THE RELATIONSHIP OF THE ViuB HYDROLASE TO IRON-LIMITED GROWTH AND VIRULENCE OF *Vibrio vulnificus*

By

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To my Mom and Dad, who have never stopped supporting me through all my endeavors

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THE RELATIONSHIP OF THE ViuB HYDROLASE TO IRON-LIMITED GROWTH AND VIRULENCE OF *Vibrio vulnificus*

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Vibrio vulnificus is an opportunistic, ferrophilic human pathogen and the most common cause of reported, fatal seafood-related bacterial infections in the U.S. This estuarine bacterium commonly infects individuals with iron-overloading disorders, such as hemochromatosis. It was previously demonstrated that the GacS/GacA two component signal transduction system regulates iron acquisition and virulence in *V. vulnificus* (Gauthier *et al.*, 2010). Multiple genes are responsible for iron acquisition and comprise the catechol and hydroxamate siderophore systems in *V. vulnificus*. The catechol siderophore system includes *venB*, *vuuA*, and *viuB* genes. The *venB* gene was previously shown to be required for virulence, and *viuB* was proposed as a virulence marker. Deduced amino acid sequences were examined for all three genes, and the sequences of *viuB* segregated most clinical strains of *V. vulnificus* into a group that correlated with increased virulence in mice. However, no correlations were observed for *venB* or *vuuA* sequences. Therefore, the specific role of the ViuB hydrolase in iron acquisition and virulence of *V. vulnificus*, and its regulation by GacA, was examined. Mutational analysis confirmed GacA was required for increased expression of all three catechol genes under iron-limiting conditions; however, significant differences in gene

expression for the hydroxamate system were only observed under iron-replete conditions. USER friendly cloning and chitin transformation were used to construct a deletion mutation of *viuB* in order to determine its role in iron uptake and virulence. Compared to wild type, the *viuB* deletion mutant was significantly deficient in growth under iron-limiting conditions. However, virulence of the *viuB* mutant in mouse infections did not differ significantly from virulence observed in the wild-type strain. Gene expression of the hydroxamate system may compensate somewhat for loss of *viuB* function, as the *viuB* deletion mutant showed significant increases in transcript levels for two of three hydroxamate genes, as compared to the wild type. In conclusion GacS/GacA differentially controls the expression of iron acquisition genes in the catechol and hydroxamate systems of *V. vulnificus.* Although allelic variation of the *viuB* gene correlated with virulence in mice and was required for growth under iron-limiting conditions, *viuB* does not appear to be a virulence factor for *V. vulnificus*.

CHAPTER 1
INTRODUCTION

Vibrio vulnificus is an estuarine pathogen and the most common cause of fatal seafood-related bacterial illnesses in the United States (CDC, 2008). This opportunistic bacterium is gram-negative, halophilic, and causes systemic disease through consumption of raw oysters or through exposure of wound infections to sea water (Jones & Oliver, 2009). Previous findings showed a strong link between salinity and temperature on numbers of *Vibrio vulnificus* in oysters and sea water, with the highest levels of this bacterium in the warmer summer months (CDC, 2008; Motes *et al.*, 1998; Randa *et al.*, 2004). Optimal conditions for recovery of this bacterium ranged from 5 to 25 ppt for salinity and 13 to 22^{o}C for temperature (Kaspar & Tamplin, 1993). Interestingly, this pathogen does not cause harm to the oysters, and the bivalve appears to be a primary habitat for *V. vulnificus*. Oysters harvested from the Chesapeake Bay, showed approximately 80% of the mollusks contained the bacterium dependent on time of the year (Wright *et al.*, 1996).

V. vulnificus disease is not spread by human-to-human transmission, and the bacterium is considered to be an opportunistic pathogen in humans, as serious infections only occur in individuals with some type of underlying condition that compromises host defenses. Transmission of disease through oyster consumption or wound infections can lead to septicemia and death in these individuals. Common conditions that are associated with increased proliferation of the pathogen in the host include hemochromatosis, diabetes, and alcoholic cirrhosis (Blake *et al.*, 1979; Tacket *et al.*, 1984). A common factor among these compromising conditions involves increased iron availability in the body (Forouhi *et al.*, 2007; Ganne-Carrie *et al.*, 2000;

Jehn *et al.*, 2007). Hemochromatosis is a condition that is specifically characterized by elevated serum iron levels: the hereditary form is primary hemochromatosis, and the developmental form is termed secondary hemochromatosis (Gan *et al.*, 2011). Primary hemochromatosis is the most common form and involves mutations in the *HFE* genes, which when mutated cause the body to absorb excess iron. This protein is a negative regulator that decreases the affinity of transferrin for free iron in the blood. Therefore, more iron is stored within the body as opposed to being lost through urine (Gan *et al.*, 2011).

Iron is important to a variety of metabolic pathways for all aerobic bacteria. The correlation of hemochromatosis with *V. vulnificus* infections suggested an association between iron availability and the ability of this organism to cause disease. Furthermore, several studies experimentally demonstrated the important role of iron in pathogenicity and virulence of *V. vulnificus* in animal models (Litwin *et al.*, 1996; Okujo *et al.*, 1996; Simpson & Oliver, 1983; Starks *et al.*, 2000; Wright *et al.*, 1981; Wright *et al.*, 1986). The purpose of the study herein was to look at global regulation of iron acquisition and its relationship to the genes involved in the *V. vulnificus* iron-uptake siderophore systems in order to better understand their role in the virulence of *V. vulnificus*.

CHAPTER 2
LITERATURE REVIEW

Iron Response in *Vibrio Vulnificus*

Iron and Virulence of *V. vulnificus*

Injection of exogenous iron during mouse infections causes a large decrease in the 50% lethal dose of *V. vulnificus*, indicating that iron is essential for virulence (Wright *et al.*, 1981). Additional research showed that the various iron acquisition genes contributed to the degree of virulence of *V. vulnificus* in mouse models. Mutation of *venB* produced strains that were defective in transferrin iron utilization and virulence, and their phenotypes were restored through complementation (Litwin *et al.*, 1996). In a more recent study (Starks *et al.*, 2000), the correlation between severity of disease in mice and the level of iron availability demonstrated that *V. vulnificus* was a ferrophilic pathogen. In this model of infection, mice were injected with iron-dextran prior to subcutaneous inoculation with the pathogen, and both clinical and environmental isolates of *V. vulnificus* were compared for virulence potential. The results showed that at higher inocula, the mice without iron-dextran treatment typically lacked any symptoms of disease. In fact, the mice required approximately one million-fold increase in inocula during the infection to replicate the same results as seen in the iron treated mice. Furthermore, this observation was true for the majority of both clinical and environmental strains and was attributed to the assumption that the iron injection compensates for any innate inability to proliferate within the host (Starks *et al.*, 2000). Not limited to these two examples, the contribution of iron acquisition on virulence of *V. vulnificus* in animal models has been studied and characterized in a variety of studies

(Litwin *et al.*, 1996; Okujo *et al.*, 1996; Simpson & Oliver, 1983; Starks *et al.*, 2000; Wright *et al.*, 1981; Wright *et al.*, 1986).

Iron Acquisition Genes

In *V. vulnificus*, multiple genes are responsible for iron acquisition, and these genes comprise both the catechol and hydroxamate siderophore systems. Siderophores are compounds that compete with the host proteins for available free iron and are capable of stripping iron away from the transferrins (Kim *et al.*, 2007b; Litwin *et al.*, 1996). The catechol siderophore iron acquisition system has been characterized in *V. vulnificus* and includes *venB*, *vuuA*, and *viuB*, among other genes (Webster & Litwin, 2000). The *venB* gene is responsible for the synthesis of the vulnibactin siderophore, which is used in high-affinity iron acquisition. The *vuuA* gene encodes a ferric vulnibactin receptor, and the expressed product is located on the outer membrane. Investigations on a homologous system in *Vibrio cholerae* showed that once the siderophore-iron complex is translocated through the inner membrane, the hydrolase product of the *viuB* gene cleaves the iron from the siderophore (Butterton & Calderwood, 1994). These three iron acquisition genes are tightly linked on chromosome 2 in *V. vulnificus* as seen in Figure 2-1 (Crosa *et al*., 2004). Also included in this grouping is the gene *entE*, which encodes a protein responsible for peptide arylation.

Figure 2-1. Iron acquisition gene arrangement in *V. vulnificus* (Crosa, 2004).

The catechol iron acquisition genes are considered to be potential virulence factors because mutations in them often result in reduced virulence of the pathogen (Litwin *et al.*, 1996). Studies have also examined *viuB* and suggested it may be a potential virulence marker, as original reports linked the presence of this gene with the clinical origin of the strain. Using a multiplex PCR assay for evaluation of clinical and environmental strains of *V. vulnificus* in shellfish, the *viuB* gene was found in all clinical isolates, while only 24% of environmental strains were positive for the gene (Panicker *et al.*, 2004). In a later study, *viuB* positive clinical isolates survived longer in human serum than *viuB* negative strains (most environmental isolates) (Bogard & Oliver, 2007). However it was later revealed that all strains of *V. vulnificus* actually contained the *viuB* gene, and the reported differences in absence or presence of the gene were the consequence of genetic polymorphisms at the primer sites used for PCR detection (Bogard & Oliver, 2008).

Other systems exist for iron acquisition in *V. vulnificus* but are much less characterized when compared to the catechol pathway and include hydroxamate (Tanabe *et al.*, 2005) and desferoxamine (Kim *et al.*, 2007a) systems. The hydroxamate system is similar to the catechol system in that siderophores are released into the environment to bind iron with high affinity, but it differs in that it uses a hydroxamic acid derived siderophore rather than a phenolate derived siderophore. In addition to this, they differ regarding he specific genes required for uptake and utilization of the iron. Meanwhile the desferoxamine system consists of just one gene, *desA*, which encodes the receptor for the desferoxamine-iron complex; *V. vulnificus* can utilize the siderophore made from other sources, but does not synthesize it (Kim *et al.*, 2007a).

The hydroxamate siderophore system includes three genes. The products of two of these genes are thought to be components in the ABC transporter system used to release the aerobactin siderophore into the environment. GenBank ID AAO07920.1 (ATP) is the ATPase component, while AAO07921.2 (PP) is the periplasmic substrate binding protein. The characterized gene *iutA* (AAO07924.2) is the aerobactin siderophore receptor.

Global Regulation of Iron Acquisition

Bacterial Iron Regulation

Iron-related virulence factors are regulated by a combination of global regulatory systems that in turn are dependent upon the availability of iron (Lapouge *et al.*, 2008). The ferric uptake regulation protein (Fur) specifically manages iron procurement through inhibition of iron acquisition genes under conditions of higher iron availability in *V. vulnificus* (Litwin & Calderwood, 1993). During a test of global gene expression based on the status of iron, Alice *et al.* in 2008 showed that high levels of serum iron could indeed influence the expression levels of many genes in *V. vulnificus* in mice. Based on their results, low iron concentrations influenced expression levels of genes required for general growth and survival, including outer membrane porins and enzymes for amino sugar biosynthesis. However, upon infection into a host, the bacterium causes disease through the expression of a variety of virulence factors including pili, RtxA1, and Group 1 capsular polysaccharide (Alice *et al.*, 2008; Liu *et al.*, 2007). Although iron plays a part in virulence, it appears as though it is not the sole reactant in activation of virulence expression (Alice *et al.*, 2008).

GacS/GacA Regulatory System

Another global regulatory system that has been related to iron acquisition in *Vibrio fischeri* is the GacS/GacA two-component regulatory system (Whistler & Ruby, 2003). GacA and GacS are common to all γ-proteobacteria examined, including *V. vulnificus* (Gauthier *et al.*, 2010). GacS is the cognate sensor kinase and GacA is the response regulator (Whistler *et al.*, 1998). The GacS/GacA regulatory system is known to act on a global scale within the cells through the small RNAs *csrB/csrC* and also through the CsrA regulatory protein, as seen in Figure 2-2 (adapted from Lapouge *et al.* 2008. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Molecular microbiology* **67**, 241-253). Both *csrB* and *csrC* bind to the small regulatory protein CsrA, and thus inhibiting its function as a transcription factor, which in turn regulates various behaviors including biofilm, quorum sensing, virulence, chemotaxis, and motility in other *Vibrio* species (Lapouge *et al.*, 2008; Whistler & Ruby, 2003).

GacS/GacA Regulation of Iron in *Vibrios*

The GacA/GacS pathway has been studied in relationship to iron acquisition in *V. fischeri* (Whistler & Ruby, 2003). In this study siderophore production in *gacA* deletion mutants was examined using low-iron media. Mutants lacking GacA expression were defective in iron acquisition that required siderophores, and this phenotype was restored through complementation (Whistler & Ruby, 2003). This result demonstrated that a closely related species, *V. fischeri*, regulates catechol siderophore iron acquisition via the GacS/GacA two-component system and suggested that a similar mechanism could potentially be present in *V. vulnificus*.

Role of GacA/Gacs in Virulence of *V. vulnificus*

In a recent study (Gauthier *et al.*, 2010), transcript levels of various genes were measured in a *gacA* deletion mutant, and compared back to expression in the wild-type strain. Results showed that *gacA* deletion mutants of *V. vulnificus* CMCP6 had decreased levels of various transcripts compared to wild type. These genes include: *csrB1*, *csrB2*, *csrB3*, *csrC*, *flaA*, *rpoS*, and *vvpE*. Altered phenotypes in the mutant included protease and cytotoxin expression, phase variation of capsular polysaccharide, biofilm formation, and virulence. The *vvpE* gene encodes protease activity in *V. vulnificus*, and the *gacA* deletion mutant showed significantly decreased levels of this activity and *vvpE* transcripton, which was complemented *in trans*. The authors concluded that GacA regulates protease expression in *V. vulnificus*. Cytoxicity related to the RTX hemolysin was tested based on the ability of *V. vulnificus* to destroy INT-407 monolayers *in vitro*. The mutant showed decreased destruction of INT-407 cells in comparison to wild type; however, expression of the corresponding *rtxA* gene was not significantly changed in mutant compared to the wild-type strain. Virulence in a mouse model was tested with and without the injection of exogenous iron. The results showed iron overloaded mice exhibited no difference between *V. vulnificus* CMCP6 wild-type strain and mutant for virulence. However without iron-loading conditions, the CMCP6 mutant was significantly decreased for virulence compared to the wild-type strain. These results were not replicated in *V. vulnificus* MO6-24, and demonstrated that although *gacA* may contribute to virulence, its role is dependent upon strain and iron availability.

In summary, our current knowledge of the relationship of GacA to the *V. vulnificus* iron response includes the following associations: 1) the ability to acquire iron is

required for virulence in *V. vulnificus* (Starks *et al*., 2000; Wright *et al*., 1981); 2) mutation of the iron acquisition gene *venB* decreased virulence in infant mice compared to wild-type response (Litwin *et al.*, 1996); 3) differences in the *viuB* alleles correlate with clinical origin in some strains (Bogard & Oliver, 2008); 4) mutations in the global regulator GacA decrease virulence and iron acquisition in *V. vulnificus* (Gauthier *et al.*, 2010); and 5) iron acquisition in *V. fischeri* is regulated by GacA (Whistler & Ruby, 2003). Therefore, I hypothesize it is likely that GacA is an essential component for iron regulation in *V. vulnificus*, and that the role of GacA in virulence is related to its regulation of iron uptake.

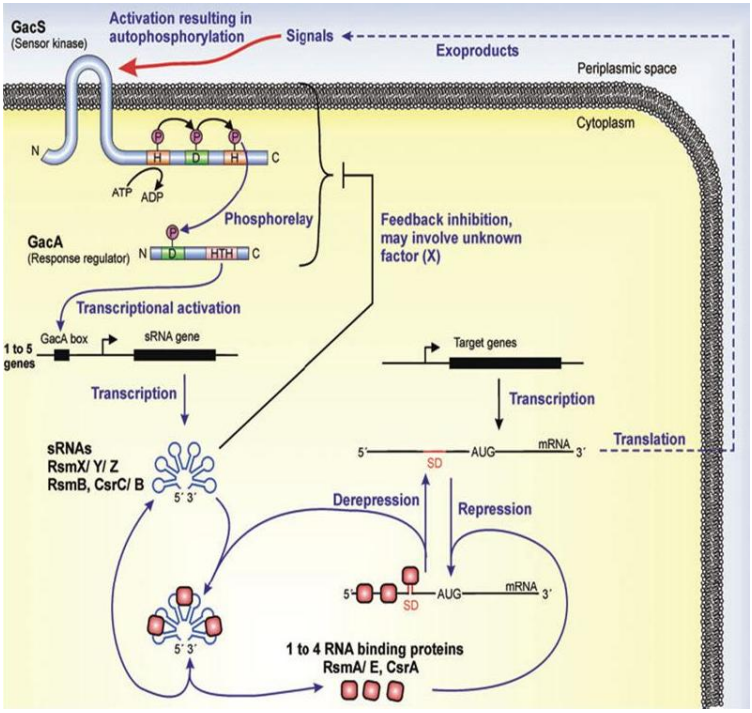


Figure 2-2. GacS/GacA signal transduction pathway in γ-proteobacteria (Lapouge *et al.*, 2008)

CHAPTER 3
OBJECTIVES AND HYPOTHESIS

The overall objective of this research was to study the relationship of GacA and the iron response of *V. vulnificus* by determining the effect of a *gacA* mutation on expression of specific iron acquisition genes, and relating any observed effects to the virulence of this bacterium. The *gacA* deletion mutants were previously shown to be more sensitive to iron-limiting growth conditions and less virulent than wild-type strains in mice that were not iron-loaded (Gauthier *et al.*, 2010). The role of GacA on the expression profile of genes related to iron uptake in *V. vulnificus* was examined in the present study during exposure to various *in vitro* conditions with or without iron-limitation. Furthermore, the relationship of GacA and iron was examined by looking at the function of the GacA-regulated *viuB* gene in iron uptake and virulence of *V. vulnificus*. The hypotheses for this project include the following:

- GacA regulates growth under conditions of iron-limitation (Gauthier *et al.*, 2010): therefore, GacA may control expression of genes (*venB, vuuA, viuB*) related to catechol siderophore uptake.
- Genetic polymorphisms in *V. vulnificus viuB* correlated with clinical origin and increased virulence, suggesting it may be a virulence marker. Similar profiles may be common to other genes in the catechol siderophore system.
- If *viuB* is a virulence factor, deletion of this gene should alter the iron response and decrease virulence in the mutant when compared to the wild-type strain.
- *V. vulnificus* iron response also involves the hydroxamate siderophore system, and these genes involved (ATP, PP, *iutA*) may also be regulated by GacA or could compensate for loss of *viuB*.

CHAPTER 4
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains of *V. vulnificus*, described in Table 4-1, were stored as frozen stocks (-70^{o}C) in Luria Burtani Broth with NaCl (LBN) and 50% glycerol and streaked onto LBN Agar (LA) for isolation overnight at 37^{o}C. LBN at pH of 7.5 was used for the iron-replete conditions because it contains iron (506.5 µg of iron per liter of LBN) from the yeast extract (1%) and tryptone (1%). For iron-limiting conditions, the iron chelator dipyridyl (Acros Organics) was added to LBN at concentrations ranging from 100 µM to 200 µM.

Table 4-1. *V. vulnificus* strains used in studies performed.

Strains Used	Description	Reference
MO6-24/0	Clinical, virulent wild-type strain	Wright *et al.*, 2001
MO6-24/0 *ΔgacA*	*gacA* deletion mutant with kanamycin inserted	Gauthier *et al.*, 2010
MO6-24/0 *ΔgacA* (pGacA)	*gacA* mutant with complemented vector containing *gacA in trans*	Gauthier *et al.*, 2010
MO6-24/0 *ΔgacA* (pGTR1160)	*gacA* mutant with empty complemented vector	Gauthier *et al.*, 2010
CMCP6/0	Clinical, virulent wild-type strain	Kim *et al.*, 2003
CMCP6/0 *ΔgacA*	*gacA* deletion mutant with kanamycin inserted	Gauthier *et al.*, 2010
CMCP6/0 *ΔgacA* (pGacA)	*gacA* mutant with complemented vector containing *gacA in trans*	Gauthier *et al.*, 2010
CMCP6/0 *ΔgacA* (pGTR1160)	*gacA* mutant with empty complemented vector	Gauthier *et al.*, 2010
CMCP6/0 Δ*viuB*	*viuB* deletion mutant with kanamycin inserted	This study
CMCP6/0 Δ*viuB* (pViuB)	*viuB*mutant with complemented vector containing *viuB in trans*	This study
CMCP6/0 Δ*viuB* (pGTR1160)	*viuB* mutant with empty complemented vector	This study
CMCP6/0 *ΔmtlA*	*mtlA* deletion mutant with kanamycin inserted	Donated by Dr. Paul Gulig, University of Florida
CMCP6/0 Δ*mtlA* (pMtlA)	*mtlA*mutant with complemented vector containing mtlA *in trans*	Donated by Dr. Paul Gulig, University of Florida
CMCP6/0 Δ*mtlA* (pGTR1160)	*mtlA* mutant with empty complemented vector	Donated by Dr. Paul Gulig, University of Florida

Phenotypic Responses of *V. vulnificus*

Individual colonies were inoculated into 250 mL flasks containing 35 mL of LBN. The flasks were incubated at 80 rpm at 37^{o}C overnight in a C24 Incubator Shaker® (New Brunswick Scientific). The absorbance at 600nm (OD_{600}) was recorded at 24 hours using the SPECTRAmax PLUS 384® (Molecular Devices), and was adjusted to an A_{600} of 0.30 in 1 mL PBS. Cells were then washed 2 times using LBN, at 8,000xg, for 90 seconds with the Centrifuge 5415D® from Eppendorf. The final pellet was suspended in 1mL LBN. Washed *V. vulnificus* cells (710 µL) were added to each 250 mL flask containing 35 mL LBN to achieve an inoculum of approximately 2.0 x 10^{6}CFU/mL. All flasks were incubated overnight at 37^{o}C, shaking at 80 rpm to achieve stationary phase. After 18-24 hours, the absorbance at 600nm was recorded. Three biological replicates of each variant were used for the experiment, with one technical replicate for each sample. Percent growth yield was calculated using the equation: % growth yield = (OD_{600} in LB:150 µM DP ÷ OD_{600} in LB) x 100.

Individual Gene Expression

Quantitative reverse transcription PCR (qRT-PCR) was the primary method used to examine gene expression of *viuB*, *venB*, and *vuuA* catechol siderophore genes and ATP, PP (both previously described and designated), and *iutA* hydroxamate genes in both the *gacA* and *viuB* deletion mutants compared to wild-type *V. vulnificus* strain CMCP6. Variations of CMCP6 described in Table 4-1 were grown in LBN under iron-replete conditions, and iron-limiting conditions used dipyridyl at concentrations of 150 µM. Preliminary data showed that for both strains of *V. vulnificus*, 150 µM dipyridyl caused a large inhibitory effect on the *gacA* deletion mutants, but allowed the wild type to grow normally. Following overnight growth, RNA was extracted as previously

described (Gauthier *et al.*, 2010). After isolation, the RNA was quantified using a Gene Spec and underwent reverse transcription through the Invitrogen cDNA synthesis kit® to a final amount of 2 µg. For qRT-PCR, a 1/20 dilution of the cDNA was used in the Express SYBR Green® qRT-PCR kit by Invitrogen with primers (Figure 4-1) designed through primer-3 software. The thermocycler used for the QPCR was the Smart Cycler II® from Cepheid. The fold differences in transcript levels were calculated from Ct values using the ΔΔCt method with 16S as the reference gene (Livak & Schmittgen, 2001).

qPCR	*Name*	*Sequence*
	VuuA-F	ctctgttgcacctggggtat
	VuuA-R	gcctatgctcaaaccgagag
	ViuB-F	ggaatcgcgtagagcttgtc
	ViuB-R	tccacgatcttgttcatcca
	VenB-F	acgttagacgccttcatgct
	VenB-R	cttttgcgaacggatttcat
	16S-F	caagtcgagcggcagca
	16S-R	tcctgacgcgagaggcc
	ATPase-F	acggtgatcatggtgctaca
	ATPase-R	ttttggggtgagcgaataac
	PP-F	agtcggtttgatcggttttg
	PP-R	cagccacaacaaaaatggtg
	iutA-F	atcgaagtgttgtcgggttc
	iutA-R	ccgccagaaatagactgagc
Sequencing		
	VenB up out F2	cctctccggttaacgttactcg
	VenB dwn out R1	gagaatgggatacgtgaaagc
	VenB in F2	gcatcaccaatcacaaacgg
	VenB F RS	*see VenB-F
	ViuB Flank F	agaaacgcttgcgtgaatct
	ViuB mj	cgatgtctgcctttagtgc
	ViuB Flank R	atgttcgttgccttggactc
	ViuB F1	cagagcgcgtctatccaatgt
	VuuA up out F1	gtgattgacggtgataagc
	VuuA dwn out R1	cagaactcaggacaatgaagg
	VuuA in F2	cgttatagaaggtgttggtc
	VuuA in F3	caggtctaaacgcttcattc
	VuuA F RS	*see VuuA-F
	VuuA in R2	gttcgaccaagagacgatag
	VuuA in F3 rev	gaatgaagcgtttagacctg
	VuuA in F2 rev	gaccaacaccttctataacg
	VuuA F2 new	ccgaaggtctgatcattag
	VuuA F3 rev new	cttgcattccgcctcagc
viuB Mutant Creation		
	ViuB up 5'	**GGAGACAU**-aacggtttttatcgcactgg
	ViuB up 3'	**ACCCGGGU**-cgcctcgaaacagaagaaag
	ViuB down 5'	**ACCCGGGU**-ggccatggtttgttcttcat
	ViuB down 3'	**GGGAAAGU**-ggcatactttcgaccacgtt
	viuB USER Comp5'	**GGAGACAU**-gataacaacgtcagctaggc
	viuB USER Comp3'	**GGGAAAGU**-ctatcgatgaacaacggaag

Figure 4-1. Primers for PCR and sequencing. Bold nucleotides indicate the USER specific sites for USER cloning.

Sequence Comparisons of Iron Response Genes

DNA from clinical and environmental strains of *V. vulnificus* (Table 4-2) was used to sequence the *venB, vuuA* and *viuB* genes. Primers (Figure 4-1) were derived using the primer-3 software and the GenBank sequences for *V. vulnificus* CMCP6, YJ016 and available MO6-24/0 sequences provided by Paul Gulig. Primers were selected from sequences that were conserved among all strains. The isolation of the amplified DNA was performed using a PCR Clean-Up kit® (Qiagen). ICBR of the University of Florida provided sequencing. Sequences were aligned using the MEGA program version 4 to generate a dendrogram to identify phlogroupings (Tamura *et al.*, 2007).

viuB Deletion Mutation and Complementation

To perform the deletion mutation of *viuB* in *V. vulnificus* strain CMCP6 (Type 1), USER® friendly cloning and chitin transformation were used to remove *viuB* and substitute the gene for kanamycin resistance, according to the protocol of Gulig *et al* (2010). 500 base-pair regions upstream and downstream of *viuB* were PCR amplified using the primers “ViuB up 5’”, “ViuBup3’”, “ViuBdown5’”, and “ViuBdown3’” found in Figure 4-1. These PCR products were combined and directionally cloned into the USER ready, broad-host-range vector pGTR1129. Included in the upstream and downstream primers was a *SmaI* site such that the kanamycin-resistance marker could be inserted into the joined regions. This construct was electroporated into *Escherichia coli* EC100D to screen for successful ligations. The extracted plasmid was then digested with *SmaI* to open the vector. A kanamycin resistance cassette was then ligated into the USER vector and electroporated into *E. coli* again. The plasmid was extracted and 2 µg of the vector was sheared and added to *V. vulnificus* CMCP6. This mixture was contained in a 12-well plate with cleaned crab shells in each well. After 24 hours the bacteria were

plated and screened for kanamycin resistance. PCR confirmation was used to verify the insertion along with sequence analysis. The complemented mutant (pViuB) was created by PCR-amplifying the *viuB* gene from *V. vulnificus* CMCP6 with USER end primers and then cloned *in trans* using pGTR1160, a USER-end modified vector containing a maker for tetracycline-resistance. Conjugation was then used to transfer the plasmid from *E. coli* S17 to a *V. vulnificus* CMCP6 *viuB* deletion mutant. Meanwhile the plasmid control was the empty vector pGTR1160 conjugated into the *viuB* mutant.

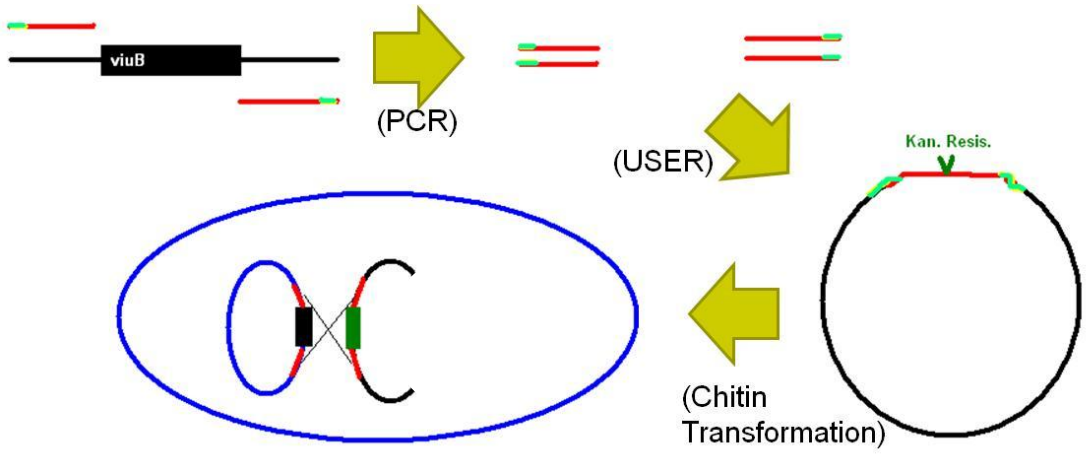


Figure 4-2. Diagram of *viuB* deletion mutation construction in *V. vulnificus* strain CMCP6 using USER cloning and chitin transformation.

Virulence Analysis

V. vulnificus strains were tested for virulence potential using a previously described mouse model (Starks *et al.*, 2000). Female mice (*n*=5) were injected sub-cutaneously with 0.1 mL of bacteria in PBS. This process used approximately 10^3 colony forming units (cfu) for iron treated mice and 10^6 cfu for mice without added iron. To iron treat the mice, 250 µg of iron dextran per gram of body weight was injected into the mice. Mice were euthanized using CO_2 asphyxiation, and a rectal temperature of

Table 4-2. Strains used for sequencing analysis.

Strain	Source
YJ016	Clinical
M06-24/O	Clinical
CDC 9053-96 (117)	Clinical
CDC 9074-96 (149)	Clinical
CDC 9076-96 (133)	Clinical
CDC 9003-97 (139)	Clinical
ATL-9824 (112)	Clinical
CMCP6	Clinical
CDC 9345-95 (105)	Clinical
99-578 DP-B1(134)	Oyster
CDC 9349-95 (102)	Clinical
CDC9352 (116)	Clinical
CDC 9340-95 (129)	Clinical
CDC 9032-95 (150)	Clinical
CDC 9070-96 (113)	Clinical
CDC 9005-97 (122)	Clinical
CDC 9038-96 (146)	Clinical
CDC 9075-96 (109)	Clinical
CDC9031-6 (141)	Clinical
99-736 DP-C7 (106)	Oyster
99-623 DP-F5 (135)	Oyster
99-520 DP-B8 (137)	Oyster
99-780 DP-E1 (123)	Oyster
ATL-9572 (126)	Clinical
99-609 DP-A4 (142)	Oyster
99-581 DP-C7 (111)	Oyster
99-738 DP-B5 (125)	Oyster
99-537 DP-G7 (127)	Oyster
ATCC 33147 (B1)	Fish
90-2-11 (B2)	Fish
ATCC33149 (B3)	Fish
NCIMB2137 (B4)	Fish
246-0058 (108)	Clinical
98-640 DP-E9 (119)	Oyster
99-509 DP-A6 (148)	Oyster

33^{o}C was the surrogate for death. Skin tissue at lesion site and liver samples were excised, homogenized and diluted in PBS, and plated on LA. Bacterial counts were calculated as log cfu per gram.

In addition to comparison of virulence assays for individual strains, competitive virulence assays were performed in the mouse model as well. An *mtlA* deletion mutant defective in mannitol utilization was used as a surrogate for wild type. Co-inoculation

studies were performed following the same procedure as before; however, the mice were infected with twice the amount of bacteria, using 10^6 of each strain per mouse. Skin tissue and liver samples were prepared as before but were plated onto LA supplemented with mannitol and phenol red as a pH indicator. On these plates *viuB* mutants are yellow colonies due to their ability to ferment the mannitol, which causes a pH change and a corresponding color change from the phenol red. Meanwhile wild-type surrogate *mtlA* mutants are pink due to their inability to utilize the mannitol as an energy source.

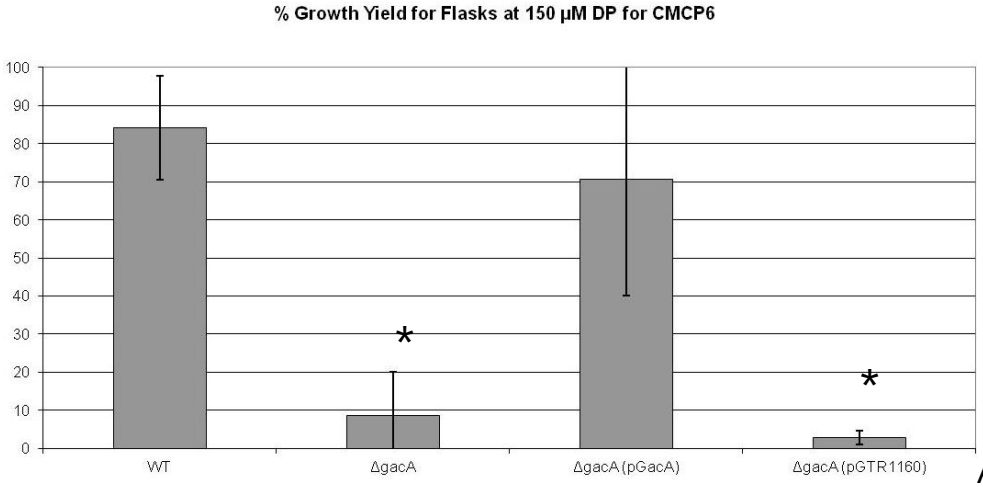
CHAPTER 5
ROLE OF GACA IN IRON-LIMITED GROWTH AND GENE EXPRESSION

GacA Regulates Iron-Limited Growth

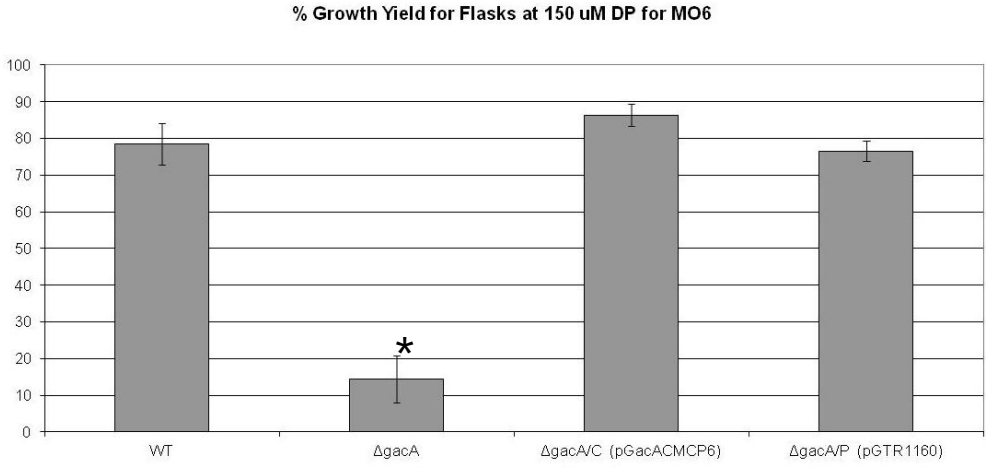
As a first step in establishing a link between GacA and the phenotypic response to iron-limitation*, V. vulnificus* was grown under of iron-limited and iron-replete conditions. Since LBN medium contains iron, both wild-type and *gacA* deletion mutant strains thrive as neither has to scavenge for available iron. However, once iron-limiting conditions are introduced by the addition of dipyridyl, the *gacA* deletion mutant was limited in growth compared to wild-type strain, indicating that the iron acquisition is regulated in part by GacA (Gauthier *et al.*, 2010). Mutants in two strains of *V. vulnificus* showed decreased growth under iron-limiting conditions, while under iron-replete conditions growth yields of all strains were approximately equal (Figure 5-1). Triplicate experiments showed wild-type strains had significantly greater growth yields under iron-limitation (84.1% and 78.6% in CMCP6 and MO6, respectively) compared to the corresponding *gacA* deletion mutants (8.7% and 14.6% in CMCP6 and MO6, respectively, with p-values 0.002 and 0.0002 respectively). Thus, iron-limitation imposed by application of a chelating agent demonstrated that the mutants were deficient in their ability to acquire iron for survival compared to wild-type. These results support Hypothesis 1 that GacA regulates the phenotypic response to iron-limitation in *V. vulnificus*.

The phenotype of mutant strains was restored when the *gacA* gene was introduced *in trans* on a plasmid vector (pGacA) and showed activity comparable to wild type. However, the vector (pGTR1129) controls showed conflicting results between the two strains. For CMCP6 *ΔgacA* (pGacA) growth yield was similar to the *gacA* deletion mutant, while MO6-24 *ΔgacA* (pGacA) growth yield was more similar to the wild-type

strain. Complementation of the mutant strain should restore the phenotype, but the plasmid control should not (Falkow, 1988). Ultimately, these results support to the conclusion that GacA regulates growth of *V. vulnificus* under conditions of iron-limitation, but vector effects cannot be explained for the one strain.



A.



B.

Figure 5-1. Growth yield under iron-limiting conditions. A) Percent growth yield of CMCP6 for each variant at 150 µM dipyridyl. B) Percent growth yield of MO6 for each variant at 150 µM dipyridyl. Significant differences are noted for $p<0.001$ (*).Percent growth yield was calculated using the equation: % growth yield = (OD_{600} in LB:150µM DP ÷ OD_{600} in LB) x 100.

GacA Regulates Gene Expression of the Catechol Siderophore System

The role of GacA was examined for its influence on the expression of various genes related to iron acquisition. *V. vulnificus* CMCP6 Wild-type strain, the *gacA* deletion mutant, the complemented mutant, and plasmid control strain (Table 4-1) were compared for gene expression by quantitative reverse transcription PCR (qRT-PCR) in order to determine differences in gene expression for *viuB*, *venB* and *vuuA* genes under iron-replete (LBN) and iron-limiting conditions (LBN with dipyridyl was added to a final concentration of 150 µM). These catechol siderophore-related genes were chosen because they are important iron acquisition, which is also important for virulence. The fold changes for these genes were compared in the *gacA* deletion mutant relative to wild-type expression.

Under iron-replete conditions the *gacA* deletion mutant showed slightly increased transcript levels compared to wild-type (Figure 5-2) while the complemented mutant and plasmid control had decreased transcript levels under iron-replete conditions (Appendix A, Figure A-1). However, none of the genes demonstrated significant changes in transcript levels for *gacA* deletion mutant vs. wild-type strain.

Conversely, under iron-limiting conditions, the *gacA* mutant and the plasmid control showed significant decreases in gene expression compared to wild-type strain for all three genes examined (Figure 5-2, p-values<0.001). Complementation restored *viuB* expression to levels that did not differ significantly from wild-type (p=0.05). Although the complementation of mutations for *venB, vuuA* (Appendix A, Figure A-1) still showed significant decreases in transcript levels (p-values 0.004 and 0.002 respectively), the phenotype was somewhat restored, as differences were greatly reduced.

These results confirm the role of GacA regulation in the iron response of *V. vulnificus* under iron-limiting conditions and are consistent with results reported for the related species of *V. fischeri,* whereby GacA regulates iron acquisition (Whistler & Ruby, 2003). Based on these results we conclude that GacA contributes to regulation of iron acquisition genes in the catechol siderophore system in *V. vulnificus*.

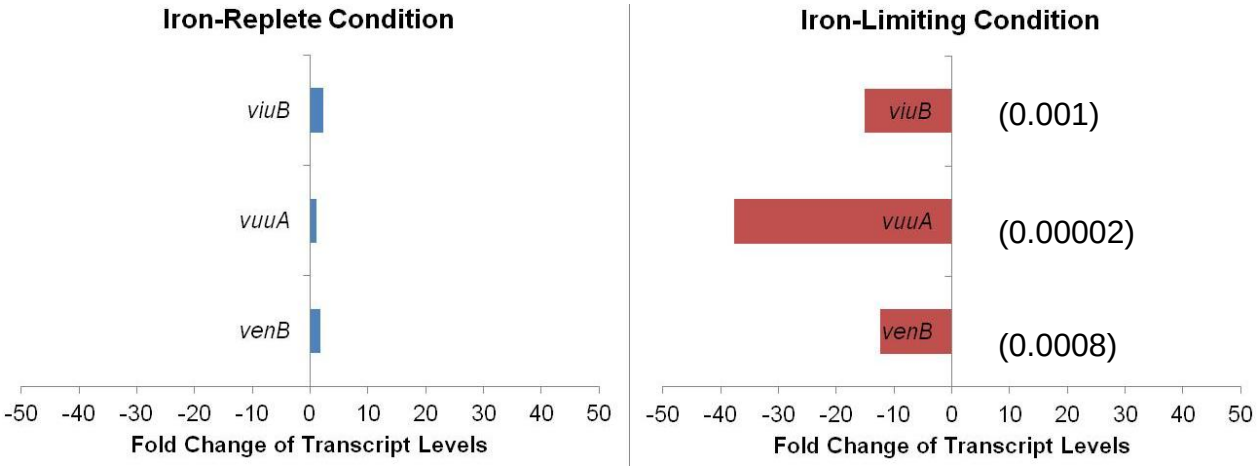


Figure 5-2. Fold changes of catechol siderophore iron acquisition genes when the *gacA* deletion mutant is compared to wild-type strain CMCP6 under iron-replete and iron-limiting conditions. Results are the average of 3 independent experiments with at least 2 technical replicates for each experiment. (P-values are indicated compared to wild-type).

Gene Expression of the Hydroxamate Siderophore System

V. vulnificus has the ability to produce and utilize more than one type of siderophore. Therefore, it was hypothesized that *gacA* may also play a role in regulation of the hydroxamate system, and the transcript levels of several genes (ATP, PP, *iutA*) related to the hydroxamate siderophore system were also analyzed.

As seen in Figure 5-3, when compared to the wild type under iron-replete conditions the *gacA* deletion mutant exhibited significant fold increases for ATP, PP, and to a lesser extent *iutA* (p-values 0.001, 0.00003, and 0.04 respectively). When *gacA* was introduced *in trans*, the same genes showed significant fold decreases compared

to wild-type (Appendix A, Figure A-2); p-values 0.0003, 0.000004, and 0.000002); however, the plasmid control did not significantly differ from wild type.

Under iron-limiting conditions (Figure 5-3), the *gacA* deletion mutant showed somewhat increased expression for ATP, PP, and *iutA* when compared to wild-type (1.07, 1.59, and 1.34 fold, respectively), but differences were not significant. Interestingly, the complemented strain (3.00, 1.36, and **2.71**, respectively) and the plasmid control (**4.49**, 1.07, and **2.12**) both exhibited decreased expression in the mutant compared to wild-type, and significant difference were noted (boldened).

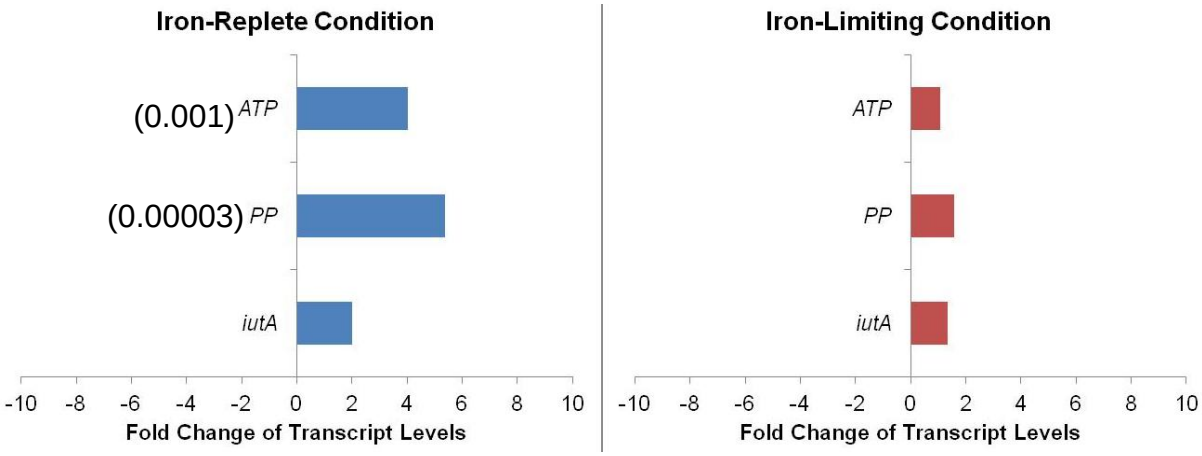


Figure 5-3. Fold changes of hydroxamate siderophore iron acquisition genes when the *gacA* deletion mutant is compared to wild-type strain CMCP6 under iron-replete and iron-limiting conditions. Results are the average of 3 independent experiments with at least 2 technical replicates for each experiment. (Significant differences are shown).

The results for hydroxamate gene expression are difficult to interpret, but they indicate that GacA does not play a role in regulating the hydroxamate siderophore system under iron-limiting conditions. Instead, the *gacA* deletion mutant had significant increases for two genes, ATP and *iutA* under iron-replete conditions, possibly through de-repression in the absence of the *gacA* gene expression. Clearly, GacA provides differential regulation for the two high-affinity iron acquisition systems examined in *V.*

vulnificus, and more research will be required to sort out these pathways and the conditions that influence their regulation.

CHAPTER 6
COMPARATIVE GENETICS OF THE CATECHOL SIDEROPHORE SYSTEM

Comparative Alignments of ViuB Sequences

Prior research (Bogard & Oliver, 2007; Panicker *et al.*, 2004) showed the correlation of a particular *viuB* sequence with all strains from clinical origin, while most oyster isolates differed from the clinically-associated sequences and were negative for PCR assays for this gene. These prior publications initially reported that only clinical isolates contained the gene, and concluded that *viuB* should be considered as a marker for virulence of *V. vulnificus*. However, both groups later published errata showing their data were incomplete, and DNA sequence comparisons instead identified the sequence in all strains but with polymorphic sites. Thus, sequence differences were attributed as the reason that the original PCR screening of the gene failed to detect its presence in environmental strains.

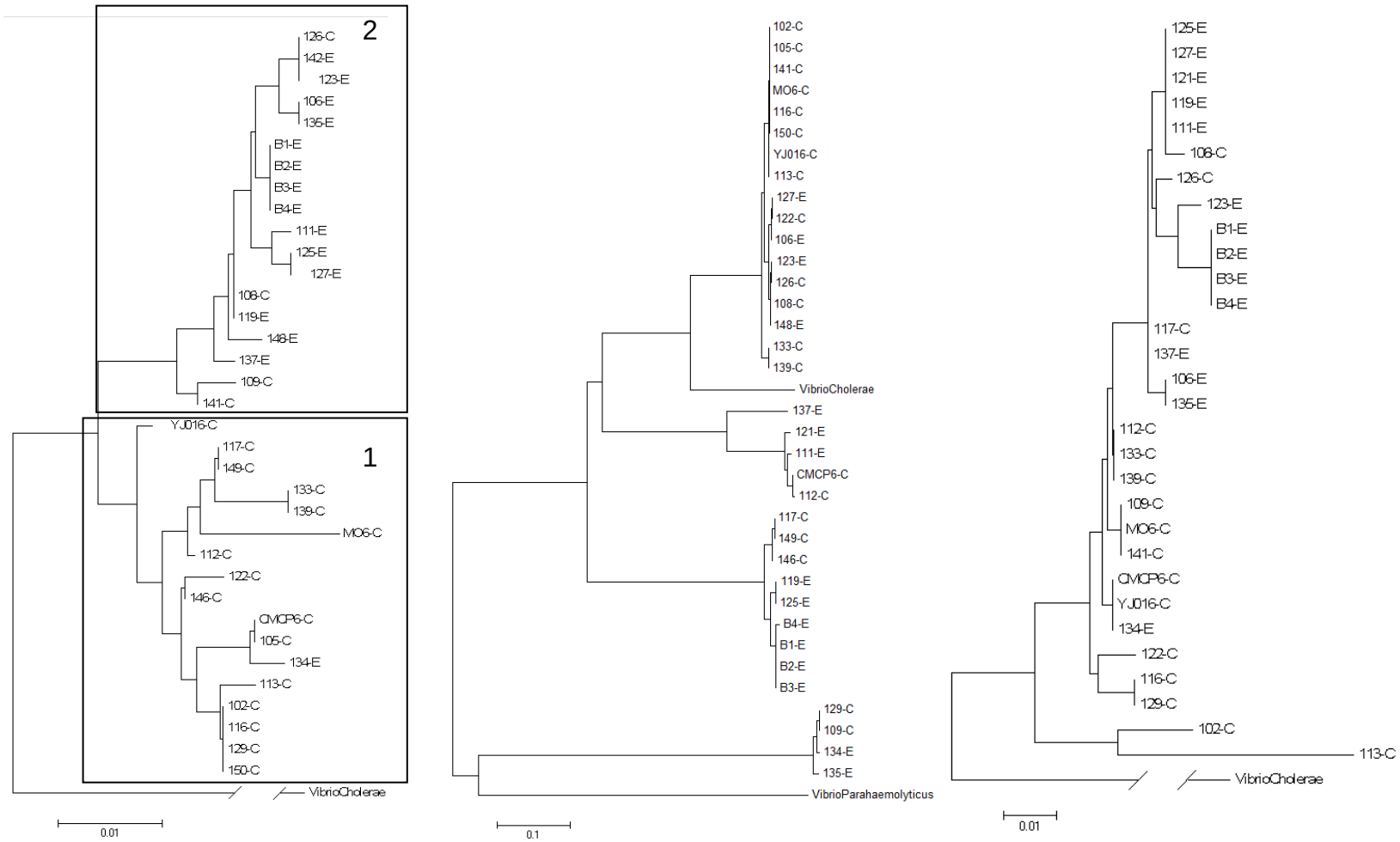
These studies described above examined complete sequence from only a limited number of strains. To further investigate Hypothesis 2, that sequence differences related to genes for iron acquisition could predict virulence, *viuB* genes from 35 additional strains (Table 4-3), as well as other genes (*venB* and *vuuA*) related to the catechol siderophore system, were sequenced, and their deduced amino acids were used to create phylogenetic trees. Sequence analysis of *viuB* genes from the 35 *V. vulnificus* strains is shown in Figure 6-1A, and segregated ViuB sequences into two phylogenetic types (phylotypes). ViuB Type 1 contained 80% of the clinical strains examined, while Type 2 was found in 93% of the environmental isolates examined. These data confirmed differences at the amino acid level and demonstrated divergent phylogenetic groups with excellent correlation between strain source and phylotype.

Alignments of ViuB identified 14 polymorphic amino acids sites (Figure 6-2), including sites with acidic residues aspartic (D) and glutamic acids (E) that were found in Type1 strains CMCP6 and YJ105, but were substituted with glycine (G, neutral) residues in Type 2 strains 109, 141, B1, and 108. These acidic residues have been shown to contribute to iron binding in other related proteins (Bailey *et al.*, 1988). We hypothesize that these amino acid substitutions may alter the iron-binding capacity and hence the function of ViuB, and future studies are planned.

Comparative Alignments of VuuA Sequences

The *vuuA* gene, encoding the outer membrane receptor for the vulnibactin-iron complex, was also sequenced in multiple strains from various sources, and the deduced amino acid sequences were aligned. The corresponding phylogenetic tree is shown in Figure 6-1B. Unlike ViuB, VuuA sequences did not segregate into two phylogroups that correlated with the strain source. Instead a multi-branching tree with much greater distances (0.1 scale compared to 0.01 for ViuB) between branches was observed, indicating greater diversity for this protein compared to ViuB. Interestingly, the *V. cholerae* sequence did not root this tree as seen with ViuB; instead, *V. parahaemolyticus* VuuA sequence was used as a root, although some *V. vulnificus* sequences showed greater similarity to those of *V. parahaemolyticus* compared to other sequences of *V. vulnificus.*

The reason that *V. cholerae* sequence did not root the tree for *V. vulnificus* VuuA sequences was due to regions with highly conserved sequences between the two species for this protein (Figure 6-3). The alignments showed a 320 amino acid insert that had 59% agreement with sequences from *V. cholerae*.



A. B. C.

Figure 6-1. Comparative alignments of deduced amino acids of ViuB (A), VuuA (B), and VenB (C), using *V. cholerae* as a root for all three, and using *V. parahaemolyticus* as a root for VuuA. ViuB Types are indicated by boxes.

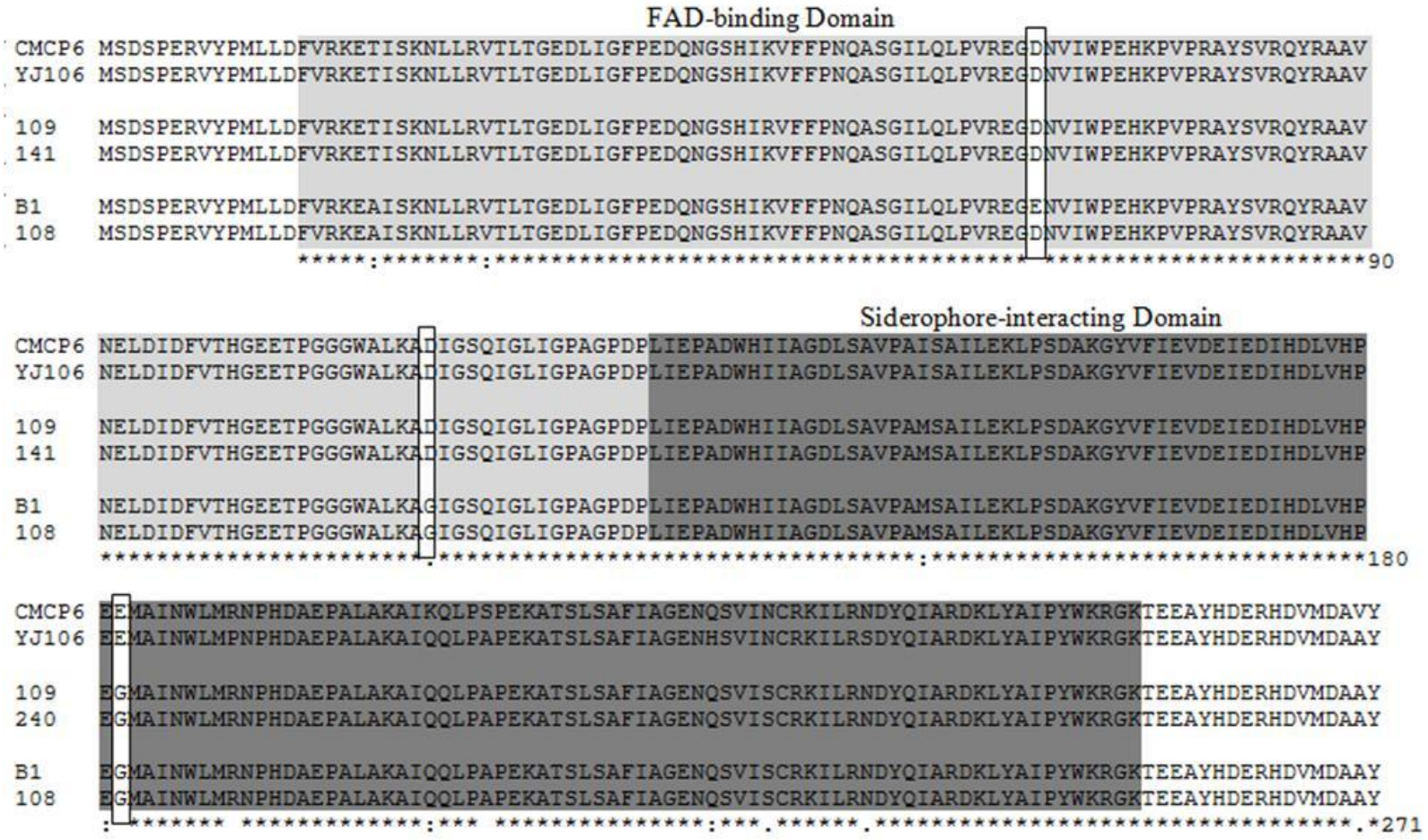


Figure 6-2. Deduced amino acid alignment of ViuB for several *V. vulnificus* strains, CMCP6 and YJ106 are Type 1 while 109, 141, B1, and 108 are Type 2. Three of the changes to acidic residues are noted in boxes.

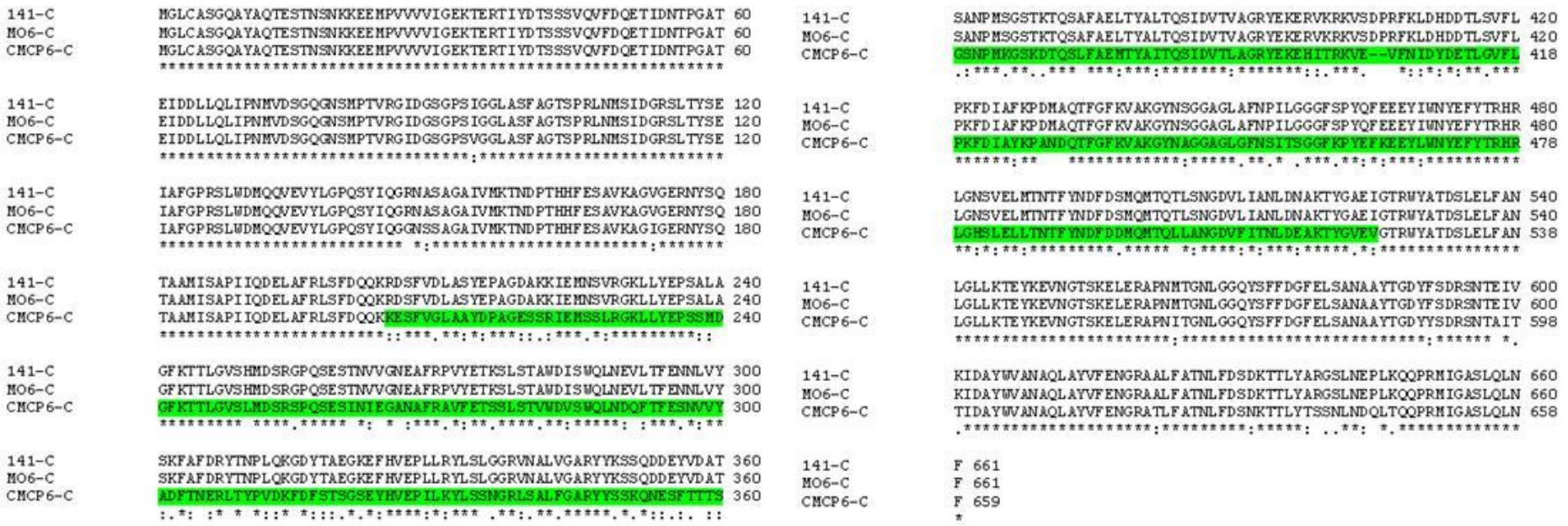


Figure 6-3. Alignment of deduced amino acids of VuuA in *V. vulnificus* with corresponding region with high affinity to *V. cholerae* sequence highlighted. "*" represents no change, ":" represents a change to an amino acid with highly similar properties, "." represents a change to an amino acid with weakly similar properties, while a space represents a change to an amino acid with highly dissimilar properties.

Conversely, this region has the highest degree of variability among some *V. vulnificus* strains (~68% agreement) when compared to the flanking regions (~98% for upstream region and ~90% for downstream region), indicating the insert was a recent acquisition.

Comparative Alignments of VenB

The final gene sequenced was *venB*, encoding an enzyme required for synthesis of the vulnibactin siderophore. Deduced amino acid sequences were aligned into a phylogenetic tree (Figure 6-1C). Approximately 93% of all environmental strains examined showed sequence that clustered into single branch with <0.01 divergence, while 81% the clinical isolates were distributed across other branches. Although the distinction between strains from different sources was not as clear as that seen with ViuB, these results also suggested a closer relationship of strains from clinical origin as compared to environmental isolates.

Relationship of Sequence Types with Virulence

Litwin *et al*. demonstrated that a *V. vulnificus venB* deletion mutant had reduced virulence compared to wild-type in the infant mouse model (1996). As iron acquisition contributes to virulence of *V. vulnificus,* the different phylotypes of the genes described above were examined for their relative virulence potential in a mouse model of disease (Thiaville *et al.*, 2011). It was shown that strains within ViuB Type 1 demonstrated increased virulence in mice when compared to strains without that sequence ($p<0.01$). As shown in Figure 6-4, significant differences were observed for the log CFU/g in the liver and for temperature (p-values 0.008 and 0.004 respectively (adapted by Thiaville *et al.*, 2011. Genotype is correlated with but does not predict virulence of Vibrio vulnificus biotype 1 in subcutaneously inoculated, iron dextran-treated mice. *Infection and*

immunity **79**, 1194-1207)). However, similar correlations were not observed for any other phylotypes and strain origin for either VuuA or VenB deduced amino acid sequences.

Table 6-1. Virulence comparison in the mouse model, compared to Type 1. Significant values indicated in parentheses. Represents 1 experiment, with each strain inoculated into mice (n=5) (Thiaville *et al.*, 2011).

	Virulence in Mice (p value relative to Type1)		
ViuB Type	Mean Log Skin CFU/g	Mean Log Liver CFU/g	Temperature (°C)
1	7.2 ± 0.90	4.40 ± 0.79	32.8 ± 1.55
2	6.68 ± 1.43	3.13 ± 0.59 (0.008)	35.4 ± 1.52 (0.004)

CHAPTER 7
ROLE OF VIUB IN IRON ACQUISITION AND VIRULENCE

Role of ViuB under Iron-Limiting Growth

Although *viuB* is part of a cluster of genes that has been demonstrated to play a role in the acquisition of iron (Litwin *et al.*, 1996), the specific contribution of this gene to iron uptake has not been previously demonstrated. Therefore, a deletion mutant in *V. vulnificus* strain CMCP6 was constructed to determine the role of this gene in growth under iron-limiting conditions. As seen in Figure 7-1, the *viuB* mutant and plasmid control had significantly lower % growth yields (17.8% and 18.1% respectively, with p-values<0.0001) when compared to the wild type and complemented mutant (90.2% and 97.9% respectively). These results verified that *viuB* is required for growth under iron-limiting conditions in *V. vulnificus* and that the phenotype could be complemented *in trans*. Results are consistent with prior reports that identified *viuB* as a gene in the catechol siderophore pathway in related bacteria (Butterton & Calderwood, 1994).

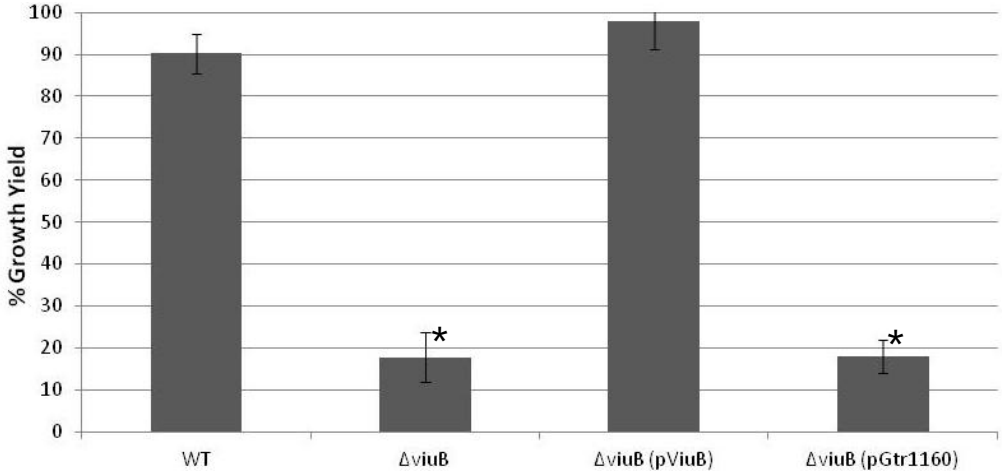


Figure 7-1. Percent growth yield under iron limitation conditions at 130 µM dipyridyl. Significant differences are noted for p<0.01 (*). Percent growth yield was calculated using the equation: % growth yield = (OD_{600} in LB:130µM DP ÷ OD_{600} in LB) x 100

Effect of *viuB* on Virulence in Mice

The *viuB* deletion mutant of *V. vulnificus* was also used to study the role of this gene in virulence in mice. Using a previously described mouse model (Starks *et al.*, 2000) but with or without exogenous iron injections, wild-type strain CMCP6 and the *viuB* mutant were infected subcutaneously, and the CFU/g in tissues that were harvested after 18 hours was compared in order to determines the relative levels of virulence between these strains. As seen in Table 7-1, two separate conditions were tested: mice (n=5) were inoculated A) with or B) without pretreatment with injected iron-dextran. The results showed that neither the iron status of the host nor the absence of the *viuB* gene in *V. vulnificus* significantly altered the virulence of *V. vulnificus*. Conditions in mice without iron pretreatment are presumed to be iron-limiting as the untreated host will sequester iron in the form of transferrin and other iron binding compounds. This condition was where differences in virulence between mutant and wild-type were expected, as the *gacA* mutant showed reduced virulence compared to wild-type under similar conditions. Therefore, the no iron added condition was repeated in two additional experiments, and similar results were obtained. The experiment with added iron was only tested once, but no significant difference was seen between *viuB* mutant and wild-type virulence under these conditions.

Table 7-1. Virulence comparison in the mouse model. A) Virulence assay in mice injected with iron. B) Virulence assay in mice without injected iron. Represents 1 experiment, but similar results were obtained on two additional experiments.

	Virulence in Mice (p value relative to Wild Type)		
	Mean Log Skin CFU/g	Mean Log Liver CFU/g	Temperature (°C)
Wild Type	7.4 ± 0.34	4.7 ± 0.70	32.3 ± 1.3
ΔviuB	7.6 ± 0.19	4.7 ± 0.74	32.6 ± 1.1

A.

	Virulence in Mice (p value relative to Wild Type)		
	Mean Log Skin CFU/g	Mean Log Liver CFU/g	Temperature (°C)
Wild Type	7.0 ± 0.2	3.7 ± 0.5	32.0 ± 0.8
ΔviuB	6.9 ± 0.2	4.1 ± 1.5	34.6 ± 2.9

B.

Competitive Virulence between Wild-Type CMCP6 and the *viuB* Deletion Mutant

In order to evaluate further the contribution of *viuB* to virulence, virulence comparisons were performed using a competitive *in vivo* analysis. In these competitive assays a *V. vulnificus* mutant defective in mannitol utilization (*ΔmtlA*),donated by the lab of Dr. Paul Gulig, was used as a surrogate for wild-type strain CMCP6 in order to mark the strain and easily distinguish it from the *viuB* mutant variant of the same strain (CMCP6) on the same medium. Equal inocula were prepared for each strain (approximately 1 x 10^6 CFU for each mutant in 100 µL) and injected sub-cutaneously into mice as previously described (Gauthier *et al.*, 2010). As seen in Table 7-2, there was no significant difference in virulence between the *viuB* mutant and wild type surrogate based on liver and skin samples (p-values of 0.99 and 0.11 respectively). This result also supports the above evidence that *viuB* does not directly play a role in virulence. As with the previous mouse assay, these results represent data from one experiment but were similar to results from two additional experiments, which also did not show significant differences.

Table 7-2. Competitive virulence assay in the mouse model, using an *mtlA* deletion mutant as a surrogate for wild-type strain. Represents 1 experiment, but similar results were obtained on two additional experiments

	Virulence in Mice (p value relative to Mannitol mutant)	
	Mean Log Skin CFU/g	Mean Log Liver CFU/g
"Wild Type" (red)	7.1 ± 0.2	3.6 ± 1.4
ΔviuB (yellow)	6.7 ± 0.4	3.6 ± 1.5

Previous data (Gauthier *et al.*, 2010) demonstrated that *gacA* did play a significant role in virulence depending on the strain examined and the host iron status, and the results herein revealed an association between *gacA* and expression of iron acquisition genes in the catechol siderophore pathway, including the *viuB* gene. However, results in a mouse model clearly showed that the *viuB* product in the catechol pathway did not contribute to virulence under these conditions. These results argue against the use of this gene as a virulence marker for *V. vulnificus* (Bogard & Oliver, 2007; Panicker *et al.*, 2004). The supposition that observed differences in phylogroups based on ViuB may be an underlying factor in evolution of more virulence strains is also not supported. Thus, the results from this experiment show that although *viuB* Type 1 correlates with clinical origin, it does not predict or contribute to virulence in this model.

Relationship of ViuB to Hydroxamate Gene Expression

Because virulence assays yielded no observed difference between the *viuB* deletion mutant and wild type in the mouse model, the specific role of iron acquisition in the virulence of *V. vulnificus* is still unclear. A prior report (Litwin *et al.*, 1996) showed small but significant reduction in virulence that was attributed to the loss of vulnibactin expression. These differences could be attributed to differences in animal models (infant vs. adult mice), but they might also be a consequence of the relative contribution of the two genes to the catechol pathway. It is also possible that alternative iron acquisition pathways (i.e. the hydroxamate pathway) may compensate for the loss of *viuB* expression. Therefore, the effects of the *viuB* mutation on transcript levels of the genes contributing to the hydroxamate siderophore system were also examined.

As seen in Figure 7-2, the *viuB* deletion mutant had some changes (0.95, 1.99, and 0.86 fold increase) for transcript levels of ATP, PP, and *iutA* respectively, compared

to wild-type; however, none of these values were significant under iron-replete conditions. Significant differences were also not observed for the complemented mutant or the plasmid control under these conditions. However, significant differences were seen between mutant and wild-type strain for iron-limiting growth. The *viuB* deletion mutant exhibited fold increases of 5.56 (0.008), 2.01, and 6.84 (0.0001) for ATP, PP, and *iutA* transcript levels, respectively, compared to wild-type. The plasmid control had similar fold increases of 4.99 (p-value 0.001), 2.88, and 4.06 (p-value 0.0005) when compared to wild type, and the complemented mutant did not differ significantly from wild-type for all three genes examined.

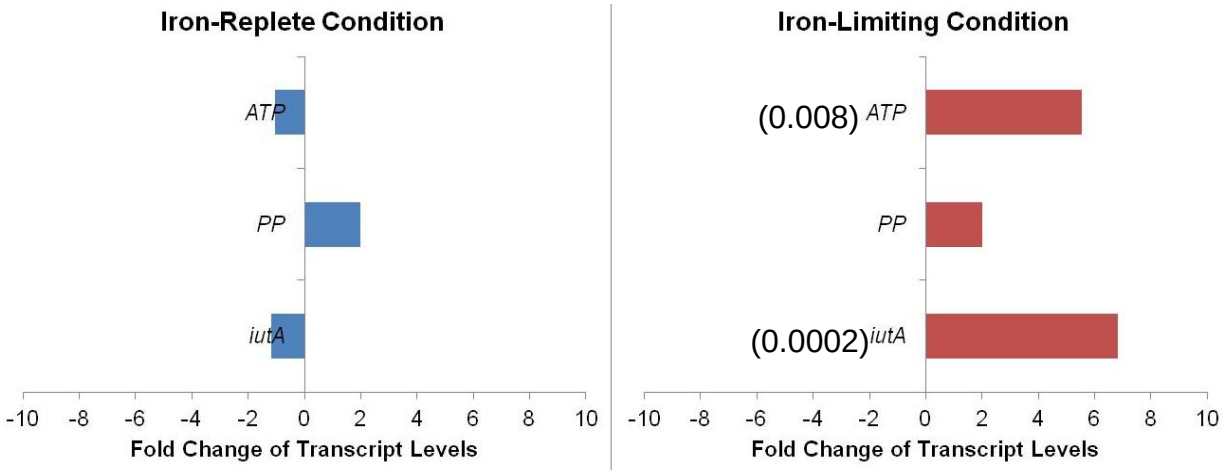


Figure 7-2. Fold changes of hydroxamate siderophore iron acquisition genes when the *viuB* deletion mutant is compared to wild-type strain CMCP6 under iron-replete and iron-limiting conditions. Results are the average of 3 independent experiments with at least 2 technical replicates for each experiment. (Significant differences are shown).

Further work is needed to understand the relative contribution of the catechol and hydroxamate pathways to survival of *V. vulnificus* under limiting conditions, but these data suggest that loss of one pathway may be circumvented by the increased activity of the alternate pathway for iron uptake. A compensatory pathway may also explain why

little or no effect on virulence in observed when components of the catechol pathway are eliminated through genetic manipulation.

CHAPTER 8
SUMMARY AND CONCLUSIONS

The GacA Regulation of Iron Acquisition in *V. vulnificus*

Vibrio vulnificus is a halophilic, opportunistic bacterium known to cause systemic disease in individuals with compromised conditions that involve altered host iron status with excess physiologically available iron. Prior experimental research demonstrated an association between iron availability in the host and the relative virulence of *V. vulnificus* in mice, whereby increased host iron status through injection of exogenous iron caused greatly increased lethality of both clinical and environmental strains of the bacterium (Litwin *et al.*, 1996; Okujo *et al.*, 1996; Simpson & Oliver, 1983; Starks *et al.*, 2000; Wright *et al.*, 1981; Wright *et al.*, 1986). The LD_{50} approached one bacterium in the first study. *V. vulnificus* contains multiple high-affinity iron acquisition systems, including the expression of both catechol and hydroxamate siderophores. In the present study, the catechol siderophore system was examined for its contribution to growth under iron-limiting conditions and virulence, as well for its interactions with the hydroxamate system. Research in the related species, *Vibrio fischeri*, demonstrated that the GacS/GacA two-component signal transduction pathway could regulate iron acquisition (Whistler & Ruby, 2003), and therefore the role GacS/A on regulation of iron acquisition genes of *V. vulnificus* was also examined.

A deletion mutation of the *gacA* gene of *V. vulnificus* significantly reduced growth yield under conditions of iron limitation as compared to wild-type strain CMCP6. The catechol system was found to be under GacA control. Under iron-limiting conditions, the *gacA* deletion mutant exhibited significant decreases of all three catechol genes tested compared to wild-type strain ($p<0.001$), and this activity was partially restored in the

complemented mutant, as the fold decreases in transcripts were significantly smaller compared to the *gacA* deletion mutant. For the hydroxamate system, the *gacA* deletion mutant showed no significant decrease compared to wild-type strain under iron-limiting conditions, and expression actually increased during iron-replete growth. These results demonstrate that the GacS/GacA system regulates the catechol siderophore system under iron-limitation through up-regulated expression of catechol-related genes for iron acquisition. However, GacA behaves differentially with respect to the hydroxamate system. Instead, loss of GacA expression did not alter regulation of the hydroxamate siderophore system under iron-limiting conditions. The increased gene expression under iron-replete conditions could be explained through possible de-repression in the absence of GacA. Clearly, GacA provides differential regulation for these two high-affinity iron acquisition systems in *V. vulnificus*.

viuB and its Role in Iron Acquisition and Virulence

The *viuB* gene has been proposed as a potential virulence factor for *V. vulnificus* and was examined as possible marker to discriminate virulent vs. avirulent strains (Bogard & Oliver, 2007; Panicker *et al.*, 2004). Comparative phylogenetic analysis of clinical and environmental isolates confirmed correlation of *viuB* gene sequence with strain origin and further demonstrated that *viuB* sequence was also associated with virulence in *V. vulnificus* in mice. However, analysis of the deduced amino acid sequences for other catechol genes did not show similar associations with clinical origins and virulence. VenB did show a branch clustered with all the environmental isolates studied, but it proved not to be significantly different from the rest of the strains. This demonstrates that although iron acquisition is important for virulence of *V.*

vulnificus, the evolution of these particular genes in clinical and environmental isolates is not the sole cause for differences in virulence.

In order to definitively ascertain the role of the ViuB hydrolase and the catechol system in iron acquisition and virulence, a *viuB* deletion mutant was constructed and examined for altered phenotypes under various *in vitro* and *in vivo* conditions. The results demonstrated that *viuB* is required for growth under *in vitro* iron-limiting conditions in *V. vulnificus*. However, regardless of host iron status or the presence or absence of the *viuB* gene, no significant differences were seen between the mutant and wild-type in virulence assessment. A competitive virulence assay yielded similar results, as nearly equal amounts of each strain was recovered from the liver and skin lesions following infections. Hypothesis 3 regarding that *viuB* was important to virulence was not supported by these results.

These data seemed to conflict with previous research demonstrating that the *venB* gene did cause a significant decrease of virulence in *V. vulnificus* (Litwin *et al.*, 1996), as it might be expected for both genes to contribute similarly regarding virulence. However, VenB is responsible for siderophore biosynthesis, and deleting this gene also prevents the function of downstream components of the pathway. Conversely, the *viuB* deletion mutants presumably synthesize the siderophore, release it, and bring in the iron-siderophore complex; but then are either not able to release the iron from the siderophore at all, or release it with much lower efficiency. In the iron-limiting growth study, the *viuB* mutant showed greatly reduced growth yield but was still able to survive and grow somewhat. In the mice the mutant was just as virulent as wild type, and demonstrated no competitive disadvantage. These results suggest that alternate

pathways may be deployed for iron acquisition *in vitro* vs. virulence in an animal host. The transcript levels of two of the hydroxamate-related genes, ATP and *iutA*, were significantly increased in the *viuB* mutant compared to wild-type strain under iron-limiting conditions. This increase, although small, was significant and could offer an explanation for the maintenance of the virulence phenotype in the *viuB* mutant. The hydroxamate siderophore system uses chemical reduction rather than a hydrolase to remove the iron from the siderophore, and thus this system cannot compensate through its own hydrolase. Rather, an alternative pathway most likely exists to explain this observation and is for future studies. Finally, different animal models were used in the Litwin *et al.* study (2000) and may also explain contradictory finding for the role of the catechol iron uptake system in virulence of *V. vulnificus*.

This research demonstrated that although *viuB* is required for growth under iron-limiting conditions, its role in virulence of *V. vulnificus* is not supported. It is likely that the multiple pathways available for high-affinity iron acquisition, contribute differentially to survival in the host and other habitats. This research confirmed the complexity of the multiple iron acquisition systems in this species, indicating co-ordinate regulation by GacA and feedback between the systems as indicated by the effects of loss of *viuB* expression on hydroxamate system. Further research is needed to define the distinct roles of different iron acquisition systems and the regulatory pathways that control their function. This research confirms the multi-factorial nature of *V. vulnificus* virulence and supports the need for additional investigations in order to discover meaningful markers for virulence in this species.

APPENDIX
ADDITIONAL MATERIALS

Wild Type vs *ΔgacA* mutant			
Genes	ΔΔCt	Fold Increase	P-Value
vuuA	0.20	1.145	0.920
venB	0.80	1.739	0.607
viuB	1.20	2.299	0.542

A.

Wild Type vs *ΔgacA* mutant			
Genes	ΔΔCt	Fold Decrease	P-Value
vuuA	-5.24	37.71	0.00002
venB	-3.63	12.40	0.00084
viuB	-3.91	15.06	0.00113

Wild Type vs Complement			
Genes	ΔΔCt	Fold Decrease	P-Value
vuuA	-0.35	1.275	0.864
venB	-0.70	1.623	0.669
viuB	-0.84	1.786	0.670

B.

Wild Type vs Complement			
Genes	ΔΔCt	Fold Decrease	P-Value
vuuA	-1.55	2.932	0.004
venB	-1.80	3.481	0.002
viuB	-1.51	2.854	0.049

Wild Type vs Plasmid Control			
Genes	ΔΔCt	Fold Decrease	P-Value
vuuA	-1.02	2.031	0.495
venB	-0.56	1.477	0.647
viuB	-1.43	2.688	0.299

C.

Wild Type vs Plasmid Control			
Genes	ΔΔCt	Fold Decrease	P-Value
vuuA	-3.26	9.57	1.08E-04
venB	-5.08	33.91	2.11E-06
viuB	-6.53	92.09	5.39E-05

IRON-REPLETE IRON-LIMITING

Figure A-1. Role of GacA in the fold changes of gene transcripts in the catechol siderophore system under iron-replete and iron-limiting conditions comparing a *gacA* deletion mutant (A), the complemented mutant (B), and the plasmid control (C) to wild-type strain CMCP6. Iron-replete conditions were Luria Burtani Broth with NaCl (LBN) and iron-limiting were LBN + 150 µM dipyridyl. Fold differences in gene expression are shown and p-values were calculated as described in Materials and Methods.

Wild Type vs *ΔgacA* Mutant			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	1.65	3.14	0.001
PP	2.43	5.37	0.00003
iutA	1.00	2.00	0.04

A.

Wild Type vs *ΔgacA* mutant			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	0.10	1.07	0.83
PP	0.67	1.59	0.12
iutA	0.42	1.34	0.20

Wild Type vs Complement			
Genes	ΔΔCt	Fold Decrease	P-Value
ATP	-2.57	5.96	3.13E-05
PP	-2.87	7.31	3.91E-06
iutA	-2.50	5.66	1.58E-06

B.

Wild Type vs Complement			
Genes	ΔΔCt	Fold Decrease	P-Value
ATP	-1.59	3.00	0.03
PP	-0.45	1.36	0.40
iutA	-1.44	2.71	0.001

Wild Type vs Plasmid Control			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	0.07	1.05	0.90
PP	0.04	1.03	0.93
iutA	-0.59	0.66	0.03

C.

Wild Type vs Plasmid Control			
Genes	ΔΔCt	Fold Decrease	P-Value
ATP	-2.17	4.49	0.001
PP	-0.10	1.07	0.85
iutA	-1.09	2.12	0.01

IRON-REPLETE IRON-LIMITING

Figure A-2. Role of GacA in the fold changes of gene transcripts in the hydroxamate siderophore system under iron-replete and iron-limiting conditions comparing a *gacA* deletion mutant (A), the complemented mutant (B), and the plasmid control (C) to wild-type strain CMCP6. Iron-replete conditions were LBN and iron-limiting were LBN + 150 µM dipyridyl. Fold differences in gene expression are shown and p-values were calculated as described in Materials and Methods.

Wild Type vs *ΔviuB* mutant			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	-0.07	0.95	0.86
PP	0.99	1.99	0.02
iutA	-0.22	0.86	0.62

A.

Wild Type vs *ΔviuB* mutant			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	2.47	5.56	0.01
PP	1.01	2.01	0.14
iutA	2.77	6.84	0.0002

Wild type vs complement			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	0.96	1.94	0.07
PP	0.61	1.53	0.17
iutA	-1.05	0.48	0.07

B.

Wild Type vs Complement			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	0.38	1.30	0.448
PP	0.68	1.60	0.23
iutA	-0.69	0.62	0.1599

Wild type vs plasmid control			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	0.11	1.08	0.74
PP	0.78	1.71	0.21
iutA	-0.61	0.65	0.27

C.

Wild type vs Plasmid control			
Genes	ΔΔCt	Fold Increase	P-Value
ATP	2.32	4.99	0.001
PP	1.53	2.89	0.06
iutA	2.02	4.07	0.0005

IRON-REPLETE IRON-LIMITING

Figure A-3. Role of ViuB in the fold changes of gene transcripts in the hydroxamate siderophore system under iron-replete and iron-limiting conditions comparing a *viuB* deletion mutant (A), the complemented mutant (B), and the plasmid control (C) to wild-type strain CMCP6. Iron-replete conditions were LBN and iron-limiting were LBN + 130 µM dipyridyl. Fold differences in gene expression are shown and p-values were calculated as described in Materials and Methods.

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BIOGRAPHICAL SKETCH

Rick Swain was born on September 9th, 1986, in Cape May, New Jersey. After graduating 3rd in his high school class, he decided to attend Coastal Carolina University in Conway, South Carolina where he majored in Biology. After one year, he transferred to the Biotechnology Department of Rutgers University in New Brunswick, New Jersey to pursue a new field of study. During the remaining three years of his undergraduate experience, Rick excelled in his academics and undertook a minor in Biochemistry to supplement his education. In addition to academics, Rick also began research at the Waksman Institute located in Piscataway, New Jersey where he studied an unknown protein found in *Arabidopsis thaliana* in the lab of Dr. Todd Michael. After graduating magna cum laude with a B.S., he was offered a graduate position under the guidance of Dr. Anita Wright in the Department of Food Science and Human Nutrition at the University of Florida. In 2011, he graduated with his masters and plans on pursuing a career in the biotechnology and biomedical industry before potentially going back to earn a Ph.D.