The iron limitation response of *Vibrio vulnificus*: genetic variation, GacA regulation, and virulence

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Introduction. *Vibrio vulnificus* is an opportunistic human pathogen that is the most frequent cause of fatal seafood related illnesses in the U.S. (CDC, 2009). This ferrophilic bacterium typically infects individuals with underlying conditions related to host iron status, such as hemochromatosis or hepatic disease (reviewed in Jones and Oliver, 2009). The GacS/GacA two-component signal transduction system was previously associated with iron acquisition in *V. fischerii* (Whistler and Ruby, 2003) and with regulation of virulence and biofilm in *V. cholerae* (Jang *et al.*, 2010). The present study investigated the relationship of the GacS/GacA system to iron acquisition and the contribution of the iron acquisition genes on virulence of *V. vulnificus*. The catechol siderophore system in *V. vulnificus* includes *venB*, *vuuA*, and *viuB* genes, which encode gene products for siderophore synthesis (Litwin *et al.*, 1996), siderophore surface receptor (Webster and Litwin, 2000), and a cytoplasmic hydrolase respectively (Butterton and Calderwood, 1994). The *viuB* was previously reported as a good marker for virulence (Bogard and Oliver, 2007) (Panicker *et al.*, 2004) because its presence correlated with most clinical strains but is less evident in environmental isolates. However, further examination revealed the gene was ubiquitous for the species but showed allelic variation.

Materials and Methods. For the phylogenetic study, DNA sequences (ICBR, University of Florida) of clinical and environmental isolates were analyzed using BLAST and ExPasy. Phylograms were constructed using MEGA version 4 (Tamura *et al.*, 2007) with *V. cholerae* sequence to anchor analyses.

Physiological responses to iron limitation was tested in Luria-Bertani broth with 1% NaCl (LBN) prepared with or without the iron chelator dipyridyl (150 μM). The OD_{600} and plate counts were used to calculate the percent growth yield (OD from growth in LBN-dipyridyl/LBN). The complemented mutant [Δ*gacA* (pGacA)] and vector control [Δ*gacA* (pGTR1160)] were also examined. Gene expression was examined in the *gacA* mutant vs. wild-type CMCP6 in LBN with or without dipyridyl from cDNA that was synthesized (Invitrogen) from extracted RNA (Qiagen) and DNase treated (Ambion). Quantitative RT-PCR (Cepheid) was performed using 16S RNA as the internal control, and fold differences between mutant and wiltype were calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

Subcutaneous inoculation was used in a mouse model to examine localized skin infection as measured by CFU/g of skin tissue following infection and systemic disease as determined by CFU/g of liver. Decreased temperature indicates systemic infection, and a rectal temperature below 33ºC is a surrogate for death (DePaola et al, 2003).

Results and Discussion.

Allelic variation of iron uptake genes. GenDNA sequences of *viuB* from strains from both clinical (n=21) and environmental (n=20) sorted into two distinct phylogroups. Group 1 strains were primarily of clinical origin (94%) while Group 2 consisted predominately of environmental isolates (78%). Interestingly, this dichotomy was not observed for either *venB* or *vuuA* gene sequences. The deduced amino acid sequence and predicted structure of group 1 and 2 revealed differences in presence of some acidic amino acid that may contribute to observed structural (Figure 1) and functional differences in the two phylogroup, as the *viuB* Group 1 was also shown to be associated with increased virulence in a mouse model (p=0.01).

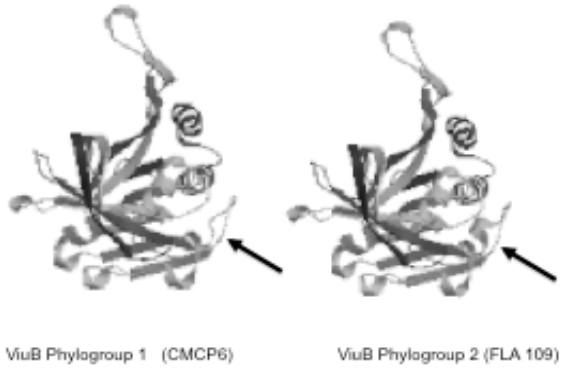

Figure 1: Predicted structure of *V. vulnificus* ViuB based on deduced amino acid sequences (arrow indicates observed differences)

Regulation of the iron limitation response of V. vulnificus. The two component GacS/GacA signal transduction system mediates iron acquisition in other *Vibrio* species. Therefore, growth and expression of iron uptake genes were examined in a *gacA* mutant compared to wild-type CMCP6 under iron-limiting conditions. Larger growth yields were found for wild type (84.1%) and complemented mutant (70.7%) as compared to the *ΔgacA* mutant (8.7%) and vector control (2.9%). The mutant also showed greatly reduced expression for iron uptake genes compared to wild-type strains($p<0.001$), and fold decreases for *viuB, venB,* and *vuuA* were 15.06, 12.40, and 37.72, respectively.

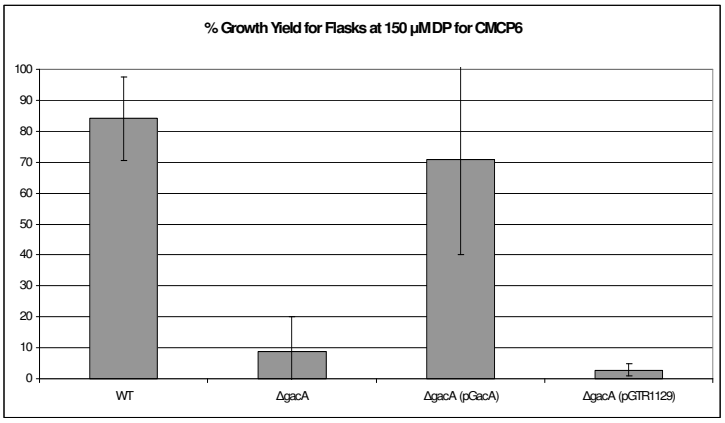

Figure 2: Percent growth yield for *V. vulnificus* strain CMCP6 under iron deplete conditions.

GacA-mediated virulence. The role of GacA in virulence was also related to the iron status of the host. The deletion of *gacA* significantly impaired the capacity of *V. vulnificus* CMCP6 to cause both localized and systemic infections in mice, and this defect was related to iron pretreatment of the host. Lesions that were apparent following wild-type infections were not evident or were greatly reduced in non-iron-treated mice that were infected with the *V. vulnificus gacA* mutant (not shown). The CFU/g recovered from

Table 1: Virulence data of *V. vulnificus* strain CMCP6 using an infant mouse model.

Strain	Mouse Virulence*					
	Not Iron-Treated			Iron -Treated		
	Skin infection (log CFU/g)	Liver Infection (log CFU/g)	Temperature (°C)	Skin infection (log CFU/g)	Liver Infection (log CFU/g)	Temperature (°C)
CMCP6	7.4±0.3	4.7±1.1	35.0±3.1	7.9±0.2	5.1±0.8	32.3±1.5
CMCP6 Δ*gacA*	3.9±1.3	2.8±0.9	38.1±0.7	8.2±0.3	4.4±1.5	33.7±3.5
CMCP6 Δ*gacA* (pGacA)	6.9±0.8	2.9±1.3	38.4±1.5	ND†	ND	ND

both skin (P=0.0003) and liver (P=0.017) tissues was also significantly lower in the mutant compared to the wild-type (Table 1). Higher body temperatures were observed with mutant compared to wild-type infections, but differences were not significant. Conversely, the mutant was not affected for any measure of virulence in mice that were pre-treated with iron dextran. Complementation of the Δ*gacA::aph* mutation with pGacA mostly restored the level of skin infection, but did not significantly change the levels of liver infection or temperature caused by the mutant strain.

These data support the hypothesis that the ability to acquire iron in the host is an important determinant of virulence for *V. vulnificus.* Ongoing experiments will examine allelic exchange in order to replicate altered virulence using *viuB* Phylogroup 1 vs. 2 genes strain background and vice versa. We also present evidence that GacA regulates iron acquisition in *V. vulnificus*, and that its contribution to virulence is dependent on the iron status of the host.

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