 0.16)
<hr/>			
<i>plp</i> RNA Copy number	M93sm	8.50×10^2 ($\pm 2.07 \times 10^2$)	3.02×10^2 (± 8)
	<i>hns</i> -	2.66×10^4 ($\pm 4.08 \times 10^3$)	1.11×10^4 ($\pm 2.43 \times 10^2$)
	<i>hns</i> +	4.21×10^2 ($\pm 1.08 \times 10^2$)	3.18×10^2 (± 73)
	<i>hlyU</i> -	1.14×10^3 ($\pm 3.38 \times 10^2$)	3.46×10^2 (± 50)
	<i>hns</i> -/ <i>hlyU</i> -	8.46×10^3 ($\pm 1.64 \times 10^3$)	1.18×10^4 ($\pm 2.15 \times 10^3$)
	<i>hns</i> -/ <i>hlyU</i> +	8.58×10^3 ($\pm 8.21 \times 10^2$)	2.39×10^4 ($\pm 8.47 \times 10^2$)
	<i>hns</i> +/ <i>hlyU</i> -	2.02×10^3 ($\pm 5.75 \times 10^2$)	3.58×10^3 ($\pm 1.54 \times 10^2$)
Relative change (fold) to M93Sm ^d	M93Sm	1.00 (± 0.24)	1.00 (± 0.03)
	<i>hns</i> -	31.27 (± 4.80)	36.88 (± 0.81)
	<i>hns</i> +	-2.02 (± 0.13)	1.05 (± 0.24)
	<i>hlyU</i> -	1.34 (± 0.40)	1.15 (± 0.17)
Relative change (fold) to <i>hlyU</i> ^{-d}	<i>hlyU</i> -	1.00 (± 0.30)	1.00 (± 0.15)
	<i>hns</i> -/ <i>hlyU</i> -	7.46 (± 1.44)	34.02 (± 6.21)
	<i>hns</i> +/ <i>hlyU</i> -	1.78 (± 0.51)	10.35 (± 0.44)
Relative change (fold) to <i>hns</i> ^{-d}	<i>hns</i> -	1.00 (± 0.15)	1.00 (± 0.02)
	<i>hns</i> -/ <i>hlyU</i> -	-3.14 (± 0.06)	1.06 (± 0.19)
	<i>hns</i> -/ <i>hlyU</i> +	-3.10 (± 0.03)	2.15 (± 0.08)

^a The data presented is a representative experiment of two independent experiments.

Each sample is the average of three replicates.

^b *V. anguillarum* strains: M93Sm (wild type), *hns*⁻ (*hns* mutant, M114), *hns*⁺ (*hns* complemente, M116), *hlyU*⁻ (*hlyU* mutant, S305), *hns*⁻/*hlyU*⁻ (*hns*/*hlyU* double mutant, ES114), *hns*⁻/*hlyU*⁺ (*hns*/*hlyU* double mutant with *hlyU* complemented, ES115), *hns*⁺/*hlyU*⁻ (*hns*/*hlyU* double mutant with *hns* complemented, ES116)

^c Standard Deviation

^d Gene expression is up-regulated (positive number) or down-regulated (negative number) compared to expression in M93Sm.

Figure S1. Supplement data for Dnase I protection assay. 6-FAM labeled DNA probes (probes 1-5) used for DNaseI protection studies of the intergenic regions between **A)** *plp* and *vah1* and **B)** *rtxH* and *rtxB* were obtained by PCR amplification using the primers in Table 2.

A.

B.

Figure S2. Supplement data for qRT-PCR analysis. Real-time qRT-PCR was performed to determine **A)** the expression level of *hlyU* in the wild-type strain (M93Sm), *hns* mutant (*hns*-), and the *hns* complement (*hns*+) during exponential and stationary growth phases. Each sample is the average of three replicates. The data presented are the averages of two independent experiments. **B)** Expression level of *hns* in the wild-type strain (M93Sm) during exponential and stationary growth phases. Each sample is the average of three replicates. Statistically significant differences between samples are marked with a bracket and an * symbol. Error bars represent 1 standard deviation.

Figure S3. Growth profiles of *V. anguillarum* strains. **A)** Growth curve of *V. anguillarum* wild type (M93Sm), *hns* mutant (*hns*-) and *hns* complemented (*hns*+) strains in LB20. **B)** Generation time of *V. anguillarum* wild type (M93Sm), *hns* mutant (*hns*-) and *hns* complemented (*hns*+) strains grown in LB20. Statistically significant differences between samples are marked with a bracket and an * symbol. Error bars represent 1 standard deviation.

A.

B.

Manuscript IV

Publication status: To be submitted to one of the following ASM journals: *Applied and Environmental Microbiology*, *Infection and Immunity*, or *m Bio*.

Title: A *Vibrio anguillarum* isocitrate dehydrogenase mutant is highly attenuated and immunogenic in rainbow trout (*Oncorhynchus mykiss*).

Authors: Xiangyu Mou, Edward J. Spinard, Shelby L. Hillman, and David R. Nelson*

Affiliation: Department of Cell and Molecular Biology. University of Rhode Island, Kingston, RI 02822

Key Words: *Vibrio anguillarum*, isocitrate dehydrogenase, virulence, vaccine, tricarboxylic acid (TCA), hemolysin, HlyU, H-NS

***Corresponding author:** David R. Nelson, Department of Cell and Molecular Biology, 120 Flagg Rd., University of Rhode Island, Kingston, RI 02881, USA; E-mail: dnelson@uri.edu; Phone: 1-401-874-5902

Abstract

We have been investigating the regulation and roles of the hemolysin/cytotoxin genes in pathogenesis by *V. anguillarum*, the causative agent of vibriosis in fish. We have shown that there are three major hemolysin genes (*vahI*, *plp* and *rtxA*) and have created an avirulent strain by knocking *rtxA*. In this study, we aimed to create avirulent and immunogenic *V. anguillarum* strains that can be used as a live vaccine for fish, without knocking out any of the hemolysin genes. For this purpose, six genes (*mdh*, *icd*, *sucA*, *sucC*, *sdhC*, and *fumA*) encoding enzymes in tricarboxylic acid (TCA) cycle and one gene (*cra*) encoding a fructose metabolism repressor were identified and mutated in wild type *V. anguillarum* M93Sm (serotype O2a). Among all mutants, *icd* mutant showed high attenuation of virulence and lowest cell density limit in two forms of rich media. All mutants exhibited the same or higher levels of hemolysin gene expression compared to wild type during exponential phase. Further, fish that were pre-treated by immersion with *icd* mutant protected rainbow trout from the subsequent challenge of *V. anguillarum* M93Sm; and fish that were pre-treated injection of *icd* mutant elicited cross-serotype immunity against the subsequent challenge of *V. anguillarum* NB10 (serotype O1). The results suggest the TCA cycle mutation approach is likely to be an easy method to construct modified live vaccine for a wide variety of pathogenic bacteria.

Introduction

Vibrio anguillarum is the causative agent of vibriosis, a fatal hemorrhagic septicemic disease. *V. anguillarum* infects more than 50 fresh and salt-water 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). The mortality rate from infections ranges from 30% to 100% (2). *V. anguillarum* also causes vibriosis in crustaceans and bivalves (2). Infections by this bacterium have resulted in severe economic losses to aquaculture worldwide (2). In Norway, killed vaccines against *V. anguillarum* are proven successful in salmon aquaculture. During the 1980s, salmon farming in Norway experienced huge losses due to vibriosis and cold water vibriosis, and a total crash in the industry was only prevented by the use of vast amounts of antibiotics (3). Currently, all farmed salmon and trout in Norway are vaccinated against vibriosis (*V. anguillarum*), cold-water vibriosis or Hitra disease (*Aliivibrio salmonicida*), and furunculosis (*Aeromonas salmonicida*), and few reports of vibriosis were observed (4). The success of these killed vaccines immediately resulted in a decline in the use of antibiotics (3).

Shoemaker (5) reported an example of a successfully commercialized modified live vaccine (AQUAVAC-ESC) currently used in US catfish industry which highlights the live vaccine strategy. Modified live vaccines have several advantages over killed vaccines: First, modified live vaccines can be administered by immersion (5), which avoids the injection procedure frequently used in killed vaccines and make it possible to apply the vaccines to the larval or fry stages or some vulnerable species

(3). Second, modified live vaccines are able to elicit humoral, mucosal and cell-mediated immunity (6), while killed or subunit vaccines are not effective at eliciting cellular responses (7). Third, modified live vaccines do not require an adjuvant, such as mineral oil, which is reported to cause side effects including decreased growth rates, chronic peritonitis, adhesions, granulomas and pigmentation in the peritoneal cavity (8). Fourth, due to omission of injection and adjuvant, modified live bacterial vaccines have a lower cost (7, 9). Currently, there are eight licensed bacterial vaccines for fish in the United States. Five of them are killed vaccines and three of them are live cultures (*Arthrobacter*, *Edwardsiella ictaluri* and *Flavobacterium columnare*) (10). While there is a great need for modified live vaccines, modification of a specific pathogenic bacterium to result in a live vaccine strain may require a comprehensive understanding of its pathogenesis.

In vibriosis, *V. anguillarum* invades its host fish through the intestine and skin (11, 12). Infected fish usually die within 1-4 days (13-16) with systemic infection of *V. anguillarum*. Chemotactic motility and the metalloprotease EmpA were shown to be important during the invasion stage, while the siderophore anguibactin, flagellin subunits, lipopolysaccharides (LPS), and the MARTX toxin RtxA were shown to be important at least for the persistence in the host during the post-invasion stage (see reviews by Frans et al, 2011, and by Naka and Crosa, 2011) (1, 17). Recently, a *V. anguillarum* mutant with a global regulator H-NS knocked-out showed attenuation in virulence when injected intraperitoneally, suggesting bacterial fitness is also a important factor during the post-invasion stage (15).

Additionally, it has been demonstrated in *Salmonella enterica*, an intracellular bacterial pathogen, that some tricarboxylic acid (TCA) cycle mutant strains were avirulent and immunogenic for subsequent wild-type *S. enterica* infection (18-20). A functional fructose repressor (Cra) is also required for *S. enterica* (21). Similar results have also been observed for the uropathogenic *Escherichia coli* (UPEC), another intracellular bacterial pathogen (22). The TCA cycle- and gluconeogenesis-defective strains demonstrate significant fitness reductions during urinary tract infections (22). These observations suggest that the central metabolism of pathogens is important for pathogenesis.

The observations concerning the effects of the TCA cycle upon virulence allowed us to hypothesize that mutations in central metabolism-related genes, such as those that encode the TCA cycle enzymes could interrupt the infection process in fish. To test the hypothesis, we identified and created six TCA cycle mutant strains plus one fructose metabolism mutant strain and tested their virulence against rainbow trout (*Oncorhynchus mykiss*). Further, we examined their ability to grow in three different media, monitored their expression of each of the three hemolysins, performed infection experiments on rainbow trout to determine the virulence of each mutant, and performed challenge experiments to demonstrate the ability of the one highly attenuated (*icd*) mutant to elicit a protective response against virulent strains of *V. anguillarum*.

Materials and Methods

Identification of genes in *V. anguillarum*. *V. anguillarum* M93Sm draft genome (unpublished data) and *V. anguillarum* 775 genome (17) were annotated by the RAST (Rapid Annotation using Subsystem Technology) service (<http://rast.nmpdr.org/rast.cgi>) using the default settings (23).

Bacterial strains, plasmids and growth conditions. *V. anguillarum* strains (Table 1) were routinely grown in Lysogeny broth containing 2% NaCl (LB20) (24), supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. *E. coli* strains (Table 1) were routinely grown in Lysogeny broth containing 1% NaCl (LB10). Antibiotics were used at the following concentrations: streptomycin, 200 µg ml⁻¹ (Sm²⁰⁰); chloramphenicol, 20 µg ml⁻¹ (Cm²⁰) for *E. coli* and 5 µg ml⁻¹ (Cm⁵) for *V. anguillarum*; kanamycin, 50 µg ml⁻¹ (Km⁵⁰) for *E. coli* and 80 µg ml⁻¹ (Km⁸⁰) for *V. anguillarum*. 3M-Glucose [Minimal Marine Medium (25) plus 0.2 % Glucose] and NSSM (Nine Salts Solution plus fish gastrointestinal mucus) (26) were used in specific experiments.

Insertional mutagenesis. Insertional mutations were made by using a modification of the procedure described by Milton *et al.* (27). Briefly, primers (Table 2) were designed based on the target gene sequence of M93Sm. Then a 200~300 bp DNA fragment of the target gene was PCR amplified and ligated into the suicide vector pNQ705-1 (Table 1) after digestion with SacI and XbaI. The ligation mixture was introduced into *E. coli* Sm10 by electroporation using BioRad Gene Pulser II (BioRad,

Hercules, CA). Transformants were selected on LB10 Cm²⁰ agar plates. The construction of the recombinant pNQ705 was confirmed by both PCR amplification and restriction analysis. The mobilizable suicide vector was transferred from *E. coli* Sm10 into *V. anguillarum* strains by conjugation. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the recombinant pNQ705 was confirmed by PCR amplification.

Bacterial conjugation. Bacterial conjugation were carried out using the procedure modified from by Varina et al (28). Briefly, 100 µl *V. anguillarum* overnight was added into 2.5 ml NSS (Nine Salts Solution) (29); 100 µl *E. coli* culture overnight was added into 2.5 ml 10 mM MgSO₄. The resulting *V. anguillarum* and *E. coli* suspension was mixed, vacuum filtered onto a autoclaved 0.22-µm-pore-diameter nylon membrane (Millipore, USA), placed on an LB15 agar plate (LB-plus-1.5% NaCl), and allowed to incubate overnight at 27°C. Following incubation, the cells were removed from the filter by vigorous vortexing in 1 ml NSS. Cell suspensions (70 µl) were spread on LB20 plated with appropriate antibiotics and the plates were incubated at 27°C until *V. anguillarum* colonies were observed (usually 24 to 48 h).

Hemolytic assay. Single *V. anguillarum* colonies were transferred onto LB20-fish blood agar (LB20 agar plus 5% rainbow trout blood with heparin), and hemolytic activity was determined by measuring the hemolysis zone surrounding the colonies after incubation at 27°C.

RNA isolation. Exponential phase cells ($\sim 0.5 \times 10^8$ CFU ml⁻¹) of various *V. anguillarum* strains were treated with RNeasy Protect Bacteria Reagent (QIAGEN), following the manufacturer's instructions. Total RNA was isolated using the 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) and stored at -75°C for future use.

Real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was used to quantify various mRNAs using an LightCycler® 480 Real-Time PCR System (Hoffmann-La Roche Inc.) and the Brilliant II SYBR Green Single-Step QRT-PCR Master Mix (Agilent Technologies), 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 stage of each cycle. Samples were run in triplicate along with the no-reverse-transcriptase control and the no-template control. All experiments were repeated at least twice.

Fish infection experiment. Various *V. anguillarum* strains were tested for virulence with rainbow trout (*Oncorhynchus mykiss*) by intraperitoneal (IP) injection or immersion. Briefly, *V. anguillarum* cells grown in LB20 supplemented with appropriate antibiotics for 19 h at 27°C were harvested by centrifugation ($9,000 \times g$, 5 min, 4°C), washed twice in NSS, and resuspended in NSS. NSS suspensions with an estimated specific cell density were prepared according to the optical density at 600 nm (OD₆₀₀) of the culture and the conversion equation: cell density (10^8 CFU ml⁻¹) =

$44.905 \times OD_{600}$, determined by experiment (Figure S1). The OD_{600} was determined by the following method: 100 μ l of NSS suspension was transferred into a 96-well plate with a clear bottom. The OD_{600} was read by a VersaMax™ Absorbance Microplate Reader (Molecular Devices). The actual cell density of injection solution was confirmed by viable count. All fish were examined prior to the start of each experiment to determine that they were free of disease or injury. For IP injection, fish were anesthetized by tricaine methanesulfonate (Western Chemical, Ferndale, WA), with 100 mg L⁻¹ for induction and 52.5 mg L⁻¹ for maintenance. *V. anguillarum* strains were IP-injected into fish in 100 μ l NSS vehicle. Fish that were between 15 and 25 cm long were injected with bacteria diluted with NSS to a specific dosage, or NSS only as negative control. For immersion, 1.5 % Instant Ocean sea salt (Pentair Aquatic Eco-Systems) water solution at 18 ± 1 °C was used as immersion liquid. Each experiment group was provided with 10 L of immersion liquid in a bucket lined with a new, clean, 13-gallon (50L) household trash bag. After the sea salt was fully dissolved, 10 ml the *V. anguillarum* suspended in NSS at a specific cell density, or 10 ml NSS only as the negative control, was added. After *V. anguillarum* was mixed, fish that were between 15 and 25 cm long were immersed for 1 h. For both methods, fish inoculated with different bacterial strains were maintained in separate 10-gallon tanks with constant water flow (200 ml/min) at 18 ± 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 confirmed by the recovery and isolation of *V. anguillarum* cells resistant to the appropriate antibiotics from the head kidney 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. Student's T-tests assuming unequal variances were used for statistical analyses for all experiments except for the fish infection experiment. *P* values of < 0.05 were considered statistically significant. A Kaplan-Meier survival analysis with log rank significance test was performed on the survival percentage in the fish infection experiment. *P* values of < 0.05 were considered statistically significant.

Results

Gene identification and mutant construction in *V. anguillarum*. The TCA cycle in *Salmonella enterica* consists of eight biochemical reactions catalyzed by nine enzymes/enzyme complexes, encoded by *gltA*, *icd*, *sucAB*, *sucCD*, *sdhCDAB*, *fumAC*, *fumB*, and *mdh* (18-20). Cra (encoded by *cra*) is a repressor of *fruA*, *fru* and *fruK*, which are required for fructose metabolism in *Salmonella enterica* (21). The nine TCA cycle enzyme genes, plus the *cra* gene, were examined in the RAST annotation results of *V. anguillarum* M93Sm draft genome (unpublished data) and *V. anguillarum* 775 genome (17). BLASTx was performed on the identified sequences against the NCBI non-redundant protein sequences database to confirm the accuracy of annotation. Among the ten examined metabolism associated genes/operons, *gltA*, *icd*, *sucAB*, *sucCD*, *sdhCDAB*, *fumA*, *mdh* and *cra* were identified in *V. anguillarum* (Fig. 1 and Table 3). The sequences encoding *icd*, *sucA*, *sucC*, *sdhC*, *fumA*, *mdh*, and *cra* in *V. anguillarum* M93Sm were used to create insertional mutations in *V. anguillarum* M93Sm. Seven metabolism mutant strains were obtained and listed in Table 1.

***icd* mutant is highly attenuated against rainbow trout.** To test the hypothesis that mutation in central metabolism could interrupt the infection process in fish, we tested the virulence of the seven *V. anguillarum* metabolism mutants along with the M93Sm wild type on rainbow trout. Groups of five fish were infected by IP injection as described in the Materials and Methods with wild type *V. anguillarum* (M93Sm) cells or with cells from one of the seven mutant strains: *icd* (XM420), *sucA* (XM440), *sucC* (XM450), *sdhC* (XM460), *fumA* (XM470), *mdh* (XM410) or *cra* (XM430) in NSS at a

dosage of $\sim 2 \times 10^5$ CFU/fish, or with NSS only as a negative control (mock). During the 14-day observation window, 40% of M93Sm infected fish survived. In comparison, the *sucA* mutant, *sdhC* mutant and *icd* mutant infected fish all had higher survival percentages compared to M93Sm (50% for *sucA* mutant, 80% for *sdhC* mutant, and 100% for *icd* mutant); however, the difference between any of the three mutants and M93Sm is not statistically significant ($P = 0.051$ to 0.913) (Fig 2A). The fish infection experiment was repeated with a higher dose ($\sim 4 \times 10^5$ CFU/fish) of the *V. anguillarum* wild type and the three mutant strains (*icd*, *sucA*, and *sdhC*) that exhibited some attenuation of virulence in the first experiment. At this higher dose of *V. anguillarum* only 20% of fish infected with M93Sm (wild type) survived. Similarly, fish infected with either *sucA* or *sdhC* mutants survived at 0% or 20%, respectively. In contrast, fish infected with the *icd* mutant had a significantly higher survival percentage (100%) compared to M93Sm ($P = 0.013$) (Fig 2B). The data indicate *icd* mutant is a highly attenuated or avirulent strain in these experimental conditions.

We then compared the virulence of M93Sm with the *icd* mutant by another infection route - immersion. Groups of 10 fish were infected by immersion as described in the Materials and Methods with either M93Sm or the *icd* mutant in a 1.5% sea salt solution at a dose of $\sim 4 \times 10^6$ CFU ml⁻¹, or 1.5% sea salt solution without *V. anguillarum* as a negative control (mock). Control experiments showed that neither the *V. anguillarum* cells nor the rainbow trout were adversely affected by the 1 h exposure to 1.5% sea salt solution (Fig. S2A and 2C). During the 14-day experiment, 30% of M93Sm infected fish survived while 90% of *icd* mutant infected fish survived.

The difference is statistically significant ($P = 0.007$) (Fig 2C). Taken together, the two infection experiments demonstrate that the *icd* mutant exhibits highly attenuated virulence against rainbow trout.

Growth rates and growth yields of *V. anguillarum* wild type and mutant strains.

In order to determine the possible cause of attenuation in the *icd* mutant, we examined the growth curves of the wild type and the seven metabolism mutants of *V. anguillarum* in one minimal medium (3M-Glucose) and two forms of rich media (LB20 and NSSM). The cell density in 3M-Glucose and LB20 was measured by spectrophotometry at 600 nm, while the cell density in NSSM was measured by viable count.

When cells were grown in Minimal Marine Medium plus 1% Glucose (3M-Glucose), the *icd* mutant and the *sucA* mutant failed to grow, since there is no alternative way to produce α -ketoglutarate or succinyl-CoA (Fig. 1). The *sucC* mutant, *sdhC* mutant, *mdh* mutant and *cra* mutant exhibited similar generation times to M93Sm ($P = 0.139$ to 0.985), while the *fumA* mutant exhibited a 33% longer generation time than that of M93Sm ($P = 0.022$) (Table S1).

In LB20 broth, M93Sm, *icd* mutant and *cra* mutant exhibited a classic bacterial growth curve with a lag phase, a log phase and a stationary phase, while the other strains *sucA* mutant, *sucC* mutant, *sdhC* mutant, *fumA* mutant and *mdh* mutant exhibited biphasic growth curves, with a lag phase then a log phase followed by slower growth (stationary/lag phase), then a second log phase, followed by a

stationary phase (Fig 3A). The log phase in the first growth stage was named log phase I and the log phase in the second growth stage was termed log phase II. The generation times during the log phase(s) of all mutants (52.4 to 115.3 min) were longer than for M93Sm (44.0 min) (Fig 3B). The generation time of the *icd* mutant (55.0 min) was only longer than M93Sm (44.0 min) and *sucC* mutant log phase I (52.4 min), and shorter than all other mutants - *sucA* mutant (64.3 and 98.5 min), *sdhC* mutant (61.19 and 101.70 min), *fumA* mutant (73.6 and 89.4 min), *mdh* mutant (67.1 and 115.3 min), and *cra* mutant (58.6 and 91.8 min). The maximum OD₆₀₀ of the *icd* mutant was the lowest among all strains (0.75 vs. 0.96-1.10). The log phase generation time of the *icd* mutant was 25% longer than M93Sm (55.0 vs. 44.0 min) while the maximum OD₆₀₀ was ~70% of that for M93Sm (0.75 vs. 1.10).

To better mimic the colonization environment in fish intestine, we examined the growth of the wild type (M93Sm) and the *icd* mutant (XM420) strains in NSS plus fish gastrointestinal mucus (NSSM) broth by viable count. In these conditions, the shortest generation time of the *icd* mutant was only ~5% longer than M93Sm (66 vs. 63 min) while the maximum cell density of the *icd* mutant was only 24% of that for M93Sm (3.27×10^8 vs. 1.37×10^9 CFU ml⁻¹). Taken together, these data indicate that the loss of *icd* severely limits the growth of *V. anguillarum*, even in nutrient-rich media.

Expression of hemolysin genes in mutants and wild type strains. The attenuation in virulence of the *icd* mutant could be the result of down-regulation of one or more of the hemolysin (*plp*, *vah1*, and *rtxA*) genes used by *V. anguillarum* to promote

virulence (22). The genes encoding the three hemolysins are expressed most strongly during log phase (15, 16, 30). To examine this possibility, we determined the expression of *vah1*, *rtxA* and *plp* during log phase using qRT-PCR (Fig 4A). Data indicate in all mutants except the *icd* mutant, expression of *vah1* and *plp* were up-regulated by 1.77-fold to 16.15-fold compared to M93Sm ($P = 0.001$ to 0.036). In the *icd* mutant, expression of *plp* was up-regulated by 1.76-fold, while the expression of *vah1* was at approximately the same level as M93Sm ($P = 0.083$). Expression of *rtxA* by all mutants was also at the same level as M93Sm ($P = 0.165$ to 0.753). Therefore, all metabolism mutants exhibit the same or higher levels of hemolysin gene expression compared to the wild type and attenuation of the *icd* mutant is not due to reduced expression from the hemolysin genes.

We have observed that Plp is the most efficient of the three hemolysins against fish erythrocytes (Li et al, unpublished data). Thus, zones of hemolysis on fish blood agar are primarily the result of Plp activity. The data indicate when compared to the wild type M93Sm, all TCA cycle mutants (but not the *cra* mutant) had significantly higher levels of hemolytic activity after 7 h of incubation on fish blood agar plates. After 23 h of incubation all TCA cycle mutants and the *cra* mutant had significantly larger zones of hemolysis on fish blood agar compared to M93Sm ($P = 0.003$ to 0.044) (Fig 4B). The hemolysis activity results correspond with the expression data for *plp* and *vah1* (Fig 4A).

Expression level of *hlyU* is higher in *sucA* mutant and *mdh* mutant than that in wild type. Previously, we demonstrated that the three hemolysin genes, *vah1*, *rtxA* and

plp, are co-regulated by a repressor (H-NS) and an anti-repressor (HlyU) (15, 30). In order to examine their possible roles in regulating hemolysin gene expression in the metabolism mutants, we examined the expression of *hlyU* and *hns* during log phase using qRT-PCR in two TCA cycle mutants – the *sucA* mutant and *mdh* mutant (Fig 4C). Data show that both *hlyU* and *hns* were expressed at significantly higher levels in both the *sucA* and *mdh* mutant compared to M93Sm ($P = 0.015$ to 0.018). The expression of *hlyU* was 76% (in the *sucA* mutant) and 63% (in the *mdh* mutant) higher than in M93Sm, while the expression of *hns* was 14% (in the *sucA* mutant) and 36% (in the *mdh* mutant) higher than in M93Sm. Compared to the increase in *hlyU* expression, the increase of *hns* was relatively small (14% vs. 76% in *sucA* mutant, 36% vs. 63% in *mdh* mutant), suggesting the possibility that the relative increase in *hlyU* expression may be responsible for the increased expression of *plp* and *vah1*.

Pre-treatment by immersion with *icd* mutant protected rainbow trout from the subsequent challenge of *V. anguillarum* M93Sm. In order to test whether rainbow trout previously exposed to the highly attenuated *icd* mutant by immersion were protected against vibriosis by the fully virulent wild type M93Sm, we performed an infection challenge on fish that had been pre-treated with the *icd* mutant. A group of five fish that survived from the initial infection experiment by immersion (labeled as “pre-treated by immersion” in Fig 5A) and group of five “untreated” fish were immersion-infected by M93Sm at a dosage of $\sim 4 \times 10^6$ CFU ml⁻¹ and observed for 14 days. The pre-treated trout were challenged with the wild type cells 6 weeks after the initial infection with the *icd* mutant to allow adaptive immunity to develop. In the untreated group, no fish survived past day 2, while in the “pre-treated by immersion”

group, all five fish survived for the entire 14-day observation period (Fig 5A). The difference between the two experimental groups is statistically significant ($P = 0.003$). The results indicate that *icd* mutant elicits protective immunity when fish are inoculated by immersion.

Pre-treatment by injection of *icd* mutant elicited cross-serotype immunity against the subsequent challenge of *V. anguillarum* NB10. In order to test whether pre-treatment of fish with the *icd* mutant was able to provide cross-serotype protection, we carried out a second infection challenge experiment using the virulent wild type strain of *V. anguillarum* NB10 (serotype O1) with a serotype different from that of the M93Sm-derived *icd* mutant (serotype O2a), on fish that had been pre-treated with the *icd* mutant. A group of three fish that survived the initial infection experiment by immersion (labeled as “pre-treated by immersion” in Fig 5B), and group of five fish that survived from the initial infection experiment by injection (labeled as “pre-treated by injection” in Fig 5B) were infected by immersion with NB10 at a dosage of $\sim 4 \times 10^6$ CFU ml⁻¹ and observed for 14 days. As in the first challenge experiment, the pre-treated trout were challenged with the wild type cells 6 weeks after the initial infection with the *icd* mutant to allow adaptive immunity to develop. A group of four “untreated” fish were also included as a control. In the untreated group, no fish survived beyond day 4, while in the “pre-treated by immersion” group, one fish (33%) survived for the entire 14-day observation period. However, there is no statistically significant difference between the two experimental groups ($P = 0.139$). In the “pre-treated by injection” group, four fish (80%) survived for the entire 14-day observation period. When compared to the untreated control group, the number of

survivors is statistically significant ($P = 0.007$) (Fig 5B). The results indicate *icd* mutant elicited cross-serotype immunity against *V. anguillarum* NB10 at least when applied by injection.

Discussion

An ideal modified live vaccine would be a strain that retains its invasive ability but does not cause further damage to the fish host, so that not only humoral but also cell-mediated immunity would be elicited, by simply applying the vaccine by immersion. For this purpose, factors that are exclusively required in the post-invasion stage, rather than the virulence factors that required for the invasive stage of the pathogen, would be the preferred targets to modify or knock out. We hypothesized that disrupting the central fueling pathways, such as the TCA cycle enzymes, were likely to result in defects that would accomplish this goal, especially since other investigators had success with this approach in both UTI *E. coli* (22) and *Salmonella enterica* (18-20). It has been suggested that intracellular pathogens inside host cell phagosomes are in nutritionally restricted sites and are likely to be starved for essential nutrients, especially when central fueling pathways are disrupted (20). However, unlike *S. enterica* and UTI *E. coli*, *V. anguillarum* is an extracellular pathogen and does not invade host cells. Thus, it was not clear that disrupting the central fueling pathways would necessarily result in *V. anguillarum* mutants unable to persist and grow in the fish host. To examine this question, we constructed 7 mutants – six TCA cycle mutants and one regulatory mutant (*cra*) and tested the mutants for virulence in

rainbow trout and, subsequently, tested the attenuated mutant for its ability to protect the trout against challenge by virulent wild type strains of *V. anguillarum*.

For all metabolism mutants, there is no correlation between their virulence and growth rate in LB20, however, there is correlation between virulence and the maximum cell density since only the *icd* mutant had both the lowest virulence and the lowest maximum cell density (compare Fig 2A with Fig 3A), suggesting that a certain cell density is a prerequisite for a successful systemic infection. It is possible that once *icd* mutant cells get into the host fish, they are unable to grow to a high enough density to overwhelm the host innate immune defenses. Thus the host has time to mount an effective adaptive immune response. This raises the question as to whether *icd* mutant growth is restricted in the GI tract of the fish and/or after it penetrates the epithelial cell layer and invades the fish host. Our data suggest that the growth restrictions may be in both environments. Specifically, the *icd* mutant cells grew with nearly the same doubling time as the M93Sm wild type cells in NSSM, but their maximum cell density was only 24% that seen in M93Sm, suggesting that the cells may not reach a critical density necessary for successful invasion of the host. However, we also injected fish with potentially lethal doses of the *icd* mutant, but all injected fish survived (unlike fish IP injected with M93Sm). This result suggests that even if *icd* mutant cells penetrate the epithelial layer and enter the fish host, they are unable to grow to a high enough density to overwhelm the host immune response.

All mutants constructed in this study exhibited the same or higher levels of hemolysin gene expression and higher hemolytic activity than M93Sm (Fig 4A and

4B), ruling out the possibility that the attenuated virulence of the *icd* mutant is due to the lack of hemolytic activity. Additionally, we suggest that the presence of secreted hemolysins will act to further stimulate the inflammatory response (31) and are likely to serve as antigens to elicit immunity against themselves, which is an advantage of this TCA cycle mutation approach over constructing avirulent strains containing mutations in specific virulence genes, such as the avirulent *rtxA* mutant obtained previously (14). The increase in expression of *vah1-plp* gene cluster (for at least the *sucA* and *mdh* mutants) is correlated to the increase in expression of *hlyU*, the transcriptional activator/antirepressor of the *vah1-plp* gene cluster (compare Fig 4A with Fig 4C). It is unclear how mutations in TCA cycle enzymes trigger increase expression of *hlyU*.

Immersion is the preferred method for vaccination in the aquaculture industry (3, 5, 32). In this study, rainbow trout pre-treated by immersion for 1 h with the *icd* mutant were fully protected from subsequent challenge with virulent wild type *V. anguillarum* M93Sm (Fig 5A). Moreover, since the TCA cycle exists in all aerobic organisms, as well as many facultative (like *Vibrio*) and anaerobic organisms, the TCA mutation approach is likely to be an easy method for the construction of modified live vaccines against a wide variety of pathogenic bacteria. Although there is a residual virulence of the *icd* mutant, a combination of *icd* and another TCA cycle gene mutation is expected to further attenuate the strain. In *Salmonella enterica*, the combination of a *sdhCDA* deletion and *frdABCD* deletion resulted in further attenuation in virulence than either single gene deletion (19, 20).

Cross-serotype protection is also highly preferred in vaccine development. Data indicate the injection of *icd* mutant that derived from M93Sm (serotype O2a) had elicited cross-serotype immunity against the subsequent challenge of NB10 (serotype O1), demonstrating the potential of the TCA cycle mutation approach (Fig 5B). Although the administration of *icd* mutant by immersion didn't show statistically significant protection against NB10 in this study, it should be noted that there were only three fish tested. More repeats need to be done to make a more solid conclusion.

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Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
<i>V. anguillarum</i> strains		
M93Sm	Spontaneous Sm ^r mutant of M93 (serotype O2a)	(33)
NB10	Clinical isolate from the Sea of Bothnia (serotype O1)	(34)
XM420	Sm ^r Cm ^r ; <i>icd</i> insertional mutant	This study
XM440	Sm ^r Cm ^r ; <i>sucA</i> insertional mutant	This study
XM450	Sm ^r Cm ^r ; <i>sucC</i> insertional mutant	This study
XM460	Sm ^r Cm ^r ; <i>sdhC</i> insertional mutant	This study
XM470	Sm ^r Cm ^r ; <i>fumA</i> insertional mutant	This study
XM410	Sm ^r Cm ^r ; <i>mdh</i> insertional mutant	This study
XM430	Sm ^r Cm ^r ; <i>cra</i> insertional mutant	This study
<i>E. coli</i> strains		
Sm10	<i>thi thr leu tonA lacY supE</i> <i>recA</i> RP4-2-Tc::Mu::Km (λ <i>pir</i>)	(35)
S100	Km ^r ; Sm10 containing plasmid pNQ705-1	This study
Q420	Km ^r Cm ^r ; Sm10 containing plasmid pNQ706- <i>icd</i>	This study
Q440	Km ^r Cm ^r ; Sm10 containing plasmid pNQ708- <i>sucA</i>	This study
Q450	Km ^r Cm ^r ; Sm10 containing plasmid pNQ709- <i>sucC</i>	This study
Q460	Km ^r Cm ^r ; Sm10 containing plasmid pNQ710- <i>sdhC</i>	This study
Q470	Km ^r Cm ^r ; Sm10 containing plasmid pNQ711- <i>fumA</i>	This study
Q410	Km ^r Cm ^r ; Sm10 containing plasmid pNQ705- <i>mdh</i>	This study

Q430	Km ^r Cm ^r ; Sm10 containing plasmid pNQ707- <i>cra</i>	This study
Plasmid		
pNQ705-1	Cm ^r ; suicide vector with R6K origin	(36)
pNQ705- <i>icd</i>	Cm ^r ; For <i>icd</i> insertional mutant	This study
pNQ705- <i>sucA</i>	Cm ^r ; For <i>sucA</i> insertional mutant	This study
pNQ705- <i>sucC</i>	Cm ^r ; For <i>sucC</i> insertional mutant	This study
pNQ705- <i>sdhC</i>	Cm ^r ; For <i>sdhC</i> insertional mutant	This study
pNQ705- <i>fumA</i>	Cm ^r ; For <i>fumA</i> insertional mutant	This study
pNQ705- <i>mdh</i>	Cm ^r ; For <i>mdh</i> insertional mutant	This study
pNQ705- <i>cra</i>	Cm ^r ; For <i>cra</i> insertional mutant	This study

Table 2. Primers used in this study.

Primer	Sequence (5'to3', underlined sequences are designed restriction sites)	Description	Reference
pr31	GGT <u>GAGCTCT</u> ATTCTTTATTG CCGATTATC	For <i>icd</i> insertional mutant, forward, SacI	This study
pr32	AAATCTAGAGTAAGTCGCTT TAATCGCTTC	For <i>icd</i> insertional mutant, reverse, XbaI	This study
pr50	AAAGAGCTCGTGATCCAGAT GTCGATGCTA	For <i>sucA</i> insertional mutant, forward, SacI	This study
pr51	GGT <u>TCTAGAGTTC</u> AGTGTCG ATAATGTGCA	For <i>sucA</i> insertional mutant, reverse, XbaI	This study
pr52	AAAGAGCTCGGTCGGATTAG TACAGCGAAG	For <i>sucC</i> insertional mutant, forward, SacI	This study
pr53	GGT <u>TCTAGACT</u> TTTTCAATTT CCACGCCGC	For <i>sucC</i> insertional mutant, reverse, XbaI	This study
pr54	AAAGAGCTCATGTTTCGTTGC GGTCGGAATT	For <i>sdhC</i> insertional mutant, forward, SacI	This study
pr55	GGT <u>TCTAGATCC</u> AACTCTTCA AAGTGGCCC	For <i>sdhC</i> insertional mutant, reverse, XbaI	This study
pr56	GGTGAGCTCTCCTTGCACCAT ATTGATATG	For <i>fumA</i> insertional mutant, forward, SacI	This study
pr57	GGGT <u>CTAGAAGG</u> CTTATCAT CGAGAAGAGAG	For <i>fumA</i> insertional mutant, reverse, XbaI	This study
pr29	GGTGAGCTCATGCCAGCGTT AACATTAAAC	For <i>mdh</i> insertional mutant, forward, SacI	This study
pr30	AAATCTAGAGCTGTATGACA TCGCACCGGT	For <i>mdh</i> insertional mutant, reverse, XbaI	This study
pr33	AAAGAGCTCGCGGCGTGAGA CTAAGGCATC	For <i>cra</i> insertional mutant, forward, SacI	This study
pr34	AAATCTAGACAATGGCAAAG CGCAGAAGTA	For <i>cra</i> insertional mutant, reverse, XbaI	This study

F <i>vah1</i> RT (BF)	GTTTGGTATGGAACACCGCT CAAG	For <i>vah1</i> qRT-PCR, forward	This study
R <i>vah1</i> RT (BF)	GGCTCAACCTCTCCTTGTAAC CAA	For <i>vah1</i> qRT-PCR, reverse	This study
plpF RT	CAGACGACCACCAGTAACCA CTAA	For <i>plp</i> qRT-PCR, forward	(16)
plpR RT	GCAATCATGATGACCCAGCA ACAG	For <i>plp</i> qRT-PCR, reverse	(16)
Pm111	GGAAATTATTCCGCCGACGA TGGA	For <i>rtxA</i> qRT-PCR, forward	(14)
Pm112	GCCGATACCGTATCGTTACCT GAA	For <i>rtxA</i> qRT-PCR, reverse	(14)

Table 3. Gene identification in *V. anguillarum*

Gene or operon	Product	Presence in <i>V. anguillarum</i> M93Sm draft genome	Locus tag in <i>V. anguillarum</i> 775 genome
<i>gltA</i>	Type II citrate synthase	Yes	VAA_03416
<i>icd</i>	Isocitrate dehydrogenase	Yes	VAA_02190
<i>sucAB</i>	2-oxoglutarate dehydrogenase (E1 component, E2 component)	Yes	VAA_03410, VAA_03409
<i>sucCD</i>	Succinyl-CoA synthetase (beta subunit, alpha subunit)	Yes	VAA_03408, VAA_03407
<i>sdhCDAB</i>	Succinate dehydrogenase (cytochrome b556 subunit, membrane anchor subunit, flavoprotein subunit, iron-sulfur protein)	Yes	VAA_03415, VAA_03414, VAA_03413, VAA_03412,
<i>fumAC</i>	Aerobic fumarate hydratase (class I, class II)	<i>fumA</i> : Yes; <i>fumC</i> : not found	<i>fumA</i> : VAA_01430; <i>fumC</i> : not found
<i>fumB</i>	Anaerobic fumarate hydratase (class I)	Not found	Not found
<i>mdh</i>	Malate dehydrogenase	Yes	VAA_01685
<i>cra</i>	Fructose repressor protein	Yes	VAA_01008

Figure 1. Glycolysis pathway, gluconeogenic pathway, TCA cycle and metabolism of fructose as identified from the sequenced genomes of *V. anguillarum* 775 and M93Sm. The arrows indicate the physiological directions of the reactions. The gene symbols of the enzyme for each reaction are listed beside the reaction. Boxed genes indicate the mutants that were created (Table 1).

Figure 2. Survival percentage of rainbow trout IP injected with various *V. anguillarum* strains at a dosage of **A)** 2×10^5 CFU/fish and **B)** 4×10^5 CFU/fish. Five fish were infected with each strain. **C)** Immersion infection survival of fish infected with M93Sm, *icd* mutant, or mock infected. *Statistically significant difference compared to M93Sm.

A

B

C

Figure 3. Growth profiles of *V. anguillarum* strains. A) Growth curves of various *V. anguillarum* strains in LB20. B) Generation time of various *V. anguillarum* strains in LB20 grown in LB20. C) Growth curves of *V. anguillarum* wild type and *icd* mutant in NSSM. The data presented are from a representative experiment of two independent experiments.

Figure 4. qRT-PCR analysis. **A)** Real time qRT-PCR analysis of expression of *rtxA*, *plp* and *vah1* in various *V. anguillarum* strains grown in LB20 in logarithmic (log) phase. **B)** Hemolytic activity of various *V. anguillarum* strains grown on fish blood agar. **C)** Real time qRT-PCR analysis of expression of *hlyU* and *hns* in various *V. anguillarum* strains grown in LB20 in log phase. *Statistically significantly higher than M93Sm.

A **B** **C**

Figure 5. Fish infection experiment. **A)** Survival percentage of untreated or treated rainbow trout immersed in *V. anguillarum* M93Sm (4×10^5 CFU ml⁻¹). Five fish were used for each group. **B)** Survival percentage of untreated or treated rainbow trout immersed in *V. anguillarum* NB10Sm (4×10^5 CFU ml⁻¹). 3~5 fish were used for each group. *Statistically significant difference compared to untreated fish.

Table S1. Generation time of various *V. anguillarum* strains in 3M-Glucose.

Strain	Generation Time (min)
M93sm	78 (± 5)
XM420 (<i>icd</i>)	no growth
XM440 (<i>sucA</i>)	no growth
XM450 (<i>sucC</i>)	76 (± 12)
XM460 (<i>sdhC</i>)	78 (± 10)
XM470 (<i>fumA</i>)	104 (± 9)
XM410 (<i>mdh</i>)	77 (± 10)
XM430 (<i>cra</i>)	84 (± 2)

Figure S1. Cell density determined by viable count vs. OD₆₀₀ of various *V. anguillarum* strains. The line indicates linear regression.

Figure S2. Supplement data for fish infection assay. **A)** Survival curve of *V. anguillarum* M93sm starved in water with various NaCl concentrations. **B)** Survival percentage of rainbow trout vs. *V. anguillarum* dosage (in CFU ml⁻¹) in immersion infection experiment. The line indicates logarithmic regression.

A

B