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Existing regulatory circuitries govern backbone and acquired host association factors in the human pathogen Vibrio parahaemolyticus

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EXISTING REGULATORY CIRCUITRIES GOVERN BACKBONE AND ACQUIRED HOST ASSOCIATION FACTORS IN THE HUMAN PATHOGEN *VIBRIO PARAHAEMOLYTICUS.*

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ABSTRACT

Existing regulatory circuitries govern backbone and acquired host association factors in the human pathogen *Vibrio parahaemolyticus*.

By

Jennifer C. Mahoney
University of New Hampshire, May 2011

Vibrio parahaemolyticus is a poorly characterized human gastrointestinal pathogen whose virulence mechanisms are not well understood. Though closely related to *Vibrio cholerae, V. parahaemolyticus* infections are inflammatory and utilize virulence traits that are unique from the Cholera toxins yet remain poorly characterized. *Vibrio spp.* in general share an extensive core genome dedicated to environmental survival and unique, often horizontally acquired, gene content that is reserved for species specific lifestyles. This diversity has resulted in a genus of highly specialized bacteria partaking in dramatically different lifestyles ranging from symbiosis to pathogenesis. We propose that a comparative genomic and transcriptomic analysis of a conserved host association regulon (GacA) in the closely related *V. fischeri* (symbiont) and *V. parahaemolyticus* (pathogen), to exploit key lifestyle differences will expose genes that are unique to pathogenesis. In this study we identified a small, core regulon that is GacA activated comprised mostly of genes involved in central metabolism. Unique gene content in the *V. parahaemolyticus* GacA regulon contained both backbone and horizontally acquired

elements that were activated or repressed in a highly selective fashion. Temperature was an important cue in virulence gene activation as determined through phenotypic and transcriptional assays and acted with GacA in an additive fashion. Specifically, expression of the classic virulence marker *tdh* was enhanced in a GacA mutant at 37°C; however the mutant was defective at initiating an infection in mice potentially discounting the relevance of TDH in disease. Though TDH is currently accepted as a clinical identifier, further work may be necessary to confirm a genuine correlation with disease causing strains. In addition, comparative genomics revealed a significant reorganization of the GacA regulon in *V. parahaemolyticus* suggesting that its control has been uncoupled from quorum sensing circuitry unlike in other characterized regulons. The GacA regulon of *V. parahaemolyticus* has undergone subtle regulatory changes resulting in GacA repression of select horizontally acquired elements and may serve as an ideal model of the evolution of regulatory networks and the emergence of novel virulence mechanisms.

CHAPTER I

INTRODUCTION

The family *Vibrio*naceae is comprised of a diverse group of aquatic organisms participating in a variety of lifestyles. Dual chromosomes and evidence of extensive horizontal gene transfer (HGT) in *Vibrio* have facilitated the expansion of this group to include related species that occupy unique niches (Okada et al. 2005, Kirkup et al. 2010). A core "*Vibrio*" genome allows members of this genus to survive in a general aquatic ecosystem including estuarine environments of moderate salinity. Occupation by specific species of more defined niches such as the light organ of a squid, deep-sea hydrothermal vents, or the gut of a human is thought to be attributed to the horizontal acquisition of symbiosis genes or virulence genes respectively (Ruby et al. 2005, Makino et al. 2003).

Genomic Plasticity within *Vibrionaceae*

Although *Vibrios* share a common evolutionary past, genomic flexibility has permitted the acquisition of a number of HGT elements that have shaped distinct species (Kirkup et al. 2010). Viruses that target bacterial cells known as bacteriophage can transport virulence genes from one bacterial host to another, thereby conferring enhanced pathogenicity. In *Vibrio cholerae* for example, the Cholera Toxin genes (ctxAB) required for virulence, were acquired horizontally in a filamentous phage (Davis et al. 20

03). Integrons, super-integrons, integrative conjugative elements, transposons, and plasmid bound genes can all be introduced by bacterial mating or conjugative transfer. Evidence of these mobile elements can be seen throughout the *Vibrio* genomes including super-integrons found in both *V. cholerae* and *V. parahaemolyticus*, and may contribute to multi-drug resistance (Rowe-Magnus et al. 2001). Natural competence is rare among bacteria; however, *Vibrio spp.* are able to take up naked DNA from the environment when in the presence of chitin. This unique ability provides additional opportunities for enhanced host association through the acquisition of new DNA (Meibom et al. 2005, Yamamoto et al. 2010).

Management of horizontally acquired elements

Once acquired, new DNA must be integrated into existing regulatory networks to ensure proper expression in coordination with the rest of the genome. Although a variety of unique regulatory mechanisms exist that direct the expression of ancient genes, the mechanisms that regulate newly acquired DNA are less clear (Lercher et al. 2008). Evidence exists to implicate the temperature-sensitive histone-like nucleoid-structuring protein (H-NS) as a predominant regulatory mechanism for both existing and horizontal genes (Dorman 2004, Banos et al. 2009, White-Ziegler et al. 2009). H-NS is a globally acting repressor that appears to target and "police" the expression of new DNA (Atlung et al. 1997). Upon exposure to a signal, such as increased temperature, H-NS silencing is relieved and the transcription of virulence genes is activated (White-Ziegler et al. 1998, Stoebel et al. 2008).

In addition to H-NS silencing, host factor 1 (Hfq), a sRNA chaperone protein, helps to facilitate communication between the core and variable genomes of several

gastrointestinal pathogens including *Salmonella* Typhimurium (Chao et al. 2010). For example, the horizontally acquired Salmonella Pathogenicity Island-1 encodes an Hfq target sRNA (InvR) that acts with Hfq to transcriptionally repress the OmpD porin gene found in the core genome (Pfeiffer et al. 2007, Chao et al. 2010). Additionally, Hfq-dependent sRNAs within the core may interact with transcripts of newly acquired genes to promote their integration into existing regulatory networks (Chao et al. 2010). In this way, Hfq may help to facilitate harmonious communication and coordinated gene expression of core and acquired genes.

Distinct Lifestyles of *V. fischeri* and *V. parahaemolyticus*

The continuous influx of new genetic elements and ongoing genomic reorganization in *Vibrio spp.* has resulted in a genus marked by unique species with distinctive lifestyles. For example, some *Vibrio spp.* such as *Vibrio fischeri* associate with their hosts in a symbiotic manner (Guerrero-Ferreira et al. 2010). Specifically, *Vibrio fischeri,* a bioluminescent marine bacterium, forms a stable, beneficial association with the Hawaiian Bobtail squid, *Euprymna scolopes* (Lee et al. 1994). Initiation of this symbiosis requires *V. fischeri* to successfully evade a gauntlet of natural host defenses much like a pathogen (Davidson et al. 2004). Once a small population of cells is able to migrate through the pores and into the highly specialized light organ, morphological changes are induced in the host and all other bacteria are excluded (Nyholm et al. 2003). Successful colonization, growth in the light organ and host signaling/association are all under the control of the GacS/A global regulator (Whistler et al. 2007).

In contrast, *Vibrio parahaemolyticus* is a known human pathogen that causes inflammatory gastrointestinal illness. It is acquired through ingestion of raw or

undercooked shellfish, typically oysters, and accounts for the majority of seafood borne illnesses in the U.S. (FDA 2001). Once ingested, *V. parahaemolyticus* must survive host immunity, fluctuations in nutrient availability and competition from other organisms. Successful colonization and pathogenicity in this organism is controlled by the GacA global regulator much like the host association capabilities of *V. fischeri* (Chapter III).

The GacS/GacA global regulator

Given their large-scale influence on both backbone and acquired genes, conserved global regulators such as the two-component GacS/A system may play a role in the regulation of horizontally acquired elements. The membrane bound sensor (GacS) and its cognate response regulator (GacA) act globally to relieve CsrA mediated repression of transcripts. When GacS/A is activated, it triggers the production of the sRNA's csrB/C which competitively bind CsrA relieving repression of transcripts and allowing translation to proceed (Figure 1.1) (Lapouge et al. 2008).

The GacS/GacA global regulator plays a known role in bacterial host association and pathogenicity of *Vibrio spp.* and other gram negative pathogens (Table 1.1. and Figure 4.1). In *Pseudomonas aeruginosa* for example, the GacA/GacS regulator is responsible for multihost virulence contributing to acute infections in both vertebrate and non-vertebrate hosts (Rahme et al. 2000) as well as chronic infections in a murine model. Interestingly, host colonization and invasion in the human gastrointestinal pathogens enteropathogenic *Escherichia coli* (Bhatt et al. 2009) and *Salmonella* Typhimurium (Mizusaki et al. 2008) are both dependent on GacS/A mediated activation of virulence genes embedded in horizontally acquired elements (BarA/UvrY in *Escherichia coli* and BarA/SirA in *Salmonella* Typhimurium). In *Vibrio cholerae* and *Vibrio vulnificus*,

mutations in GacS/GacA (VarS/VarA in *Vibrio cholerae*) results in decreased production of virulence factors such as the Cholera Toxin, proteases and cytotoxic proteins and ultimately attenuation of virulence in mice (Wong et al. 1998, Jang et al. 2010, Gauthier et al. 2010). In addition to pathogenic interactions, GacS/A is an important regulator of general host-microbe interactions including symbiotic relationships. *Vibrio fischeri* for example, requires GacA to establish a beneficial association with its squid host, *Euprymna scolopes*. Specifically, a GacA mutant in this organism is unable to initiate and maintain an infection of the light organ, possibly as a result of defective production of the specific symbiosis factor bioluminescence (*lux)* (Whistler et al. 2003). This highlights that GacS/GacA is an important regulator not only of pathogenic associations, but of host-microbe interactions in general. Given that host specificity and association traits are often the result of horizontally acquired genes, it is reasonable to consider the GacS/A system as a potential regulatory control mechanism of newly acquired host association genes.

Unique and Shared Characteristics of Distinct Host Associations within *Vibrio*

Despite the unique outcome of their respective host associations, *V. fischeri* and *V. parahaemolyticus* share several interesting similarities in gene content and host environment. For example, the light organ of the squid is lined with polarized epithelial cells whose brush borders resemble those of the human gastrointestinal tract. Once colonized with *V. fischeri*, predictable changes to the host microvillar surfaces mimic those seen in pathogenic infections of the human gut (Lamarcq et al. 1998) and may be the result of either host or bacterial induced ß-actin reorganization (Lamarcq et al. 1998, Aktories et al. 2011). Indeed both *V. fischeri* and *V. parahaemolyticus* contain a toxin

termed RTX (repeats in toxin) that is involved in the reorganization of ß-actin in human gut epithelial cells (Ruby et al. 2005, Makino et al. 2003). This well characterized toxin has known roles in virulence in *V. cholerae* and *V. vulnificus* making its presence in the non-pathogenic *V. fischeri* all the more note-worthy (Fullner et al. 2000, Liu et al. 2007). In fact, RTX is only one of several toxin genes found in *V. fischeri*. Others include hemolysin genes, CNF (cytotoxic necrotizing factor), ace (accessory cholera enterotoxin), and zot (Zonula occludens toxin) highlighting that, if functional, these "toxins" are more likely related to host-association rather than virulence per se (Ruby et al. 2005). In addition, pathogenic infections are typically marked by an immediate morphologic response to the presence of the pathogen such as membrane ruffling in *Salmonella* Typhimurium (Finlay et al. 1991) and the attachment and effacement of host brush borders in *Escherichia coli* (Finlay et al. 1992). Alterations to the squid light organ are not as immediate and tend to be observable over several days of colonization. These slower changes are akin to commensal associations and chronic infections of the human gut and their study may shed light on the contribution of single species colonization in a host (Lamarcq et al. 1998).

Despite similarities seen in *V. fischeri* and gut pathogens including *V. parahaemolyticus*, *V. fischeri* ultimately forms a beneficial relationship with its host. Historically, unique genes acquired horizontally have been theorized to confer distinctive host association properties such as symbiosis or pathogenesis. However, the presence of several toxins in *V. fischeri* and its resultant lack of virulence have forced us to consider alternative possibilities. Conserved core genes may play more of a role in host species specificity than previously thought, or perhaps gene content alone may not be enough to

explain differing lifestyles. It is possible that unique gene regulation or global regulatory patterns of core genes may account for specific, highly specialized lifestyles. Ultimately, it may not be what they have but how they use it that defines the outcome of *Vibrio* host associations.

Objectives and Goals

Chapter I: What defines virulence in *Vibrio parahaemolyticus*?

Hypothesis: In addition to unique genome content, specific regulatory responses of core and acquired genes to host cues are required for virulence.

Approach: Compared known pathogenic strains of *V. parahaemolyticus* to environmental isolates in a suite of virulence-associated phenotypes at both environmental and human host temperatures (28°C and 37°C respectively) to identify group specific trends.

Chapter 2: Do the novel virulence mechanisms of *V. parahaemolyticus* represent an evolutionary re-wiring of the conserved GacA pathway?

Hypothesis: Virulence mediated by both core and horizontally acquired genes in *V. parahaemolyticus* is dependent upon the GacS/GacA pathway.

Approach: A mutation in *gacA* was generated and examined for defects in virulence associated phenotypes consistent with *gacA* mutations in other organisms. In addition, microarray analysis was performed at both 28°C and 37°C to examine this regulon at

both environmental and human host temperatures and comparatively with previously characterized *gacA* regulons.

Chapter 3: Are horizontally acquired elements incorporated into existing regulatory networks that have known roles in host association in *Vibrio spp.*?

Hypothesis: Comparative analysis of the GacA host association regulons in a pathogen and symbiont (*V. parahaemolyticus* and *V. fischeri*) will reveal lifestyle specific gene content and regulatory patterns.

Approach: Microarray analysis of wild type and a *gacA* mutant regulons was performed in both organisms (*V. parahaemolyticus* at human body temperature 37° and *V.fischeri* at 28°C, as close to squid temperature (23°C) as can be maintained in the laboratory). A core "*Vibrio*" genome was identified as was unique gene content in each organism under GacS/GacA control. The potential for a gene(s) to have been acquired through horizontal gene transfer was assessed informatically based on %GC content, dinucleotide bias and the presence of mobility genes.

Table 1.1. Summary of GacA regulons in other bacteria

Organism	Altered Motility	Iron	Protease	Biofilm	Host Associatior	Quorum Sensing[a]	Cytotoxicity
Vibrio							
[b]*fischeri*	X	X			X	X	X*
[c]*cholerae*			X	X	X (T3SS)	X	
[d]*harveyi*					X (T3SS)	X	
[e]*vulnificus*	-	X	X		X		X
Erwinia							
[f]*carotorva*	X		X		X		
[f]*chrysanthemi*	-		X		X (T3SS)	-	
[f,g]*Salmonella*	X		X		X		
[h]*Escherichia coli*	X			X	X		
[f]*Legionella pneumophila*	X				X		X
[f,i]Pseudomonads	X	X	X		X (T3SS)	X	
[f]*Pseudomonas aeruginosa*	X	X		X	X	X	

Blank spaces denote "not evaluated"
- not influenced by GacA
X influenced by GacA
[a]A GacA mutant of Vibrio fischeri had a very modest (and not considered biologically relevant) influence on AHL signal production, though microarrays show a general integration of GacA with Quorum Sensing.
Adapted from [b]Whistler et al. 2003 and Chapter IV, [c]Wong et al. 1998, Jang et al. 2010 and Tsou 2011, [d]Henke et al. 2004 and Lenz et al. 2005, [e]Gauthier et al. 2010 [f]Lapouge et al. 2008, [g]Goodier et al. 2001 [h]Bhatt et al. 2009.
[i]Summary of the following species: *chlororaphis(aureofaciens),entomophila, fluorescens, marginalis syringae* pv.,*syringae, syringae* pv. *Tomato, tolaasii, viridiflava*
*APPENDIX II

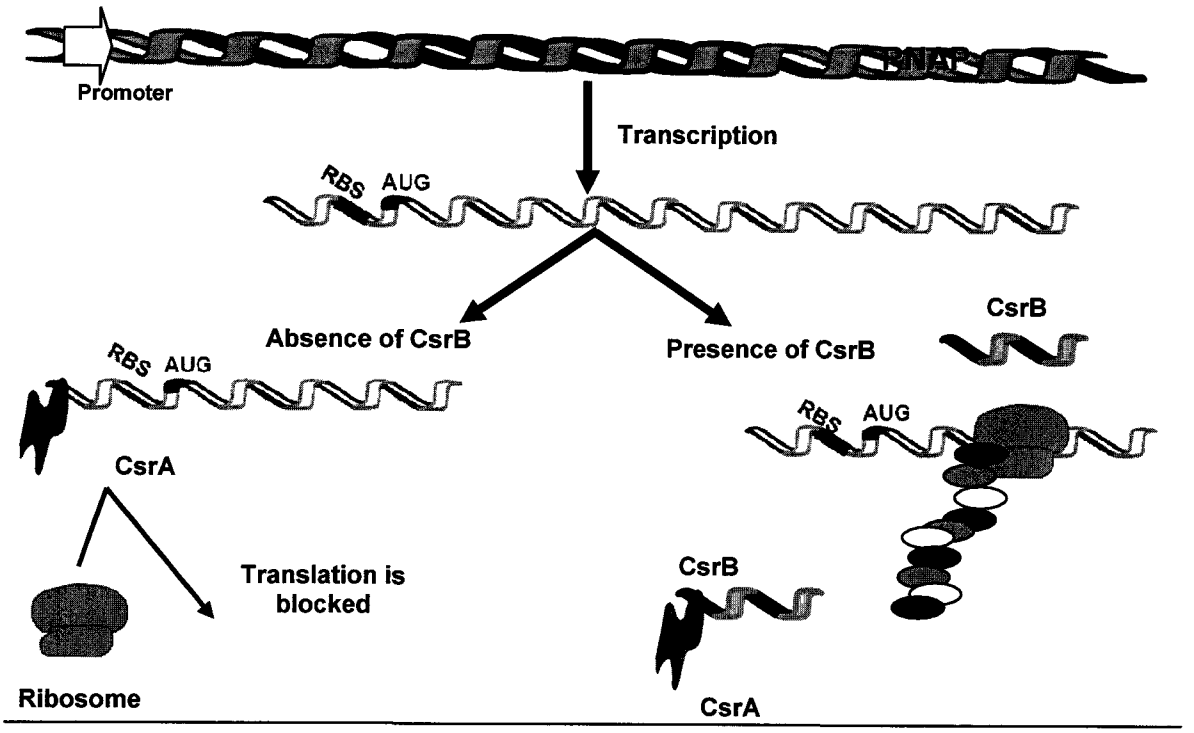

Figure 1.1. Model of GacA mediated translational regulation (adapted from Ballok 2007).

CHAPTER II

COMPARISON OF THE PATHOGENIC POTENTIALS OF ENVIRONMENTAL AND CLINICAL *VIBRIO PARAHAEMOLYTICUS* STRAINS INDICATES A ROLE FOR TEMPERATURE REGULATION IN VIRULENCE

Although the presence of pathogenic *Vibrio spp.* in estuarine environments of northern New England has been known for some time (Bartley et al. 1971, O'Neil et al. 1990), their virulence and the relative threat they may pose to human health has yet to be evaluated. In this study, the virulence potential of 33 *Vibrio parahaemolyticus* isolates collected from the Great Bay Estuary of New Hampshire was assessed in comparison to that of clinical strains. The environmental isolates lack thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH), which are encoded by *tdh* and *trh*, respectively. Though not hemolytic, they do possess putative virulence factors, such type III secretion system 1, and are highly cytotoxic to human gastrointestinal cells. The expression of known and putative virulence-associated traits, including hemolysin, protease, motility, biofilm formation, and cytotoxicity, by clinical reference isolates correlated with increased temperature from 28°C to 37°C. In contrast, the environmental isolates did not induce their putative virulence-associated traits in response to a temperature of 37°C. We further identified a significant correlation between hemolytic

activity and growth phase among clinical strains, whereby hemolysin production decreases with increasing cell density. The introduction of a *tdh*::*gfp* promoter fusion into the environmental strains revealed that they regulate this virulence-associated gene appropriately in response to temperature, indicating that their existing regulatory mechanisms are primed to manage newly acquired virulence genes.

Despite relatively cool water temperatures, estuarine environments in northern New England are a known niche for pathogenic species of the genus *Vibrio* (Bartley et al. 1971, O'Neil et al. 1990, Striplin et al. 2008). Shellfish beds within these habitats serve both as a natural reservoir where *Vibrio spp.* accumulate and as vectors for human infection. Recent outbreaks of *Vibrio parahaemolyticus* in NewYork, New Jersey, Connecticut, Washington, and Alaska highlight the importance of further research to understand the resident strains in these potentially significant habitats for this emergent pathogen (CDC 2006, McLaughlin et al. 2005, Nolan et al. 1984).

Infections by *V. parahaemolyticus* are often acquired through the ingestion of raw or undercooked shellfish, including oysters. Upon colonization of the intestine, the products of the hemolysin genes (*tdh* and *trh*, encoding thermostable direct hemolysin [TDH] and TDH-related hemolysin) are believed to rapidly induce inflammatory gastroenteritis (Honda et al. 1993, Raimondi et al. 1995, Shiraih et al. 1990). Recently, a more infectious pandemic serotype (O3:K6) emerged that is often identified by the presence of the ORF8 gene (Hurley et al. 2006). Not all strains of *V. parahaemolyticus* are human pathogens. Historically, acquisition by horizontal gene transfer (HGT) of the *tdh* gene within *Vibrio* pathogenicity island-7 (VPaI-7) (Makino et al. 2003) and/or the *trh* gene within the recently identified Vp-PAITH3996 (Okada 2009) has been credited

with the transition from non-infectious environmental to human pathogenic clinical strain. However, *V. parahaemolyticus* remains pathogenic even in the absence of these two thermostable hemolysins, indicating that other virulence traits exist (Nishibuchi et al. 1992, Park et al. 2000, Xu et al. 1994). Furthermore, recent outbreaks have been caused by strains lacking *tdh* and/or *trh* (Garcia 2009). Although other virulence factors have been investigated, including type III secretion systems 1 and 2 (T3SS1 and T3SS2) and biofilm formation, the understanding of virulence remains incomplete (Hurley et al. 2006, Tamayo et al. 2010). Moreover, the contribution to pathogenicity of conserved virulence-associated factors present in a wide range of bacterial pathogens (e.g., protease, siderophore, and motility), the ability to regulate newly acquired virulence factors, and the capacity to adjust to a human host environment have yet to be revealed.

In order to assess the virulence potential of environmental *V. parahaemolyticus* isolates lacking the characteristic *tdh/trh* hemolysin genes, we examined their ability to express alternative, conserved virulence-associated factors at environmentally relevant and human body temperatures. The "environmental" strains in this study were isolated from environmental samples and have further been defined by the absence of either *tdh* or *trh*, whereas "clinical" strains were isolated either from patients or from the environment during an outbreak and harbor *tdh* and/or *trh* genes. Among the clinical strains examined, we found a strong correlation of the production of potential virulence-associated factors with human body temperature and an inverse correlation of hemolysin with cell density. In contrast, the environmental strains do not express their conserved virulence-associated traits in response to body temperature, but many of the environmental isolates were highly cytotoxic to human gastrointestinal cells. In addition, the environmental isolates

lack the *tdh* gene but were able to express this gene's promoter appropriately in response to a temperature of 37°C. Although these environmental isolates are unlikely to cause a human infection, they do represent a population of bacteria whose existing regulatory networks can appropriately control newly acquired virulence genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Thirty-three environmental strains of *Vibrio parahaemolyticus* isolated from New Hampshire's Great Bay Estuary from May through December 2007 (Striplin et al. 2008) were used in this study, along with an additional two strains from other sources (Table 2.1). All New Hampshire isolates were negative for both *tdh* and *trh* based on multiplex PCR analysis (Panicker et al. 2004, Striplin et al. 2008). The presence of various other proposed virulence genes, including T3SS2 apparatus gene *vscC2* and effector *vopP*, a putative DNA methyltransferase, a homolog of the *Escherichia coli* cytotoxic necrotizing factor *vopC*, and a homolog of *V.cholerae* pathogenicity island gene VPA1376, was determined by PCR with published methods (4). A prepandemic *tdh*-positive strain, BB22, was used as a reference, and nine clinical strains from outbreaks worldwide were used for comparison (Table 2.1).

Bacteria were grown in heart infusion (HI) medium at a pH of 7.3 (Fluka, Buchs, Switzerland) at 28°C, a temperature often used to cultivate many environmental *Vibrio spp.*, and 37°C, human body temperature, for all phenotypic assays. Uniform growth patterns were observed for environmental and clinical strains grown in rich medium, as determined from growth curves in HI medium at 28°C and 37°C using a Tecan infinite M200 plate reader (Männedorf, Switzerland) for 24 h. For experiments

measuring *tdh* promoter activity, plasmid pVCW10C1 was maintained by the addition of chloramphenicol (CHL) at a final concentration of 25 μg/ml. Triparental matings were performed on super optimal broth (SOB) plates containing 2% Bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgS04, and 1.5% agar at 37°C for 12 h, and outgrowth was performed on HI-CHL at 28°C. For quantitative hemolysin assays conducted in conditioned broth, strain BB22 was grown to an optical density at 600 nm (OD_{600}) of 1.0, the cells pelleted by centrifugation and discarded, and the cleared broth filter sterilized. Strains were inoculated into a 1:1 mix of rich HI medium and BB22 cell-free supernatant.

Characterization of virulence-associated phenotypes. All phenotypic characterization assays were conducted a minimum of three times. Plate assays were conducted for motility, protease, and siderophore in cultures grown in HI with shaking and then standardized to an OD_{600} of 0.5 prior to inoculation. Throughout our phenotypic characterization experiments, the OD_{600} was often determined from 150μl of culture in a 96-well plate, and the value converted to a standard OD_{600} per ml based on an empirically determined conversion factor so that our data would be comparable to those of other studies. Motility was measured using 25-ml soft agar plates containing 10 g tryptone, 20 g NaCl, and 3.35 g Bacto agar per liter as previously described (Jaques 2006). The plates were inoculated with 2 μl of each isolate in triplicate. After 24 h, the diameter of the zone of bacterial migration was measured in millimeters. Protease production was assessed with HI plates to which 4 g/liter sterilized skim milk was added following autoclaving and cooling to 50°C. The plates were inoculated in triplicate with 5μl of each isolate. A positive result was indicated by a clearing around the inoculum after 24 h. Siderophore

production was determined from cultures spotted onto artificial-seawater-based chrome azurol S (CAS) agar buffered with 100 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], pH 6.8 (Payne 1994). A positive result was indicated by an orange halo around siderophore-producing cells. Hemolytic activity was determined qualitatively using Wagatsuma agar (Miyamoto et al. 1969). Cultures were grown either to mid-log or overnight, and 5µl amounts were spotted on the plates in triplicate. Plates were examined at 24 h, and a betahemolytic clearing around the colony indicated a positive result.

Biofilm assays were performed according to a standard protocol (O'Toole et al. 1999), with slight modifications. Strains were inoculated into 200 µl of HI medium per well of a polystyrene microplate (Costar, St. Louis, MO) and grown for 4h at either 28°C or 37°C with shaking at 200 rpm. Four hours was empirically determined to be the point at which biofilm could be reliably measured without biofilm "clumps" releasing from the glass tube and introducing significant variation. The OD_{595} was determined for baseline optical density. The cultures were then expelled from the plate, which was washed twice with 200 µl of distilled water per well. The plates were allowed to dry, and then the cells were fixed at 80°C for 30 min. Following staining with 0.1% crystal violet for 20 min, the wells were destained with 200 µl 95% ethanol. The OD_{595} was measured, and the biofilm quantified as absorbance normalized to the cell density of the initial culture (OD_{600}). When cultures were grown for more than 4 h, the biofilm production was so great that clumped cells were sometimes expelled with the medium, making biofilm measurements highly variable.

A quantitative hemolysin assay was adapted from a previously published assay (Fan et al. 2001). In brief, 150 µl amounts of cultures taken throughout the growth cycle

were combined with 800 µl of a blood mix of 40 ml of 1X phosphate-buffered saline (PBS) plus 500 µl defibrinated sheep blood (Northeast Laboratories Services, Winslow, ME), and cells were pelleted by centrifugation at 9,000 rpm (6,605X*g*) for 4 min. The tubes were incubated for 18 h, and the cell wall debris removed by centrifugation at 13,000 rpm. The amount of heme present in cleared cell lysate as determined by the OD_{415} was used as a measure of red blood cell lysis and normalized to the bacterial cell density (OD_{600}).

In order to determine the potential role of late-growth-phase extracellular or cell-associated proteases in the degradation of hemolysins, we performed a time course hemolysin assay with exposure to either late-phase cell-free supernatant or sonicated whole cells. Strain MDOH-03-17282-Vp was grown to an OD_{600} value of 1.0, when hemolysin is optimally produced, and an OD_{600} value of 2.5, when hemolysin activity was substantially decreased. The cells were pelleted, and the supernatants were purified with a 0.2µm polyethersulfone filter (VWR, West Chester, Pennsylvania). The cell pellet was then sonicated in 1 ml of sterile 1X PBS to release cell-associated proteases. The supernatant or sonicated cells were then combined with 1 ml of extracted hemolysin, and hemolytic activity against blood mix was assayed as described above every 30 min for 3 h. as a control, cell-free hemolysin was also incubated in HI.

Cytotoxicity assays were developed using a human gastrointestinal epithelial cell line (CaCo-2) grown in Eagle's minimal essential medium (Mediatech, Inc., Herndon, VA) modified with HEPES (J.T.Baker, Mumbai, India) and supplemented with 0.7% Leibovitz's L-15 (Mediatech, Inc., Herndon, VA), 0.03% L-glutamine (Lonza, Basel, Switzerland), 0.08% sodium bicarbonate (J.T.Baker,Phillipsburg, NJ), 1% nonessential

amino acids (Thermo Scientific HyClone,Logan, UT), 10% fetal bovine serum (Lonza, Basel, Switzerland), and 1% penicillin-streptomycin-amphotericin B (100X) (Lonza, Basel, Switzerland) at a pH of 7.0 with no CO_2. Preliminary experiments were conducted to determine the optimal multiplicity of infection (MOI), using strain BB22 as a positive control, because it was previously determined to be highly cytotoxic (L. L. McCarter, personal communication), and HI broth as a negative control. Exposure to each *V. parahaemolyticus* strain, grown overnight with shaking at an MOI of 100, was carried out in triplicate in a 384-well plate. Following incubation for at least 5 hours at 37°C with no CO_2, the relative cytotoxicity was measured by lactate dehydrogenase release, quantified by using a Cytotox-Fluor cytotoxicity assay, following the manufacturer's protocols (Promega, Madison, WI), and read on a Tecan Infinite M200 plate reader. The experiment was conducted four times. Each experiment had low within-experiment variability, and the data from one representative experiment are presented.

Analysis of significance of differences in cytotoxicity and correlations of phenotypes with temperature using replicate experiments were conducted by oneway analysis of variance (ANOVA) using the statistical software SPSS (SPSS, Inc., Chicago, IL).

Expression of *tdh*. To measure *tdh* gene expression, a *tdh*::*gfp* promoter fusion was generated by splicing-by-overlap extension (SOE)-PCR following published protocols (Horton 1990), using the Expand long template PCR system (Roche Applied Science, Indianapolis, IN). The outer forward primer (tdhF1, TATGTCGACCAGATT TCTCGCTTGTGC) was designed to the *tdh* gene (VPA1314) from the RIMD2210633 published sequence. The outer reverse primer (gfpR1, TATCTGCAGGTTGTACAGT

TCATCCATGC) was designed to the *gfp* gene from pQBI63 (Quantum Biotechnology, Montreal, Canada). Internal forward and reverse SOE primers were designed to fuse the *gfp* gene to the *tdh* promoter at its ribosomal binding site, ensuring correct spacing of the gene within the transcript (tdhSOEF6, AAGTTATTAATCAATTCAGAGGAG GAGAATACTAATGGC, and tdhSOER6, GCCATTAGTATTCTCCTCCTCTGAATTG ATTAATAACTT). The first PCR amplicons were generated with primers tdhF1 and tdhSOER6 using strain BB22 as a template and with tdhSOEF6 and gfpR1 using pVSV102 as a template for *gfp* (Dunn 2006), and the second round of SOE was performed with the outer primers (tdhF1 and fgpR1) only. The amplicon was directionally cloned into the low-copy-number plasmid pVSV105 (Dunn 2006) so that expression was driven by the native *tdh* promoter only. The newly generated construct (pVCW10C1) was then introduced into strains BB22, DAL1094, MDOH-03-17282-Vp, MDOH-04-5M732, KE9967, G1, G91, and G145 by conjugation.

The expression of the *tdh* gene was determined throughout the growth cycle of each strain inoculated in quadruplicate into 150µl HI-CHL in the wells of a clear-bottom black polystyrene microplate (Costar, St. Louis, MO) and grown at either 28°C or 37°C for 24 h. At each time point, the OD_{600} was used to determine cell growth, and *tdh* expression was determined by emission at a wavelength of 515 nm following excitation at a wavelength of 480 nm using a Tecan Infinite M200 plate reader with a manual gain of 175. The background fluorescence produced by reference strain BB22 was subtracted from each value, and the fluorescence normalized to the OD_{600}. Upon determination of the induction profile of *gfp* in each strain, a single post-induction time point (OD_{600} =1) was chosen for direct comparison between strains at each temperature.

RESULTS AND DISCUSSION

Although the environmental strains examined in this study were confirmed to lack the *tdh/trh* virulence-associated genes, we further genotypically characterized these strains for five genes putatively involved in virulence, as previously described, most notably in other environmental isolates without the classical virulence markers *tdh* and *trh* (Caburlotto 2009). Although none harbored the putative T3SS2 effector protein *vopP*, some strains were positive for a gene involved in its secretory apparatus, as well as a homolog of a gene found within a *Vibrio cholerae* pathogenicity island (Table 2.1). Despite the presence of one or more of these putative virulence genes in a small subset of environmental strains, which could prove to be relevant to their virulence potential, this cohort did not display enhanced or altered virulence patterns in the phenotypic characterization assays. This may be the result of genes that are present in a genome but not functionally expressed or, possibly, a lack of connection between these putative virulence genes and the phenotypes for which we screened.

The environmental strains did show evidence of more conserved virulence-associated mechanisms, such as motility and biofilm, siderophore, and protease production (Luan et al. 2007, Tamayo et al. 2010, Tsou et al. 2008, Wang et al. 2010). Specifically, the environmental and clinical strains were motile and produced extracellular proteases, biofilm, and siderophore at both 28°C and 37°C (Table 2.S1). Unlike the environmental isolates, the clinical group showed a notable difference in response to the human host temperature of 37°C (Table 2.2). In particular, we observed a significant increase in motility and in biofilm and protease production (1.2, 9 and 4 fold change respectively) at 37°C compared to the results at 28°C in the clinical strains,

whereas the environmental isolates showed no significant difference in motility or biofilm production and showed a significant decrease in protease production at 37°C. Further underscoring the difference between the environmental and clinical strains, a direct comparison of each population at 37°C indicates that the environmental group produces significantly less protease ($P > 0.001$) and less biofilm ($P = 0.02$) than the clinical group, although due to high variability among strains, they were not significantly different in motility. Siderophore was detected in all strains at both 28°C and 37°C, with the exception of the reference strain BB22 (Table 2.S1). Although siderophore production in other *Vibrio spp.*, specifically *Vibrio* (*Aliivibrio*) *salmonicida*, has been observed only in response to disease-permissive temperatures (Colquhoun et al. 2001), the production of siderophore by *V.parahaemolyticus* at both temperatures indicates that it may play a role both in infection and in environmental fitness or may simply be required for general survival.

Bacterial pathogens that oscillate between the natural habitat and a mammalian host commonly utilize environmental cues to coordinate virulence-associated gene expression (Roux et al. 2009). Though a variety of signals exist within the human host, temperature has been implicated as an important activator of virulence gene expression in several gastrointestinal pathogens, including *Shigella spp.*, *Yersinia spp.*, and pathogenic *Escherichia coli* (Herbst et al. 2009, Maurelli et al. 1984, Olsen et al. 1998). Pathogenic *Shigella spp.*, for example, are unable to successfully penetrate human epithelial cells and have attenuated virulence at 30°C that is fully restored upon a temperature increase to 37°C (Maurelli et al. 1984). Furthermore, *Vibrio parahaemolyticus* strains that are positive for the virulence-associated gene urease exhibit an increase in urease expression

at 37°C compared to its expression at 30°C (Park et al. 2009). The specific response of virulence phenotypes, including hemolysin (Table 2.S1), to host temperature seen in this study may be reflective of the fundamental regulatory differences between environmental and clinical strains. This unique regulatory switch could provide a selective advantage in the human host environment, promoting infection and disease. Despite the presence of some functional virulence-associated factors in the environmental population, the strains are apparently unable to coordinate the production of these factors in response to this host cue. The data presented herein supports previous work implicating temperature dependent regulation in the appropriate expression of putative virulence genes in other bacterial pathogens and further clarify the differences between environmental and clinical strains of *V.parahaemolyticus* (Herbst et al. 2009, Maurelli et al. 1984, Olsen et al. 1998, Park et al. 2009).

Cytotoxicity toward human gastrointestinal cells is widely used to assess bacterial virulence and was used here to assess the virulence of both clinical and environmental strains (Hiyoshi et al. 2010). Clinical isolates of *V. parahaemolyticus* contain a conserved T3SS1 (Makino et al. 2003) and horizontally acquired genes, including *tdh* and T3SS2 (Kodama et al. 2007, Park et al. 2004), that are known to contribute to the lysis of epithelial cells through disruption of cytoskeletal structures and a loss of membrane integrity, possibly leading to secretory diarrhea (Caburlotto et al. 2010, Nishibuchi et al. 1992). The clinical strains all displayed significant cell lysis ability at both 28°C and 37°C, which perhaps implies that this mechanism is important both in the natural environment and during human infection (Figure 2.1(A)). In comparison, the majority of environmental strains were as cytotoxic as a reference strain at 37°C but had overall

increased cytotoxicity at 28°C (Figure 2.1(B)). The upregulation of a virulence mechanism at a more environmentally relevant temperature further conflicts with the behavior of the virulent clinical strains. Strains G91 and G149 represent a small subgroup of environmental isolates that showed a different trend, with low cytotoxicity at both temperatures (Figure 2.1 (B)). The elevated cytotoxicity seen in the environmental group in response to a cooler temperature (28°C) may imply the utility of cytotoxicity in the natural environment, perhaps during interactions with a eukaryotic marine host. However, the high level of cytotoxicity in environmental strains may also indicate that cytotoxicity is not an accurate measure of the virulence capacity of a given strain of *V. parahaemolyticus* and that the development of other *in vitro* or *in situ* models is critical for understanding the pathogenic capability of this emergent pathogen.

Unlike the conserved, virulence-associated traits we investigated in both environmental and clinical strains, the putative virulence factor hemolysin is likely the result of horizontal acquisition of the *tdh* gene on *Vibrio* pathogenicity island-7 (Chun et al. 1975, Myers et al. 2003) and/or the *trh* gene on Vp-PAITH3996,. These pore-forming toxins are solely responsible for the Kanagawa phenomenon that is the defining characteristic of many clinical strains (Chun et al. 1975). The environmental isolates used in this work lacked *tdh* and *trh* (Table 2.1), and as expected, they showed no hemolytic activity at either 28°C or 37°C on Wagatsuma agar (Table 2.S1) (Miyamoto et al. 1969). The clinical strains were not hemolytic at 28°C but were strongly hemolytic at 37°C (Table 2.S1). Although no comprehensive study has been completed on temperature regulation of hemolysin in *V. parahaemolyticus*, some observations have been made as part of other studies. For instance, *V. parahaemolyticus* induced to the viable but

nonculturable state by cold and then exposed to temperature upshifts to 37°C did not show *tdh* expression (Coutard et al. 2007), but heat shock at 42°C did induce TDH production in a separate study (Chiang et al. 2008). The dramatic response of this putative virulence phenotype to human host temperature across a group of diverse clinical strains agrees with its role in pathogenesis and provides us with new insights about host cues, specifically temperature, involved in hemolysin activation.

In addition to activation in response to increased temperature, hemolysin production correlated inversely with culture density (Figure 2.2). Specifically, hemolysins are present at high levels during early log phase and decrease as the OD_{600} increases. Virulence gene repression at high densities in both *Vibrio harveyi* and *Vibrio parahaemolyticus* is known to result from quorum-sensing regulation (Henke et al. 2004). In order to assess the possible involvement of quorum sensing in the decrease of hemolysin during late growth phase, hemolysin assays were completed with cultures grown in medium conditioned with cell-free supernatants from late-log-phase cultures of the reference strain BB22. We anticipated that if the repression of hemolysin was in response to quorum-sensing signals that accumulate in the medium, then hemolytic activity would decrease more rapidly in conditioned broth. We saw no effect on hemolysin production (data not shown), indicating that hemolysin regulation is likely the result of a cue(s) separate from quorum sensing. Next, we examined the transcription of *tdh* using a *tdh*::*gfp* promoter fusion normalized to cell density, which showed no decline in expression as cell density increased but, rather, showed a plateau in expression, supporting the idea that the decrease in hemolysis was not transcriptional (data not shown).

Finally, we tested the role of extracellular and/or cell-associated proteases in hemolysin degradation. Complete loss of hemolysis was achieved after incubation with late-log-phase cell-free supernatants (data not shown), indicating that proteolysis is the likely mechanism of hemolysin turnover.

Not only do the environmental strains lack the *tdh/trh* putative virulence genes, but they also lack the ability to regulate other conserved virulence-associated traits in response to human body temperature. The underlying questions of how existing and acquired virulence genes are appropriately regulated in response to temperature and, more specifically, whether this regulation is ancient or obtained horizontally with acquired virulence genes remain unanswered. We addressed this question by introducing a *tdh*::*gfp* promoter fusion into various environmental and clinical strains and assessing the activity of the promoter at the two experimental temperatures. Green fluorescent protein (GFP) was only detected above background levels at 37°C and not at 28°C degrees in all environmental and clinical strains tested (Figure 2.3). The early induction of GFP in all strains by the time the OD_{600} reached 0.5 and the rapid plateau of GFP by the time the OD_{600} reached 1.0 are in agreement with the hemolysin phenotype seen in the quantitative assays (Figure 2.2) and could reflect the importance of TDH in early infection and colonization. Virulence traits that are acquired horizontally must be regulated in coordination with the rest of the genome in order to confer enhanced pathogenicity. During the transition from an environmental setting to a host environment, the bacterial cell must integrate an assortment of signals and respond with appropriate activation or repression of both conserved and newly acquired genes. Although a variety of unique regulatory mechanisms exist that direct the expression of ancient genes, the

mechanisms that regulate newly acquired HGT components are less clear (Lercher et al. 2008). In recent years, a body of evidence has grown showing that a predominant regulatory mechanism for both existing and horizontal genes is the temperature-sensitive histone-like nucleoid-structuring protein (H-NS) (Banos et al. 2009, Dorman et al. 2004, White-Ziegler et al. 2009). H-NS is a globally acting repressor that appears to target and "police" the expression of new DNA (Atlung et al. 1997). Upon exposure to a signal, such as increased temperature, H-NS silencing is relieved and the transcription of virulence genes is activated (Stoebel et al. 2008, White-Ziegler et al. 1998). Given that the *tdh* gene is located within a horizontally acquired pathogenicity island (Hurley et al. 2006) and is induced at higher temperatures, H-NS silencing is a probable mechanism for the temperature-dependent expression we observed. If all virulence phenotypes are under the regulatory control of H-NS, then the disparity between environmental strain regulation of *tdh* but not conserved virulence-associated traits in response to temperature indicates that the regulation of virulence is more complex, perhaps suggestive of different niche adaptations.

Since the majority of the environmental strains in this study were isolated from the same cooler Northern population, it is possible that the temperature dysregulation we observed in environmental strains is unique to this population. It is, however, notable that the one clinical strain isolated from Washington, also a cooler climate, did trend more closely with the clinical strains and not with the cool-water environmental strains (Table 2.S1), supporting the hypothesis that temperature regulation is an attribute of clinical strains. Still, the two environmental strains isolated from warmer climates (both of which were environmentally isolated but associated with an outbreak) did show some

temperature regulation similar to that in the clinical group (Table 2.S1). A broader sampling of environmental strains will be necessary to fully address the question of whether northern strains are unique in their regulation or if this trait is specific to isolates from the Great Bay Estuary. Ultimately, temperature regulation could still be an important consideration in determining the virulence potential of strains from such climates.

Although the environmental strains do not contain *tdh* or the VPaI-7 island, they do have the capacity to regulate *tdh* in response to an increase in temperature and growth phase. Thus, the regulatory mechanisms involved in managing newly acquired virulence elements are present even in nonclinical strains of *V. parahaemolyticus.* This provides evidence that the control of new virulence genes is ancient and that horizontally acquired components are incorporated into existing regulatory networks for proper expression.

ACKNOWLEDGMENTS

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TABLE 2.1. Bacterial strains with relevant pathogenicity markers

Strain	Origin	*tdh*	*trh*	ORF8[a]	*vscC2*	*vopP*	MTase	*vopC*	VPA1376	Source
Clinical										
BB22	Reference strain Water[b] (India)	+	-	-	+	+	+	+	+	(McCarter 1998)
MDOH-03-17294-Vp	Patient (USA, FL)	+	-	-	+	+	+	+	+	(Davis 2007)
AQ4037	Patient (Japan)	-	+	-	+	-	-	-	+	(DePaola 2003)
MDOH-03-17282-Vp	Patient (USA, FL)	+	-	+	+	-	-	-	-	(Davis 2007)
KE9967	Patient (Japan)	+	-	-	+	+	-	+	+	(Osawa 2002[c])
TX2103	Patient (USA, TX)	+	-	-	+	+	+	+	-	(DePaola 2003)
VP81	Clinical (India)	+	-	+	+	+	+	+	-	(Martinez-Urtaza 2004[c])
MDOH-04-5M732	Patient (USA, FL)	+	-	+	+	+	+	+	-	(Davis 2007)
BAC98-03255	Patient (USA, NY)	+	+	+	+	+	+	+	+	(Myers 2003)
F11-3A	Clam[b] (USA, WA)	+	+	-	+	+	+	+	-	(DePaola 2003)
Environmental										
EnvDOH-04-001	Oyster (USA, FL)	-	-	-	-	-	-	+	-	(Striplin 2008)
DAL1094	Oyster[b] (USA, TX)	-	-	-	+	-	-	-	-	(Striplin 2008)
G46, G151, G227	Oyster (USA, NH)	-	-	-	+	-	-	+	+	(Striplin 2008)
G246, G69	Water (USA, NH)	-	-	-	+	-	-	+	+	(Striplin 2008)
G10, G31, G43, G91, G251	Oyster (USA,NH)	-	-	-	+	-	-	-	+	(Striplin 2008)
G149	Water (USA, NH)	-	-	-	+	-	-	-	+	(Striplin 2008)
G26	Clam (USA, NH)	-	-	-	+	-	-	-	+	(Striplin 2008)
G74, G95, G237	Oyster (USA,NH)	-	-	-	-	-	-	-	-	(Striplin 2008)
G1, G7, G23, G242	Oyster (USA, NH)	-	-	-	-	-	-	-	+	(Striplin 2008)
G4, G6, G8, G235	Water (USA, NH)	-	-	-	-	-	-	-	+	(Striplin 2008)
G25	Mussel (USA, NH)	-	-	-	-	-	-	-	+	(Striplin 2008)
G12, G13, G79, G145, G259, G277	Oyster (USA, NH)	-	-	-	-	-	-	-	-	(Striplin 2008)
G61, G62, G255	Water (USA, NH)	-	-	-	-	-	-	-	-	(Striplin 2008)

[a] associated with pandemic isolates; [b] associated with outbreak; [c] provided by EF Boyd.

TABLE 2.2. Phenotypic characterization of the environmental and clinical groups

Clinical Isolates from Outbreaks			
Phenotype	**Effect of temperature shift (28° to 37°)**	**Average Fold Δ**	**p-Value**
Motility	Significant increase at 37°	1.2	<0.0001
Biofilm	Significant increase at 37°	9	0.01
Siderophore	No trend observed	0	-
Protease	Significant increase at 37°	4	0.001
Environmental NH Isolates			
Phenotype	**Effect of temperature shift (28° to 37°)**	**Average Fold Δ**	**p-Value**
Motility	No trend observed	0	0.336
Biofilm	No trend observed	0	0.092
Siderophore	No trend observed	0	-
Protease	Significant Decrease at 37°	2	<0.0001

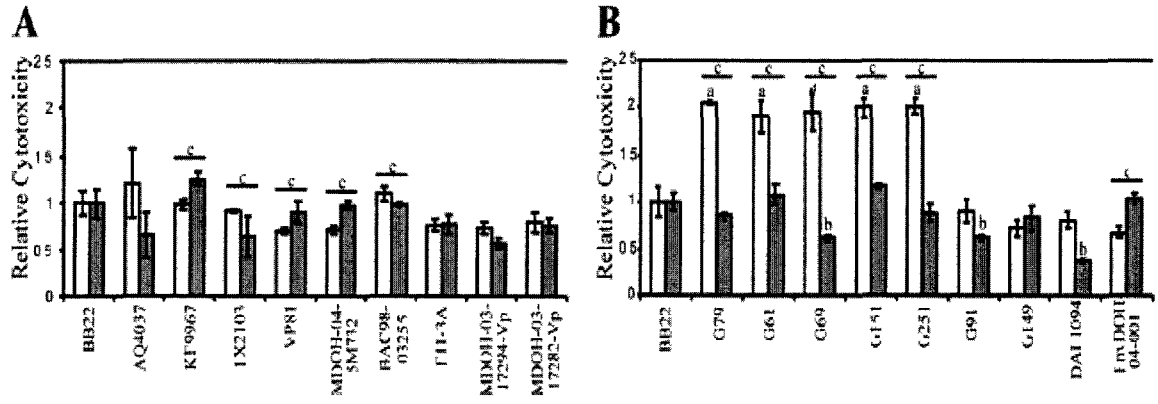

Figure 2.1. Cytotoxicity of clinical strains and select environmental isolates relative to reference strain BB22. Average cytotoxicity of clinical (A) and environmental (B) towards human Caco-2 cells at 28°C (white bars) and 37°C (grey bars) relative to BB22. Cytotoxicity was determined in four separate experiments, however given a high level of variability the values for one representative experiment are shown (trends were consistent for the strains shown here through out all experiments). Values represent the average of three replicates, error bars are SE. One-way ANOVA's were performed on all strains and a significant differences relative to BB22 is denoted by (a) for significant increase in relative cytotoxicity at 28°C, (b) for significant decrease at 37°C, and (c) for statistically significant changes in a single strain at 37°C compared to 28°C.

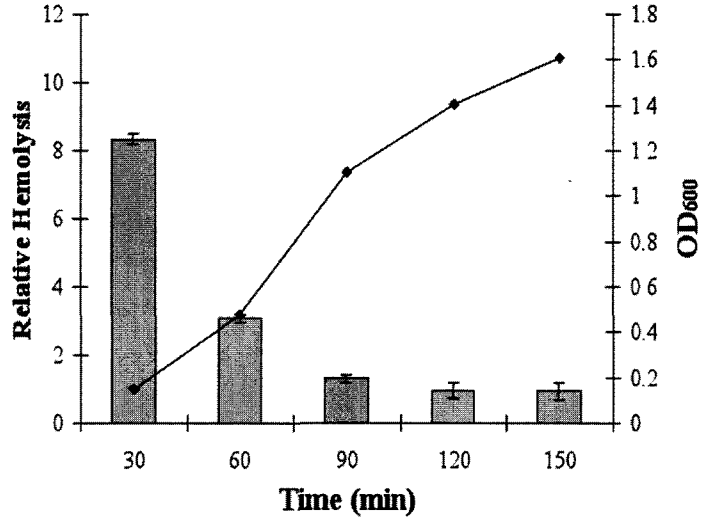

Figure 2.2. Quantitative hemolysis by a select clinical strain at 37°C compared to cell density. Red blood cell lysis was determined over time, and relative hemolytic activity was determined (OD_{415}/OD_{600}) for one representative strain, MDOH-03-17282-Vp (bars), throughout the growth cycle (black line; OD_{600}).

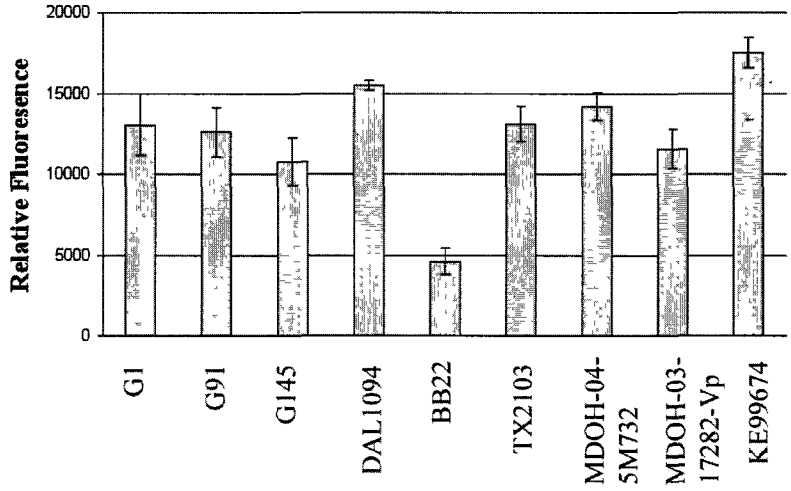

Figure 2.3. Induction of the *tdh* promoter in clinical and environmental strains. Promoter activity of a *tdh*::*gfp* promoter fusion harbored *in trans* in a low copy number plasmid is presented as relative fluorescence (fluorescence/OD_{600}) for various strains during maximal production (OD_{600} ~1.0) as determined from time course assays for cultures grown at 37°C.

TABLE 2.S1. Virulence-associated phenotypes for environmental and clinical strains

Strain	Biofilm[a]		Protease[b]		Siderophore		Motility[c]		Hemolysin	
	37°[d]	28°	37°	28°	37°	28°	37°	28°	37°	28°
BB22	0.08(0.02)	0.03(0)	1.0(0)	2.0(0)	-	-	1.00	1.00	+	-
MDOH-03-17294-Vp	0.14(0.02)	0.09(0.02)	0.8(0.4)	1.0(0)	+	+	1.50	1.84	+	-
AQ4037	0.04(0)	0.04(0)	0.0(0)	0.50(0)	+	+	1.25	1.32	+	-
MDOH-03-17282-Vp	0.03 (0)	0.03(0)	1.8(0.6)	1.2(0.3)	+	+	0.92	1.05	+	-
KE9967	0.93(0.42)	0.04(0.02)	1.8(0.2)	1.0(0)	+	+	1.25	1.16	+	-
DAL1094	0.05(0.01)	0.05(0)	1.2(0.2)	1.0(0)	+	+	1.08	1.79	-	-
TX2103	0.68(0.27)	0.04(0)	2.2(0.4)	0.75(0.3)	+	+	1.33	1.53	+	-
VP81	0.62(0.37)	0.03(0)	1.8(0.2)	0.50(0)	+	+	0.75	0.84	+	-
EnvDOH-04-001	0.94(0.49)	0.04(0.02)	1.8(0.2)	1.0(0)	+	+	0.67	0.90	-	-
MDOH-04-5M732	0.43(0.24)	0.05(0)	1.3(0.2)	0.50(0)	+	+	1.00	1.11	+	-
BAC98-03255	0.70(0.41)	0.03(0)	1.0(0)	0.50(0)	+	+	1.25	1.37	-	-
F11-3A	0.44(0.20)	0.05(0)	1.3(0.3)	1.0(0)	+	+	1.08	1.16	+	-
G1	0.04(0.02)	0.03(0)	1.3(0.2)	2.7(0.3)	+	+	0.74	0.75	-	-
G4	0.05(0)	0.03(0.01)	1.2(0.2)	2.7(0.3)	+	+	1.00	1.00	-	-
G6	0.04(0)	0.04(0)	1.2(0.2)	2.7(0.3)	+	+	1.16	1.08	-	-
G7	0.04(0)	0.03(0)	1.2(0.2)	2.0(0.6)	+	+	1.16	1.17	-	-
G8	0.04(0)	0.04(0.01)	1.3(0.2)	3.0(0.6)	+	+	1.00	1.04	-	-
G10	0.05(0)	0.03(0.01)	0.5(0)	2.3(0.3)	+	+	0.84	1.08	-	-
G12	0.06(0.02)	0.06(0.02)	1.2(0.2)	3.0(0.6)	+	+	1.05	1.08	-	-
G13	0.04(0)	0.05(0)	0.8(0.2)	1.7(0.3)	+	+	0.95	0.75	-	-
G23	0.04(0)	0.04(0.01)	0.7(0.2)	2.3(0.3)	+	+	0.95	0.92	-	-
G25	0.04(0)	0.03(0)	0.7(0.2)	1.3(0.3)	+	+	1.37	1.17	-	-
G26	0.04(0)	0.05(0)	0.9(0.1)	1.3(0.3)	+	+	1.37	1.50	-	-
G31	0.04(0)	0.04(0)	0.8(0.2)	1.7(0.3)	+	+	0.95	1.17	-	-
G43	0.03(0)	0.03(0)	1.2(0.2)	3.0(0.6)	+	+	1.63	1.67	-	-
G46	0.049(0)	0.03(0)	0.5(0)	2.7(0.7)	+	+	0.90	1.08	-	-
G61	0.08(0.2)	0.04(0)	0.8(0.2)	3.0(0.6)	+	+	1.11	1.00	-	-
G62	0.06(0.05)	0.04(0)	0.5(0)	1.3(0.3)	+	+	0.79	0.75	-	-
G69	0.03(0)	0.03(0)	0.7(0.2)	2.3(0.3)	+	+	0.84	0.92	-	-
G74	0.02(0)	0.07(0.03)	1.0(0)	2.0(0)	+	+	0.84	0.75	-	-
G79	0.06(0.03)	0.04(0.02)	0.7(0.2)	2.0(0)	+	+	1.00	1.00	-	-

G91	0.04(0.02)	0.04(0)	0.7(.2)	2.3(0.3)	+	+	0.84	0.92	-	-
G95	0.06(0)	0.03(0)	1.3(0.2)	2.3(0.7)	+	+	1.32	1.42	-	-
G145	0.04(0.01)	0.03(0)	1.2(0.2)	2.0(0)	+	+	0.79	0.83	-	-
G149	0.08(0.03)	0.03(0)	0.7(0.2)	1.3(0.3)	+	+	1.11	0.92	-	-
G151	0.09(0.03)	0.04(0)	0.7(0.2)	2.00(0)	+	+	0.79	0.58	-	-
G227	0.06(0.01)	0.03(0)	1.5(0)	2.3(0.3)	+	+	1.00	1.00	-	-
G235	0.03(0)	0.03(0)	1.2(0.2)	1.7(0.7)	+	+	1.21	1.25	-	-
G237	0.04(0.01)	0.10(0.08)	1.0(0)	2.7(0.3)	+	+	1.26	1.33	-	-
G242	0.02(0)	0.05(0.02)	0.50(0)	3.0(0)	+	+	1.26	1.42	-	-
G246	0.03(0)	0.03(0)	0.6(0.1)	2.7(0.3)	+	+	1.21	1.25	-	-
G251	0.03(0)	0.03(0)	0.7(0.2)	2.3(0.3)	+	+	0.90	0.83	-	-
G255	0.09(0.01)	0.04(0.01)	0.7(0.2)	1.7(0.3)	+	+	1.16	1.00	-	-
G259	0.09(0.04)	0.08(0.05)	0(0)	4.0(0.6)	+	+	1.84	0.92	-	-
G277	0.03(0)	0.03(0)	0(0)	3.7(0.3)	+	+	0.79	0.83	-	-

[a]Biofilm formation was quantified from crystal violet staining determined at OD_{595} normalized by cell density of culture (OD_{600}) for three replicates per treatment which were averaged. The experiment was performed a total of three times and the data for one representative experiment is presented with standard error (SE).

[b]Protease was determined as the diameter of the zone of clearing around each of three replicate spots per strain which were averaged. The experiment was performed a total of three times and the data for one representative experiment is presented with standard error (SE).

[c]Motility is reported as velocity (mm/hr) of movement from the edge of the spotted culture for each of three replicates, which were averaged. The experiment was performed a total of two times and the data for one representative experiment presented. The experiments were highly reproducible and the standard deviations for all treatments were less than 0.01.

[d]All temperatures are °C.

CHAPTER III

GacA DIFFERENTIALLY REGULATES BACKBONE AND ACQUIRED VIRULENCE FACTORS IN RESPONSE TO A HUMAN HOST TEMPERATURE

Although the horizontally acquired hemolysin gene *tdh* is a commonly used marker for human pathogenic strains of *Vibrio parahaemolyticus*, little evidence exists definitively linking this or other traits to disease. In an effort to better characterize disease mechanisms in *V. parahaemolyticus*, we examined a conserved global regulator, GacA, which regulates virulence and host association in many bacterial species including *Vibrio*. A GacA mutant of *V. parahaemolyticus* was defective in several traits previously associated with this regulon including protease production and metabolism. However, other traits that may play roles in infection, including motility and biofilm formation were uninfluenced. The mutant was defective in functional assays of virulence, including cytotoxicity against human colon cells and disease in mice. The cytotoxicity defect mediated by GacA was temperature dependent consistent with previous work implicating this as an important signal in virulence in *V. parahaemolyticus.* In contrast to these expected defects, the mutant also displayed a surprising hyper-hemolysis phenotype, and microarray analysis revealed *tdh* and most of the VPaI-7 island was upregulated in the mutant compared to wild-type. This was mediated by the RNA chaperone Hfq, and

translational repressor CsrA, suggesting that some of the established regulatory circuitry is conserved. However, unlike in some other *Vibrio spp.*, phenotypic and transcriptional changes did not mirror those observed in an OpaR mutant, which based on existing paradigms was predicted to mediate GacA regulation. This work provides a foundation for understanding regulatory changes and candidate traits required during infection.

INTRODUCTION

Despite extensive research to define virulence in *Vibrio cholerae*, the mechanisms that underlie the unique infectious processes in other human pathogenic *Vibrio spp.*, such as *Vibrio parahaemolyticus*, remain elusive. Once thought to be requisite virulence genes, thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) have been conspicuously absent from strains involved in recent outbreaks (Garcia et al. 2009). Though regulation of *tdh* is known to be under the negative control of the small RNA chaperone protein Hfq, little is known about how this contributes to overall pathogenicity (Nakano et al. 2008). In addition, a singular role in virulence seems unlikely as when all copies of *trh* and *tdh* are mutated the organism remains cytotoxic, enterotoxic, and lethal to mice indicating that these hemolysins likely act in concert with other as yet unidentified virulence traits (Xu et al. 1994, Park et al. 2004, Hoashi et al. 1990, Hiyoshi et al. 2010). Without a more comprehensive understanding of fundamental virulence mechanisms in *V. parahaemolyticus*, we lack the ability to target genetic markers of virulence in environmental surveillance and are ill-equipped to predict and reduce outbreaks.

Several approaches have been employed to find candidate virulence factors for this organism, most of which seek to identify unique gene content of clinical strains. For example, the *tdh* and *trh* hemolyisin genes, located on VPaI-7 and Vp-PAI$_{TH3996}$ respectively, were found by their association with clinical strains (Makino et al. 2003, Okada et al. 2009). Similarly, seven key pathogenicity islands linked to a highly infectious pandemic clone of *V. parahaemolyticus* were discovered through comparative genomics across several *Vibrio spp.* (Boyd et al. 2008). Complete genome sequencing revealed additional putative virulence factors including exoenzyme T, cytotoxic necrotizing factor (CNF), VPA1376 (homolog of gene found in *V. cholerae* pathogenicity island), VPA1357 (protein with extensive repeats likely involved in immune evasion or host cell attachment) and repeats in toxin (RTX), though none have been evaluated in disease (Makino et al. 2003). Alternatively, two different type three secretion systems, one found on chromosome I (T3SS1) and the other in a pathogenicity island on chromosome II (T3SS2) have been investigated and are known to contribute to cytotoxicity and enterotoxicity respectively (Park et al. 2004). However, T3SS1 is found in all strains of *V. parahaemolyticus* including non-pathogenic environmental isolates and may be more important in survival in a natural habitat (Mahoney et al. 2010). Interestingly, although T3SS2 has been linked to enterotoxicity, a T3SS2 defective strain showed little attenuation in virulence in a mouse model (Hiyoshi et al. 2010). In addition, increased production of these known and putative virulence associated traits in response to a human host temperature of 37° is an important characteristic of pathogenic strains of *V. parahaemolyticus* (Mahoney et al. 2010). Ultimately, the inflammatory gastroenteritis seen in human infections with *V. parahaemolyticus* cannot be attributed to

any single effector molecule or toxin. Pathogenicity in this organism is likely the result of large-scale programming of virulence and colonization genes by global regulators in response to a multitude of signals, including human host temperature (Mahoney et al. 2010).

Pathogenic and symbiotic host-microbe associations in *Vibrio spp.* and other gram negative pathogens have long been known to require the GacS/GacA global regulator (Figure 4.1) (Whistler et al. 2003, Lenz et al. 2005, Wong et al. 1998, Gauthier et al. 2010). This regulatory system coordinates expression of general colonization traits (e.g. motility) with more specific colonization or virulence factors (e.g. toxins), even when they have been acquired horizontally. For example, in enteropathogenic *Escherichi coli*, the GacS/GacA orthologs (BarA/UvrY) control host colonization via regulation of the locus of enterocyte effacement embedded within a pathogenicity island (Bhatt et al. 2009). *Salmonella* Typhimurium requires the GacS/GacA orthologs (BarA/SirA) to properly activate transcription of the salmonella pathogenicity island-1 and therefore invade host epithelial cells (Mizusaki et al. 2008). In the absence of a functional GacS/GacA system, *V. cholerae* is unable to produce sufficient levels of the virulence factors toxin co-regulated pilus and cholera toxin, is attenuated in virulence towards infant mice and does not properly regulate Hap proteases (Wong et al. 1998, Jang et al. 2010, Jang et al. 2011). A *gacA* mutant of *V. vulnificus* is defective in cytotoxicity, protease, and siderophore production and virulence in mice (Gauthier et al. 2010). Even in the beneficial association of *Vibrio fischeri* with its squid host GacA is required for colonization and production of the specific symbiosis factor bioluminescence (*lux*), thus

demonstrating that this two component system plays a role not just in virulence, but in host microbe relationships in general (Whistler et al. 2003).

The mechanism by which the GacS/GacA system activates the necessary machinery for appropriate global gene expression has been well studied (Figure 1.1). The membrane bound sensor GacS and its cognate response regulator GacA act to relieve repression of translation exerted by CsrA, through the production of several antagonistic sRNA's including CsrB1/2/3/C which competitively bind CsrA (Lapouge et al. 2008). In several *Vibrio spp.* studied thus far, GacS/A is intimately tied to regulation of quorum sensing by acting upon LuxO upstream of the quorum sensing master regulator HapR in *V. cholerae* and LuxR in *V. harveyi* (Lenz et al. 2004, Tu et al. 2008). The homologous regulator in *V. parahaemolyticus* (OpaR) has been under intense study for its role in colony opacity and motility (Jaques et al. 2006, Enos-Berlage et al. 2005). The sRNA chaperone protein host-factor 1Hfq (encoded by *hfq*) works downstream of luxO to destabilize mRNA of *hapR* and other genes in *V. harveyi* and *V. cholerae*, and is subject to direct CsrA binding and repression in *Escherichia coli* (Lenz et al. 2004, Baker et al. 2007). Given the role that GacS/GacA is known to have in virulence in other *Vibrios spp.*, characterization of this regulon in *V. parahaemolyticus* may reveal important insights into the unique virulence mechanisms of this organism.

In the present study, we establish that GacS/GacA mediates the regulation of host association and virulence in *V. parahaemolyticus*, although a *gacA* mutation had some unexpected effects. Consistent with its role in virulence, we expected reduced production of known and candidate virulence factors in the GacA mutant. Indeed, the mutant was altered in many traits, and impaired in disease in an infant mouse model for infection.

However, in sharp contrast to its reduced virulence, the mutant displayed enhanced hemolysis and enhanced *tdh* expression mediated by CsrB1, CsrA and Hfq. This work further brings into question the role of *tdh* as an authentic virulence factor in this organism. Further studies within the GacS/GacA regulon could help to identify potential virulence mechanisms unique to this organism and to elucidate how closely related organisms evolve to utilize the same pathways with novel outcomes.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 3.1. Strains of *Vibrio parahaemolyticus* were routinely grown with shaking at 37°C (or 28°C where indicated) in Heart Infusion (HI) broth pH 7.3 which contained 12.5 g heart infusion (Fluka, Buchs, Switzerland), 20g NaCl per liter and for plates 15g/liter granulated agar. *E. coli* was maintained in Luria-Bertani (LB) broth. Swimming motility was assessed with plates containing 10 g tryptone, 20 g NaCl, and 3.35 g Bacto agar per liter as previously described (Jaques et al. 2006). Swarm plates contained HI media with 10 g Bacto agar per liter. Protease activity was measured using standard HI plates to which 4 g/L of sterilized skim milk was added following autoclaving and cooling to 50°C. Lipase activity and exopolysaccharide differences were evaluated qualitatively with plate assays as previously described (Mahoney et al. 2010). Hemolysin activity was measured with Wagatsuma agar plates as previously described (Miyamoto 1969). For carbon utilization experiments, IM media and a single carbon source (fumurate, Casamino acids, glucose, galactose or glycerol) were used. Tri-parental matings were performed on Super Optimal Broth (SOB) plates

containing 2% Bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgS0_4$ and 1.5% agar. Where required, HI media was supplemented with antibiotics specifically, erythromycin (EM) 12 μg/ml for *V. parahaemolyticus* and BHI media with 150 μg/ml for *Escherichia coli* and LB with chloramphenicol (CH) 25 μg/ml or kanamycin (KM) 50 μg/ml.

Strain and plasmid construction. The low copy plasmid pEVS79 which contained the wild-type *gacA* open reading frame was mutagenized using the EZ::TN<KAN-2> insertion kit following manufacturer protocols (Epicentre, Madison,Wis.). Disruption of the *gacA* gene was identified by PCR amplification and confirmed by sequence analysis. The mutagenized *gacA* gene was introduced into the genome by allelic exchange as previously described (Stabb et al. 2002). Strain JM1F3 was confirmed to have the gacA::EZ::TN<KAN-2> mutation by sequence analysis.

Multicopy plasmids for complementation analysis were constructed by standard methods. A *gacA* complementation construct was created using a plasmid pJM1D4 containing the full length *gacA* gene (VP1945) and was digested with PstI and SalI to generate a 2.2kb fragment that was ligated into pVO8 in the forward direction to create pVCW9G3. An *hfq* complementation construct was also created by standard methods using primers designed to the VP2817 locus of the RIMD2210633 genome. The forward primer 5'TATTCCCGGGCGATAACAT TGAGCAGGCAC-3') and reverse primer (5'-TAATGCATGCGTCTCAACCCCAG CGGAA-3') with an SphI restriction enzyme site engineered into the 5'end were used to amplify a 683bp amplicon from BB22 genomic DNA. The amplicon was then cloned directly into pCR®2.1-TOPO® (Invitrogen,

Carlsbad, CA). After confirmation by sequence analysis the newly cloned *hfq* was subject to a standard digest with EcoRI and ligated into the low copy number plasmid pEVS79. Subcloning into pVO8 was achieved by standard double digest of pJM3D6 with SphI and SmaI to create pJM3E1.

***In vitro* phenotypes.** All phenotypic assays were done in triplicate with replicate experiments using cultures grown in HI (EM^{12}) when necessary and standardized to an OD_{600} of 0.5 prior to inoculation. Protease, lipase and hemolysin (Wagatsuma agar) plates were inoculated with 10 µl and incubated at 37° for 24 hours. A clearing around the inoculum was considered a positive reaction and the diameter was measured. Swimming motility was measured with 25ml plates inoculated with a toothpick just below the surface of the media. After incubation at 37° measurements were taken of the diameter at 3 hour intervals and velocity was calculated. Swarming motility was measured using 25 ml swarm plates inoculated with 10 µl and incubated for up to 72 hours at 28°C. Biofilm production was assessed with glass tubes (12 x 75mm) inoculated with 3 mls of HI EM^{12} and incubated at 37°C for 20 hours. An initial OD_{595} was taken and cultures were dumped and incubated upside down at 80°C for 30 minutes. The tubes were then stained with 5mls of 0.1% crystal violet for 20 minutes. Tubes were then gently washed 4 times with dH_2O then allowed to dry for 10 minutes. The tubes were then de-stained with 5 mls of 95% EtOH and 200 µl was used to read a final OD_{595} in a Tecan infinite M200 plate reader (Männedorf, Switzerland). Biofilm production was plotted as final OD_{595}/initial OD_{595}. An acid challenge assay and quantitative hemolysin assay were performed as previously described (Whitaker et al. 2010, Mahoney et al.

2010). Starvation survival was assessed by CFU counts after 24 hours of growth in HI media. They were then normalized to CFU counts at log phase to account for the growth defect of the gacA mutant. Oxidative stress was measured at both exponential and stationary phase by the disk diffusion method using both bleach and H_2O_2. Diameters were measured after overnight incubation at 37°C. Cytotoxicity towards human CaCo-2 cells was assessed as previously described at both 28°C and 37°C at an MOI of 100 for 4 hours (Mahoney et al. 2010).

Animal studies. An assay for induction of symptoms in an infant mouse model was assessed as previously described (Chiang 1995). Briefly, cohorts of 6-5 day old CD-1 mice were inoculated by oral gavage with 50 µl of 1XPBS containing 10^6 cells of wild type BB22 or JM1F3 or without bacteria. Each group contained 6 mice including a no bacteria control group. The mice were monitored every hour for 24 hours or until a change in pallor was observed at which point the mice were sacrificed. The intestines were then aseptically harvested and homogenized in 5 mls of LB broth and plated onto HI to determine output counts. All experiments were conducted with approval from the Institutional Animal Care and Use Committee (IACUC Approval 08-02-05).

RNA purification. BB22 and JM1F3 were grown at both 28° and 37° in HI broth to an OD_{600} of ~0.5 and immediately harvested with RNA Protect (Qiagen, Valencia, CA) for microarray experiments. RNA was purified using an RNeasy minikit (Qiagen) following manufacturer's instructions and cDNA synthesis for RT-PCR experiments was carried out using a BioRad iScript cDNA Synthesis Kit (Hercules, CA, USA). For microarray

experiments, cDNA synthesis, labeling and DNA hybridization were all performed by the Microarray Facility at the University of California Irvine.

Microarray analysis. An Affymetrix custom 3' GeneChip containing all ORF's from the *Vibrio parahaemolyticus* and *Vibrio fischeri* genome was designed as previously described (Gode-Potratz et al. 2010). Gene Chip-Robust Multiarray Averaging was used to background correct, normalize across replicate chips, and to calculate and summarize probe level intensity. A Cyber T analysis was performed to identify differentially expressed genes (Long et al. 2001). Only those genes with a PPDE(<p) value of ≥ 0.90 and a fold change in gene expression of ≥ 2.0 in a *gacA* mutant compared to wild type in either *V. fischeri* or *V. parahaemolyticus* were selected for further analysis.

Microarray confirmation by Quantitative Real Time-PCR (qRT-PCR). Three genes were chosen from the microarray analysis for further confirmation by quantitative real time PCR. The *tdh* and *spa*24 genes were selected for confirmation because they were significantly upregulated (2.4 and 4.9 fold change respectively) in a *gacA* mutant compared to wild type at 37°. In addition, VP2448, a putative protease, was down regulated -3.5 fold at 37°, though it lacked strong statistical values to support this fold change (PPDE(<p)=0.51). Despite the lack of statistical support, VP2448 was selected for confirmation by qRT-PCR because it is annotated as a putative protease and may be correlated with the protease defect seen in a *gacA* mutant. All assays were performed twice with ±2% difference in PCR efficiency between the gene of interest and a calibrator gene (16s). All genes of interest, including 16s, were cloned into a pCR®2.1-TOPO® vector for use in a standard curve as a clone of interest.

RESULTS

A *gacA* mutant of *Vibrio parahaemolyticus* is altered in multiple phenotypes. If the function of GacA as a global virulence regulator is conserved in *V. parahaemolyticus*, then the mutant should display multiple defects related to host association. Following generation of a *gacA*::TnKan-2 insertion mutant (JM1F3), the mutant was examined for a suite of phenotypes representative of previously characterized GacA regulons, and potentially involved in virulence (Table 3.2). As expected, the mutant had many general growth and metabolic defects, including a temperature dependent growth rate phenotype. Though no change in doubling time was detectedat 37°C (wild type= 2 hours, GacA= 2 hours), the GacA mutant had an enhanced growth rate at 28°C compared to wild type (wild type= 3.5 hours, GacA= 2.8 hours). In addition, GacA was impaired in some traits that may impact virulence, including starvation survival, protease, lipase, altered surface properties (opacity and uptake of Congo red) and swarming ability. However, several other traits that are potentially important for disease by *V. parahaemolyticus*, including swimming motility, biofilm production and acid tolerance were not affected by the *gacA* mutation, and oxidative resistance was only impaired in exponentially growing cells. We observed plaque-like clearings in lawns of the GacA mutant but not the wild-type after several days of growth consistent with the induction of a temperate phage in the absence of GacA. Finally, the GacA mutant was less cytotoxic to human gastrointestinal cells only at 28°C and not 37°C. All mutant phenotypes were fully complemented by the *gacA* gene *in trans* (pVCW9G3) with the exception of growth yield, growth on single carbon sources and swarming ability. These phenotypes were only partially complemented, which may have resulted from cellular toxicity due to multicopy *gacA*. For example,

complementation of defective growth on single carbon source agar plates was observed but growth was weak.

The majority of GacA-phenotypes were expected given the context of previously published GacA regulons in this and other species (Whistler et al. 2003, Lenz et al. 2005, Wong et al. 1998, Gauthier et al. 2010). Although GacA functions upstream of the quorum sensing regulator OpaR in multiple *Vibrio* sp., GacA in *V. parahaemolyticus* appears not to work solely via OpaR unlike in *V. fischeri* (Whistler et al. 2003, Ballok 2007). Comparison with an existing OpaR mutant revealed a dramatically different profile of phenotypes, especially swarming motility, with few in common including opacity and cytotoxicity (Table 3.2).

The *gacA* mutant has a hyper-hemolysis phenotype that is mediated by CsrB, CsrA and Hfq. Perhaps the most striking and unexpected phenotype in the GacA mutant was the overproduction of the presumptive virulence factor hemolysin, a phenotype that was not observed in the OpaR mutant (Table 3.3). Notably, overproduction only occurred at 37°C, and both the wild-type and GacA mutant produced very low levels of hemolysin at 28° C (data not shown). Complementation of this phenotype was not achieved with *gacA in trans* (pVCW9G3), but the wild-type also exhibited enhanced hemolysis with multicopy *gacA* suggesting gene dosage affects on this phenotype. As expected, hemolysis was restored to wild type levels when the GacA mutant was complemented with *csrB1* (pAEB2H8) and in the spontaneous *csrA* mutant JM1G1(Table 3.3).

Previous work implicates the sRNA chaperone protein Hfq in CsrA-mediated repression in other organisms at two different junctions: CsrA directly binds to and blocks translation of the *hfq* transcript in *E. coli* (Baker et al. 2007), and Hfq acts

downstream of LuxO in the CsrA regulatory cascade by mediating *qrr* destabilization of *hapR/luxR* (homologs of *opaR*) (Lenz et al. 2004). In addition, hyper-hemolysis is reported in an *hfq* mutant in *V. parahaemolyticus* (Nakano et al. 2008). Therefore, enhanced hemolysis in the GacA mutant could be mediated via reduced Hfq levels resulting from its sequestration by enhanced qrr1-5 production, or via direct CsrA repression. Indeed, a CsrA binding site (AAATAAGGAAAA) closely matching the consensus sequence (RUACARGGAUGU) (Baker 2007) was identified in *hfq* and introduction of *hfq in trans* (pJM3E1) fully restored normal hemolysis in the GacA mutant suggesting that reduced Hfq activity influenced this phenotype (Table 3.3.). Notably, steady state levels of *qrr1-5* were not enhanced as one might expect based on existing paradigms (fold change in a GacA mutant compared to wild type of *qrr1-5* with corresponding PPDE(<p) values: 1.1 (.21), 1.5 (.34), 1.3 (.23), -1.1 (.15), -1.2 (.20)). Additionally, *hfq* was not reduced significantly in the GacA mutant compared to the wild-type indicating that if GacA exerts an influence on this regulator, it does so post-transcriptionally (Table 3.S1)(Tu et al. 2008).

GacA-mutant infected infant mice have a delay in onset of symptoms despite hyper-hemolysis. If the hemolysin TDH functions as a virulence determinant, the hyperhemolysis of the GacA mutant may enhance disease; however, we observed a reproducible delay in the onset of diarrhea and change in pallor in GacA-mutant infected mice compared to wild type (1.5 fold change, chi^2=11, p=<0.001) (Figure 3.1). Bacteria were recovered indicating that *V. parahaemolyticus* is able to survive and possibly replicate in the gut (wild type input: $2x10^5$ n=1 output: $7x10^5$ n=1 and GacA input:

$1.8x10^7$ n=1 output: $5.57x10^5$ n=2 mice). Even with the consistent delayed onset of symptoms in multiple experiments, this model was inherently variable and subtle differences in development of mice within cohorts required that onset of symptoms and not mortality (LD_{50}) be used as a measure of disease. Specifically, infant mice that were slightly more developed (exhibiting hair growth) often became symptomatic, and then recovered when infected with either the wild type or the GacA mutant, rather than succumbing to disease. Future work to elucidate disease may require further optimization or an alternative model for infection.

The GacA hyper-hemolysin phenotype is attributed to upregulation of *tdh* (VPaI-7) at 37°C. Regulation of virulence genes in response to human host temperatures is an associated characteristic of clinical strains of *V. parahaemolyticus* (Mahoney et al. 2010). Therefore, we examined the GacA regulon in this organism at both 28°C and 37°C to identify GacA regulated genes that may contribute to observed phenotypes and attenuated virulence. Arrays completed with mid-log phase cells (OD_{600} 0.5) at 28°C revealed a small set of genes influenced by *gacA* (Figure 3.2). Of the 20 genes within this group (.41% of the overall genome), many were hypothetical proteins while others were related to general cellular metabolism and physiology (Table 3.S1). In terms of putative virulence gene expression, an RTX toxin (VPA1633) was upregulated in the mutant despite the down regulation of the ABC transporters thought to be involved in RTX export (Delepelaire et al. 2004). In comparison, examination of GacA-regulated genes at 37° revealed a sizeable regulon (202 genes or 4.2% of the overall genome) of both up and down regulated genes (Figure 3.2 and Table 3.S1). Specifically VPaI-7 which contains both the *tdh* and T3SS2 genes was significantly upregulated (2.0-7.6 fold change),

whereas the genes immediately flanking the island were down regulated (Figure 3.3). This increase in *tdh*-gene expression is consistent with the hyper-hemolysin phenotype observed in both quantitative and qualitative assays. Interestingly, this regulatory change is dependent on a defective GacA as there is no significant regulatory change in wild-type VPaI-7 at 37°C compared to 28°C. This is contradictory to previous findings that expression of a *tdh-gfp* gene fusions is dependent on temperature (Mahoney et al. 2010), though the construct used in these experiments would be subject to both native transcriptional and translational regulation. All genes in the capsular polysaccharide (CPS) island (VPA1403-VPA1412) were down regulated in the absence of GacA unlike VPaI-7. A small number (18 genes or 3.8% of genome) were influenced at both temperatures (Figure 3.2).

Quantitative Real-Time PCR anlaysis confirmed the upregulation of *tdh* at 37°C ($2^{-\Delta\Delta Ct}$= 3.0 average fold change; WT $2^{-\Delta\Delta Ct}$= 0.799 SD=0.178, GacA $2^{-\Delta\Delta Ct}$= 0.198 SD=0.046) in a GacA mutant compared to wild type. The T3SS *spa24* gene found within the same pathogenicity island as *tdh* (VPaI-7) was also confirmed to be differentially regulated at 37°C ($2^{-\Delta\Delta Ct}$= 7.0 fold change; WT $2^{-\Delta\Delta Ct}$= 0.727 SD=0.386, GacA $2^{-\Delta\Delta Ct}$= 0.080 SD=0.037) while both *tdh* and *spa24* were uninfluenced at 28°C in qRT-PCR assays (*tdh* WT $2^{-\Delta\Delta Ct}$= 0.592 SD=0.532, *tdh* GacA $2^{-\Delta\Delta Ct}$= 0.523 SD=0.247 and *spa24* WT $2^{-\Delta\Delta Ct}$= 0.427 SD=0.498, *spa24* GacA $2^{-\Delta\Delta Ct}$= 0.152 SD=0.072. The -3.5 fold downregulation of the putative protease VP2448 observed in the microarray studies was not observed in qRT-PCR assays(WT $2^{-\Delta\Delta Ct}$= 0.645 SD=0.074, GacA $2^{-\Delta\Delta Ct}$= 0.776 SD=0.194), therefore this change was likely an artifact of the assay and may explain the weak statistical values (PPDE(<p)=0.51) associated with this gene.

DISCUSSION

In order to properly function as virulence factors, the hemolysins, and other horizontally acquired island bound traits, must be incorporated into existing regulatory networks to ensure coordinated and timely expression with the entirety of the cell. Hemolysin activity and *tdh*-gene expression is only detected from cultures grown at human host temperature (Mahoney et al. 2010), and microarray analysis indicates that this response is not specific to *tdh*, but is a property of the majority of genes within the VPaI-7 island. However, we only detected differences in *tdh* steady-state transcript levels between the two temperatures in the absence of GacA, which leads to overproduction of hemolysin. This suggests that temperature regulation interfaces with GacA repression to control hemolysin and VPaI-7 island expression. Array analysis further revealed that temperature and GacA regulation were not general attributes of all islands. Though temperature has previously been implicated as an indirect cue in GacS/GacA regulation in *Pseudomonas fluorescens*, biocontrol factors in this organism were down regulated at higher temperatures contrary to the data shown here (Humair et al. 2009). This work illustrates how conserved regulatory pathways evolve to selectively include horizontally acquired genes which potentially contribute to virulence and host association, and highlights the importance of combining comparative genomics with functional expression analysis.

Once integrated into the conserved GacA regulon, multiple levels of regulatory control are likely utilized by the cell to ensure proper expression of VPaI-7 and horizontally acquired elements in general. In addition to GacA (and its downstream regulators CsrB and CsrA), Hfq mediates the hyper-hemolysin phenotype (Table 3.3).

Hfq is a small protein that chaperones non-coding sRNAs with the appropriate mRNA transcripts and can act alone to influence regulation by polyadenlaytion or direct interaction with transcripts (Valentin-Hansen et al. 2004). These interactions can either stabilize or de-stabilize mRNA and therefore influence expression. In addition, the Hfq protein is proposed to operate as an intermediary of communication between core and horizontally acquired genes, likely facilitating the incorporation of horizontal elements into existing networks such as GacA (Chao et al. 2010). Hfq also mediates the activity of the qrr RNAs in the GacA regulon in *V. harveyi* and has a similar hyper-hemolysin phenotype when mutated in *Vibrio parahaemolyticus* (Tu et al. 2008, Nakano 2008). The most likely model for regulation based on our data and that from other organisms is that GacA represses hemolysin activity by relieving CsrA repression of *hfq* transcripts (Figure 3.3 and Figure 1.1). This results in elevated Hfq protein which lowers hemolysin activity either by directly destabilizing the *tdh* transcript preventing translation, or through an undefined and indirect mechanism (Baker et al. 2007, Nakano et al. 2008). If hemolysins are required during infection and are produced in response to host temperature, then this model would require that GacA either be inactivated or that repression be overridden *in vivo*. Importantly, this model implicates temperature and GacA as jointly acting regulators that converge to control hemolysin production in an additive fashion. This dynamic process of signal interpretation and integration followed by an intricate regulatory response, mediated by conserved circuitry yet involving newly acquired DNA, represents a curious adaptation that may provide an advantage within a, likely coincidental, host.

Despite the classification of TDH as a virulence factor in *V. parahaemolyticus* and an increase in hemolysin activity seen in both quantitative and qualitative assays at human body temperature, a hyper-hemolytic GacA mutant was impaired at symptom induction in infant mice. This was a surprising result as the entire VPaI-7 island containing the T3SS2 thought to be involved in enterotoxicity was upregulated in the mutant. An interpretation of this data is that TDH and hemolysin activity in general may not play as large a role in *V. parahaemolyticus* infections as previously thought, or alternatively that the regulation observed *in vitro* does not reflect that *in vivo*. The delay in symptom onset when T3SS2 is over expressed is more difficult to interpret. It is possible that over-expression of this island in general is toxic to the bacterial cell and thereby inhibits fitness in an intestinal environment. Alternatively, GacA may not be able to effectively colonize due to defects in other host association traits under its control and may be inhibited from reaching the appropriate stage of infection where T3SS2 effector proteins are most useful. Though defective at initiating disease, preliminary experiments indicated that the GacA mutant was more resistant to complement mediated killing and had enhanced fitness in a mouse intestine compared to wild type in a competition assay (3-fold increased survival over wild-type based on % CFU surviving after 1 hour, APPENDIX III and IV).

Enhanced survival with reduced symptoms may allude to important functions and mechanisms during disease development, such as immune activation, consistent with the inflammatory nature of gastroenteritis caused by this pathogen (Qadri et al. 2003). It is possible that GacA was recovered in higher numbers compared to wild type because it was better able to colonize the mouse gut but had a lack of proper symptom induction as

a result of phenotypic defects detected *in vitro*. For example, a GacA mutant had cytotoxicity, protease and lipase defects that could possibly have hindered gastrointestinal cell death and enterotoxicity. Additionally, the GacA mutant may lack a mucosal escape response whereby regulatory changes occur that result in the detachment of bacterial cells from the intestine for eventual release into the external environment (Nielson et al. 2006). As wild type cells leave the intestine at a normal rate, GacA may accumulate in the intestine and therefore be recovered at higher numbers. Alternatively, though wild type may induce a more potent infection, it may also be more susceptible to the very host response that it elicits. Initial experiments showed that GacA is more resistant to complement mediated killing (APPENDIX III). Though preliminary data, this may point to a possible mechanism of immune evasion and may explain why fewer wild type cells were recovered from mouse intestines despite a more potent infection. Further analysis of gene expression within the host context, and host responses will be essential and is an important future direction of this work.

Clearly a GacA mutation results in extensive global programming deficiencies with far reaching implications. Differences observed in the presence/absence of unanticipated and expected phenotypes in the GacA regulon compared to other organisms provides further evidence that this conserved network has been rewired in this organism. Specifically, GacA exerts its control through the regulator HapR in *V. cholerae* (homologous to LuxR in *V. harveyi*, LitR *V. fischeri,* and OpaR in *V. parahaemolyticus*). However, simultaneous evaluation of an OpaR mutant of *V. parahaemolyticus* revealed different phenotypic profiles in comparison to GacA (Table 3.2) confirming that organizational changes have occurred. Despite this rewiring, host association traits were

conserved in this regulon as a GacA mutant was impaired in symptom induction in mice. Animal experiments combined with microarray analysis of this regulon identified a valuable subset of genes influenced by both human host temperature and GacA that may contribute to virulence and/or colonization. Specifically, 202 genes in the GacA regulon were influenced by temperature (37°C) (Table 3.S1). Within this cohort, a large number of genes are downregulated, annotated as "hypothetical protein" and may be important candidate virulence factors that could account for the defective host association of the GacA mutant. Additionally, GacA enhanced fitness in a mouse gut could be attributed to an upregulation of hypothetical genes within the backbone or in the horizontally acquired VPaI-7. Given the now confirmed role in host association, characterization of the GacA regulon could begin to clarify the unique virulence mechanisms and inflammatory host response seen in infections of *V. parahaemolyticus*. Further analysis of this regulatory cascade may help to clarify the complex mechanisms involved in the evolution of regulatory pathways to include horizontal elements that may confer enhanced niche or lifestyle specialization traits, and clarify the elusive virulence of this organism.

ACKNOWLEDGEMENTS

We thank Linda McCarter for kindly providing *V. parahaemolyticus* strains (BB22 and OpaR). We also thank Deena Small for allowing us to perform cytotoxicity assays in her cell culture facility and Ronald Taylor for valuable training and oversight of mouse work.

Table 3.1. Bacterial strains and plasmids

Strain or Plasmid	Description	Source or Reference
E. coli strains		
DH5α	F⁻ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1Δ* (*argF-lacZYA*) *U169* ϕ80*lacZ*M15λ⁻	(Gibco-BRL, Inc.)
V. parahaemolyticus strains		
LM5312 (BB22)	Wild type clinical strain *tdh+/trh-*	(McCarter 1998)
LM5674 (OpaR)	ΔopaR1, BB22 derivative permanently locked in translucent phase	(McCarter 1998)
JM1F3 (GacA)	BB22 with EZ::TN<KAN-2> insertion in *gacA* at P602 *gacA*::Tn, Km^r	(This study)
JM1G1 (GacA;CsrA)	Spontaneously arising derivative of JMIF3; *gacA*::TNKm *csrA* C17T $GacA^{Sup1}$	(This study)
Plasmids		
pCR®2.1-TOPO®	Km^r, Ap^r multicopy cloning vector	(Invitrogen, Inc)
pEVS79	pBCSK derivative, Mob^+, Chr^r Km^r	(Stabb 2002)
pEVS104	R6Kγ derivative of pRK2013; ΔColE1, *oriT tra trb* ΔTn*903* Km^r	(Stabb 2002)
pVO8	pACYC, Ch^r, Em^r; multicopy complementation vector	(Visick 1997)
pJM1D4	pCR®2.1-TOPO® derivative containing 2.45kb wild type *gacA* gene	(This study)
pJM1C9	pEVS79 derivative containing 2.45kb EcoRI fragment of *gacA*	(This study)
pJM1C8	JM1C9 derivative with random EZ::TN<KAN-2> insertion in *gacA*	(This study)
pVCW9G3	pV08 derivative containing 2.19kb pstI, sallI fragment of *gacA* from JM1C9	(This study)
pJM3D4	Wild type putative host factor-I (*hfq*) in pCR®2.1-TOPO®	(This study)
pJM3D6	pEVS79 derivative containing *hfq* from pJM3D4	(This study)
pJM3E1	pV08 derivative containing 671bp EcoRI fragment with *hfq* from pJM3D6	(This study)
pAEB2H8	pV08 derivative containing *csrb1* from *V.fischeri*	(Ballok 2007)
pJM2G5	VP16SB (16s) in pCR®2.1-TOPO® Km^r, Ap^r	(This study)
pJM2G7	VP1247 (*serC)* in pCR®2.1-TOPO® Km^r, Ap^r	(This study)
pJM2F5	VPA1314 (*tdh)* in pCR®2.1-TOPO® Km^r, Ap^r	(This study)
pJM2H7	VPA1342 (putative Type III secretion protein *spa24)* in pCR®2.1-TOPO® Km^r, Ap^r	(This study)
pJM2F8	VP2448 (protease) in pCR®2.1-TOPO® Km^r, Ap^r	(This study)

Ch, chloramphenicol; Em, erythromycin; Km, kanamycin; Ap, Ampicillin

Table 3.2. Metabolic and virulence associated phenotypes in a *gacA* mutant[a]

	Cytotoxicity[b]				Survival		Growth	Rate[g]		
	28°	37°	Lipase	Protease[c]	Oxidation[d]	Starvation[e]	Yield[f]	28°	37°	Sugars[h]
BB22	1.0 (0.02)	1.0 (0.36)	+	2.5 (0.5)	6.0 (0)	306%	3.3	3.5	2.0	+
JM1F3	0.6 (0.04)	0.96 (0.33	-	1.0 (0)	7.3 (0.33)	0.28%	1.8	2.8	2.0	-
LM5674	0.7 (0.02)	0.77 (0.13	+	24.5 (1.5)	7.3 (0.33)	279%	2.5	3.8	2.5	+
BB22+pV08	1.0 (0.06)	1.0 (0.27)	+	2.0 (0)	21.3 (0.67)	16.6%	1.7			+
JM1F3+pV08	0.079 (0.16)	1.0 (0.08)	-	0 (0)	8.3 (0.33)	0.15%	1.2			-
BB22+pVCW9G3	0.9 (0.01)	1.12 (0.51	+	2.0 (0)	23.0 (1.00)	10.8%	1.8			+
JM1F3+pVCW9G3	1.0 (0.06)	1.06 (0.18	+	2.0 (0)	21.0 (1.00)	10%	1.4			+

[a]All phenotypes done at 37° unless otherwise specified
[b]Fluoresence relative to BB22 (SE)
[c]Diameter of clearing in mm (SE)
[d]diameter in mm (SE) from 5µl bleach
[e]% survival after 24 hours of growth
[f]growth yield is maximum OD_{600}
[g]values are in hours
[h]growth on glucose, galactose, fumarate and glycerol

Table 3.3. Complementation of hyper-hemolysis at 37°

Strain	Qualitative[a]	Quantitative[b]
BB22 (Wild type)	1.0	3.0 (0.01)
JM1F3 (*gac* ::Tn)	3.0	5.5 (0.03)
LM5674 (*opaR*)	2.0	3.2 (0.64)
JM1G1 (*csrA,gacA* ::Tn)	0.5	2.5 (0.16)
BB22+pVO8 (empty vector)	1.0	2.9 (0.04)
JM1F3+pVO8 (empty vector)	2.0	7.0 (0.04)
BB22+pVCW9G3 (*gacA in trans*)	2.0	5.1 (0.20)
JM1F3+pVCW9G3 (*gacA in trans*)	3.0	4.5 (0.12)
BB22+pAEB2H8 (*csrb1 in trans*)	0.5	4.3 (0.06)
JM1F3+pAEB2H8 (*csrb1 in trans*)	1.0	2.8 (0.03)
BB22+pJM3E1 (*hfq in trans*)	0.5	4.1 (0.27)
JM1F3+pJM3E1 (*hfq in trans*)	1.0	3.3 (0.52)

Hemolysin activity was very low at 28°

[a]Kanagawa Phenomenon (KP) Diameter of clearing in mm from triplicate samples on Wagatsuma agar, SE=0

[b]Absorbance (OD_{415}) normalized by cell density (OD_{600}) of triplicate cultures ± (SE).

Table 3.S1. Fold change of genes at 28° and 37° in JM1F3 compared to BB22

Locus	Fold Change		Annotation
	37°	28°	
VP0047	-2.4		peptide ABC transporter, ATP-binding protein
VP0048	-6.7	-6.7	peptide ABC transporter, periplasmic peptide-binding protein
VP0049	-5.4	-6.9	peptide ABC transporter, permease protein
VP0050	-2.5		peptide ABC transporter, permease protein
VP0054	2.3		hypothetical protein
VP0055	2.2		putative RNA polymerase ECF-type sigma factor
VP0056	2.1		hypothetical protein
VP0111	-6.6	-6.1	hypothetical protein
VP0170	-2.2		putative transmembrane ABC transporter protein
VP0293	-2.2		sulfate adenylate transferase, subunit 1
VP0294	-2.9		hypothetical protein
VP0294_x	-3.0		hypothetical protein
VP0295	-2.7		putative sodium sulfate symporter
VP0296	-3.7		adenylylsulfate kinase
VP0311	-2.1		inorganic pyrophosphatase
VP0320	-3.3	-4.6	conserved hypothetical protein
VP0321	-3.7	-3.2	hypothetical protein
VP0321	-3.9	-3.6	hypothetical protein
VP0323	-2.9	-3.0	immunogenic protein
VP0331	-2.8		conserved hypothetical protein
VP0332	-2.4		conserved hypothetical protein
VP0355	2.0		putative LuxZ
VP0363	-3.9		glycerol dehydrogenase GldH
vp0365a	-2.8		
VP0366	-2.8		putative phosphoenolpyruvate-protein phosphotransferase (phosphotransferase system, enzyme I)
VP0376	-2.7		hypothetical protein
VP0378	-4.8		hypothetical protein
VP0379	-5.0		putative ABC transporter substrate binding protein
VP0482	-2.6		glutamate synthase, large subunit
VP0484	-3.8		glutamate synthase, large subunit
VP0515	-2.1		conserved hypothetical protein
VP0593	-3.1		inositol monophosphate family protein
VP0625	-2.7		hypothetical protein
VP0629	-2.0		homocysteine synthase
VP0634	-2.6		hypothetical protein
VP0752	-5.6		conserved hypothetical protein
VP0797	-2.8		cysteine synthase A
VP0817	-2.1		selenoprotein W-related protein

VP0842	-3.0		citrate synthase
VP0997	-2.3		hypothetical protein
VP0998	-3.2		amino acid ABC transporter, ATP-binding protein
VP0999	-3.0		amino acid ABC transporter, periplasmic amino acid-binding protein
VP1011	-2.3		isocitrate dehydrogenase
VP1012	-2.1		cold shock-like protein CspD
VP1076	2.8		hypothetical protein
VP1103	-2.9		alanine dehydrogenase
VP1252	-2.8		hypothetical protein
VP1253	-2.2		NifS-related protein
VP1256	-2.4		transporter, NadC family
VP1264	2.1		hypothetical protein
VP1318	-3.0		putative flippase
VP1319	-3.0		hypothetical protein
VP1320	-2.9		putative CDP-ribitol pyrophosphorylase
VP1321	-2.7		putative dTDP-glucose 4-6-dehydratase
VP1322	-2.4		putative LicD1 protein
VP1325	-2.2		hypothetical protein
VP1326	-2.3		hypothetical protein
VP1374	-6.1		putative transporter
VP1433	-2.0		hypothetical protein
VP1434	-2.4		putative V10 pilin
VP1516 to VP1517	-3.8		Intergenic region
VP1517	-2.2		putative Rhs-family protein
VP1519	-2.9		hypothetical protein
VP1521	-2.6		hypothetical protein
VP1751	-4.4		homoserine O-succinyltransferase
VP1752	-2.2		putative fimbrial biogenesis and twitching motility protein
VP1861	-2.5		hypothetical protein
VP1879	2.3		serine transporter
VP1944	-4.5		excinuclease ABC, subunit C
VP1945 to VP1946	4.6	19.4	Intergenic region
VP1975	-3.6		hypothetical protein
VP1999	3.3		conserved hypothetical protein
VP2002	-2.3		hypothetical protein
VP2011	2.9		tetrathionate reductase, subunit B
VP2014	3.7		putative tetrathionate reductase, subunit A
VP2015	3.9	13.4	putative cytochrome c
VP2016	27.4	156.1	hypothetical protein
VP2080	-2.2		ABC transporter substrate-binding protein
VP2087	-4.9	-6.1	oligopeptide ABC transporter, ATP-binding protein
VP2088	-3.9	-5.5	oligopeptide ABC transporter, ATP-binding protein

VP2089	-6.5	-6.7	oligopeptide ABC transporter, permease protein
VP2090	-9.7	-7.8	oligopeptide ABC transporter, permease protein
VP2091	-2.9	-2.6	oligopeptide ABC transporter, periplasmic oligopeptide-binding protein
VP2122		3.9	putative channel protein
VP2201		5.4	conserved hypothetical protein
VP2326	-12.6		acetyltransferase-related protein
VP2329	-4.1		efflux pump component MtrF
VP2495	-2.3		aconitate hydratase 2
VP2544	-2.7		oxaloacetate decarboxylase, alpha subunit
VP2545	-3.7		putative oxaloacetate decarboxylase gamma chain 1
VP2593	-4.3		D-3-phosphoglycerate dehydrogenase
VP2717	-2.5		cobalamin-dependent methionine synthase
VP2761	-2.7		phosphoenolpyruvate carboxylase
VP2765	-2.4		cystathionine gamma-synthase
VP2792	-3.3		phosphoribulokinase
VP2794	-3.8		conserved hypothetical protein
VP2795	-4.1		succinylglutamate 5-semialdehyde dehydrogenase
VP2796	-7.9		putative arginine ornithine succinyltransferase
VP2797	-2.7		acetylornithine aminotransferase
VP2917	-2.5		hypothetical protein
VP2918	-2.1		hypothetical protein
VP3012	-2.1	-4.0	hypothetical protein
VPA0096	-4.6		outer membrane protein OmpW
VPA0101	2.4		hypothetical protein
VPA0102	3.9		putative isomerase
VPA0125	-2.9		oxidoreductase, short-chain dehydrogenase reductase family
VPA0126	-2.4		putative acetyltransferase
VPA0175to VPA0176C			
OMP	-4.5		Intergenic region
VPA0192	-2.7		tryptophanase
VPA0285	4.2		hypothetical protein
VPA0286	5.5		chaperonin, 10 kDa subunit
VPA0287	4.8		chaperonin, 60 kDa subunit
VPA0319_s	3.5		hypothetical protein
VPA0319	4.0		hypothetical protein
VPA0328	-3.6		putative translation elongation factor G
VPA0391 to VPA0392	2.2		Intergenic region
VPA0393	2.7		hypothetical protein
VPA0588	-4.3		hypothetical protein
VPA0638	-2.2		arginine ABC transporter, permease protein
VPA0737	2.1		putative two-component response regulator
VPA0738	2.0		putative fimbrial protein Z, transcriptional regulator

		(LuxR UhpA family)
VPA0851	-6.3	putative formate transporter 1
VPA0859	-2.9	putative lipase
VPA0933	2.0	conserved hypothetical protein
VPA0994	3.7	putative membrane protein
VPA1000	3.0	methyl-accepting chemotaxis protein
VPA1025	-2.3	hypothetical protein
VPA1027	-2.8	hypothetical protein
VPA1030	-2.7	hypothetical protein
VPA1031	-3.2	hypothetical protein
VPA1032	-3.6	hypothetical protein
VPA1033	-2.8	hypothetical protein
VPA1034	-3.9	conserved hypothetical protein
VPA1035	-3.8	conserved hypothetical protein
VPA1036	-3.2	hypothetical protein
VPA1037	-2.6	probable phosphoprotein phosphatase
VPA1038	-2.7	hypothetical protein
VPA1039	-2.3	hypothetical protein
VPA1041	-3.7	hypothetical protein
VPA1042	-2.9	hypothetical protein
VPA1043	-2.8	hypothetical protein
VPA1044	-3.9	hypothetical protein
VPA1073	-2.2	putative alkaline phosphatase
VPA1204	-2.1	acetyl-CoA acetyltransferase
VPA1215	-2.4	hypothetical protein
VPA1278	2.3	hypothetical protein
VPA1279	2.9	diacylglycerol kinase
VPA1314_s	2.4	thermostable direct hemolysin A
VPA1323	2.0	hypothetical protein
VPA1324	3.3	hypothetical protein
VPA1328	2.8	hypothetical protein
VPA1329	3.5	putative traA protein
VPA1331	2.0	putative OspC2
VPA1334	2.5	hypothetical protein
VPA1335	2.3	putative type III secretion apparatus protein
VPA1336	2.8	hypothetical protein
VPA1339	2.5	putative type III secretion system EscC protein
VPA1340	3.9	hypothetical protein
VPA1341	5.1	putative Spa29, component of the Mxi-Spa secretion machinery
VPA1342	4.9	putative Type III secretion protein Spa24
VPA1345	2.1	hypothetical protein
VPA1346	2.5	putative targeted effector protein YopP
VPA1346 to VPA1347	2.1	Intergenic region
VPA1346 to	2.8	Intergenic region

VPA1347			
VPA1348	3.0		putative transcriptional activator ToxR
VPA1349	2.7		putative Type III secretion protein Spa33
VPA1350	2.5		hypothetical protein
VPA1351	3.0		hypothetical protein
VPA1352	3.4		hypothetical protein
VPA1353	3.1		putative outer membrane protein
VPA1354	2.7		putative type III secretion system EscU protein
VPA1355	3.5		putative type III secretion system EscV protein
VPA1356	2.7		hypothetical protein
VPA1358	3.2		putative dimethyladenosine transferase
VPA1359	3.0		hypothetical protein
VPA1360	2.9		hypothetical protein
VPA1361	2.5		hypothetical protein
VPA1362	3.8		putative secreted protein EspD
VPA1363	3.7		putative chaperone
VPA1364	7.6		hypothetical protein
VPA1365	5.0		putative two-component response regulator
VPA1366	3.3		hypothetical protein
VPA1367	6.4		putative type III secretion system lipoprotein precursor EprK
VPA1368	7.2		hypothetical protein
VPA1370	3.3		hypothetical protein
VPA1372	3.6		hypothetical protein
VPA1380	3.2		putative OspB protein
VPA1403	-2.4		putative capsular polysaccharide biosynthesis glycosyltransferase
VPA1404	-2.4		hypothetical protein
VPA1405	-3.9		putative polysaccharide export-related protein
VPA1406	-3.6		putative exopolysaccharide biosynthesis protein
VPA1407	-3.0		hypothetical protein
VPA1408	-2.3		putative lipopolysaccharide biosynthesis protein
VPA1409	-2.5		hypothetical protein
VPA1410	-3.4		hypothetical protein
VPA1411	-3.0		putative glycosyltransferase
VPA1412	-2.7		hypothetical polysaccharide biosynthesis related protein
VPA1413	-2.3		hypothetical protein
VPA1432	2.3		putative two-component response regulator
VPA1444	-2.1		putative transport protein
VPA1498	-9.1	-6.7	putative L-lactate permease
VPA1499	-9.9		L-lactate dehydrogenase
VPA1609	-9.3	-5.6	proton glutamate symporter
VPA1621	2.9		hypothetical protein

All genes at both temperatures, PPDE (>p)=≤ 0.90

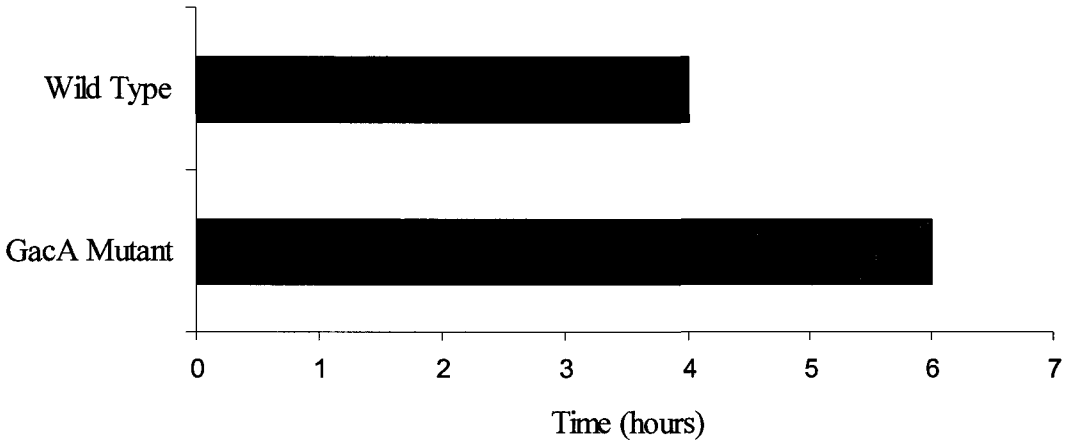

Figure 3.1. Infection and onset of symptoms in mice. The GacA mutant was delayed at initiation of symptoms in infant mice compared to wild type (6 mice per treatment p=<0.001).

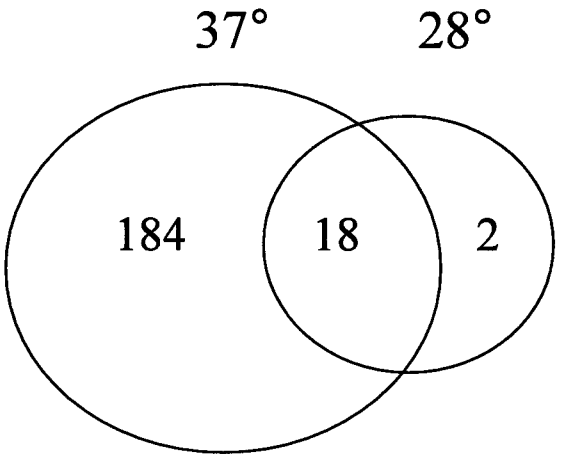

Figure 3.2. GacA regulon at two temperatures. Number of genes differentially regulated at two temperatures (28° and 37°) in a GacA mutant compared to wild type with a PPDE (p<1) cut off of 0.90. Out of all ORF's in the genome, 4.2% were influenced by a GacA mutation at 37°, 0.41% at 28°, and 0.37% genes were influenced at both temperatures.

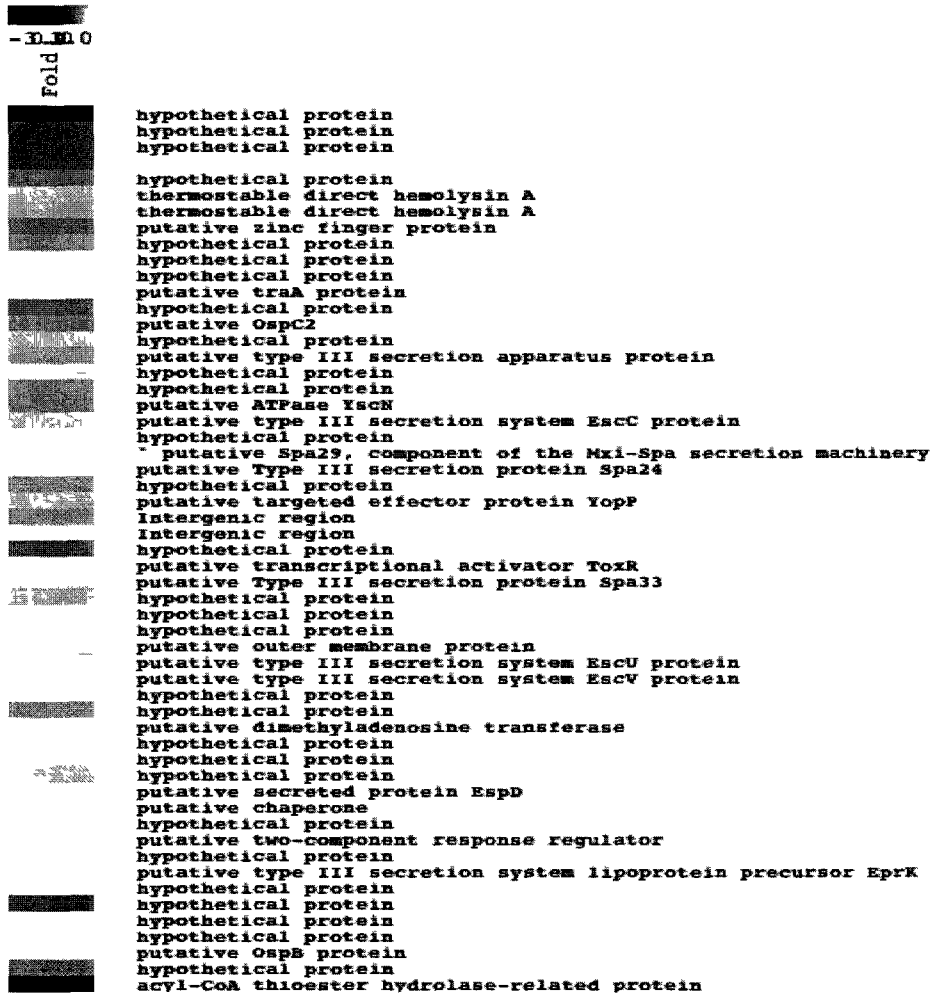

Figure 3.3. Heat map of VPaI-7. The fold change of VPaI-7 and its flanking genes at 37° C in a GacA mutant compared to wild type represented by heat map. Black represents downregulation and white is upregluation in a microarray analysis.

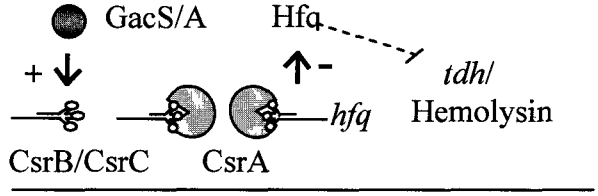

Figure 3.4. Proposed model of GacA repression of *tdh,* mediated by CsrA and Hfq.

CHAPTER IV

COMPARATIVE GENOMICS AND TRANSCRIPTOMICS OF THE GacA REGULONS IN *VIBRIO FISCHERI* AND *VIBRIO PARAHAEMOLYTICUS* REVEAL IMPORTANT EVOLUTIONARY CHANGES AND NOVEL VIRULENCE MECHANISMS

ABSTRACT

Vibrio spp. are closely related and share a conserved core genome that likely allows for general aquatic survival. Genes that have been acquired horizontally by individual species may contribute to the unique lifestyles (i.e. symbiosis or pathogenesis) seen in *Vibrio spp.* A better understanding of how new genes are acquired and assimilated into an existing and conserved host association pathway may shed light on the process that underlies the divergence of closely related species. We propose that exploitation of an overlapping core genome and transcriptome shared by a human pathogen and squid symbiont will reveal insights into the evolution of species specific host association and the emergence of novel virulence mechanisms. In this study we utilized a combined approach of comparative genomics and transcriptomics to examine the conserved GacA regulons in a pathogen and symbiont. *V. parahaemolyticus*, is an emerging human pathogen whose virulence mechanisms are unique from other *Vibrio*

spp. and are poorly characterized. *Vibrio fischeri* is the mutualistic symbiont of *Euprymna scolopes* and shares a long evolutionary history with its host. These two organisms were selected for comparative analysis as they are closely related but take part in dramatically different lifestyles. This work revealed a small GacA activated transcriptional core comprised of a majority of genes involved in central metabolism, consistent with previously characterized GacA regulons. However several distinct alterations specifically within the *V. parahaemolyticus* GacA circuitry allude to a rewiring of this regulon to exclude biofilm and iron metabolism genes and possibly circumvent quorum sensing machinery. This reorganization was not seen in *V. fischeri* in which the GacA regulon remains intact compared to other characterized regulons. In addition, the GacA regulon of *V. parahaemolyticus* had a larger subset of unique genes (not found within the core shared by *V. fischeri*) including select GacA repressed horizontally acquired genes. Unlike in the pathogenic *V. parahaemolyticus*, the symbiont, *V. fischeri* had a much smaller group of unique GacA regulated genes but had a much larger GacA differentially regulated core. Interestingly, we observed a trend in GacA mediated gene expression whereby genes with a %GC content lower than the genome average (including VPaI-7 containing *tdh* and T3SS components) were subject to GacA repression (while the shared core of orthologous genes were GacA activated) in the pathogen but not in the symbiont. A fundamental understanding of how related bacteria evolve to take part in symbiosis or pathogenesis could provide important information regarding the evolution of novel virulence mechanisms that utilize existing cellular circuitry.

BACKGROUND

The family *Vibrionaceae* has evolved to encompass diverse species that specialize in the occupation of specific niches and lifestyles. Bacterial host association within this group can span from vertebrate and invertebrate symbiosis to human pathogenesis (Thompson et al. 2004). Several of these associations have co-evolved to be highly specific and exclusionary to other microbes. *Vibrio fischeri*, for example, is housed in a specialized light organ within its mutualistic symbiont the Hawaiian Bobtail squid. This symbiosis is initiated by a handful of cells that are able to survive a gauntlet of host defenses, enter the light organ and induce morphological host changes that prevent any further colonization (Whistler et al. 2003). This intimate association provides the host with protection from predation and likely supplies a nutrient rich, protected niche for the bacterial population (Jones et al. 2006).

In contrast to a symbiotic lifestyle, *Vibrio parahaemolyticus* causes gastroenteritis when introduced into a human host. This, likely unintentional, infection occurs upon ingestion of raw or undercooked seafood, particularly oysters (Shiraih et al. 1990). Unlike the precision of the *V. fischeri* relationship with the squid, *V. parahaemolyticus* does not share an extensive co-evolutionary history with its human host. Consequently, this interaction from unintentional ingestion results in virulence facilitated by mechanisms truly intended for an as yet unidentified natural host(s).

Despite differences in host association and outcomes, *V. fischeri* and *V. parahaemolyticus* face similar obstacles to colonization of their respective hosts. Both species exist in a dynamic aquatic environment requiring flexibility in adaptation to changing conditions such as fluctuations in, among others, nutrient availability,

temperature, osmolarity, and pH. During colonization, both *V. fischeri* and *V. parahaemolyticus* must survive entry into the host environment and resist host immunity within the human gut or squid light organ (Lamarcq et al. 1998). In addition, both species must be able to effectively recognize and communicate with a suitable host and to respond with appropriate changes in gene expression once an association has been established. It is likely that general host association capabilities, along with conserved central metabolic process are a function of the core "*Vibrio*" genome thought to support general aquatic fitness, while host-specific functions may be reserved for genomic material acquired horizontally (Reen et al. 2006).

Extensive work has been done to describe the genomic plasticity of *Vibrio*s. All members of this genera possess two chromosomes, the larger, Chromosome I is comprised of mainly housekeeping genes and is involved in cellular survival and reproduction. Chromosome II is smaller and appears to be the initial resting place for DNA acquired horizontally through bacteriophage, natural competence or bacterial conjugation (Gu et al. 2009, Han et al. 2008). For example, vibriophage was recently found to facilitate the transfer of a major *Vibrio cholerae* virulence gene (CTX□) from one strain of *Vibrio cholerae* to another through transduction (Choi et al. 2010). Mobile segments of DNA including integrons, super-integrons, Integrative Conjugative Elements, transposons, and plasmid bound genes can all be introduced by bacterial mating or conjugative transfer. Evidence of these mobile elements can be seen throughout the *Vibrio* genomes including super-integrons found in both *V. cholerae* and *V. parahaemolyticus* (Rowe-magnus et al. 2001). Though rare among bacteria, *Vibrio spp.* are able to take up naked DNA from the environment when in the presence of chitin

through natural competence. This unique ability provides additional opportunities for enhancement of existing host associations and a potential increase in the breadth of available hosts (Meibom et al. 2005).

Although Chromosome II houses many horizontally acquired genes and is correlated with host specificity (Izutsu et al. 2008, Boyd et al. 2008), Chromosome I is not entirely divorced from this process. Embedded within this portion of the genome exist important conserved regulatory mechanisms that manage not only housekeeping genes, but also virulence and host association traits acquired horizontally (Makino et al. 2003, Ruby et al. 2005). This communication between new and old genes is critical for coordinated gene activation and repression in response to host and environmental cues. For example the Ler regulator found in the locus of enterocyte effacement island of Enterohemorrhagic *Escherichia coli* influences both horizontally acquired and backbone genes through relief of H-NS repression. Globally, the Ler regulator acts to integrate acquired genes into existing virulence regulons for proper expression in response to environmental signals (Abe et al. 2008). Additionally, in *Salmonella* Typhimurium, the ancient two component regulator SirA (GacA in *V. parahaemolyticus* and *V. fischeri*) coordinately regulates both conserved motility traits as well as acquired virulence genes within a pathogenicity island (Goodier et al. 2001). GacA in *V. fischeri* and *V. cholerae* is found on Chromosome I and has been linked to regulation of both conserved and horizontally acquired genes including the Cholera Toxin (Ruby et al. 2005, Wong et al. 1998). Interestingly, colonization is mediated by GacA in both *V. fischeri* and *V. parahaemolyticus*, despite the dramatically different outcomes of host associations (Whistler et al. 2003, Chapter III).

In addition to GacA, a large number of conserved genes and regulatory circuitry exist across the genus, yet *Vibrio spp.* utilize unique mechanisms of symbiosis and pathogenicity within their respective host. This illustrates the possibility that orthologous genes that have undergone subtle changes in gene expression patterns within a single species, could contribute to novel host associations and outcomes. GacA has been tightly linked to host association in several *Vibrio spp.* including, *V. fischeri* (Whistler et al. 2003), *V. cholerae* (Wong et al. 1998), *V. vulnificus* (Gauthier er al. 2010) and *V. parahaemolyticus* (Chapter III), and would therefore serve as an ideal regulon to interrogate for unique GacA mediated expression patterns that may have conferred enhanced colonization traits. These important evolutionary changes in gene activation or repression would not be captured by simple genomic comparisons. Therefore, a transcriptomics approach would help to identify global changes in GacA mediated transcriptional activation or repression and may reveal some of the resulting downstream transcriptional changes (Hassan et al. 2010, Sahu et al. 2003). Though GacA is known to exert its regulatory control at the level of translation, the genes in this regulon that are subject to CsrA mediated repression may themselves be transcriptional activators or repressors. We propose that a comparative examination of the conserved GacA regulons in both *V. fischeri* and *V. parahaemolyticus* will reveal important insights into the regulatory mechanisms involved in each lifestyle. We theorize that both acquired and conserved genes will be involved in both lifestyles and that unique patterns of gene activation or repression will govern species specific host association. This approach will provide a valuable perspective on the evolution of regulatory networks that would be missed through comparison of genomic content alone.

In this study we utilized both comparative genomics and comparative transcriptomics to identify shared core and unique acquired genes potentially involved in host association in two *Vibrio*s that participate in different lifestyles. *V. fischeri* strain ES114 is a very well characterized mutualistic symbiont, was the first sequenced strain of *V. fischeri*, and has been used in a number of microarray studies (Lupp et al. 2005, Chun et al. 2008, Antunes et al. 2007). Though not currently sequenced, pre-pandemic *V. parahaemolyticus* strain BB22 has 95% similarity to the sequenced pandemic strain RIMD2210633, is well characterized especially for motility, is amenable to genetic manipulation, and has also been the subject of microarray analysis (Chapter II). We focused this study on genes regulated by the conserved GacA global regulator which influences a variety of functions in cells, but is notably involved in host association in both *V. fischeri* and *V. parahaemolyticus* (Whistler et al. 2003, Chapter III). Due to their association with GacA in other organisms, some of the core genome involved in metabolism, iron acquisition and transport, oxidative stress, motility and protease production were expected to be GacA-regulated and part of a GacA shared core (Whistler et al. 2003, Chapter III, Lenz et al. 2005, Wong et al. 1998, Gauthier et al. 2010, Heeb et al. 2001, Hassan et al. 2010). However, we anticipate that some of the core genome may be regulated in only one organism, reflective of the loss of ancient regulatory connections in one organisms or the development of new circuitry as a result of adaptation and specialization by each organism, specifically a GacA differentially-regulated core. Additionally, a portion of the unique genomes, some of which are horizontally acquired elements, is also likely to have been incorporated into this regulon deemed the GacA unique gene regulon.

Through direct comparison, we have defined the conserved GacA regulatory core, and identified species-specific GacA regulated genes that may play important roles in symbiosis and pathogenesis. We have also identified a unique GacA controlled subset of genes that are likely to have been horizontally acquired and integrated into this conserved regulon. This subset of genes may be relevant to virulence in *V. parahaemolyticus* and are ideal candidates for further evaluation for a role in host association. In addition, we have characterized the GacA regulon in *V. parahaemolyticus* and found that some components have been rewired while others remain conserved alluding to the evolutionary origin of this regulon, only some of which has remained intact in both organisms.

METHODS

Bacterial Strains A wild type strain of *Vibrio fischeri* ES114 and *Vibrio parahaemolyticus* BB22 were used as control strains in all microarray experiments. A deletion in the *gacA* gene of *Vibrio fischeri* (VCW2F5) was created as previously described (Whister et al. 2003). The *gacA* gene of *Vibrio parahaemolyticus* was mutated with an EZ::TN<KAN-2> kit from Epicentre, (Madison, Wisconsin) to create JM1F3 in previous work (Chapter III).

Microarray growth conditions and RNA purification. BB22, ES114, JM1F3 and VCW2F5 were grown at both 28° and 37° in HI broth to an OD_{600} of ~0.5 and immediately harvested with RNA Protect (Qiagen, Valencia, CA) as previously described (Chapter III). RNA was purified using an RNeasy minikit (Qiagen) following

manufacturer's instructions and cDNA synthesis, labeling and DNA hybridization were all performed by the Microarray Facility at the University of California Irvine.

Microarray analysis. An Affymetrix custom 3' GeneChip containing all ORF's from the *Vibrio parahaemolyticus* and *Vibrio fischeri* genome was designed as previously described (Gode-Potratz, 2010). Gene Chip-Robust Multiarray Averaging was used to background correct, normalize across replicate chips, and to calculate and summarize probe level intensity. A Cyber T analysis was performed to identify differentially expressed genes (Long et al. 2001). Only those genes with a PPDE(<p) value of $\geq$ 0.90 and a fold change in gene expression of $\geq$2.0 in a *gacA* mutant compared to wild type in either *V. fischeri* or *V. parahaemolyticus* were selected for further analysis.

Identification of putative horizontally acquired elements through IslandPath analysis. The IslandPath program identifies genes that may have been acquired horizontally based on specific sequence signatures including, dinucleotide bias, abnormal %G+C compared with overall genome %G+C content, proximity to transfer and structural RNA and the presence of mobility genes (transposase and integrase) (Hsiao 2003). All genes within both chromosomes of *V. parahaemolyticus* and *V. fischeri* have been characterized using the IslandPath program (http://www.pathogenomics.sfu.ca/islandpath/update/IPindex.pl) and are publicly available through the IslandPath website.

RESULTS AND DISCUSSION

A combined approach of comparative genomics and transcriptomics reveals a surprisingly small GacA-activated core shared by *V. fischeri* and *V. parahaemolyticus*. Comparative genomics is a useful tool in the examination of existing

genetic material across two or more organisms. This approach can reveal important information regarding the evolutionary history of genes; specifically those acquired horizontally or through duplication and specialization, and those lost, and can offer valuable insight into the divergence of closely related species. A comparative genomic analysis of *V. parahaemolyticus* with *V. fischeri* revealed a large core genome containing 3153 orthologous genes (Cooper et al. 2010), hereafter referred to as the "core genome." This analysis compared (by BLASTp) the genomes of *V. parahaemolyticus* and *V. fischeri* to establish a true *Vibrio* core genome that is likely essential for general survival in an aquatic habitat. Given that GacA is highly conserved across *Vibrio* spp, we expected to find many genes previously associated with this global regulator in the core genome. Indeed, genes involved in metabolism, iron acquisition, oxidative stress, motility and protease production were identified as part of the core genome. Though this work defined a large number of shared genes, the remaining 1679 unique genes in *V. parahaemolyticus* and 649 unique genes in *V. fischeri* represent a sizable group too large to interrogate on a gene by gene basis for possible roles in symbiosis or pathogenesis. In addition, though gene content may be exclusive to one species, this does not guarantee that it plays a role in host association, nor does it provide any information regarding functional expression of these genes.

Limitations in the type and breadth of analysis available from comparative genomics can be addressed with comparative transcriptomics providing a powerful two fold analysis of genome content and regulation. For example, detection of a newly acquired pathogenicity island through genomics as well as characterization of its functional expression in response to a host cue through transcriptomics can expose and

define newly acquired virulence mechanisms. In addition, genomes with nearly identical content can function differently as a result of subtle changes in gene activation or repression that would be missed in stand alone genomics. Therefore, we utilized a comparative trancriptomics approach focused on a global regulon of colonization, layered upon the comparative genome to reveal important insights into host association, mechanisms of specialization, and the evolution of gene networks.

Our examination of the GacA transcriptomes revealed a small set of core orthologous genes regulated in both organisms that represents only a fraction of the core genome (19 genes in the GacA shared core compared to 3153 in the core genome). This is an important and more focused subset of genes that are functionally expressed in each species and may be relevant to general aquatic survival. Given the similarities in natural habitat, the size of the GacA shared core was unexpectedly small though the genes present in this group were expected given other characterized regulons. The majority of these 19 genes (Table 4.1.) are annotated as regulators or enzymes involved in central metabolism responsible for general survival and reproduction, and many have been identified in other GacA regulons (Wong et al. 1998, Whistler et al. 2003, Lupp et al. 2005, Lenz et al. 2005, Chun et al. 2008, Antunes et al. 2007, Gauthier et al. 2010, Chapter III). The inclusion of central metabolism as a core component of the *gacS/gacA* regulon has been confirmed by microarray analysis in many different species and genera including, but not limited to, Vibrio (Chapter III), Pseudomonas (Hassan et al. 2010, Girard et al. 2006), Escherichia (Sahu et al. 2003), and Xylella (Shi et al 2009). It is important to note that GacA regulation of central metabolism has been conserved despite other, sometimes dramatic, changes in the organization of this regulon. When *V. fischeri*

and *V. parahaemolyticus* were grown *in vitro* (at 28° and 37° respectively), all genes in this regulatory core were GacA activated. This implies that the GacA shared core supports essential cellular functions, not specific host association.

Even though only a small number of true orthologs are GacA-controlled, further comparisons of the transcriptomes revealed a number of genes that were annotated with similar, if not identical, functions and so are tentatively identified as analogs (Table 4.2.). An additional 18 genes with roles in metabolism, transport, and chemotaxis were identified through this analysis. With the exception of the chemotaxis protein which was GacA repressed (in both species), all other functional analogs were GacA activated; consistent with the GacA shared core. The incorporation of analogous genes into the appropriate regulatory network is a striking example of the efficiency and precision with which the cell manages its regulatory components. Specifically, orthologous genes and networks that are conserved across all *Vibrio* species are likely the result of a common ancestor and have been maintained throughout evolution. However in the case of analogous genes, the cell must be able to recognize gene function and therefore sort analogs into the appropriately regulated networks. One interpretation of this process is that GacA regulation can not precede function in the case of the 18 analogs identified in this regulon and must come after the convergent evolution of function. This fascinating and intricate system of regulatory management highlights the complex and ever changing regulatory infrastructure of a bacterial cell. This work underscores the importance of supplementing comparative genomics with functional analyses to identify those genes that may lack sequence similarity or history but are analogous in function.

In addition to the GacA shared core, we also identified two other categories of interest within this regulon. First, a "GacA differentially regulated core" that consisted of genes within the *Vibrio* core that lack a true ortholog but are still under GacA control (Table 4.3). This group in *V. fischeri* consisted of 275 GacA activated and repressed genes involved in metabolism, motility, iron acquisition and other genes common to this regulon. In *V. parahaemolyticus* this group was small (96 GacA activated and repressed genes) but had similar content to the *V. fischeri* group with more unannotated or hypothetical proteins. The second category of interest was the "GacA unique gene regulon" consisting of genes that were found only in one species and were GacA regulated (Table 4.4). *V. fischeri* had 52 genes within this group while *V. parahaemolyticus* had 128 genes. It is interesting to note that *V. parahaemolyticus* contains a larger GacA unique gene regulon which may allude to enhanced virulence potential or, alternatively, may reflect the loss of gene content within the *V. fischeri* regulon as it has co-evolved to a mutualistic lifestyle with its squid host (Figure 4.2). A better understanding of how these genes might have been horizontally acquired and ultimately co-opted into the GacA regulon may reveal important information regarding the evolution of unique lifestyles.

GacA acts as an activator of backbone genes and repressor of horizontally acquired genes in the GacA shared core and GacA unique gene regulons. In order to better understand how the genes of the GacA shared core and GacA unique gene regulons are composed and regulated, we examined each group for traits associated with horizontal gene transfer. In combination with comparative genomics and transcriptomics, an

applied informatics analysis aimed at identifying horizontal genes based on GC content (Island Path analysis) revealed an interesting regulatory pattern that was for the most part, specific to the pathogen *V. parahaemolyticus*. Specifically, all 19 genes in the GacA shared core regulon were GacA activated. The majority of these 19 orthologous genes (57.9% in *V. fischeri* and 84.2% in *V. parahaemolyticus*) had %GC content that was similar to the overall genome average (respectively), in partial agreement with the comparative genomic analysis (Table 4.5). Differences seen in the GacA shared core reported by Cooper et al. and by an informatics approach (Islandpath program) likely reflect differences in the techniques used to generate them. Specifically, the default settings in Islandpath may not have been stringent enough, or the analysis based in part on %GC content, may have been inhibited by the high %AT content of *V. fischeri*. However within the GacA differentially regulated cores (orthologs) we identified a portion of genes with a %GC content differing from the genome average suggesting that they may have been horizontally acquired. This group was larger in the symbiont (*V. fischeri* 42.1%) compared to the pathogen (*V. parahaemolyticus* 15.8%). This is a curious result given that these genes have been classified as core and are orthologous, it may be that acquisition of these genes occurred prior to the divergence of species or may reflect artifacts of the different parameters of the different analyses.

Despite categorization as part of the GacA unique gene regulons, just under half of the genes in *V. parahaemolyticus* (40.6%) and a majority in *V. fischeri* (69%) had %GC content consistent with the genome average by informatics analysis. A large majority of these genes were subject to GacA activation (88.9% in *V. fischeri* and 86.5% in *V. parahaemolyticus*) consistent with the GacA shared core regulon. In addition, *V.*

parahaemolyticus had a much larger group of unique genes in the GacA regulon than *V. fischeri* (54.7% and 30.5% respectively), precisely the opposite of our findings for the GacA differentially regulated core. Interestingly, less than half of the unique genes in *V. parahaemolyticus* (35.3%) whose % GC content fell below the lower limit of the overall genome were subject to GacA activation, with the remaining 67.4% of genes under GacA repression in contrast to the GacA activated orthologous or core genes (Table 4.5).

The specific activation or repression of backbone and horizontal genes in the GacA regulon is an impressive example of a sophisticated bacterial response system. GacA mediates the global activation of conserved genes involved in central metabolism and general cell survival while repressing virulence and other acquired elements. Our work shows that this correlated, at least in part, to differences in the % GC content of acquired genes through a currently undefined mechanism. We propose that GacA selectively represses virulence traits whose expression is resource intensive and too costly for constitutive expression until the appropriate host cues are present. It is plausible that transcriptional activation or repression directed by host signaling and mediated by GacA, allows the cell to down regulate housekeeping genes and redirect cellular resources towards virulence traits once in a host. We have shown that temperature is important in this signaling, though it works additively with GacA and may be reflective of the multi-layered tight transcriptional regulation of acquired elements. Further analysis of the subset of genes subject to GacA repression in the *V. parahaemolyticus* GacA unique gene regulon may yield important virulence and host association factors.

Comparative transcriptomic analysis reveals a reorganization of the conserved GacA circuitry in *V. parahaemolyticus*. The conserved *gacA* regulon is well characterized in a number of pathogenic and symbiotic bacterial species. Genes involved in central metabolism, iron acquisition, motility and oxidative stress resistance are all GacA regulated in *Vibrio spp* and other organisms (Whistler et al. 2003, Chapter III, Lenz et al. 2005, Wong et al. 1998, Gauthier et al. 2010). A combined comparative analysis of the GacA genome and transcriptome in *V. parahaemolyticus* and *V. fischeri* revealed that a significant reorganization has occurred within the *V. parahaemolyticus* regulon. Though some traits remain conserved including swarming motility, oxidative stress and central metabolism, others appear to have been lost. Specifically, biofilm production, iron acquisition and quorum sensing circuitries have been eliminated from the *V. parahaemolyticus* regulon, yet are conserved in *V. fischeri,* based on microarray analysis (Table 4.1. and Table 4.3.).

The loss of quorum sensing components in the V. *parahaemolyticus* GacA regulon is particularly striking given the consistent correlation with GacA in other organisms including *V. cholerae* (Tsou et al. 2011) (Table 4.6 and Figure 4.1). In *V. parahaemolyticus*, quorum sensing is known to regulate expression of targeted genes including biofilm and cytotoxicity mediated by T3SS1 on Chromosome I (Enos-Berlage et al. 2005, Henke et al. 2004). Whereas biofilm production has historically been linked to environmental fitness, recent work has shown that cytotoxicity as a result of T3SS1 is may play a larger role in the natural habitat (Mahoney et al. 2010). Interestingly, T3SS2 found within the VPaI-7 island on Chromosome II is correlated with virulence and regulated by human host temperature and GacA but not by quorum sensing machinery

(Mahoney et al. 2010, Hyoshi et al. 2010). It appears that regulation by quorum sensing may be more valuable for traits important in coordinated behavior that promote survival in the natural habitat rather than in the human host. We propose that a separation of GacA from the quorum sensing circuitry in *V. parahaemolyticus* may represent a significant divergence within this regulatory network. It is possible that the GacA global regulator has become (or is in the process of becoming) a specialized regulator of horizontally acquired elements and therefore virulence mechanisms in *V. parahaemolyticus*.

CONCLUSIONS

Subtle alterations in host association regulons such as GacA could potentially lead to novel symbiosis or virulence mechanisms and might help to explain the emergence of pathogens. V. *cholerae, V. fischeri* and *V. parahaemolyticus* are closely related organisms that all contain the conserved GacA regulator, yet all have distinct virulence, symbiosis and general host association traits (Wong et al. 1998, Whistler et al. 2003, Chapter III). We have shown with comparative genomics and transcriptomics that while portions of the GacA regulon are conserved in *V. parahaemolyticus* an obvious reorganization resulting in the elimination of quorum sensing circuitry has occurred (Figure 4.1). The loss of this significant connection may be indicative of an evolutionary change in the GacA regulon whereby regulation of environmental fitness traits is lost while management of horizontal elements and host association factors is enhanced. In addition, differential gene activation or repression seen in the *V. parahaemolyticus* GacA unique gene regulon provides an example of selective and potentially specialized GacA

transcriptional control. The majority of unique, likely acquired genes in this group were GacA repressed while the core elements were GacA activated. Interestingly, GacA repression does not apply to all horizontally acquired elements but is selectively utilized to control specific genes such as the VPaI-7 island. Overall, this work has revealed unique evolutionary alterations in the GacA regulon in *V. parahaemolyticus* and highlights the importance of conserved global regulators networks in the emergence of novel host association mechanism.

Table 4.1 True orthologs regulated by GacA in both *V.fischeri* and *V. parahaemolyticus*

Probe ID	FoldΔ[a]	Annotation	Probe ID	FoldΔ[a]	Annotation
VF2528	-6.0	Ketol-acid reductoisomerase 2-dehydropantoate 2-reductase	VP0035	-2.2	ketol-acid reductoisomerase
VF2518	-9.5	Dipeptide-binding protein	VP0048	-6.7	peptide ABC transport
VF2111	-4.0	Hypothetical protein	VP0502	-2.6	hypothetical protein
VF0818	-3.8	Citrate synthase	VP0842	-3.0	citrate synthase
VF1775	-4.4	No Annotation	VP1011	-2.3	isocitrate dehydrogenase
VFA0769	-4.1	Agglutination protein	VP1252	-2.8	hypothetical protein
VF1627	-930.1	Response regulator, GacA	VP1945	19.4	response regulator
VF1722	-2.4	Transcriptional activator protein MetR	VP1976	-2.8	transcriptional activator MetR
VF0509	-4.1	Phosphoserine phosphatase	VP2431	-2.4	phosphoserine phosphatase
VF2158	-3.2	Aconitate hydratase 2	VP2495	-2.3	aconitase
VF2106	-2.5	D-3-phosphoglycerate dehydrogenase	VP2593	-4.3	D-3-phosphoglycerate dehydrogenase
VF0337	-2.9	5-methyltetrahydrofolate--homocysteine methyltransferase	VP2717	-2.5	methionine synthase
VF2309	-4.3	Methylenetetrahydrofolate reductase	VP2763	-2.3	5,10-methylenetetrahydrofolate reductase
VF2267	-2.5	Cystathionine gamma-synthase	VP2765	-2.4	cystathionine gamma-synthase
VF2281	-9.0	<not provided>	VP2794	-15.0	hypothetical protein
VF2282	-10.2	Succinylglutamic semialdehyde dehydrogenase	VP2795	-4.1	succinylglutamic semialdehyde dehydrogenase
VF2283	-14.7	Arginine N-succinyltransferase, beta chain	VP2796	-8.4	arginine succinyltransferase
VF2284	-15.7	Acetylornithine Aminotransferase N-SUCCINYL-L,L-DAP Aminotransferase	VP2797	-4.1	acetylornithine aminotransferase apoenzyme
VF2404	-2.6	Transcriptional regulator	VP2920	-3.2	regulator of sigma D

[a]PPDE (<p) values were all $\geq$.90

Table 4.2. GacA regulated analogs defined by functional similarity

	Probe Set ID	FoldΔ	PPDE (<p)
ABC Transporter Components	VFA1043	-2.43	0.99
	VP2080	-2.18	0.91
	VP0379	-4.96	0.98
Cold Shock Proteins	VF1767	-9.80	1.00
	VP1012	-2.11	0.91
Cysteine Synthase	VF1579	-2.90	0.98
	VP0797	-2.80	0.97
Homoserine-O- succinyltransferase	VP1751	-4.38	0.91
	VF2063	-4.82	1.00
Methyl-accepting chemotaxis protein	VPA1000	2.99	0.99
	VF1091	2.74	0.99
	VFA0170	3.56	0.99
	VFA0169	5.46	0.99
Phosphoribulokinase	VP2792	-3.30	0.99
	VF0214	-4.52	1.00
	VF2374	-6.63	1.00
Proton Glutamate Symport Protein	VF1155	-2.12	0.99
	VPA1609	-9.31	0.99

Analogous genes within the GacA regulon identified by functional similarity expanding the GacA shared core regulon.

Table 4.3. Orthologous genes that are GacA regulated in only one species

Probe Set ID	Fold Δ	PPDE(<p)	Annotation
VP0331	-2.45	0.95	conserved hypothetical protein
VP0056	2.10	0.90	hypothetical protein
VPA0391to VPA0392	2.22	0.90	Intergenic region
VP0515	-2.12	0.91	conserved hypothetical protein
*VP1012	-2.11	0.91	cold shock-like protein CspD
VP2011	2.87	0.91	tetrathionate reductase, subunit B
VPA1432	2.32	0.91	putative two-component response regulator
*VP1751	-4.38	0.91	homoserine O-succinyltransferase
VPA0328	-3.63	0.92	putative translation elongation factor G
VP0366	-2.82	0.92	putative phosphoenolpyruvate-protein phosphotransferase (phosphotransferase system, enzyme I)
VP2495	-2.35	0.92	aconitate hydratase 2
VP0293	-2.18	0.92	sulfate adenylate transferase, subunit 1
VP0593	-3.07	0.92	inositol monophosphate family protein
VP0055	2.17	0.93	putative RNA polymerase ECF-type sigma factor
VP0355	2.02	0.93	putative LuxZ
VP0629	-2.04	0.93	homocysteine synthase
VP1879	2.27	0.93	serine transporter
VP0295	-2.72	0.94	putative sodium sulfate symporter
*VP1011	-2.28	0.94	isocitrate dehydrogenase
VP0482	-2.57	0.94	glutamate synthase, large subunit
VPA1444	-2.09	0.94	putative transport protein
VP2765	-2.42	0.94	cystathionine gamma-synthase
VP0054	2.27	0.94	hypothetical protein
VP2761	-2.69	0.95	phosphoenolpyruvate carboxylase
VP0332	-2.36	0.95	conserved hypothetical protein
VP1516 to VP1517	-3.81	0.95	Intergenic region
VP0047	-2.37	0.95	peptide ABC transporter, ATP-binding protein
VP2717	-2.45	0.95	cobalamin-dependent methionine synthase
VP1752	-2.20	0.96	putative fimbrial biogenesis and twitching motility protein
VP1374	-6.12	0.96	putative transporter
VPA1039	-2.33	0.96	hypothetical protein
VPA0638	-2.21	0.96	arginine ABC transporter, permease protein
VPA0588	-4.33	0.96	hypothetical protein
VPA1204	-2.09	0.96	acetyl-CoA acetyltransferase
VP1256	-2.35	0.96	transporter, NadC family
VP1434	-2.40	0.96	putative V10 pilin
VPA1025	-2.33	0.97	hypothetical protein
VP2544	-2.68	0.97	oxaloacetate decarboxylase, alpha subunit
VPA1037	-2.60	0.97	probable phosphoprotein phosphatase

VPA0851	-6.32	0.97	putative formate transporter 1
VPA0101	2.43	0.97	hypothetical protein
VP0376	-2.75	0.97	hypothetical protein
*VP0797	-2.80	0.97	cysteine synthase A
VPA0125	-2.94	0.98	oxidoreductase, short-chain dehydrogenase reductase family
VPA1404	-2.40	0.98	hypothetical protein
VP1999	3.26	0.98	conserved hypothetical protein
VPA0393	2.74	0.98	hypothetical protein
VP2797	-2.72	0.98	acetylornithine aminotransferase
VPA0737	2.11	0.98	putative two-component response regulator
VPA1033	-2.84	0.98	hypothetical protein
VPA1339	2.53	0.98	putative type III secretion system EscC protein
VPA0175to VPA0176	-4.55	0.98	Intergenic region
VPA1034	-3.87	0.98	conserved hypothetical protein
*VP0379	-4.96	0.98	putative ABC transporter substrate binding protein
VP1253	-2.24	0.99	NifS-related protein
VPA1042	-2.89	0.99	hypothetical protein
VP0050	-2.46	0.99	peptide ABC transporter, permease protein
VP1103	-2.85	0.99	alanine dehydrogenase
VPA1348	3.02	0.99	putative transcriptional activator ToxR
VPA0102	3.94	0.99	putative isomerase
*VPA1000	2.99	0.99	methyl-accepting chemotaxis protein
VPA1041	-3.65	0.99	hypothetical protein
VP0320	-3.30	0.99	conserved hypothetical protein
VPA1279	2.87	0.99	diacylglycerol kinase
VP0170	-2.20	0.99	putative transmembrane ABC transporter protein
*VP2792	-3.30	0.99	phosphoribulokinase
VPA1324	3.28	0.99	hypothetical protein
VP0296	-3.69	0.99	adenylylsulfate kinase
VP0999	-2.98	0.99	amino acid ABC transporter, periplasmic amino acid-binding protein
VP1252	-2.75	0.99	hypothetical protein
VPA1044	-3.93	1.00	hypothetical protein
VPA0287	4.77	1.00	chaperonin, 60 kDa subunit
VPA1353	3.12	1.00	putative outer membrane protein
VPA1355	3.47	1.00	putative type III secretion system EscV protein
VP2329	-4.12	1.00	efflux pump component MtrF
VP2794	-3.78	1.00	conserved hypothetical protein
VPA1035	-3.79	1.00	conserved hypothetical protein
*VP0842	-3.01	1.00	citrate synthase
VP2015	3.90	1.00	putative cytochrome c
*VP0323	-2.95	1.00	immunogenic protein
VP2091	-2.93	1.00	oligopeptide ABC transporter, periplasmic

			oligopeptide-binding protein
VP2593	-4.29	1.00	D-3-phosphoglycerate dehydrogenase
VP1944	-4.53	1.00	excinuclease ABC, subunit C
VP2087	-4.95	1.00	poligopeptide ABC transporter, ATP-binding protein
VPA0286	5.52	1.00	chaperonin, 10 kDa subunit
VP0484	-3.78	1.00	glutamate synthase, large subunit
VP1945 to VP1946	4.65	1.00	Intergenic region
VP2795	-4.11	1.00	succinylglutamate 5-semialdehyde dehydrogenase
VP2088	-3.92	1.00	oligopeptide ABC transporter, ATP-binding protein
VP0998	-3.18	1.00	amino acid ABC transporter, ATP-binding protein
*VPA1609	-9.31	1.00	proton glutamate symporter
VP0049	-5.36	1.00	peptide ABC transporter, permease protein
*VP0048	-6.71	1.00	peptide ABC transporter, periplasmic peptide-binding protein
VP2089	-6.54	1.00	oligopeptide ABC transporter, permease protein
VP2796	-7.94	1.00	putative arginine ornithine succinyltransferase
VP2090	-9.68	1.00	oligopeptide ABC transporter, permease protein
VF1427	-9.84	1.00	Sulfatase family protein
VF1459	-3.46	1.00	Translation initiation inhibitor
VF1479	-3.89	1.00	Aquaporin
VF1488	-2.06	1.00	Aspartate aminotransferase
VF1506-1	-4.24	1.00	Iron-regulated protein FrpC, first half , RTX
VF1506-2	-2.65	1.00	Iron-regulated protein FrpC, second half
VF1570	2.32	1.00	TorCAD operon transcriptional regulatory protein TorR
*VF1579	-2.90	0.98	Cysteine synthase
VF1585	-4.07	1.00	Arginine ornithine transport system protein AotM
VF1586	-5.82	1.00	Arginine ornithine transport system protein AotQ
VF1587	-8.56	1.00	Arginine-binding protein
VF1588	-6.79	1.00	Arginine ornithine transport ATP-binding AotP
VF1593	-17.70	1.00	Oligopeptide transport ATP-binding protein OppF
VF1594	-14.60	1.00	Oligopeptide transport ATP-binding OppD
VF1595	-30.80	1.00	Oligopeptide transport system permease OppC
VF1596	-56.73	1.00	Oligopeptide transport system permease OppB
VF1597	-17.76	1.00	Oligopeptide-binding protein OppA
VF1597 to VF1598	-9.36	1.00	intergenic region
VF1605	-3.04	1.00	Hypothetical protein
VF1627	-930.08	1.00	Response regulator, GacA
VF1644	2.15	1.00	L-asparaginase
VF1645	-2.23	1.00	Aspartate-semialdehyde dehydrogenase
VF1646	2.52	1.00	MALATE-2H+ LACTATE-NA+ ANTIPORTER
VF1646 to	-2.53	1.00	intergenic region

VP1647			
VF1722	-2.38	1.00	Transcriptional activator protein MetR
VF1729	-2.31	1.00	<not provided>
VF1730	-3.47	1.00	Sodium proline symporter
*VF1767	-9.80	1.00	Cold shock protein
VF1771	-2.87	1.00	Sensor protein kinase
VF1772	-3.55	1.00	Hypothetical protein
VF1773	-2.08	0.99	Glycosyltransferase
VF1774	-2.24	1.00	Stage V sporulation protein R
*VF1775	-4.42	1.00	Isocitrate dehydrogenase [NADP]
VF1792 to VF1793	-2.10	1.00	intergenic region
VF1795	2.42	1.00	Outer membrane protein C
VF1811	-3.16	1.00	3-ketoacyl-CoA thiolase
VF1889 to VF1890	-2.79	1.00	intergenic region
VF1942	-2.19	1.00	Nitrogen regulatory protein GlnK
VF1964	-2.18	1.00	GlnD, [protein-PII] uridylyltransferase
VF1979	-2.93	1.00	Transcriptional regulators, LysR family
VF2059	-2.73	1.00	Na(+)-linked D-alanine glycine permease
*VF2063	-4.82	1.00	Homoserine O-succinyltransferase
VF2065	11.90	1.00	Nucleoside permease NupC
VF2104	-2.74	1.00	5-formyltetrahydrofolate cyclo-ligase
VF2106	-2.53	1.00	D-3-phosphoglycerate dehydrogenase
VF2110	2.81	1.00	Na(+)-linked D-alanine glycine permease
VF2111	-3.98	1.00	Hypothetical protein
VF2116	-4.18	1.00	Threonine synthase
VF2117	-2.98	1.00	Homoserine kinase
VF2118	-2.80	1.00	Aspartokinase Homoserine dehydrogenase
VF2123 to VF2124	-4.90	1.00	intergenic region
VF2123 to VF2124	-8.64	1.00	intergenic region
VF2124	-10.88	1.00	Glutamate synthase [NADPH] large chain
VF2125	-7.76	1.00	Glutamate synthase [NADPH] small chain
VF2133	-2.40	1.00	periplasmic component of efflux system
VF2138	-2.67	1.00	Two-component sensor histidine kinase
VF2139	-3.59	1.00	Chitooligosaccharide-binding protein
VF2140	-4.03	1.00	Chitooligosaccharide transport system permease protein
VF2158	-3.18	1.00	Aconitate hydratase 2
VF2175	-2.35	1.00	Hypothetical protein
VF2175 to VF2176	-4.23	1.00	intergenic region
VF2217	-5.48	1.00	Cytochrome c1
VF2218	-7.36	1.00	Cytochrome b

VF2219	-8.41	1.00	Ubiquinol-cytochrome c reductase iron-sulfur subunit
VF2219_x	-8.30	1.00	Ubiquinol-cytochrome c reductase iron-sulfur subunit
VF2220	-6.38	1.00	Ubiquinol-cytochrome c reductase iron-sulfur subunit
VF2255	-2.41	1.00	<not provided>
VF2262	-7.67	1.00	Acetolactate synthase small subunit
VF2263	-7.21	1.00	Acetolactate synthase large subunit
VF2266	-2.39	1.00	Aspartokinase Homoserine dehydrogenase
VF2267	-2.48	1.00	Cystathionine gamma-synthase
VF2275	-2.07	0.99	Transcriptional repressor CytR
VF2281	-9.00	1.00	<not provided>
VF2282	-10.17	1.00	Succinylglutamic semialdehyde dehydrogenase
VF2283	-14.74	1.00	Arginine N-succinyltransferase, beta chain
VF2284	-15.74	1.00	Aetylornithinge aminotransferase N-Succinyl-L, L-DAP Aminotransferase
VF2309	-4.33	1.00	Methylenetetrahydrofolate reductase
VF2334	2.41	1.00	Fumarate reductase flavoprotein subunit
VF2335	2.74	1.00	Fumarate reductase iron-sulfur protein
VF2336	2.99	1.00	Fumarate reductase, 15 kDa hydrophobic protein
VF2337	3.07	1.00	Fumarate reductase, 13 kDa hydrophobic protein
VF2362	-2.64	1.00	GGDEF family protein
*VF2374	-6.63	1.00	Phosphoribulokinase
VF2404	-2.58	1.00	Transcriptional regulator
VF2435	-3.08	1.00	Vitamin B12 receptor
VF2439	-2.77	1.00	Soluble pyridine nucleotide transhydrogenase
VF2516	-4.63	1.00	Dipeptide transport system permease,DppC
VF2517	-7.77	1.00	Dipeptide transport system permease, DppB
VF2518	-9.55	1.00	Dipeptide-binding protein
VF2519	-9.77	1.00	Dipeptide transport ATP-binding protein dppD Dipeptide transport ATP-binding protein DppF
VF2520	-4.33	1.00	Cytochrome c5
VF2526_s	-6.58	1.00	Ketol-acid reductoisomerase -dehydropantoate 2-reductase
VF2527_x	-5.84	1.00	<not provided>
VF2551	-5.20	1.00	Hypothetical protein
VF2556	-5.05	1.00	Acetolactate synthase large subunit
VF2557	-4.80	1.00	Hypothetical protein
VF2558	-4.91	1.00	Branched-chain amino acid aminotransferase
VF2559	-4.67	1.00	Dihydroxy-acid dehydratase
VF2560	-4.26	1.00	Threonine dehydratase
VFA0005	-2.07	0.96	Peptide methionine sulfoxide reductase MsrA MsrB
VFA0059	2.61	1.00	TonB-dependent outer membrane receptor
VFA0071	-5.69	1.00	<not provided>

VFA0072	-3.07	1.00	Sensor protein LuxQ
VFA0092	3.72	1.00	Aerotaxis receptor
VFA0106	-3.61	1.00	Beta-mannosidase
VFA0159	-2.30	1.00	Ferrichrome-binding protein
VFA0160	-2.10	1.00	Ferrichrome transport system permease protein FhuB
VFA0165	-2.43	1.00	Ferric aerobactin receptor precursor
*VFA0169	5.46	1.00	Methyl-accepting chemotaxis protein
*VFA0170	3.56	1.00	methyl-accepting chemotaxis protein
VFA0201	2.88	1.00	Hypothetical protein
VFA0272	-2.35	1.00	Integral membrane protein
VFA0274	-4.02	1.00	Acyl-CoA desaturase
VFA0283	-4.76	1.00	Aromatic-amino-acid aminotransferase
VFA0284	-2.96	1.00	ATP-dependent Zn proteases
VFA0285	-3.14	1.00	Amion-acid ABC transporter binding protein transglycosylase
VFA0337	2.62	1.00	Putative glucosyl hydrolase precursor
VFA0338	2.82	1.00	Putative glucosylhydrolase precursor
VFA0340	2.61	1.00	Amino acid permease
VFA0345	3.86	1.00	Amino acid permease
VFA0346	4.78	1.00	Evolved beta-galactosidase beta-subunit
VFA0347	3.25	1.00	Beta-galactosidase
VFA0363	-4.18	1.00	Sodium proton-dependent alanine carrier protein
VFA0378	-4.43	1.00	Glycine betaine transporter
VFA0379	-5.06	1.00	Fusaric acid resistance protein FusE
VFA0380	-4.70	1.00	Hypothetical protein
VFA0389	-4.87	1.00	Methyl-accepting chemotaxis protein
VFA0394	-2.02	1.00	Phospho-2-dehydro-3-deoxyheptonate aldolase
VFA0397	2.14	0.99	<not provided>
VFA0475	2.72	1.00	Sensory box GGDEF family protein
VFA0476	2.15	0.99	Hypothetical protein
VFA0507	4.41	1.00	Sodium glutamate symport carrier protein
VFA0551	-2.34	1.00	Hypothetical protein
VFA0583	2.68	1.00	Hypothetical protein
VFA0620	-2.53	1.00	<not provided>
VFA0695	-2.40	1.00	GMP reductase
VFA0757	-9.66	1.00	Hypothetical membrane protein
VFA0758	-6.21	1.00	Hypothetical membrane protein
VFA0760	-4.52	1.00	Cyclopropane-fatty-acyl-phospholipid synthase
VFA0761	-3.51	1.00	Plasmid partition protein ParA
VFA0762	-3.77	1.00	Putative dehydrogenase
VFA0763	-4.09	1.00	Short chain dehydrogenase
VFA0764	-3.54	1.00	Hypothetical transcriptional regulatory protein
VFA0767	-2.03	1.00	Cytochrome c-554(548)
VFA0768	-3.88	1.00	Cysteine desulfhydrase Selenocysteine lyase
VFA0769	-4.14	1.00	Agglutination protein

VFA0786	-5.03	0.98	PTS system, glucose-specific II BC component
VFA0794	2.54	1.00	Acetate kinase
VFA0795	-2.04	1.00	Two-component response regulator
VFA0799	-2.12	1.00	Maltose maltodextrin-binding protein
VFA0808	-2.48	1.00	1,4-alpha-glucan branching enzyme
VFA0816	-4.21	1.00	Hypothetical protein
VFA0817	-3.77	1.00	SCO2 protein precursor
VFA0818	-5.11	1.00	Hypothetical exported protein
VFA0829	-2.02	0.99	Sodium proline symporter
VFA0830	-2.42	1.00	Delta-1-pyrroline-5-carboxylate dehydrogenase
VFA0831	-3.92	1.00	Proline dehydrogenase Delta-1-pyrroline-5-carboxylate dehydrogenase
VFA0832	-3.46	1.00	Proline dehydrogenase
VFA1014	-5.68	1.00	Hypothetical protein
VFA1015	-4.59	1.00	RpoS-like sigma factor
VFA1016	-4.01	1.00	Sensory transduction protein kinase
VFA1026	-2.99	1.00	Transcriptional regulator, LuxO family
VFA1027	-2.19	1.00	Glycosyltransferase
VFA1038	-2.22	1.00	Sensory box GGDEF family protein
*VFA1042	-2.39	1.00	ABC transporter component, lysophospholipase L1 transport
*VFA1043	-2.43	1.00	ABC transporter ATP-binding protein
VFA1062	-2.39	1.00	Transporter, NRAMP family
VFA1162-1	-4.94	1.00	Iron-regulated protein FrpC, first half
VFA1162-2	-2.68	1.00	Iron-regulated protein FrpC, second half
VFA1162 to VF1163	-2.73	1.00	intergenic region
VFA1162 to VF1163	-2.32	1.00	intergenic region
VFA1163	-4.64	1.00	Type I secretion outer membrane protein
VFA1164	-5.36	1.00	Outer membrane porin F
VFA1165	-2.64	1.00	Type I protein secretion ATP-binding protein Type I protein secretion transmembrane subunit Type I secretion processing peptidase
VFA1166	-2.75	1.00	Diguanylate cyclase phosphodiesterase domain 1 (GGDEF)
VFA1167	-2.66	1.00	Hypothetical protein
VFA1168	-2.42	1.00	Type I secretion adaptor protein (HlyD family)
VFB46	-2.45	0.95	DNA topoisomerase III

Table 4.4. Unique genes regulated by GacA in each species

Probe Set ID	FoldΔ	PPDE(<p)	Annotation
VPA1499	-9.85	1.00	L-lactate dehydrogenase
VPA1498	-9.09	1.00	putative L-lactate permease
VP3012	-2.15	0.97	hypothetical protein
VP2014	3.69	0.98	putative tetrathionate reductase, subunit A
VP2016	27.40	1.00	hypothetical protein
VPA0276	-2.15	0.84	putative integral membrane protein
VPA1357	2.09	0.87	hypothetical protein
VP2326	-12.58	1.00	acetyltransferase-related protein
VP0111	-6.59	1.00	hypothetical protein
VPA0096	-4.60	0.99	outer membrane protein OmpW
VP0363	-3.87	0.92	glycerol dehydrogenase GldH
VP0321	-3.74	1.00	hypothetical protein
VP2545	-3.68	0.99	putative oxaloacetate decarboxylase γ chain 1
VPA1406	-3.57	1.00	putative exopolysaccharide biosynthesis protein
VPA1032	-3.55	0.97	hypothetical protein
VPA1036	-3.22	0.99	hypothetical protein
VP0294	-3.02	0.99	hypothetical protein
VPA1411	-3.01	0.98	putative glycosyltransferase
VPA1407	-2.96	0.99	hypothetical protein
VPA0859	-2.93	0.95	putative lipase
VP0294	-2.91	0.98	hypothetical protein
VP1519	-2.87	1.00	hypothetical protein
VPA1412	-2.73	0.99	hypothetical polysaccharide biosynthesis related protein
VPA1030	-2.71	0.92	hypothetical protein
VPA1038	-2.69	0.98	hypothetical protein
VPA0192	-2.66	0.91	tryptophanase
VP0634	-2.57	0.91	hypothetical protein
VP1861	-2.50	0.90	hypothetical protein
VPA1409	-2.48	0.93	hypothetical protein
VP2917	-2.48	0.97	hypothetical protein
VPA0126	-2.42	0.90	putative acetyltransferase
VPA1215	-2.41	0.99	hypothetical protein
VPA1403	-2.37	0.98	putative capsular polysaccharide biosynthesis glycosyltransferase
VP1326	-2.33	0.94	hypothetical protein
VPA1413	-2.33	0.97	hypothetical protein
VPA1408	-2.27	0.96	putative lipopolysaccharide biosynthesis protein
VP2002	-2.26	0.95	hypothetical protein
VP1325	-2.23	0.95	hypothetical protein
VP2080	-2.18	0.91	ABC transporter substrate-binding protein
VPA1073	-2.15	0.95	putative alkaline phosphatase
VP0311	-2.08	0.92	inorganic pyrophosphatase

VP0817	-2.06	0.98	selenoprotein W-related protein
VP1264	2.07	0.94	hypothetical protein
VPA1278	2.26	0.94	hypothetical protein
VPA1361	2.45	0.96	hypothetical protein
VPA1362	3.79	1.00	putative secreted protein EspD
VPA1368	7.23	1.00	hypothetical protein
VPA0772	-3.12	0.55	hypothetical protein
VPA1045	-2.14	0.60	hypothetical protein
VPA1020	-2.08	0.69	hypothetical protein
VP1410	2.04	0.71	hypothetical protein
VPA0243	2.22	0.80	putative virK protein
VP2003	-2.20	0.84	hypothetical protein
VP0365	-2.65	0.86	putative dihydroxyacetone kinase
VP1534	-2.29	0.86	hypothetical protein
VPA1029	-2.60	0.86	hypothetical protein
VP1235	-3.61	0.88	iron-containing alcohol dehydrogenase
VPA1443	-2.02	0.89	putative protein secretion protein
VP0364	-3.06	0.89	putative dihydroxyacetone kinase
VP0752	-5.57	0.94	conserved hypothetical protein
VP0378	-4.79	0.93	hypothetical protein
VPA1405	-3.93	1.00	putative polysaccharide export-related protein
VP1975	-3.64	0.94	hypothetical protein
VPA1410	-3.36	0.99	hypothetical protein
VPA1031	-3.24	0.94	hypothetical protein
VP1319	-3.01	0.98	hypothetical protein
VP1318	-2.95	0.99	putative flippase
VP1320	-2.94	0.97	putative CDP-ribitol pyrophosphorylase
VPA1027	-2.84	0.99	hypothetical protein
VPA1043	-2.76	0.92	hypothetical protein
VP1321	-2.70	0.97	putative dTDP-glucose 4-6-dehydratase
VP0625	-2.68	0.98	hypothetical protein
VP1521	-2.57	0.99	hypothetical protein
VP1322	-2.42	0.92	putative LicD1 protein
VP0997	-2.33	0.99	hypothetical protein
VP1517	-2.23	0.95	putative Rhs-family protein
VP2918	-2.15	0.91	hypothetical protein
VP1433	-2.01	0.92	hypothetical protein
VPA1323	2.00	0.97	hypothetical protein
VPA1331	2.02	0.98	putative OspC2
VPA1345	2.07	0.92	hypothetical protein
VPA1335	2.26	0.93	putative type III secretion apparatus protein
VPA1346	2.46	0.97	putative targeted effector protein YopP
VPA1350	2.47	0.97	hypothetical protein
VPA1334	2.51	0.98	hypothetical protein
VPA1349	2.73	0.96	putative Type III secretion protein Spa33
VPA1354	2.74	0.99	putative type III secretion system EscU protein

VPA1356	2.75	0.98	hypothetical protein
VP1076	2.76	0.96	hypothetical protein
VPA1336	2.77	0.98	hypothetical protein
VPA1328	2.83	0.99	hypothetical protein
VPA1360	2.87	1.00	hypothetical protein
VPA1621	2.93	0.97	hypothetical protein
VPA1351	2.96	0.99	hypothetical protein
VPA1359	2.99	1.00	hypothetical protein
VPA1380	3.19	1.00	putative OspB protein
VPA1358	3.23	1.00	putative dimethyladenosine transferase
VPA1370	3.34	0.99	hypothetical protein
VPA1366	3.35	0.99	hypothetical protein
VPA1352	3.45	1.00	hypothetical protein
VPA1329	3.46	1.00	putative traA protein
VPA0319_s	3.47	0.99	hypothetical protein
VPA1372	3.62	0.98	hypothetical protein
VPA0994	3.71	0.97	putative membrane protein
VPA1363	3.73	1.00	putative chaperone
VPA1340	3.91	1.00	hypothetical protein
VPA0285	4.16	1.00	hypothetical protein
VPA1342	4.86	1.00	putative Type III secretion protein Spa24
VPA1365	5.00	1.00	putative two-component response regulator
VPA1341	5.13	1.00	putative Spa29, component of the Mxi-Spa secretion machinery
VPA1367	6.39	1.00	putative type III secretion system lipoprotein precursor EprK
VPA1364	7.56	1.00	hypothetical protein
VP2904	-2.08	0.51	hypothetical protein
VPA0523	-3.49	0.54	hypothetical protein
VPA0956	-2.68	0.59	hypothetical protein
VPA1007	2.50	0.60	hypothetical protein
VP2962	2.93	0.61	hypothetical protein
VP1784	-2.07	0.77	hypothetical protein
VP1081	-2.44	0.82	hypothetical protein
VP2024	-3.82	0.85	hypothetical protein
VPA1314	2.48	0.86	thermostable direct hemolysin A
VPA1337	2.07	0.89	hypothetical protein
VP0321	-3.85	1.00	hypothetical protein
vp0365a	-2.82	0.92	
VPA1346to VPA1347	2.11	0.97	Intergenic region
VPA1314_s	2.44	0.92	thermostable direct hemolysin A
VPA1346 to VPA1347	2.83	0.97	Intergenic region
VPA0319	3.96	1.00	hypothetical protein
VF0710	-2.23	1.00	conserved hypothetical protein

VF0923	2.08	1.00	<not provided>
VF0988	-2.92	1.00	<not provided>
VF1010	-25.27	1.00	<not provided>
VF1010 to VF1011	-6.02	1.00	intergenic region
VF1037	-2.21	1.00	autoinducer synthase AinS
VF1246	-2.13	0.99	putative omptin family serine protease
VF1271	3.15	1.00	Hypothetical membrane spanning protein
VF1273	-2.70	1.00	Hypothetical protein
VF1325	-2.07	1.00	<not provided>
VF1371	-4.04	1.00	Permease
VF1372	-4.15	1.00	Permease
VF1377	-10.89	1.00	Hypothetical cytosolic protein
VF1458	-3.43	1.00	Threonine synthase
VF1473	-3.94	0.95	<not provided>
VF1474	-3.64	0.95	putative exonuclease
VF1493	-2.20	1.00	<not provided>
VF1615	-2.19	1.00	Hypothetical protein
VF2013	-3.68	0.93	Probable capsid scaffolding protein
VF2031	-2.02	0.92	conserved hypothetical protein
VF2033	-3.63	0.99	hypothetical bacteriophage protein
VF2034	-3.76	1.00	Hypothetical protein
VF2035	-4.89	1.00	Hypothetical protein
VF2036	-5.43	1.00	possible phage regulatory protein (CII)
VF2036 to VF2037	-4.42	1.00	intergenic region
VF2043	-2.50	1.00	Maltose maltodextrin-binding protein
VF2270	-2.13	0.94	Hypothetical protein
VFA0161	-2.15	0.98	Aerobactin siderophore biosynthesis protein IucA
VFA0162	-2.39	1.00	N(6)-hydroxylysine O-acetyltransferase
VFA0164	-2.18	1.00	L-lysine 6-monooxygenase
VFA0254	3.34	1.00	Hypothetical protein
VFA0254 to VF0255	4.28	1.00	intergenic region
VFA0339	3.02	1.00	Probable transcriptional regulator SyrB
VFA0364	-2.01	0.99	Transporter
VFA0406	-5.70	1.00	Possible hydrogenase cytochrome b-type
VFA0407	-5.99	1.00	Cytochrome c
VFA0473	2.72	1.00	Transcriptional regulatory protein TcpP
VFA0474	2.65	1.00	TcpH
VFA0480	2.57	1.00	<not provided>
VFA0655	-2.03	1.00	<not provided>
VFA0693	-3.47	1.00	<not provided>
VFA0847	-4.33	1.00	Succinylarginine dihydrolase
VFA0885	-2.10	1.00	Hypothetical protein

VFA0920	-2.88	1.00	LuxB, luciferase beta chain
VFA0921	-3.12	1.00	LuxA, luciferase alpha chain
VFA0922	-4.03	1.00	LuxD. acyl transferase
VFA0923	-3.55	1.00	LuxC, acyl-CoA reductase
VFA0924	-5.51	1.00	LuxI, autoinducer synthesis protein
VFA0925	-2.58	1.00	LuxR, transcriptional regulator
VFA0983	-5.67	0.99	Hypothetical protein
VFA1017	-3.19	1.00	Two-component response regulator
VFA1058	-10.92	1.00	Hypothetical protein
VFB20	-2.63	1.00	<not provided>
VFB21	-3.03	1.00	<not provided>
VFB22	-2.86	0.99	Hypothetical protein
VFB23	-2.58	0.98	Hypothetical protein
VFB24	-2.55	0.98	Hypothetical protein
VFB25	-2.69	0.98	Hypothetical protein
VFB26	-2.53	0.99	Hypothetical protein
VFB27	-2.47	0.97	Hypothetical protein
VFB28	-2.46	0.97	Hypothetical protein
VFB29	-2.46	0.96	Exonuclease SbcC
VFB30	-2.57	0.98	Hypothetical protein
VFB31	-2.38	0.94	Hypothetical protein
VFB32	-2.16	0.91	Transporter
VFB37	-2.10	0.96	<not provided>
VFB38	-2.23	0.96	Attachment Mediating Protein VIRB2 Homolog
VFB40	-2.45	0.98	Channel protein VirB8
VFB41	-2.37	0.96	Channel protein VirB9
VFB42	-2.14	0.91	Channel protein VirB10
VFB44	-2.16	0.93	Protein VirD4
VFB47	-2.53	0.91	Outer membrane protein
VFB50	-2.11	0.91	Relaxase

Table 4.5. Summary of microarray and Islandpath analyses.

	GacA Shared Core					GacA Unique Gene Regulon						
	Total Genes	Activated	Avg %GC	<Avg %GC	> Avg %GC	Total Genes	Avg %GC	Activated	< Avg %GC	Repressed	> Avg %GC	Activated
Symbiont (V. f.)	19	100%	11 (57%)	6 (32%)	2 (11%)	52	36 (69%)	88.9%	10 (19%)	90%	6 (12%)	50%
Pathogen (V. p.)			16 (84%)	1 (5%)	2 (11%)	128	52 (41%)	86.5%	63 (49%)	35.3%	7 (6%)	75%

All orthologous genes in the GacA Shared Core were GacA activated and the majority fell within the average %GC, for each organism.

Table 4.6. Summary of GacA regulons in *V. parahaemolyticus* and other bacteria

	Altered Motility	Iron	Protease	Biofilm	Host Association	Quorum Sensing[a]	Cytotoxicity
Vibrio							
[b]*parahaemolyticus*	-	-	X	-	X (T3SS)	-	X*
[c]*fischeri*	X	X			X	X	X*
[d]*cholerae*			X	X	X (T3SS)	X	
[e]*harveyi*					X (T3SS)	X	
[f]*vulnificus*	-	X	X		X		X
Erwinia							
[g]*carotorva*	X		X		X		
[g]*chrysanthemi*	-		X		X (T3SS)	-	
[g,h]*Salmonella*	X		X		X		
[i]*Escherichia coli*	X			X	X		
[g]*Legionella pneumophila*	X				X		X
[g,j]Pseudomonads	X	X	X		X (T3SS)	X	
[g]*P. aeruginosa*	X	X		X	X	X	

Blank spaces denote "not evaluated"
- not influenced by GacA
X influenced by GacA
[a]A GacA mutant of Vibrio fischeri had a very modest (and not considered biologically relevant) influence on AHL signal production, though microarrays show a general integration of GacA with Quorum Sensing.
Adapted from [b]this study, [c]Whistler et al. 2003 and Chapter IV, [d]Wong et al. 1998, Jang et al. 2010 and Tsou 2011, [e]Henke et al. 2004 and Lenz et al. 2005, [f]Gauthier et al. 2010, [g]Lapouge et al. 2008, [h]Goodier et al. 2001, [i]Bhatt et al. 2009.
[j]Summary of the following species: *chlororaphis(aureofaciens),entomophila, fluorescens, marginalis syringae* pv.,*syringae, syringae* pv. *Tomato, tolaasii, viridiflava.*
*APPENDIX II

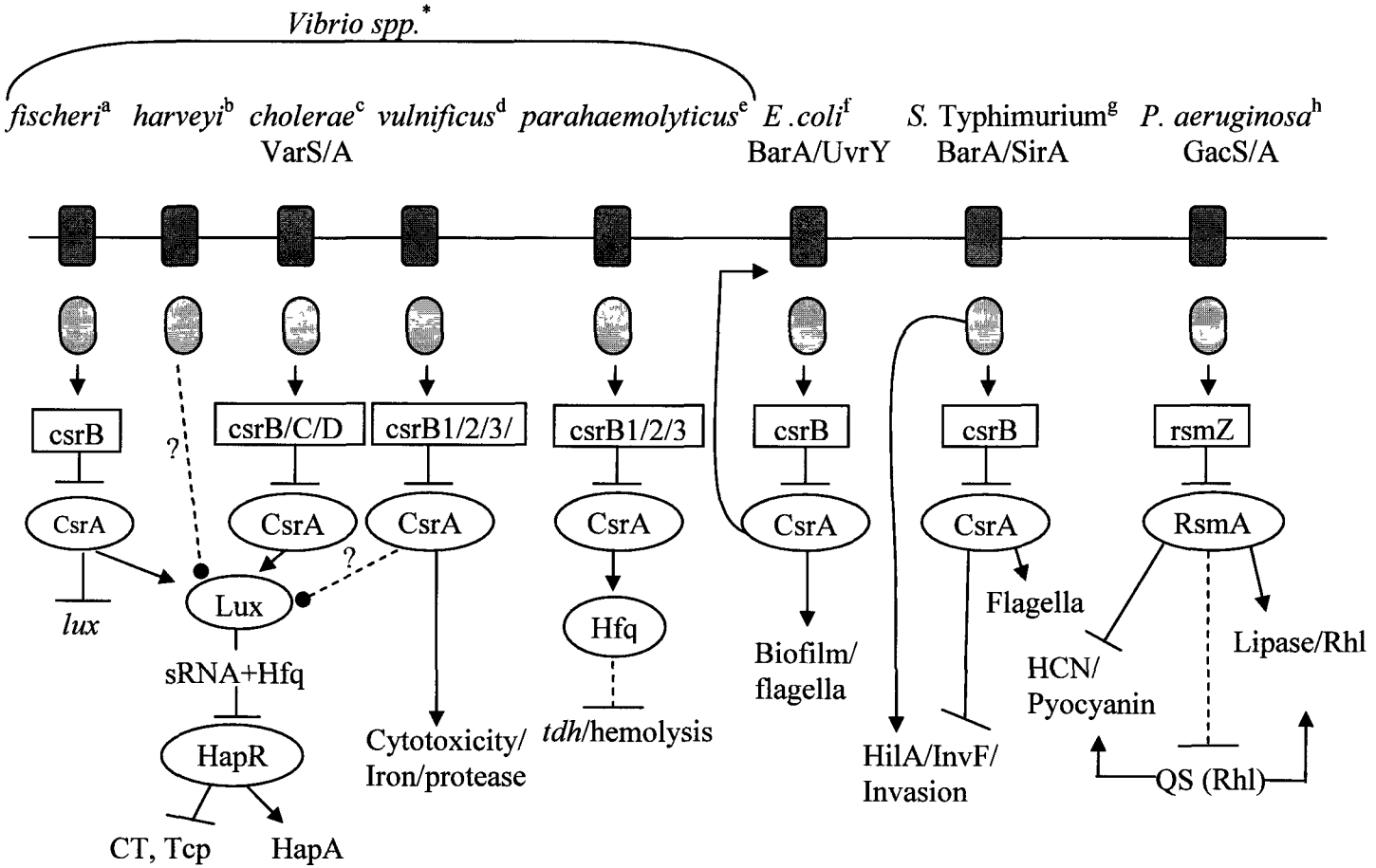

Figure 4.1. Summary of GacA regulatory cascades in *V. parahaemolyticus* and other organisms. *Annotated as GacS/GacA unless otherwise denoted, (adapted from). Dotted lines with circles represent connections that have not been evaluated (adapted from: [a]Ballok 2007, [b]Lenz 2004, [c]Jang 2011, [d]Gauthier 2010, [e]Chapter 3, [f]Pernestig 2003, [g]Teplitski 2006, [h]Heurlier 2004).

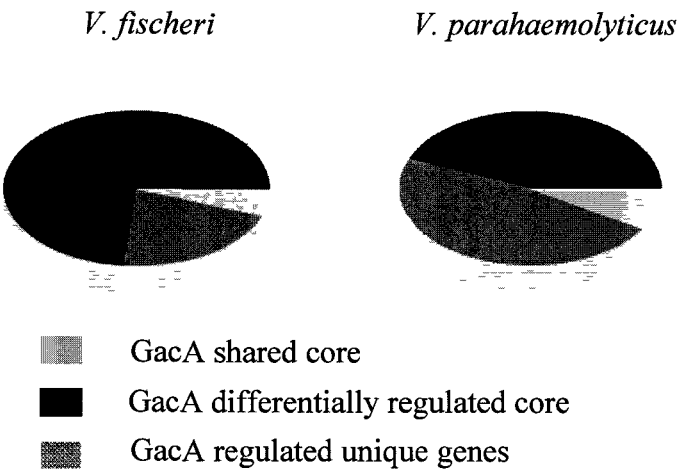

Figure 4.2. Distribution of GacA-regulated genes by genomic conservation. The GacA regulon is comprised of a small shared core of orthologous genes, core genes regulated in only one species and genes that are unique to species.

CHAPTER V

DISCUSSION

Water and foodborne illnesses remain a constant problem in the United States despite sophisticated waste water treatment facilities and extensive efforts to maintain a sanitary food and water supply. Though multiple bacterial and viral pathogens contribute to the steady rate of these outbreaks, infections with *Vibrio spp.* have increased 85% in the last ten years (in 2009 from 1996-1998) (MMWR 2010). This alarming rise in confirmed human cases may be the result of global climate change whereby water temperatures slowly increase. Consequently, waters once too cold to support certain human pathogens now provide novel habitats ideal for bacterial colonization and persistence. In addition *Vibrio spp.* are well known to have a high degree of genomic flexibility allowing rapid adaptation to fluctuating conditions or new environments (Han et al. 2008, Gu et al. 2009, Boyd et al. 2008, Hurley et al. 2006), Therefore, detection of *Vibrio spp.* in northern, historically cooler waters may be evidence of newly created habitats, or of newly cold-adapted *Vibrio spp.*

V. parahaemolyticus has been detected as far north as Alaska (McLaughlin et al. 2005) and is responsible for the majority of shellfish borne infections in the US (MMWR 2010).

Surveillance projects aimed at characterizing the prevalence of *V. parahaemolyticus* are ongoing in many geographic locations. However, *V. parahaemolyticus* is a challenging organism to characterize and currently used clinical markers are unreliable as indicators of virulence potential. Specifically, the classic virulence markers, *tdh* and *trh* are sometimes absent from outbreaks or infections (Garcia et al. 2009, Bhoopong et al. 2007). In addition, we have shown that when *tdh* is over expressed (in a GacA mutant), the organism is still defective in colonization of infant mice (Chapter II). This further blurs the line between benign and pathogenic strain and likely eliminates *tdh* as a stand alone virulence gene.

Unfortunately, in the absence of a dependable virulence marker, we are unable to effectively identify pathogenic *V. parahaemolyticus* and to therefore predict and prevent future outbreaks. In addition, molecular detection of specific "virulence" genes provides no information regarding their functional expression. Presence of these genes alone may not adequately describe the pathogenic potential of a given strain. Interestingly, our work has shown that precise regulatory patterns in response to a host cue may provide a novel way to define virulent versus environmental strains of *V. parahaemolyticus* (Chapter II). Specifically, clinical strains up-regulate several of their backbone virulence traits in response to human host temperature (37°C) while environmental strains do not (Chapter II). Instead of surveying strains for a specific gene, we propose that phenotypic characterization at two temperatures would provide a suitable alternative for virulence detection. These media based assays could be performed in a high throughput fashion to characterize entire populations of *V. parahaemolyticus* and would be more informative than marker detection alone. Additionally, this approach relies on global changes within

the cell that influence both backbone and horizontally acquired elements and may circumvent the issue of variably present genetic markers of virulence.

Horizontal gene transfer is a major force that drives the genomic diversity in both chromosomes of *V. parahaemolyticus.* The overall plasticity of the genome further confounds efforts to identify a single genetic marker present in all clinical strains (Han et al. 2008, Gu et al. 2009, Boyd et al. 2008, Hurley et al. 2006). Once in the cell, newly acquired DNA must be incorporated into existing regulatory networks to ensure coordinated expression with the entirety of the cell. Our work has shown that this is accomplished, at least in part, through the global regulator GacA. Though conserved across all *Vibrio spp.* we have shown that the GacA regulon has undergone subtle re-wiring in *V. parahaemolyticus* compared to other characterized regulons (Chapter III and IV). Specifically, GacA appears to simultaneously exert both positive and negative control, mediated by CsrA, CsrB and Hfq, over conserved and horizontally acquired elements important in metabolism and host association. Interestingly, the VPaI-7 containing *tdh* and T3SS is tightly regulated by both GacA repression and response to temperature in an additive fashion (Chapter III). Therefore, *V. parahaemolyticus* represents a unique model to study the evolution of regulatory networks in the context of the GacA two component response system.

Temperature is an important cue in the regulatory transition between natural habitat and host environment. Specifically, both backbone and acquired virulence associated traits are upregulated at 37°C compared to 28C°. This includes the horizontally acquired VPaI-7 which is subject to multiple layers of regulatory control. Though incorporated into and negatively regulated by the conserved GacA network, this

pathogenicity island is also transcriptionally activated by temperature (Chapter III) when GacA is not functional. Interestingly, VPaI-7 contains a multitude of genes putatively involved in human infection that have a specific response to 37°C, a temperature too high to exist in a natural aquatic habitat. It seems curious that such a large island is maintained in the apparent absence of continuous selective pressure, given that the time *V. parahaemolyticus* actually spends in a human gut is likely far less than that in the environment. Despite this, previous work has shown that *in vivo* seroconversion as a result of an unidentified host pressure occurs in humans infected with *V. parahaemolyticus* (Bhoopong et al. 2007). This may be evidence that, though time in a human gut is short, it may be sufficient to influence adaptation of this organism. In addition, we have shown through preliminary work that the acquisition and maintenance of this island may provide enhanced fitness within the gut (Chapter III). When VPaI-7 is upregulated (in a GacA mutant), *V. parahaemolyticus* displays defective pathogenesis in mice, however in a head to head competition, the GacA mutant is more fit in a mouse gut (APPENDIX II). It is interesting to consider that the human environment may contribute specific selective pressure for horizontally acquired elements to be maintained.

A thorough understanding of host selective pressures and the host response to a *V. parahaemolyticus* infection is impeded by the lack of an adequate model system. Though a defect in pathogenicity in a GacA mutant was detected though our work with infant mice, ultimately this model poses inherent challenges and high levels of variability. Infant mice lack a fully developed immune system, allowing us to artificially induce a bacterial infection; however this results in two specific problems. First, if mice are slightly more developed (indicated by hair growth), they become symptomatic but then

fight off the infection and fully recover. This can lead to variability within experimental groups and precludes the use of an LD_{50} as death can no longer be used as the study endpoint. Secondly, though the lack of immunity allows bacterial colonization, it prevents a true study of the interaction between a pathogen and the host immune system. This makes an authentic evaluation of the dynamic process of host-microbe association impossible. Future work to define *V. parahaemolyticus* infections will require a model system that effectively mimics both the infectious process and the host response.

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APPENDIX I

Dartmouth College

HANOVER • NEW HAMPSHIRE • 03756

TELEPHONE (603) 650-7592

Animal Care and Use Program
Protocol Approval Notice

March 11, 2008

Steven Fiering
Microbiology & Immunology
Hinman Box 7936

Dear Dr. Fiering:

Your protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Dartmouth College on February 26, 2008. It was voted APPROVED PENDING REVISION. Your revision addressed the points raised by the IACUC. That revision has been reviewed and your project has **FULL APPROVAL** effective 3/6/2008.

TITLE: Role of GacA in Viibrio parahaemalyticus virulence

SPECIES & QUANTITY: Mice - 1132, -

IACUC PROTOCOL NUMBER: 08-02-05

PRINCIPAL INVESTIGATOR: Steven Fiering

INSTITUTION: Dartmouth College

FUNDING SOURCE: COBRE in Immunology, core C P20 RR15639

This Institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance number is A3259-01.

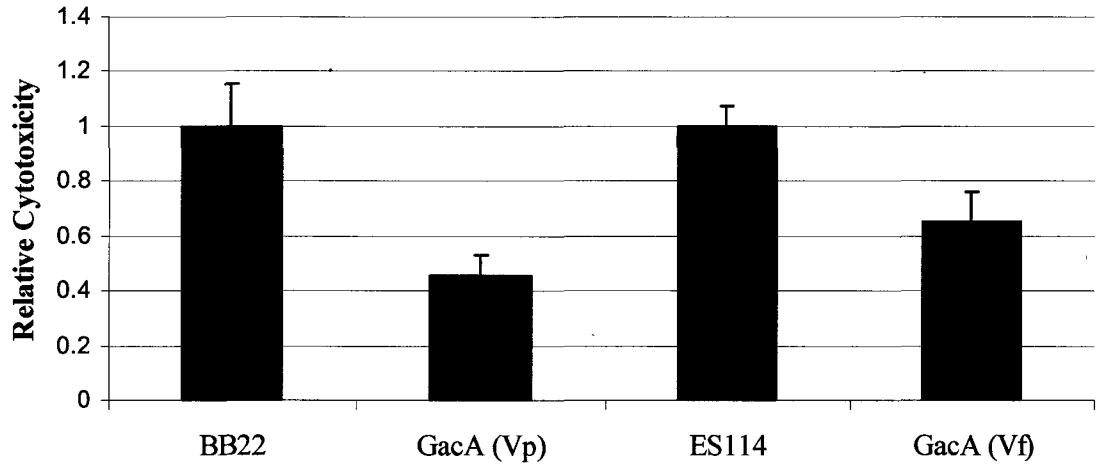
Sincerely,

Michele Martino

Michele Martino, D.V.M.
For the IACUC

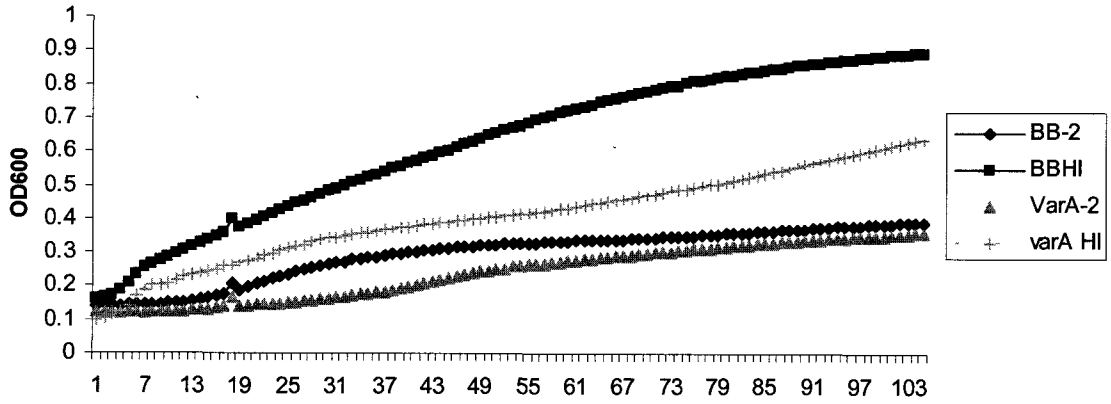
SNF/jlw

APPENDIX II



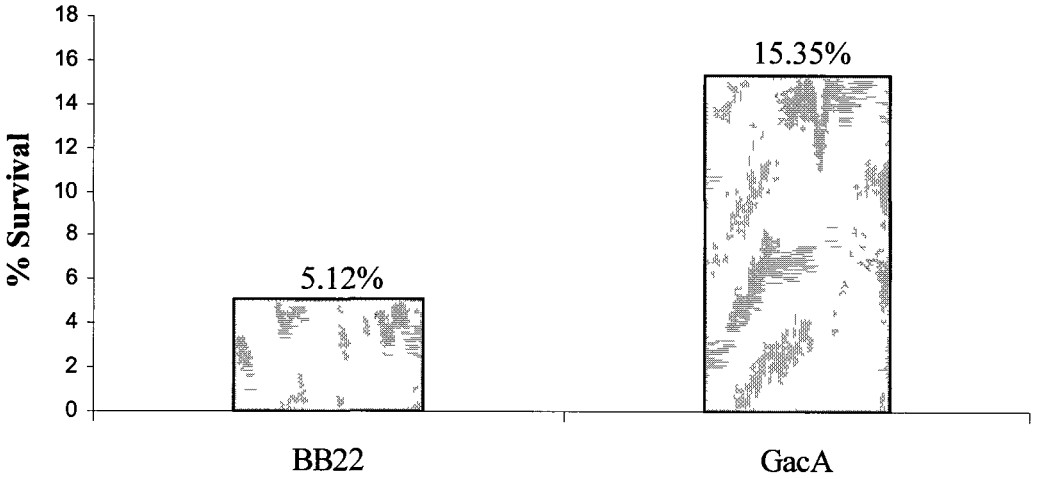
APPENDIX II. Cytotoxicity of *Vibrio parahaemolyticus* and *Vibrio fischeri*. A GacA mutation in each organism results in a loss of cytotoxic activity towards human CaCo-2 Cells.

APPENDIX III



APPENDIX III. Resistance to complement mediated killing in *V.parahaemolyticus.*

APPENDIX IV



APPENDIX IV. Competitive colonization. Competetive survival in a mouse intestine presented as % survival.