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Genes and Regulatory Mechanisms Controlling Environmental Survival Strategies of the Waterborne Pathogen *Vibrio cholerae*

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Ryan S. Mueller

Committee in charge:

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Professor Farooq Azam
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2007

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Chair

University of California, San Diego

2007

DEDICATION

To my mother,

who did not believe in idle summers.

To my father,

who did not believe in days off.

To my grandparents,

who always put such a great emphasis on family, education and hard work.

EPIGRAPH

To be without tranquility and contentment is to lack virtue.

Without virtue no man on Earth can survive for long.

Chuang Tzu

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LIST OF ABBREVIATIONS

Ap:	Ampicillin
BLAST:	Basic local alignment search tool
bp:	base pair
cdiGMP:	3'-5'-cyclic diguanylic acid
Cm:	Chloramphenicol
CSLM:	Confocal laser scanning microscopy
CTXϕ:	Cholera toxin phage
CT:	Cholera toxin
diSC3(5):	3,3′-dipropylthiacarbocyanine
EPS:	Exopolymeric substance
GFP:	Green fluorescent protein
Gm:	Gentamycin
HPLC:	High pressure liquid chromatography
IAA:	Indole-3-acetic acid
Km:	Kanamycin
LB:	Luria-Bertani growth media
LPS:	Lipopolysaccharide
mL:	milliliter
MCP:	Methyl-accepting chemotaxis protein
MSHA:	Mannose sensitive hemagglutinin pilus
nm:	nanometers
OD:	Optical density
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
Rif:	Rifampicin
RT-PCR:	Reverse-transcriptase polymerase chain reaction

TCBS:	Thiosulfate citrate bile salts sucrose growth media
TCP:	Toxin co-regulated pilus
TTSS:	Type III secretion system
VPI-1:	Vibrio pathogenicity island-1
VPS:	Vibrio polysaccharide
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µL:	microliter

LIST OF SYMBOLS

ϕ -	Bacteriophage
Δ*xxxX* -	Genetic deletion of gene *xxxX*
*xxxX::*Tn5 -	Transposon insertion in gene *xxxX*
σ -	Sigma factor subunit of RNA polymerase that enhances transcription at specific promoters

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ACKNOWLEDGEMENTS

Chapter 2: The text of chapter 2, in full, is a reprint of the material as it appears in the Journal of Bacteriology (Volume 189, pg. 5348-5360) entitled "*Vibrio cholerae* Strains Possess Multiple Strategies for Abiotic and Biotic Surface Colonization", with co-authors Diane MacDougald, Danielle Cusumano, Nidhi Sodhi, Staffan Kjelleberg, Farooq Azam and Doug Bartlett. The dissertation author was the primary investigator and author on this paper.

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ABSTRACT OF THE DISSERTATION

Genes and Regulatory Mechanisms Controlling Environmental Survival Strategies of the Waterborne Pathogen *Vibrio cholerae*

by

Ryan S. Mueller

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2007

Douglas H. Bartlett, Chair

Vibrio cholerae, the etiological agent of the severe diarrheal disease cholera, is a common inhabitant of the world's temperate and tropical waters. To cope the multitude of stresses found in these aquatic environments, *V. cholerae* has evolved many different survival mechanisms. Variations in gene content and regulation between strains allow for niche specialization within the species, and are possibly a determining factor behind fluxes of environmental populations. The work in this dissertation examines the underlying genetic and

molecular components that are responsible for these mechanisms, focusing on biofilm formation.

It was found that while the morphology of the biofilms of environmental and clinical strains of *V. cholerae* are distinct from one another; the genes driving these processes are generally conserved between the strains. Also, many new genes involved in the biofilm formation process on abiotic surfaces in *V. cholerae* have been identified, including those known to have a role in molecular transport, vibrio polysaccharide (VPS) production and its regulation, amino acid metabolism, among others. However, many of these same genes are not required when biofilms are formed on biotic surfaces common in the marine environment (e.g. copepods and dinoflagellates). Under these conditions, only genes responsible for chemotaxis and motility are essential for *V. cholerae's* ability to colonize biotic surfaces. These results suggest that the distinct biofilms of different strains and within various environments is due to variability in the regulation of the expression of genes involved in biofilm formation and not variability in gene content.

One of the genes identified to be involved for abiotic biofilm formation in multiple strains of *V. cholerae* was the tryptophanase gene. This work has shown that this gene's role appears to be indirect, as it is the indole by-product of the tryptophanase reaction, which can act as an extracellular signaling molecule that triggers an intracellular regulatory cascade and ultimately influences the expression of genes responsible for biofilm formation. This regulatory cascade is comprised of previously identified regulators of VPS production and the DksA protein, which appear to affect global gene regulation through altering intracellular concentrations of the second messenger, cyclic diguanulate (cdiGMP).

Chapter 1

Introduction: *Vibrio cholerae* as an Autochthonous Member of Aquatic Environments and the Role of Biofilms in Survival

Introduction

In the summer of 1854, the residents of London were gripped with fear, as the severe diarrheal disease cholera was rapidly spreading throughout the population leaving thousands dead. The overcrowded Soho section of town was especially ripe for an outbreak of the disease. Animal waste filled the Soho streets, slaughterhouses and cowsheds mixed with storefronts, and beneath many of the old houses lay squalid and seldom drained cesspools. On August 31st 1854, this volatile mix of a concentrated population and unsanitary conditions led to what burgeoning epidemiologist Dr. John Snow referred to as "the most terrible outbreak of cholera which ever occurred in the kingdom." Within three days the disease had swept through Soho leaving 127 residents dead and few families in the area unaffected.

Dr. Snow's studies of the previous outbreaks of cholera in London had led him to hypothesize that the disease was transmitted through sewage-contaminated water. This latest outbreak in his neighborhood presented an opportunity to test his hypothesis. In examining the various water sources in the area, he discovered that water from the Broad St. pump contained "white, flocculent particles" he felt were responsible for the disease. After inquiring into people's water usage habits and correlating them to instances of cholera, he quickly made the realization that a majority of the infected patients used water from the Broad St. pump. This observation coupled with his previous hypothesis led to the conclusion that the initial source of the disease was contaminated water from this pump (Snow 1855). The fabled outcome to this episode of history, recounts Dr. Snow triumphantly removing the handle to the Broad St. pump, effectively ending the Soho cholera epidemic of 1854.

One hundred and fifty years have passed since Dr. Snow linked cholera to contaminated water supplies and vast amounts of information pertaining to this disease have been collected. In 1883, Robert Koch identified the bacterium *Vibrio cholerae* as the causative agent of cholera. Seven pandemics of cholera have been recorded and of over 200 known serotypes of *V. cholerae* only two, O1 and O139, appear to be responsible for epidemic outbreaks of cholera. In addition to epidemiological work, the advent of microarray technology

and the completion of the *V. cholerae* strain N16961 genome (Heidelberg et al. 2000) and ensuing molecular studies have furthered our understanding of the genetic regulation and mechanism of pathogenesis (see reviews Skorupski and Taylor 1997; De Haan and Hirst 2004).

Ecology of *Vibrio cholerae*: The Influence of Environmental Factors on the Epidemiology of Cholera and the Genetic Diversity of *V. cholerae*

Although studies on the pathogenesis of *V. cholerae* have flourished, a clear understanding of the ecology and the survival mechanisms of this organism are just starting to materialize. Strains of *V. cholerae* can be found throughout the globe in tropical and temperate coastal waters of moderate salinity. Toxigenic strains, though, are more prevalent in cholera-endemic regions, such as Southeastern India, Bangladesh, Africa and South and Central America (Cockburn and Cassanos 1960; Kaper et al. 1995; Borroto and Martinez-Piedra 2000). In these regions cholera outbreaks generally demonstrate a distinct seasonality and emerge from multiple foci simultaneously, suggesting that infectivity by *V. cholerae* populations is highly dependent on environmental factors.

Bangladesh demonstrates a bimodal seasonality in the recorded outbreaks of cholera throughout each year, with a small peak occurring in the springtime and a larger peak in the fall following the monsoon season (August – September) (Kaper et al. 1995). Various studies have attempted to examine this seasonality further to determine the physical and biological parameters driving it. In a recent study by Lobitz et al (Lobitz et al. 2000) remote sensing data from the Bay of Bengal was used to show that sea surface temperature was significantly correlated to the rise and fall of recorded cholera cases in the surrounding regions between 1992 – 1995. These results were later expanded upon using direct measurements of physical and biological parameters to show that increased water temperatures in freshwater systems in Bangladesh also led to subsequent increases in cholera cases (Huq et al. 2005). Even in non-endemic regions this trend appears to hold true, as yearly detection of toxigenic and non-

toxigenic *V. cholerae* rises significantly during the summer months in the Chesapeake Bay of Maryland (Heidelberg et al. 2002; Louis et al. 2003).

One explanation for these results is that specific physical constraints within the ecosystems where *V. cholerae* live are influencing the population dynamics of this organism. Support for this idea has been presented by whole genome hybridization experiments that have examined the correlation between genomic diversity of *V. cholerae* isolates and environmental factors. In a comparison of 41 non-toxigenic isolates from California, it was shown that a core set of 2,787 genes were conserved between >95% of these strains. Due to their conservation, these genes have been hypothesized to be essential for environmental survival. Another 446 genes of the *V. cholerae* genome, though, were only conserved in <5% of the isolates (Keymer et al. 2007). These genes were considered dispensable and coded for many peripheral or variable functions, including pathogenesis, lipopolysaccharide (LPS) biosynthesis, chemosensing, cell surface modification and transport. When the presence or absence of these genes within isolates was correlated to the environmental conditions of where each strain was found, significant relationships were found. For instance, isolates from colder waters tended to contain genes for chitin metabolism, iron transport and chemotaxis; while, isolates from warm water were found to have more genes involved in exopolysaccharide production, superoxide stress protection and fructose metabolism. It may be that cold-water conditions favor *V. cholerae* isolates able to associate with chitinaceous particles or organisms, whereas organisms more accustomed to environments containing high amounts of labile nutrients and oxidative stress may be favored in sun-bathed warmer waters. Hence, niche specialization dictated by environmental parameters may be influencing the genomic diversification of populations of *V. cholerae* leading to observed fluxes in community structure.

Another consequence of environmentally driven genomic diversification may be the emergence of traditionally defined toxigenic strains during specific environmental conditions, or the evolution of “new” toxigenic strains of *V. cholerae* through the acquisition of foreign

DNA encoding novel pathogenesis functions. The emergence of toxigenic strains in a traditionally defined sense is dependent on the acquisition of two specific genetic elements involved directly in the disease process. The first of these is the termed the Vibrio Pathogenicity Island-1 (VPI-1) and carries genes coding for the production of the toxin-coregulated pilus (TCP), which has been shown to be essential for intestinal colonization during the initial stages of cholera (Herrington et al. 1988; Karaolis et al. 1998). The second set of genes (*ctxAB*) encodes the two subunits of the cholera toxin protein (CT), which is responsible for the symptomatic diarrhea of the disease (Gill and Meren 1978; Mekalanos et al. 1983). Both of these elements appear to reside in genomic islands and contain distinct features of horizontally transferred DNA (e.g. *att* sites, abnormal GC content, etc.). In fact, it has been shown that the *ctxAB* genes are transferred by a filamentous phage (CTXϕ) of *V. cholerae* and this phage uses the TCP pilus as a receptor (Waldor 1996). This illustrates the co-dependence of these features for the virulence of the organism.

Studies have shown that there is no detection of CTXϕ from patients suffering from cholera (Faruque et al. 1998). Hence, it has been speculated that CTXϕ induction does not occur in the human gastrointestinal tract and may take place between *V. cholerae* strains within natural aquatic habitats. In support of this, sunlight has been shown to induce the production and transfer of CTXϕ between *V. cholerae* strains in aquatic microcosms, and that once produced the phage is most stable in conditions mirroring seawater environments (Faruque et al. 2000). Implied within these results is the concept that both the TCP pilus and CT have functionally defined roles in environmental settings. Due to its ability to affect the efflux of Na^+ and other electrolytes from human epithelial cells, it has been proposed that CT can function to osmoregulate the intestinal environment within organisms, such as copepods, although no direct evidence for this has been provided (Colwell 1996; Lipp et al. 2002). For the TCP pilus, however, a clear role in enhancing the ability of *V. cholerae* to attach to chitinaceous surfaces has been defined. Coincidentally, this also provides an environmental setting where the TCP pilus is produced and can act as a receptor for the CTXϕ (Reguera and

Kolter 2005). These results offer evidence for some of the virulence factors *V. cholerae* possessing a dual role in both pathogenesis and environmental survival. Also, these findings may explain the continued selection for these genes in reservoirs where cholera is not endemic, allowing for the future emergence of toxigenic strains under the appropriate environmental conditions.

Given that horizontal gene transfer of pathogenic functions occurs between diverse organisms, it has been proposed that "new" toxigenic strains of *V. cholerae* can also arise from environmental sources. Recently, direct evidence in support of this idea was provided in a study by Dziejman, et al (Dziejman et al. 2005). In this work it was shown that a non-toxigenic strain of *V. cholerae*, which does not contain the genetic elements responsible for disease (i.e. *tcp* and *ctx* genes), demonstrated similar virulence levels in cholera-infection models to known toxigenic strains. Through genomic sequencing of this strain it was determined to carry a horizontally transferred genetic element coding for a type III secretion system (TTSS), and it was proposed that the acquisition of these foreign genes led to the ability of a heretofore avirulent strain to cause disease. Another example of the emergence of a non-toxigenic strain as an infectious agent has been shown recently with *V. cholerae* strain TP, isolated from the Torrey Pines State Reserve. This strain does not contain the known cholera virulence factors, but does have other toxin genes which are thought to illicit dramatic toxicity in mouse models of lung infection (Purdy et al. 2005; Makri et al. 2007).

This hypothesis has also been proposed to be responsible for the recent emergence of a new serogroup of *V. cholerae* as an etiological agent of cholera. Prior to 1992 all epidemic outbreaks of the disease cholera were attributed to the O1 serogroup. However, in the latter part of that year new outbreaks of the disease caused by a previously unknown serogroup of *V. cholerae* were recorded in regions of India and Southern Bangladesh (Albert et al. 1993; Ramamurthy et al. 1993). This epidemic quickly spread through these countries and the surrounding areas, and in some regions this new serogroup actually displaced the *V. cholerae* O1 El Tor serogroup as the main causative agent of disease for a short period of

time (Albert et al. 1993). Subsequent serotyping of this strain identified it as O139 Bengal, and revealed that its most probable origin was a El Tor Vibrio which had swapped its O1 LPS biosynthetic genes through a horizontal gene transfer event for new genes capable of producing the O139 antigen (Dumontier and Berche 1998; Li et al. 2002). Aside from producing an altered LPS moiety, this bacterium was now able to produce a capsule (Johnson et al. 1994; Waldor et al. 1994), which is essential for biofilm development in seawater (Kierek and Watnick 2003).

From these examples it is clear that environment factors can have significant effects on microbial populations leading to the establishment of genetically dynamic communities. In the case of *V. cholerae*, it appears that the fluidity of its genome can allow for the acquisition of new virulence attributes, which may aid in survival within both human and aquatic habitats. As these attributes may lead to new pathogenic strains with enhanced virulence and/or resistance to common treatment methods, the potential for environmentally driven evolution of these organisms to impact human disease cannot be overlooked.

Ecology of *V. cholerae*: Trophic Interactions and Survival Mechanisms within Aquatic Habitats

Although many years have passed since *V. cholerae* was identified as an indigenous member of aquatic environments, its role in these habitats has remained poorly defined. Fundamental studies on its growth capabilities in aquatic systems and mechanisms for combating environmental pressures have started to reveal how this organism survives in a vastly different environment from that of the human gastrointestinal tract. By gaining a deeper appreciation for the roles and niches that *V. cholerae* occupy in the water, we can also obtain a better understanding of our potential interactions with this pathogen allowing for informed steps to be taken to effectively prevent the spread of disease.

One of the first questions asked in this regard was simply whether or not *V. cholerae* can grow in water from environmental reservoirs either alone or in competition with natural

assemblages of aquatic bacteria. To investigate this, it was initially examined whether *V. cholerae*, which is traditionally thought of as a marine bacterium, could grow in low salinity environments where humans are more likely to come in contact with it. Multiple studies have addressed this issue (Singleton et al. 1982; Vital et al. 2007) and have found that while *V. cholerae* appear to favor conditions with moderate salt concentrations (5-25‰), it can grow in freshwater conditions having much lower NaCl concentrations. Aside from solitary growth under these conditions, it appears that *V. cholerae* can also compete fairly well against a natural assemblage of freshwater bacteria. For instance, it has been to shown that *V. cholerae* has similar growth rates to a natural bacterial assemblages grown in lakewater microcosms, and can constitute ~10% of the final population in competition experiments with these organisms (Vital et al. 2007). These findings provide direct evidence for *V. cholerae's* ability to persist in the many lakes and rivers where this pathogen is found. In addition to being found in freshwater environments, *V. cholerae* can often persist in marine habitats in association with phyto- and zooplankton blooms. Thus, it was proposed that the high dissolved organic carbon (DOC) provided during a phytoplankton bloom could be an excellent substrate for growth of this bacterium. A study by Mouriño-Pérez, et al (Mouriño-Pérez et al. 2003) provided support for this hypothesis by showing that *V. cholerae* can grow in bloom waters containing DOC produced by the red tide organism, *Lingulodinium polyedrum*. Taken together, the above studies have shown that *V. cholerae* is indeed an active member of aquatic ecosystems. As such, this organism is subject to the many pressures found within these low-nutrient, highly predatory environments. Therefore, it is important to understand how this organism copes with these pressures in order to define its population dynamics and the patterns of disease outbreaks.

One mechanism that has been proposed to aid in survival of *V. cholerae* is its ability to colonize and form biofilms on biotic and abiotic surfaces within aquatic ecosystems. Biofilms, which are encapsulated clusters of cells living together attached to a surface, are ubiquitous throughout the microbial world. It is thought that the structure of these films and

the physiological state of the cells within these communities contribute to the protection that biofilms provide from many adverse conditions imposed within natural and host-associated environments (e.g. UV radiation, acid stress, phagocytosis, salinity and antimicrobial agents, reviewed in Hall-Stoodley et al. 2004).

With regard to *V. cholerae*, multiple studies have found that these bacteria are commonly associated with particles or within biofilms growing on abiotic surfaces in various environmental reservoirs (Gil et al. 2004; Islam et al. 2007). Therefore, it has been proposed that biofilms play a major role in the environmental survival and infection process of this organism (Yildiz 2007). Evidence linking biofilms to disease transmission has been recently presented in two separate studies. In the first, it was shown that the stools of cholera patients contain both planktonic and aggregated clumps of *V. cholerae*, which can be released back into environmental reservoirs. These clumps, which can range upwards of 20μm in diameter, can persist in aquatic environments and, although they are not cultivatable by standard techniques, they remain highly infective (Faruque et al. 2006). These results are quite interesting in light an earlier study by Colwell, et al (Colwell et al. 2003) which showed that removal of the particulate fraction of water samples (>20μm) using a simple cloth filtration technique can effectively prevent transmission and outbreaks of the disease in endemic areas. These findings suggest that *V. cholerae* does commonly persist in the biofilm state within environmental reservoirs and that these biofilm-associated cells may be the progenitors of disease.

V. cholerae can also colonize and associate with many different zooplankton and phytoplankton in the water sources where it is present. In doing so, it is thought that the bacteria receive various benefits from the plankton, such as nutrients and protection from stresses. For example, it has been shown that *V. cholerae* cells can persist within associations with algae for significant periods of time (>15 months), and that these associations can actually enhance the survivability of these organisms (Islam et al. 1990; Islam et al. 2004). Evidence suggesting that *V. cholerae* is utilizing nutrients provided by the

algae for growth was shown in a subsequent study which demonstrated that the Hap protein, which is a quorum sensing controlled protease of *V. cholerae*, is essential in maintaining this association (Islam et al. 2002). Therefore, it is possible that lengthy persistence of toxigenic strains of *V. cholerae* in aquatic habitats could be facilitated through these relationships.

Although *V. cholerae* is found in association with phytoplankton in environmental reservoirs, there is also believed to be a strong interaction with zooplankton (Lipp et al. 2002). This idea was originally based on results which found that *Vibrio* spp. were the most prevalent organisms found within the guts of marine copepods (Sochard 1979). Further evidence in support of this association was presented in studies looking at the effects copepods had on survival of *V. cholerae* (Huq et al. 1983; Huq et al. 1984). Here it was shown that *V. cholerae* can attach to the oral region, anus and egg sacs of live copepods, and that the survival of the bacteria was increased under these conditions significantly. Beyond these associations with live copepods, it has also been shown that *V. cholerae* can also colonize the chitinaceous surfaces of dead zooplankton, which can presumably act as a food source for the chitinolytic *V. cholerae* (Tamplin et al. 1990). Additionally, the biofilms that these bacteria form on chitin surfaces are highly resistant to acid stress and can enhance DNA recombination events within mixed populations of cells (Nalin et al. 1979; Meibom et al. 2005). These findings may greatly effect *V. cholerae's* ability to cause disease, since gastric acid stress is a major barrier to infection (Cash et al. 1974) and DNA recombination appears to be a driving factor in the emergence of new toxigenic strains of *V. cholerae* (discussed above).

Obviously, in resource limited aquatic habitats not all of the interactions between *V. cholerae* and other organisms are beneficial, though. Much like any other bacteria caught up in the microbial loop, *V. cholerae* is subject to fierce competition for resources and constant attacks from predators. For example, beyond the mere competition for transport and utilization of limiting resources, the microbial world is filled with examples of organisms that use chemical warfare to limit the growth of their neighbors for their own benefit. Recently, an antagonism of this sort was shown between *V. cholerae* and another surface-associated

Vibrio species (Long et al. 2005). By producing a growth-inhibiting metabolite, this antagonistic bacterium was able to significantly inhibit the growth and colonization ability of *V. cholerae* on surfaces; thereby, illustrating the sensitivity of *V. cholerae* populations to these types of attacks in specific microenvironments.

Aside from threats by other bacteria, aquatic microbes face intense pressure to survive the effects of protozoan grazing and phage lysis. In the case of the latter, many temperate and lysogenic phages have been isolated that are specific to *V. cholerae* (Siddiqui and Bhattacharyya 1982; Waldor 1996), and it has been shown that natural phage populations can effectively modulate *V. cholerae* levels. For instance, a long term study examining the epidemiology of cholera and its relationship to natural populations of O1/O139 *V. cholerae* and their associated phages found that phage-mediated lysis had significant effects on the recorded cases of disease (Faruque et al. 2005). It was shown that increases in the detection of phages in environmental water samples almost always coincided with an inability to isolate susceptible toxigenic *V. cholerae* strains from the same samples. In very few instances were phages found in water with the *V. cholerae* strains they are known to infect, and in most of these cases the *V. cholerae* had evolved resistance to the phage and were no longer susceptible to phage attack. In this three-year study it was also found that increases in the number of cholera cases generally coincided with periods when toxigenic *V. cholerae* could be isolated from water samples but no phage was detected, showing a link between the prevalence of disease and environmental phage populations. In a subsequent study, phage populations were also monitored in the stools of cholera patients, and these same trends were apparent. Therefore, it was suggested that ingestion of both phage and its *V. cholerae* host allows for amplification of the phage populations during the explosive growth phase of the pathogen in the intestinal tract. In doing so, massive inoculums of phage can be reintroduced into the environment and effectively prevent continuation of a cholera epidemic by wide-spread phage-mediated lysis of the etiological agent of the outbreak (Faruque et al. 2005). From these results it can be concluded that an inverse correlation appears to exist between *V.*

cholerae and its phage populations, and this interaction directly affects the outbreaks of cholera in humans possibly through a self-limiting mechanism, which can end epidemics prematurely.

In addition to the role of phages in controlling bacterial populations, protozoan grazing can greatly influence their numbers in the wild. An initial study examining the effects of protozoan grazing on *V. cholerae* populations suggested that ciliates could effectively diminish the numbers of this pathogen (Perez et al. 2004). However, these results were inconclusive since the natural assemblage of bacteria was also able to decrease *V. cholerae* numbers. In a subsequent study, though, it was shown that the protozoan fraction of seawater mesocosms can exert significant grazing pressure on *V. cholerae* populations under specific conditions, severely limiting their growth (Worden et al. 2006). For example, in non-bloom seawater mesocosms similar growth rates were observed for both the *V. cholerae* and the natural bacterial assemblages, but protozoan grazing was able to greatly reduce the overall numbers of *V. cholerae* in these mesocosms leading to a negative net growth rate of the population. Conversely, in experiments conducted under intense bloom conditions net growth of *V. cholerae* was observed despite grazing pressures, suggesting that excess DOC during a phytoplankton bloom may provide for high growth rates exceeding the grazing rates within this system. It was also shown that during an intense bloom *V. cholerae* populations switch from a free-living to a particle-attached state. Therefore, an alternative explanation could be that these biofilms may also provide protection from grazing in these experiments allowing for the recorded net growth.

Evidence of the protective nature of biofilms was provided in a report by Matz, et al (Matz et al. 2005). Here, it was found that *V. cholerae* biofilms are virtually unaffected by protozoan grazing, whereas free-living populations were reduced by grazers >94% after three days. It was also shown that under intense grazing pressure clonal populations of cells that normally cannot form thick biofilms can switch to a rugose phase variant able form thick, grazing-resistant biofilms. Also, in a classic example of the hunted becoming the hunter, it

was illustrated that *V. cholerae* strains possess mechanisms to actively kill their protozoan predators. Although the mechanism was not fully elucidated, it was shown that when *V. cholerae* strains are growing within a biofilm they can control gene expression in a coordinated manner through quorum sensing mechanisms and direct the secretion of a factor that appears to kill the eukaryotic grazers. In subsequent study by an unrelated group a secreted protease PrtV was identified, which is controlled by quorum sensing, to be toxic to the nematode *Caenorhabditis elegans*, in addition to natural, unicellular ciliate and flagellate grazers (Vaitkevicius et al. 2006). Therefore, *V. cholerae* can use quorum sensing mechanisms to drive biofilm formation and secrete a toxic protease in order to defend against protozoan grazing.

In addition to this mechanism, certain strains of *V. cholerae* have been shown to utilize another newly defined secretion system termed Virulence Associated Secretion (VAS) to avoid grazing (Pukatzki et al. 2006). The VAS system is encoded by multiple genes of a conserved operon found in many different species of proteobacteria (Das 2003) and has been proposed to comprise an entirely new secretory system in bacteria. Through the secretion of two putative toxins that appear to have a role in pore formation within membranes (Mougous et al. 2006) and cross-linking actin polymers (Sheahan et al. 2004) *V. cholerae* is able to successfully kill the amoeboid grazer *Dictyostelium discoideum* and avoid predation.

The examples listed in the preceding section show that *V. cholerae* is well suited to live in a variety of aquatic habitats, including marine, brackish and freshwater environments. This is not only a result of the successful utilization of nutrients within these systems, but also from the various evolved mechanisms of this organism for the avoidance of predation by phage and protozoa. For resistance to viral attack it is proposed that *V. cholerae* can modify its surface structures to block or change the receptors of specific phages. In contrast, the ability to defend itself against protozoan raids seems to lie in the ability of the *V. cholerae* population to coordinate its gene expression through quorum sensing mechanisms which leads to the production of protozoan resistant biofilms and eukaryotic toxins. By these

combined processes *V. cholerae* strains are able to successfully combat the antagonistic pressures of aquatic environments and maintain their population levels during the periods between outbreaks.

Signaling Mechanisms and Transcriptional Regulation of Environmental Survival Strategies

From the preceding section it can be concluded that quorum sensing is a major regulator of some of the survival mechanisms *V. cholerae* possesses (i.e. biofilm formation and toxin production). In many strains of *V. cholerae* quorum sensing controls changes in gene expression by altering the production of the master transcriptional regulator HapR. In this system, two distinct types of autoinducer molecules are produced and excreted from individual cells. These two molecules are cholera autoinducer 1 (CAI-1), whose structure remains undefined, and autoinducer 2 (AI-2), a furanosyl borate diester also produced by many different bacteria. As the cells divide, these autoinducers begin to build in concentration in the extracellular environment and subsequently bind to receptors on the cell surface. Once a high cell density is reached, a threshold concentration of autoinducer molecules bound to receptors is achieved and two parallel and convergent intracellular phophorelay cascades, which control the production of active HapR, are triggered. Once active, HapR can regulate the expression of many genes within the cell including those involved in virulence towards humans and protozoa, biofilm formation and motility (For schematic see figure 3.7 of Chapter 3, reviewed in Milton 2006). In this manner, individual cells of *V. cholerae* can monitor the environment for specific chemical signals that relay information about the population around them. This allows individual cells within a population to co-ordinate their gene expression with those surrounding them. This ability is essential for mechanisms like virulence and biofilm formation where haphazard expression could lead to dire consequences, such as premature detection during infection and elimination by the immune system.

For this reason, the regulatory patterns, which are controlled by quorum sensing in V. cholerae, are quite peculiar. When cells are in a low-density state quorum sensing appears to activate genes involved in virulence and biofilm formation. Then, once a high cell density is reached these genes are repressed and the Hap protease is produced along with various other proteins (Zhu et al. 2002; Zhu J 2003). This runs contrary to the idea that there is strength in numbers, as the bacteria in this scenario are prepared to initiate disease when populations are still mustering and biofilm formation is just beginning. However, it does provide a nice framework for why the progression of cholera disease happens as it does. One of the reasons that this disease is so debilitating is that the symptoms run their course extremely fast, and without proper treatment patients can die very quickly. Therefore, it seems likely that when *V. cholerae* are first exposed to the intestinal environment they can colonize epithelial cells rapidly while secreting virulence factors, such as cholera toxin. Then, as the biofilm matures and high cell densities are reached, *V. cholerae* can use quorum sensing to turn off virulence and biofilm gene expression and activate Hap expression. Hap can cut the sticky polymers of the biofilm allowing for individual cells to detach and be removed as the cholera toxin is triggering the massive release of water and solutes into the intestinal lumen and subsequently the environment.

Despite these obvious benefits of quorum sensing, many strains of *V. cholerae* appear to carry mutations with the *hapR* gene rendering it and quorum sensing ineffective (Heidelberg et al. 2000; Kovacikova and Skorupski 2002). The reasons for the prevalence of these spontaneous mutant strains are not known, but under certain environmental conditions strains that do not respond to quorum sensing signals may be favored. For example, in aquatic environments where there are not many nutrients and high grazing pressure exists this genotype might be selected for, since it is advantageous to remain within a biofilm for a prolonged period of time.

Just as HapR acts to repress biofilm formation, many activators of this trait have also been discovered. One of these mechanisms is another signaling system of *V. cholerae*, albeit

an intracellular one. In this system, gene expression is responsive to the intracellular concentrations of the second messenger, cyclic diguanylate (cdiGMP). The prevailing concentration of this molecule is controlled by multiple GGDEF- and EAL-domain containing proteins coded by *V. cholerae*. The GGDEF domain has been shown to have diguanylate cyclase activity, which can direct the synthesis of cdiGMP, and EAL domains antagonize this activity by breaking down cdiGMP through its phosphodiesterase activity. It is not known how these proteins interact to balance cdiGMP concentrations, but many also carry sensor motifs that have been hypothesized to be involved in environmental sensing and response (reviewed in D'Argenio and Miller 2004). Therefore, it seems that much like quorum sensing this system interprets specific environmental cues and directs coordinated control of gene expression within the cell. While the nature of the extracellular cues driving this response is largely unknown, recent studies have started to define the gene expression changes, which are produced by this system. For example, in *V. cholerae*, it appears that low concentrations of cdiGMP can drive the expression of known human virulence factors such as cholera toxin. Conversely, high levels of cdiGMP control genes involved in grazing resistance, such as biofilm genes and *prtV* (Tischler and Camilli 2004; Tischler and Camilli 2005; Beyhan et al. 2006). Therefore, it is possible that cdiGMP levels may be modulated by GGDEF and EAL proteins that can sense environmental changes, allowing cells to control gene expression accordingly.

Interconnected with these signal systems are transcriptional regulators, which can also control the expression of the same virulence and biofilm genes. Two of these regulators, VpsR and VpsT, were initially discovered to activate the expression of two operons within the *V. cholerae* genome known to code for proteins involved in vibrio polysaccharide (VPS) biosynthesis (Yildiz et al. 2001; Casper-Lindley and Yildiz 2004). VPS is the major component of the extracellular matrix of *V. cholerae* biofilms and is responsible for their three-dimensional structure (Watnick and Kolter 1999; Yildiz and Schoolnik 1999). Also, the ability of strains of *V. cholerae* to switch to a grazing resistant rugose variant, as mentioned above, is directly

attributable to a spontaneous genetic mutation which causes cells to over-produce VPS (Beyhan and Yildiz 2007). Although, these two regulators interact with both quorum and cdiGMP signaling it appears that they can function independently to drive gene expression (Beyhan et al. 2007). Therefore, it appears that *V. cholerae* have many different methods of regulating peripheral activities such as virulence and biofilm formation. This overlap of regulation could prove useful to fine tune the expression of these factors in different environments where metered expression is important. It is also possible, though, that this redundancy provides for subpopulations of cells using different regulations mechanisms within a given community, ensuring sufficient diversity in the face of rapid environmental change.

Conclusion

The findings presented above provide an overview of the various stresses and environmental factors, which can influence populations of *V. cholerae* in aquatic habitats. *V. cholerae* can effectively cope with these stresses through its ability to grow under low nutrient conditions, evolve by DNA recombination, form stress-resistant biofilms and produce toxins. In my thesis, I have attempted to elucidate the molecular and genetic mechanisms that are controlling some of these survival strategies. In doing so, I have mainly focused on the molecular mechanisms behind biofilm formation in *V. cholerae.* Chapter 2 examines the genetic basis for biofilm formation in non-toxigenic strains of *V. cholerae*, which form very distinct biofilms from toxigenic ones. Also, this chapter examines whether biofilms formed on abiotic surfaces is the similar to biofilms on biotic surfaces found in marine environments, such as copepods and dinoflagellates. This work was undertaken to understand whether different strains of *V. cholerae* have acquired new genes, which are responsible for observed biofilm differences, or whether these strains are simply regulating the same genes differently to produce different biofilms. Chapter 3 then investigates the tryptophanase gene, in particular, for its role in biofilm formation. This chapter specifically examines how this protein and its enzymatic by-product, indole, are influencing a newly characterized signaling system in *V.*

cholerae. This work not only details the molecular relationships in this new regulatory network, but also shows that indole can influence the expression of many genes involved in environmental survival, such as motility, biofilm formation and virulence towards protozoa. This work adds to the findings presented in this introduction chapter to provide a more comprehensive view of some of the adaptive strategies that *V. cholerae* has evolved for survival in both human and aquatic environments. In doing so it better defines our relationship with this waterborne pathogen.

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CHAPTER 2

Vibrio cholerae Strains Possess Multiple Strategies for Abiotic and Biotic Surface Colonization

Abstract

Despite its notoriety as a human pathogen, *Vibrio cholerae* is an aquatic microbe suited to live in fresh water, estuarine and marine environments where biofilm formation may provide a selective advantage. This study reports the characterization of biofilms formed on abiotic and biotic surfaces by two Non-O1/O139 *V. cholerae* strains, TP and SIO, to the O1 *V. cholerae* strain N16961, and the discovery of 44 transposon mutants of SIO and TP impaired in biofilm formation. During the course of characterizing the mutants, thirty loci, which have not previously been associated with V. cholerae biofilms, were identified. These encode for proteins which perform a wide variety of functions including amino acid metabolism, ion transport and gene regulation. Also, when the plankton colonization abilities of strains N16961, SIO and TP were examined, each showed increased colonization of dead over live plankton (the dinoflagellate *Lingulodinium polyedrum* and the copepod *Tigriopus californicus*). Surprisingly, most of the biofilm mutants were not impaired in plankton colonization. Only mutants impaired in motility or chemotaxis showed reduced colonization. These results indicate the presence of both conserved and variable genes, which influence the surface colonization properties of different *V. cholerae* subspecies.

Introduction

The ecology of the cholera-causing bacterium, *Vibrio cholerae*, has been considered since the 1850's when contaminated water sources were first implicated by Dr. John Snow as a key factor associated with its epidemiology (Snow 1855). However, many of the strategies this organism employs for survival outside the human host have only come to light recently. Perhaps, one of the most important discoveries is the role plankton have in aiding the survival of *V. cholerae* within aquatic environments. Seminal work by Huq, *et al.* (Huq et al. 1983), demonstrated that the presence of copepods improved the survival of *V. cholerae* populations. Analogous experiments performed by Islam, *et al.* (Islam et al. 1990; Islam et al. 2004) showed that *V. cholerae* survival can be enhanced via association with cyanobacteria. In both cases, surface colonization by *V. cholerae* was evident. The propensity of *V. cholerae* for epibiosis is also evident in its attachment to chironomid egg masses and vascular plants (Shukla et al. 1995; Halpern et al. 2004).

The significance of *V. cholerae* higher order assemblages can be further illustrated in two ways. First, there is a correlation between *V. cholerae* population levels and phytoplankton and zooplankton blooms (Epstein 1993; Huq et al. 2005) and the physical factors which drive them (Colwell 1996; Lobitz et al. 2000). The biofilms formed by *V. cholerae* during these associations may aid in survival, as biofilms have been shown to offer shelter from many different environmental stresses including protozoan grazing (Matz et al. 2005), UV light (Espeland and Wetzel 2001), oxidants (Elkins et al. 1999) and toxic metals (Teitzel and Parsek 2003). Second, filtration of drinking water through sari cloth (>20µm pore size) is an effective method for removal of *V. cholerae* assemblages and reducing the incidence of cholera (Colwell et al. 2003).

The biofilm characteristics of clinical isolates of *V. cholerae* have been examined previously (Watnick and Kolter 1999; Yildiz and Schoolnik 1999). Cells of *V. cholerae* O1 El Tor strain N16961 form biofilms in a step-wise manner, first contacting the surface using flagellar motility and the mannose-sensitive hemagglutinin (MSHA) type IV pilus. After surface

contact occurs, a three-dimensional biofilm structure is established by growth of the sessile population and production of an exopolymer matrix composed of Vibrio polysaccharide (VPS). In addition, it has been noted that *V. cholerae* can exhibit a diverse range of growth phenotypes on surfaces, including rugose and smooth colony variation on agar surfaces (Wai et al. 1998; Mizunoe et al. 1999), or distinct biofilms ranging from thick, mature biofilms to flat single layers of cells colonizing a surface (Yildiz and Schoolnik 1999).

The MSHA pilus also contributes to the attachment of *V. cholerae* to the carapaces of the common water flea, *Daphnia pulex* (Chiavelli et al. 2001). *mshA* mutations diminish attachment of *V. cholerae* O1 El Tor strains and O139 strains, but have no significant effect on the attachment of O1 classical strains, due to the fact that the vast majority of classical strains do not elaborate functional MSHA (Finkelstein 1963). This suggests that additional factors outside of the MSHA pilus mediate attachment to biotic surfaces, and that different strains of *V. cholerae* may employ different strategies to attach to surfaces. In fact, further work has identified an additional colonization factor of *V. cholerae*, GbpA, which is an adhesin that binds to sugars present on both chitin containing surfaces and mammalian cells membranes (Kirn et al. 2005).

This current study examines biofilm formation by two environmental, non-toxigenic strains of *V. cholerae*, SIO and TP. The phenotypic differences of the biofilms made by each strain were examined with respect to O1 El Tor clinical strains, and SIO and TP genes important in biofilm formation have been identified using a transposon mutagenesis screen. To understand biofilm formation within a more environmentally relevant context the biofilm forming abilities of these strains on biotic surfaces such as dinoflagellates and copepods was also examined.

Materials and Methods

Strains, plasmids and growth conditions. All strains and plasmids used in this study are listed in Table 2.1. Wild-type strains SIO and TP of *V. cholerae* were isolated from

southern California coastal waters. All *Escherichia coli* and *V. cholerae* strains were grown in LB broth (Miller 1972) supplemented with appropriate antibiotics at 37°C, except when stated otherwise. Antibiotics used in this study were kanamycin (Km, 50μg/mL for *E. coli*; 200μg/mL for *V. cholerae*) and chloramphenicol (Cm, 20μg/mL for *E. coli*; 5μg/mL for *V. cholerae*) and rifampicin (Rif) at a concentration of 100μg/mL.

***V. cholerae* mating.** *E. coli* strain S17-1λ*pir* (Simon et al. 1983) was used as a donor strain for all conjugation experiments using strains of *V. cholerae*. All strains were grown overnight to stationary phase at 37°C. *V. cholerae* strains were subcultured at 1:100 dilution and grown in LB medium at 22°C until the mid-log phase of growth (OD_{600nm} ~ .7) was reached. Both *E. coli* and *V. cholerae* were washed of antibiotics by centrifugation (2 minutes, 13,000 x g) and concentrated 5x and 20x in LB, respectively. Fifteen microliters of each were transferred to a .22μm polycarbonate filter atop of a LB agar plate and mixed together. The conjugations were left overnight at 37°C. Filters were then transferred to tubes containing 1 mL of LB medium and cells were removed by vortexing and plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS, 89g/L, Difco) or LB supplemented with appropriate antibiotics and grown at 37°C overnight.

Transposon mutagenesis library and biofilm screening. The suicide vector pRL27 (Larsen et al. 2002), containing the hyper-transposable mini-Tn5 element, was introduced in V. cholerae strains SIO and TP via conjugation. Transposon mutant libraries were made by selecting individual colonies from the TCBS selective plates containing Km 200μg/ml. These colonies were then inoculated into 200 μL of LB medium in 96-well microtiter dishes, grown overnight and subsequently made into frozen stocks by adding glycerol to a final concentration of 15% to each well.

An initial screen searching for biofilm mutants was adapted from the method presented by O'Toole (O'Toole et al. 1999). Individual transposon mutants were arrayed in 96-well polystyrene microtiter dishes and grown overnight in 200 μL fresh LB at 37°C with

moderate shaking. Crystal violet was then added to each well to allow for staining of the adherent cells. The medium was then washed from each well and replaced with 200 μL of 95% ethanol and the OD_{570nm} of individual wells were recorded. Wells with absorbance readings below wild-type levels were rescreened for biofilm forming ability using borosilicate glass tubes. Cultures were grown in triplicate in 5 mL of LB overnight and retested using the crystal violet method described above. The biofilm accumulation of mutant strains was normalized to the respective parental strain by dividing the OD_{570nm} of each mutant strain by the OD_{570nm} of the wild-type to obtain a percentage. Pair-wise comparisons of the data of each mutant strain and wild-type were then analyzed for significance using a student's t-test ($\alpha = .05$). Mutants displaying significant reduction in biofilm accumulation were also tested for possible growth differences from the wild-type strain, which may have contributed to the observed biofilm forming deficiencies. Here, growth curves for each mutant and wild-type strain grown in 10mL of LB were recorded over a 24 hour period. No gross differences in the growth of the mutant strain and wild-types were noted for any of the mutants reported in this study (data not shown).

Transposon flanking DNA amplification and sequencing. The protocol for arbitrary Polymerase Chain Reaction (PCR) as described by O'toole (O'Toole et al. 1999) was followed in order to obtain sequence data from the loci disrupted by the transposon insertions in each biofilm mutant (for primers see Appendix I Table S2.1). PCRs resulting in amplicons of approximately 500 base pairs were cleaned and sequenced using an Amersham MegaBace 100 sequencer. Recovered sequence data was analyzed on 12/06 by performing BLASTX analysis (Altschul et al. 1990) against the Genbank non-redundant database (http://www.ncbi.nlm.nih.gov). In some cases additional flanking sequence was obtained using the panhandle PCR method adapted from Jones and Winistorfer (Jones and Winistorfer 1992). Several rounds of chromosome walking were used in these instances to obtain additional sequence data within these undetermined regions.

Biofilm formation under continuous flow conditions. Biofilms were formed in flow cells covered with glass cover slips. The continuous flow cell systems were prepared and assembled as previously described (Moller et al. 1998) with minor modifications. The flow cells were constructed from polycarbonate with each flow chamber measuring 1 by 4 by 40 mm and covered by a glass microscope cover slip, which had been glued on top of the flow cell to serve as the substratum for biofilm growth. All tubing and flow cells were autoclaved prior to assembly.

V. cholerae strains were grown overnight in 2M minimal medium (Paludan-Muller et al. 1996). Approximately 1 ml of the overnight culture was inoculated into the flow channels and cells were allowed to attach under conditions of no flow for one hour, during which time the tubing upstream and downstream of the flow cell was clamped. After attachment, the flow was adjusted to approximately 18 ml h^{-1} and the cells perfused with 2M minimal medium with a glucose concentration of 0.04% at room temperature supplied by a peristaltic pump for up to 10 days.

Biofilms formed in flow cell chambers were observed by confocal laser scanning microscopy (CLSM) over a period of 10 days in order to assess biofilm formation and maturation. At the appropriate times, the flow was stopped, flow cells were clamped and biofilms were stained with the LIVE/DEAD® *Bac*Light bacterial viability kit (Molecular Probes Inc., Eugene, OR). This kit contains two fluorescent dyes that stain living cells green (SYTO 9) and dead cells red (propidium iodide). Images were obtained with a BioRad MRC 1024 CLS microscope.

In-frame deletion constructs. To engineer in-frame deletions of specific genes within the *V. cholerae* genomes Gene Splicing by Overlap Extension (SOEing) PCR was performed (Horton 1997). For each deletion approximately 500 bp at the 5' and 3' end of the desired gene were amplified (For primers see Appendix I Table S2.1). These amplicons included approximately 50 bp of the ends of each gene that, when fused by SOEing PCR, created in-frame deletions of the interior portions of the gene of interest. The resulting

products were then cloned into pGPKm (This study) and transformed into S17-1λ*pir* cells. Inserts were PCR-verified using pGPkm-based primers and each suicide plasmid was conjugated into the appropriate Rifampin-resistant *V. cholerae* strain and plated onto LB agar (Rif 100μg/ml, Km 200μg/ml). Resulting colonies were verified for single crossover events using selective medium containing Rif, Km and sucrose and subsequently grown in LB containing no antibiotics allowing for a second crossover to occur. After growth in LB, selection for loss of the genome integrated *sacB*-containing plasmid was performed on LB plates containing 10% Sucrose in the place of NaCl. After overnight incubation at 37°C, colonies were picked and PCR-screened for conversion to the deletion construct using primers external to the original amplicons (see Appendix I Table S2.1). These PCR products were sequenced to verify each in-frame deletion.

Colonization assays. For copepod experiments, the GFP-expressing plasmid, pFL*gfp* (this study), was introduced into various strains of *V. cholerae* via conjugation. These strains were grown in LB medium (Cm 5μg/ml) overnight and verified for fluorescence using epifluorescence microscopy. After growth to stationary phase, the cells were washed two times with autoclaved and filtered seawater, and diluted to a concentration of 10^6 cells/mL in sterile seawater amended with Cm 5μg/ml. Copepods were then washed on GFF filters as 30-50 mL of sterile seawater were filtered over the organisms to remove bacteria from the zooplankton surface and surrounding media. Two live or dead (heat-killed, 65^{o}C-10 min.) copepods were then added to microcosms containing 10^6 washed *V. cholerae*. Each experiment was performed in triplicate and incubated overnight at room temperature in the dark. Individual copepods were then collected and washed again 3x with 15-20 ml of sterile seawater to remove unattached *V. cholerae*. These specimens were then observed using epifluorescence microscopy to detect the level of surface colonization by the bacteria on the copepod. At specific time-points, quantification of colonization to *Tigriopus californicus* was performed by homogenizing individual washed copepods in sterile seawater, and plating serially-diluted homogenates onto TCBS containing Cm 5μg/μl.

Colonization experiments with axenic *Lingulodinium polyedrum* were done in a similar manner. Axenic *L. polyedrum* CCMP strain 1932 (gift of P. Von Dassow, M. Latz, X. Mayali and F. Azam) were cultured at 16°C in F/2 medium (Guillard 1975) under a 12/12 LD cycle illuminated with cool white fluorescent tubes. *V. cholerae* strains carrying pFL*gfp* were grown overnight in LB media, washed in sterile seawater and diluted as above. *L. polyedrum* was taken from exponentially growing cultures and diluted 1:10 in either 1 mL of sterile seawater or artificial seawater (30 p.s.u) in 24-well microtiter dishes. *V. cholerae* were then added to the wells at a concentration of 10^6 cells/mL. Each experiment was performed in triplicate and incubated overnight. After incubation the 1 mL microcosms were filtered onto 5μM polycarbonate filters and washed 3 times with 10 mL of sterile artificial seawater to collect the fraction of *V. cholerae* attached to the *L. polyedrum*. Epifluorescence microscopy was then used to examine the samples for their attachment to the dinoflagellate. Quantification of colonization was performed by manually counting individual attached bacteria to washed dinoflagellates. For each sample at least 20 fields were counted and averages were used for comparison between strains. For each colonization experiment which was quantified pair-wise comparisons of the data of each mutant strain and wild-type were analyzed for significance using a student's t-test ($\alpha = .05$). If the data did not meet initial assumptions of normality and homogeneity of variance it was transformed to perform the analysis.

Colonization experiments were carried out in a similar manner as above using both types of plankton and *gfp*-tagged wild-type strains in the presence of 100μM phenamil, which has been shown to inhibit motility in Vibrios by binding to the sodium-driven motor of the polar flagella (Muramoto et al. 1996). Loss of motility was examined in wild-type strains throughout each experiment using epifluorecent microscopy and colonization phenotypes of *V. cholerae* strains were monitored as described above.

Results

Surface attachment properties of clinical and environmental isolates of *V. cholerae*. The surface-associated growth characteristics of two Southern California non-pathogenic *V. cholerae* isolates, SIO and TP (Purdy et al. 2005), were examined and compared to the O1 serotype, El Tor biotype strain, N16961 (Heidelberg et al. 2000). When grown on LB agar plates strain TP grew as smooth, opaque colonies similar to the smooth variant of the toxigenic strain N16961; whereas, strain SIO grew as a compact, rugose colony (figure 2.1), presumably due to an increase in the production of VPS (Yildiz and Schoolnik 1999). The biofilms elaborated by each strain were also very different. When strains were grown in LB overnight in borosilicate glass tubes it was noted that the biofilms accumulated by the environmental strains, SIO and TP, were thicker and more robust than those formed by strain N16961 (figure 2.1).

SIO and TP biofilms were examined in finer detail using flow cells and confocal laser scanning microscopy (CSLM). Here it was discovered that the morphology and kinetics of biofilm formation between the environmental and clinical strains of *V. cholerae* also differed. Under these conditions, all three strains rapidly colonized the coverslip surfaces, but strains TP and N16961 appear to form more differentiated structures than strain SIO, which colonized the entire surface in a homogeneous manner (figure 2.2). Within the first three days of the experiment, strain TP aggregated into discrete cell clusters on the glass substratum, however these did not resemble the typical rounded microcolonies found in N16961 biofilms, and never reached the biomass of the N16961 microcolonies. Despite this, both N16961 and TP demonstrated a similar pattern in the death of the biofilms, where the centers of these cell clusters demonstrated significant death after days five and seven, respectively. After this event, the death of the remaining biofilm occurs followed by sloughing of the attached cells from the substratum between days seven and ten of the experiment.

SIO biofilms formed in a very different manner. In contrast to the other strains tested, SIO cells never differentiated into clusters of cells and remained attached to the glass surface

in a homogeneous manner. After the initial colonization of the flow cell surface, the undifferentiated biofilm of SIO grew until day five of the experiment where significant death of the entire biofilm was seen. Unlike strain TP and N16961, this death event did not seem to coincide with a dispersal event, rather the cells remained attached to the surface until gradually sloughing by day 10.

Biofilm mutant screen. Based on the above results indicating that both TP and SIO form distinctive biofilms, an inquiry into the genetic basis for these phenotypic differences was conducted. Mini-Tn5 transposon mutagenesis was performed, followed by screening for biofilm defective phenotypes and identification of transposon insertion sites using arbitrary PCR. Forty-four unique mutations conferring a deficiency in biofilm formation were identified between strains TP and SIO, representing ~1% of the total collection of mutants screened. The nature of the biofilm loci identified based upon TBLASTX analysis (Altschul et al. 1990) is presented in figure 2.3A and Table 2.2. Figure 2.3C shows the biofilm formation by each mutant relative to the corresponding parental strain after 16 hours as measured with crystal violet staining.

Mutants involved in surface attachment and biofilm structure. Many of the mutations recovered in this screen resided in genes important for initial surface attachment, microcolony formation and subsequent biofilm maturation. Mutations within VPS genes, which comprised a large segment of the biofilm mutants of this category, were recovered in both of the VPS biosynthetic operons of the V. cholerae genome, with multiple transposon insertions occurring in four of the seven mutated *vps* genes. Microarray studies have shown that rugose colonies of *V. cholerae* up-regulate the expression of both operons compared to smooth colonies (Yildiz et al. 2004). Accordingly, *vps* mutations occurring within the SIO parental strain demonstrated a switch in colony morphology from rugose to smooth. In addition to *vps* gene insertions, numerous mutations were found in genes responsible for chemotaxis, flagella production and biosynthesis of the MSHA pilus. These types of genes have been

hypothesized to aid in V. cholerae biofilm formation by facilitating initial attachment of cells to surfaces (Watnick and Kolter 1999; Moorthy and Watnick 2004).

Interestingly, though, while mutations in the MSHA biosynthetic operon were obtained in strain TP, no such insertions were obtained in the biofilm mutant screen for strain SIO. In order to assess the role of the MSHA in SIO biofilms, an in-frame deletion of the *mshA* gene, a pilin subunit of the pilus, was constructed and its biofilm forming ability was assessed. Surprisingly, it was discovered that the Δ*mshA* mutant exhibited increased surface accumulation compared to the wild-type. Thus, the MSHA pilus is not essential in all strains for the formation of biofilms under the conditions tested.

Finally, two genes were found in loci that appear to be important in lipopolysaccharide (LPS) production. One transposon insertion disrupted the *rfaD* gene (VC0240), which has been shown in *Salmonella* to be involved in the biosynthesis of a conserved sugar within the LPS molecule (Lehmann et al. 1973). The second mutation was contained within a region of the TP chromosome not found in the N16961 genome. DNA sequencing of this region revealed that this mutation resides in a locus that contains sequences with high similarity to LPS biosynthesis genes from various organisms, including *V. cholerae* serogroup O37 (for sequence data see Appendix I Table S2.2).

Biofilm regulatory mutants. Many regulators of biofilm formation have recently been discovered in *V. cholerae*, with most of these directly or indirectly acting to affect the transcription of the VPS genes (Yildiz et al. 2001; Haugo and Watnick 2002; Zhu et al. 2002; Casper-Lindley and Yildiz 2004). For example, the modulation of cytosolic cyclic diguanylate (cdiGMP) levels by a family of proteins containing GGDEF and/or EAL domains appears to be one way in which *V. cholerae* cells regulate *vps* gene expression (Tischler and Camilli 2004).

In accordance with the importance of this signaling system, two of the genes (VC1934 and VCA0075) disrupted in the mutagenesis screen were within syntenic and paralogous operons containing enzymes of this protein family. It should be noted that although these operons are similar in their arrangement, VC1934 contains both GGDEF and EAL domains,

implying a role in both cdiGMP breakdown and synthesis, while its paralog, VCA0074, contains only the GGDEF domain. Interestingly, microarray studies have shown that genes within both of these operons are up-regulated in rugose *V. cholerae* colonies when compared to smooth variants, suggesting a possible role in modulating *vps* expression (Yildiz et al. 2004). Consistent with this regulatory role the SIO-derived mutant VCA0075 lost the rugose phenotype of its parental strain, which is similar to a published report by Lim, *et al.* (Lim et al. 2006) that notes a similar phenotypic shift in the colony morphology of mutants of this operon in a rugose variant of *V. cholerae* strain 92A1552.

Another biofilm mutant contained a transposon insertion within VCA0051, a hypothetical protein containing a tetratrico peptide repeat (TPR) motif. The TPR domain appears to coordinate protein-protein interactions between the motif containing protein and a target protein (For review see D'Andrea and Regan 2003). VCA0051 is located within a highly conserved operon within the Vibrionaceae and contains a downstream gene coding for another GGDEF/EAL protein.

In addition to these types of regulators, two mutations were found to be located within genes encoding transcriptional regulators. One of these insertions is located in the *lacI* gene (VC2337). The second of these interruptions is within a gene containing a conserved PadR transcriptional regulator domain. This family of regulators has been implicated in response to phenolic acid compounds (Gury et al. 2004).

Amino acid metabolism mutants. Various mutations were found within genes responsible for amino acid metabolism, including the *leuC* and *lysC* genes. Although the role of these genes with respect to biofilm formation is unknown, their recovery is consistent with other mutagenesis screens which have discovered similar amino acid mutants that are unable to form proper biofilms (Sauer and Camper 2001).

Both TP and SIO strains contained a mutation in the peptidase N operon (VC1494, VC1495). The peptidase N product is related to proteins in the KEGG database (Kanehisa and Goto 2000) which are involved in glutathione catabolism by catalyzing the breakdown of

L-cysteinyl-glycine to cysteine and glycine. Similar to other SIO mutants, the *pepN* mutant grew as a smooth colony on LB agar plates, indicating a possible deficiency in polysaccharide production, although it is unknown how PepN enzymatic activity may be governing VPS production.

Another gene, which is important for both SIO and TP biofilm formation, is the tryptophanase gene, *tnaA*, which is a pyridoxyl 5'-phosphate (PLP) dependent enzyme that catalyzes the breakdown of tryptophan. Recent work has shown that indole, a product of this reaction, is a key factor in *E. coli* biofilm formation. The addition of this molecule to *E. coli tnaA* mutants causes significant changes to the proteome of the cells (Collet et al. 2007), and can complement biofilm deficiencies in various bacterial strains simply through its exogenous addition (Martino et al. 2003). In the same way, indole was added to liquid cultures of the *tnaA* transposon mutants of SIO and TP. Crystal violet staining of the biofilms after overnight growth in 5 mL of LB broth at 37°C demonstrated that indole was able to complement the biofilm formation defect in both the SIO and TP mutants at concentrations $\geq$100μM (data not shown), which is in accordance with observed extracellular indole concentrations for *E. coli* grown in culture (Wang et al. 2001).

The tryptophanase mutant of SIO also grew as a smooth, opaque colony on LB agar implicating a role for this gene in VPS production. However, this mutant was not identical to the smooth, translucent colonies of the VPS mutants of SIO, suggesting that *vps* expression may not be completely eliminated in the *tnaA* mutant. To determine whether indole was also affecting rugosity in this strain, the SIO *tnaA* mutant was grown on plates containing indole. After growth overnight at 37°C, rugosity was restored in the mutant similar to the SIO wild-type. These results indicate that indole does indeed influence the production of VPS in strain SIO.

Transporter mutants. The biofilm mutant screen also uncovered numerous genes that have been annotated to function in transport of various organic compounds and ions. Two insertions occurred in genes that have putatively been annotated to be involved in the

transport of carbohydrates. The assigned function of the first of these, VC1437, is an E1-E2 family transporter, which has been annotated by the KEGG database as an isomaltose transporter. Studies examining the transcriptome of *E. coli* biofilms have discovered that a maltose permease transporter and maltose binding protein are among the genes significantly increased in expression in biofilm cells versus planktonic cells (Schembri et al. 2003). The second mutation is within the last gene of an operon coding for an ABC carbohydrate transporter, VCA1100. This operon is up-regulated by in the presence of chitin, suggesting its role in a surface attached lifestyle (Meibom et al. 2004).

A second group includes mutations within an E1-E2 family putative Cu^{2+} transporter (VC2215), a putative Zn^{2+} uptake regulation protein (VC0378), and an operon coding for a mechanosensitive channel and a Mg^{2+} transporter (VCA0817) (Heidelberg et al. 2000). These loci are noteworthy given the connection between divalent cation transport and biofilm formation uncovered in other bacteria. A magnesium transporter is required for biofilm formation in *Aeromonas hydrophilia* (Merino et al. 2001), and studies by Loo, *et al*. (Loo et al. 2003) have shown that various proteins involved in zinc and manganese uptake are important for biofilm formation in *S. gordonii*.

The last mutant recovered in this class, is in a gene coding for an Iron (III) ABC transporter (VC0609). It has recently been reported that low iron environments inhibit the normal formation of biofilms on surfaces by interfering with normal motility during biofilm development (Singh et al. 2002).

Unclassified Mutants. Numerous mutations occurred in genes without clear connections to specific functions in biofilm formation. Among these were various genes that have been annotated to encode hypothetical proteins; some of which appear to be regulated by RpoN, which has been shown in *V.* cholerae to regulate many genes involved in biofilm formation including *vpsR* and *hapR* (Yildiz et al. 2004). Included in these are two conserved hypotheticals, VCA0936 and VCA0109. The latter of these has also been shown to be located within a conserved operon essential for the secretion of factors involved in Eukaryotic cell

death (Pukatzki et al. 2006). Another gene within this group is VCA0859, which encodes a putative Family 2 Aldo/Keto reductase. While the exact role of this protein in biofilm formation is unclear, it is notable that an oxidoreductase, PA3701, was also found to be essential for biofilm formation in *P. aeruginosa* (Finelli et al. 2003).

Other genes included in this group are *hemB* (VC0105), various other hypotheticals and two putative mobile genetic elements not found within the N16961 genome. The first of these is closely related to a gene encoding a regulatory protein of the *V. cholerae* phage VGJϕ (Campos et al. 2003), and the second is a putative Tn5 transposase gene located within the intragenic region between VC0307 and VC0308 (figure 2.3B; for sequence data see Appendix I Table S2.2). When this second mutant was examined under flow cell conditions it was noticed that it was unable to form microcolonies on the glass surface, preventing subsequent three-dimensional development of the biofilm (data not shown). It has recently been demonstrated that mobile genetic elements may play a large role in biofilms, as phage-related genes have been shown to be activated in biofilms and, in some cases, dictate phenotypic variation in cells resulting in differences in biofilm development (Webb et al. 2004).

Phenotypic verification of genetic disruptions. Many of the biofilm mutants contained insertions within putative operons. Consequently, the biofilm defect could be derived from the loss of function of the disrupted gene or because of a polar effect on downstream gene expression. In order to discriminate between these possibilities, in-frame deletions were made for a select group of SIO and TP mutants and the biofilm forming ability of these strains was measured. This method was chosen over standard plasmid complementation because of the difficulty in creating suitable and stable expression vectors for strains SIO and TP (unpublished results).

Table 2.3 lists the in-frame deletions that were created. Also, shown are the relative biofilm forming abilities of some transposon mutants along with their in-frame deletion counterparts. As a control, in-frame deletions were created in the *vpsL* gene (VC0934) of SIO and TP, which is essential for VPS production and proper biofilm formation (Yildiz et al. 2001).

As expected, this mutation greatly reduced biofilm formation within these strains. Among the other in-frame deletions tested, all exhibited biofilm defects similar to the strains carrying transposon mutations within the same genes. These results indicate that the biofilm deficiency of these mutants is most likely derived from the loss of function of the disrupted genes.

Colonization of plankton by *V. cholerae* wild-type strains. To determine whether diminution of attachment to abiotic surfaces translates into an impairment in biotic attachment, the ability of various strains to colonize marine plankton was evaluated. Initial colonization experiments examined the ability of *gfp*-tagged strains of N16961, SIO and TP to attach to the marine copepod, *T. californicus*, and to the marine dinoflagellate, *L. polyedrum*. For each experiment approximately 10^6 cells per milliliter were incubated with copepods or dinoflagellates and colonization was allowed to proceed overnight at room temperature.

When attachment to copepods was examined it was noted that all strains of *V. cholerae* demonstrated an ability to colonize *T. californicus*, but that colonization was greatly enhanced on heat-killed copepods versus live animals (data not shown). On live copepods colonization was greatest on the oral and anal regions and the egg sacs, as previously reported (Huq et al. 1983). In contrast, after the copepod was killed the entire carapace was quickly colonized by all three *V. cholerae* strains tested.

Similar results were obtained when attachment to *L. polyedrum* was investigated. Each strain exhibited a strong affinity to dead organisms, and displayed little attachment to live, exponentially growing phytoplankton. Attachment to both live and dead *L. polyedrum* was dependent on the phase of growth of the dinoflagellates, as no strains of *V. cholerae* were able to attach appreciably to live or dead organisms when the *L. polyedrum* was taken from older cultures in the final stages of growth. Unlike colonization of copepods, there were no distinct differences in the ability of any of the strains of *V. cholerae* to attach to the dinoflagellates.

The biofilms formed on dead dinoflagellates and copepods under seawater conditions were different than those formed on glass or plastic surfaces by LB cultures. Similar to biofilms formed on glass under artificial seawater conditions (Kierek and Watnick 2003), the biofilms on biotic substrates consisted mainly of a single layer of cells which eventually covered the entire surface of the organism (figures 2.4B and E). When time-course experiments were performed using *T. californicus* as the substrate for colonization, rapid initial attachment occurred, with significant numbers of *V. cholerae* attaching after four hours of incubation at 30^{o}C. At this point the numbers of attached bacteria increased from ~10^3 cells per copepod to ~10^5. After 24 hours the amounts of SIO and TP cells attached to copepods were ~5x and ~10x greater than N16961, respectively (data not shown).

Colonization of plankton by biofilm mutants. In order to evaluate the plankton colonization ability of the SIO and TP biofilm mutants, a selection of *gfp*-tagged biofilm mutants were also tested. In these experiments, most of the biofilm mutants tested were found to be able to colonize both *L. polyedrum* and *T. californicus* at levels similar to the wild-type SIO and TP strains (Table 2.4). However, when the motility or chemotaxis mutants of strain TP and SIO were tested for their colonization of plankton, little to no visible attachment by the bacteria was observed (figures 2.4C and F). In the few instances where attachment was observed with these mutants, only small clusters of cells were found attached to a surface, which differed dramatically from the biofilms formed by the wild-type strains (figures 2.4B and E).

The graph in figure 2.4A illustrates quantitatively the inability of the motility mutants of strain TP to attach to *L. polyedrum.* When compared to the wild-type, the motility mutant of TP was impaired approximately 10-fold in colonization of dinoflagellates. Under the conditions used it was estimated that approximately 3.5x10^2 cells of *V. cholerae* wild-type cells were able to colonize the surface of a single *L. polyedrum* cell, whereas only forty cells of the mutants impaired in motility were able to attach. Similar results were found when attachment to copepods was quantified. Motility mutants of strain TP were on average 100-fold less

proficient in colonization after overnight incubation, with only ~10^3 cells attaching to an entire copepod compared to >10^5 cells attached for the wild-type (figure 2.4D). These results were further verified when the wild-types of SIO and TP were tested for their colonization ability in the presence of phenamil, which poisons the Na^+-driven motor of the *Vibrio* polar flagellum. In the presence of 100μM phenamil it was observed that wild-type cells of SIO and TP were non-motile and were not able to significantly colonize the carapaces of copepods after overnight incubation.

Discussion

The ability of *V. cholerae* to concentrate on surfaces in aquatic environments has been hypothesized to be one of the main factors controlling environmental survival and disease transmission (Colwell et al. 2003). This results of this study that have discovered many new genes not previously implicated in *V. cholerae* biofilm formation and demonstrated differences in abiotic and biotic biofilm formation, show that strains of *V. cholerae* are highly variable in both genotype and phenotype with regard to surface attachment.

A clear illustration of this variability was seen when different strains of *V. cholerae* were observed to form distinct biofilms even when grown in flow-cells under the same experimental conditions. Here, TP mirrored the clinical strain N16961 in its surface colonization profile, while SIO formed very unique biofilms, which were absent of distinct microcolonies late into the experiment and demonstrated near simultaneous death of the entire biofilm, which then remained attached to the substratum for multiple days. Similarly, data from separate biofilm flow-cell experiments noted that another rugose variant of *V. cholerae* (strain 91A1552) displayed the same biofilm phenotype as SIO (D. McDougald, unpublished data). Therefore, it is possible that an over-production of VPS may lead to the observed lack of water channels, which may be essential for metabolite transport and sustained growth of the biofilms, accounting for the phenotypic differences recorded.

In order to identify genes responsible for these biofilm structure differences transposon mutagenesis screens in the SIO and TP strains of *V. cholerae* were performed. While these screens were not saturating, the genes found to be responsible for biofilm formation in each strain did not fully overlap and a distinct bias was found in the types of genes recovered for each strain. Whereas mutations in strain TP comprised a wide variety of genes including VPS production, flagella and MSHA pilus biosynthesis, etc., 75% of the mutations recovered for SIO appeared to directly or indirectly influence VPS production. Also, no mutations were recovered for SIO in genes responsible for flagella or MSHA biosynthesis. In fact, in-frame deletions of the *mshA* and *flaA* genes did not significantly diminish abiotic biofilm formation of this strain, with the former actually resulting in greater surface accumulation than the wild-type. This mechanism is not unique to SIO, as a study by Watnick, *et al.* (Watnick and Kolter 1999) has shown that a *V. cholerae* O139 clinical isolate also does not rely on flagella or MSHA pili to form biofilms. The dispensable biofilm role of the MSHA pilus is further reflected by the fact that Δ*mshA* mutants of both SIO and TP are not impaired in their ability to colonize copepods. This is not entirely surprising since Chiavelli, *et al.* (Chiavelli et al. 2001) showed that while the MSHA pilus is required for attachment of *V. cholerae* to exoskeletons of *Daphnia pulex* by the MSHA-expressing strains O139 strain, it has little relevance in attachment by O1, classical strains, which are not believed to produce MSHA.

In addition to demonstrating the differences in significance of certain genes in SIO and TP to biofilm formation, the transposon mutagenesis screen identified many new genes not previously described as important for *V. cholerae* biofilms. Six transposon insertions were identified which disrupted the genes involved in the transport of carbohydrates or metal ions, such as Zn^{2+}, Mg^{2+} and Fe^{3+}. Numerous studies have suggested that in addition to being essential to many fundamental cellular processes various transporters perform a critical role in microbial biofilm formation (Merino et al. 2001; Loo et al. 2003). It has been shown that divalent cations demonstrate a strong affinity for the exopolymer matrix of biofilms and have

been hypothesized to support the biofilm by acting as ionic crosslinks between molecules of EPS (Chen and Stewart 2002; Toner et al. 2005). It is possible that under this scenario, trace metals are sequestered within the EPS matrix and become limiting within the cytosol of the bacterial cells. Therefore, the role of ion transport systems may become more critical within EPS-containing biofilms.

A second important class of biofilm mutants contained disruptions in genes involved in amino acid metabolism. These types of genes have also been found to be important for biofilms of other species of bacteria in studies using microarray and mutagenesis analyses (Sauer and Camper 2001; Whiteley et al. 2001). From this work it has been hypothesized that these genes may have an essential role in the physiological shift occurring within cells, which accompanies the transition from planktonic to biofilm cells. One gene in particular that was found to be important for biofilm formation in multiple species of bacteria was the tryptophanase gene (Martino et al. 2003). In these bacteria the *tnaA* gene has been proposed to regulate biofilm formation through a signaling mechanism involving a by-product of the tryptophanase reaction, indole. The findings of this study are consistent with these studies, and further implicate indole as a regulator of VPS expression, since indole is able to complement the loss of rugosity of the SIO *tnaA* transposon mutant. While other studies have shown that indole can modulate the expression of genes involved with a variety of cellular functions (Collet et al. 2007), the results here provide the first evidence that indole can function as an extracellular signaling molecule influencing the expression of genes involved in the production of the exopolymeric matrix extruded by cells within a biofilm.

Many genes were also found which have a role in biofilm formation at the level of gene and protein regulation, including genes coding for GGDEF/EAL family proteins. Research by Tischler, *et al.* (Tischler and Camilli 2004) has shown that proteins of this family can modulate intracellular concentrations of the second messenger 3',5'-cyclic diguanylic acid (cdiGMP), which appears to act as a signaling molecule in *V. cholerae* affecting the transcription of *vps* genes. In their work, the GGDEF domain containing protein VCA0956,

was discovered to increase intracellular cdiGMP levels through its diguanylate cyclase activity, which causes a subsequent induction of VPS transcription. Observations of reduced biofilm formation and smooth colony morphology by a mutant in the VCA0075 operon which contains another GGDEF domain-containing protein in this study and another (Lim et al. 2006) suggest a similar deficiency in VPS synthesis.

It is clear from these results that SIO and TP, much like other strains of *V. cholerae*, are relying on disparate sets of genes and regulatory mechanisms in order to produce biofilms on both biotic and abiotic surfaces. While some fundamental genetic mechanisms used to form biofilms may be shared between strains of *V. cholerae*, there appears to be no set of "universal" biofilm genes for all strains. It is also possible that the same strain will rely on many different genes to form distinct biofilm morphologies when exposed to a new environment.

This is indeed seen in the comparison between the biofilms of the wild-type strains grown on abiotic surfaces in nutrient-replete rich media (LB) and the biofilms grown on biotic surfaces in unmerited seawater microcosms. In these experiments *V. cholerae* cells formed an almost exclusive monolayer biofilm on plankton in the seawater microcosms and never produced a three-dimensional film characteristic of LB-grown biofilms. Therefore, genetic mutants involved in initial surface attachment could be important for these biofilms, whereas genes involved in producing the three-dimensional structure of biofilms (*e.g. vps* genes) could have reduced importance. Consistent with this possibility, only mutants affected in motility and chemotaxis, which have previously been shown to be responsible for defects in the monolayer stage of biofilm formation in *V. cholerae* (Moorthy and Watnick 2005), were impeded in attachment to *L. polyedrum* and *T. californicus*, whereas none of the VPS mutants tested demonstrated appreciable reduction in attachment. This result is supported by a previous finding that motility mutants of *Silicibacter* sp. TM1040 are also not able to colonize the marine dinoflagellate *Pfesteria piscicida* (Miller and Belas 2006).

It should be noted that it is not merely the presence of a flagellum, which is required for attachment to plankton, but also its proper rotation. Flagellated strains grown in the presence of phenamil, an inhibitor of the sodium-driven flagella motor (Atsumi et al. 1992), were also unable to attach appreciably to copepods. Therefore, it may be the motive force a functional flagellum provides which is needed to overcome the electrostatic repulsive forces found on surfaces (Watnick and Kolter 1999).

Another result of this study is that under the conditions tested all of the strains of *V. cholerae* were dramatically better at attaching to dead versus live organisms. This coincides with previous mesocosm experiments which showed that *V. cholerae* preferentially associate with detrital rather than free-living phase during the demise of intense phytoplankton blooms (Worden et al. 2006). Therefore, in the context of the true environment, quantification of *V. cholerae* populations associated with detritus and within sediments as a function of plankton bloom cycles would be helpful in assessing the ecological significance of these colonization preferences. With this knowledge we may be better able to identify the exact nature of the environmental reservoir of *V. cholerae*.

This study has provided evidence that different strains of *V. cholerae* possess overlapping but distinctive sets of genes for biofilm formation. In addition, clear differences have been noted in the genetic requirements for biofilm formation on abiotic and biotic surfaces and in the characteristics of these biofilms.

Table 2.1 Strains and plasmids used in chapter 2.

Strain or plasmid	Relevant Genotype or Description	Source
E. coli		
S17-1λ*pir*	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	(Simon et al. 1983)
V. cholerae		
SIO	Wild-type	(Purdy et al. 2005)
TP	Wild-type	(Purdy et al. 2005)
N16961	Wild-type O1 El Tor	(Heidelberg et al. 2000)
S2151	SIO Δ*mshA*	This study
S2153	SIO Δ*vpsL*	This study
S2163	SIO Δ*pepN*	This study
S2164	SIO ΔVC2215	This study
S2101	SIO Δ*tnaA*	This study
S2123	SIO Δ*pomA*	This study
S2104	SIO Δ*flaA*	This study
T2114	TP Δ*zur*	This study
T2151	TP Δ*mshA*	This study
T2153	TP Δ*vpsL*	This study
T2127	TP ΔVC1437	This study
T2112	TP Δ*lacI*	This study
T2101	TP Δ*tnaA*	This study
T2130	TP ΔVCA0859	This study
T2123	TP Δ*pomA*	This study
T2104	TP Δ*flaA*	This study
Plasmids		
pRL27	Tn5-Rl27 *ori*R6K Km^r	(Larsen et al. 2002)
pFL122	RSF1010 derivative lacZ MCS *mob* Sm^r	(Lauro et al. 2005)
pFL122L	Linker w SacI site ligated into BglII site of pFL122	This study
pBSL181	*ori*R6K Ap^r Cm^r	(Alexeyev and Shokolenko 1995)
pFLcm	pFL122L containing *cat* gene from pBSL181	This study
p519n*gfp*	RSF1010 derivative containing *pnptII* promoter before *gfp* gene *mob* Km^r	(Matthysse et al. 1996)
pFL*gfp*	pFLcm122L containing PCR amplicon of pnptII-*gfpmut2* of p519ngfp	This study
pGP704sac28	pGP704 derivative containing *sacB* gene of *B. subtillus ori*R6K *mob* Ap^r	(Casper-Lindley and Yildiz 2004)
pMB2190	pBR327 derivative Km^r Ap^r	(Arps 1987)
pGPKm	pGP704sac28 with Km^r of pMB2190	This study
pGPVX0408	Δ*mshA* in pGPKm	This study
pGPVX0892	Δpom*A* in pGPKm	This study
pGPVX0934	Δ*vpsL* in pGPKm	This study
pGPVX1494	Δ*pepN* in pGPKm	This study
pGPVX2188	Δ*flaA* in pGPKm	This study
pGPVX2215	ΔVC2215 in pGPKm	This study
pGPVCX0161	Δ*tnaA* in pGPKm	This study
pGPVX0378	Δ*zur* in pGPKm	This study
pGPVX1437	ΔVC1437 in pGPKm	This study
pGPVX2337	Δ*lacI* in pGPKm	This study
pGPVCX0859	ΔVCA0859 in pGPKm	This study

Table 2.2 Genes important for biofilm formation in *V. cholerae* strains SIO and TP.

Strain Designation	TIGR Gene/ Genome region	Gene Name	Common Name/Conserved Domains[2]	Predicted Function
Cell motility				
T1104	VC2188	*flaA*	Flagellin subunit A	Flagellar motility
T1106	VC2131	*fliH*	Flagellar assembly protein	Flagellar motility
T1108	VC2190	*flgL*	Flagellar hook associated protein	Flagellar motility
T1109	VC2194	*flgH*	Flagellar L-ring protein precursor	Flagellar motility
T1116	VC2125	*fliN*	Flagellar motor switch	Flagellar motility
T1123	VC0892	*pomA*	Flagellar motor protein	Flagellar motility
T1139	VC2059	*cheW*	Purine-binding chemotaxis protein	Chemotaxis
T1141	VC0414	*mshQ*	MshQ	MSHA pilus biosynth.
Amino acid transport and metabolism				
S1163	VC1494	*pepN*	AminopeptidaseN	Glutathione metabolism
S1168	VC2492	*leuC*	3-isopropylmalate dehydratase	Leucine metabolism
S1165	VCA1100		ABC transporter	Peptide/nickel transport
S/T1101	VCA0161[1]	*tnaA*	Tryptophanase	Tryptophan metabolism
T1124	VCA0160	*mtr*	Mtr	Tryptophan transport
T1133	VC0391	*lysC*	Aspartokinase III	Amino acid metabolism
Inorganic ion transport and metabolism				
S1164	VC2215		Cation transporter ATPase, E1-E2 family	Divalent ion transport
T1114	VC0378		Zinc uptake regulation protein	Zinc transport
T1127	VC1437		Cation transporter ATPase, E1-E2 family	Divalent ion transport
T1132	VC0609		ABC transporter	Iron (III) transport
Cell wall/membrane biogenesis				
S/T1105	VC0927[1]	*cpsF*	UDP-N-acetyl-D-mannosamine transferase	Exopolysaccharide biosynth.
S/T1128	VC0921[1]		Polysaccharide export protein	Exopolysaccharide biosynth.
T1102	VC0918	*epsD*	UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase	Exopolysaccharide biosynth.
T1142	VC0240	*rfaD*	ADP-L-glycero-D-mannoheptose-6-epimerase	Lipopolysaccharide biosynth.

Table 2.2 continued.

Strain Designation	TIGR Gene/ Genome region	Gene Name	Common Name/Conserved Domains[2]	Predicted Function
General function prediction only				
S1150	VC0920[1]	*epsF*	Glycosyl transferase	Exopolysaccharide biosynth.
T1130	VCA0859[1]		Aldo/keto reductase 2 family	Phosphate starvation
Coenzyme transport and metabolism				
T1118	VC0105	*hemB*	δ-aminolevulinic acid dehydratase	Porphyrin metabolism
Transcription				
T1112	VC2337	*lacI*	LacI family transcriptional regulator	Transcription regulation
Replication, recombination and repair				
T1122	VC0345	*mutL*	DNA mismatch repair protein	Hfq operon
Signal Transduction Mechanisms				
S1166	VC1934		Diguanylate cyclase/phosphodiesterase	Exopolysaccharide biosynth./signaling
Conserved hypothetical				
S1162	VCA0094		MarR transcriptional regulator domain[2]	Transcription regulation
T1121	VC0661		O-methyltransferase domain[2]	General function
T1131	VCA0524		Tellurite resistance protein domain[2]	Inorganic ion transport
Hypothetical				
S1154	VCA0051-GGDEF operon		TPR transcriptional regulator domain[2]	
S1160	VCA0075-GGDEF operon			
S/T1103	VC0922[1]-VPS operon			
T1111	VC1495- *pepN* operon			
T1115	VCA0817-*mgtE* operon		Small mechanosensitive channel domain[2]	
T1120	VCA0213		Na/H transporter domain[2]	Energy production
T1125	VC0926-VPS operon			
T1134	VC0935-VPS operon			
T1144	VCA0109		Conserved Domain: DUF1316[2]	Function unknown
T1146	VCA0936		Deacylase/carboxypeptidase domain[2]	General function

Table 2.2 continued.

Strain Designation	TIGR Gene/ Genome region	Gene Name	Common Name/Conserved Domains[2]	Predicted Function
Other				
T1143	Tn5 transposase			
T1140	Vibrio phage VJK locus			
T1138	Putative LPS locus			

[1] designates multiple mutations in the same locus
[2] designates function assignment using KEGG database

Table 2.3. Verification of biofilm defects in selected mutants.

Strain Number	Genetic Disruption	Predicted Function	Biofilm Formation [ab]	
			Transposon Insertion	In-Frame Deletion
V. cholerae SIO				
SIO	Wild-type		1.00 ± .192	1.00 ± .192
S2151	VC0408	MSHA Pilin	NR	1.17 ± .294
S2123	VC0892	Motility	NR	.919 ± .070
S2104	VC2188	Flagellin	NR	.054 ± .033
S1155/S2153	VC0920/VC0934	VPS Genes	.018 ± .004	.014 ± .015
S1163/S2163	VC1494	Aminopeptidase	.034 ± .005	.045 ± .200
S1164/S2164	VC2215	Cation Transport	.598 ± .793	.645 ± .147
S1101/S2101	VCA0161	Tryptophanase	.157 ± .046	.169 ± .097
V. cholerae TP				
TP	Wild-type		1.00 ± .182	1.00 ± .182
T1114/T2114	VC0378	Zinc Transport	.025 ± .004	.133 ± .073
T1141/T2151	VC0414/VC0408	MSHA Pilus Genes	.081 ± .040	.051 ± .064
T1123/T2123	VC0892	Motility	.161 ± .117	.009 ± .001
T1102/T2153	VC0935/VC0934	VPS Genes	.154 ± .053	.026 ± .012
T1127/T2127	VC1437	Cation Transport	.134 ± .094	.045 ± .029
T1104/T2104	VC2188	Flagellin	.076 ± .012	.013 ± .001
T1112/T2112	VC2337	Transcription	.490 ± .272	.136 ± .059
T1101/T2101	VCA0161	Tryptophanase	.076 ± .035	.057 ± .050
T1130/T2130	VCA0859	Oxidoreductase	.240 ± .230	.453 ± .558

NR-Not recovered by transposon mutagenesis screen

[a] Biofilms were quantified using the crystal violet staining technique descibed in the materials and methods section

[b] Errors associated with values represent one standard deviation from the mean.

Table 2.4 Qualitative evaluation of the colonization ability of *gfp*-tagged biofilm mutants on dinoflagellate and copepod surfaces. (Strain numbers followed by f contain the *gfp*-expressing plasmid pFL*gfp.*)

Strain Number	Genetic Disruption	Predicted Function	Colonization Substrate	
			Dinoflagellate	Copepod
V. cholerae SIO				
SIOf	SIO-Wild-type		+	+
S2151f	VC0408	MSHA pilin	++	+
S2123f	VC0892	Flagellar motor	-	-
S1155f	VC0920	VPS gene	+	++
S1103f	VC0922	VPS gene	+	+
S1166f	VC1934	GGDEF/EAL	+	+
S1154f	VCA0051	Hypo (GGDEF/EAL Operon)	+	+
S1160f	VCA0075	Hypo (GGDEF Operon)	+	+
S1162f	VCA0094	Hypo/Transcriptional regulator	+	+
S1101f	VCA0161	Tryptophanase	+	+
SIOf + Phenamil		Flagellar motor inhibitor	NT	-
V. cholerae TP				
TPf	TP-Wild-type		+	+
T1118f	VC0105	Porphyrin metabolism	+	+
T1142f	VC0240	Lipopolysaccharide biosynthesis	+	+
T1114f	VC0378	Ion Transport	+	+
T1130f	VC0391	Lysine metabolism	+	+
T2151f	VC0408	MSHA pilin	++	+
T1141f	VC0414	MSHA pilus biosynthesis	+	+
T1132f	VC0609	Ion Transport	+	+
T1123f	VC0892	Flagellar motor	-	-
T2123f	VC0892	Flagellar motor	-	-
T1102f	VC0918	VPS gene	++	++
T1103f	VC0922	Hypo (VPS operon)	+	+
T1111f	VC1495	Hypo (*pepN* operon)	+	+
T1139f	VC2059	Chemotaxis	-	-
T1116f	VC2125	Flagellar motor switch	-	-
T1106f	VC2131	Flagellar assembly	-	-
T1104f	VC2188	Flagellin	-	-
T1108f	VC2190	Flagellar hook protein	-	-
T1112f	VC2337	Transcriptonal regulator	+	+
T1101f	VCA0161	Tryptophanase	++	+
T1120f	VCA0213	Hypo/Ion transporter	+	+
T1131f	VCA0524	Hypo/Ion transporter	+	+
T1115f	VCA0817	Hypo/Mechanosensitive transporter	+	+
T1130f	VCA0859	Oxidoreductase	+	++
T1138f		Putative LPS loci	+	+
T1140f		Vibrio phage loci	+	+
T1143f		Tn5 Transposase	+	++
TPf + Phenamil		Flagellar motor inhibitor	NT	-

+ represents wild-type colonization levels, ++ exceeds wild-type levels, - denotes greatly reduced colonization compared to the wild-type; NT-not tested.

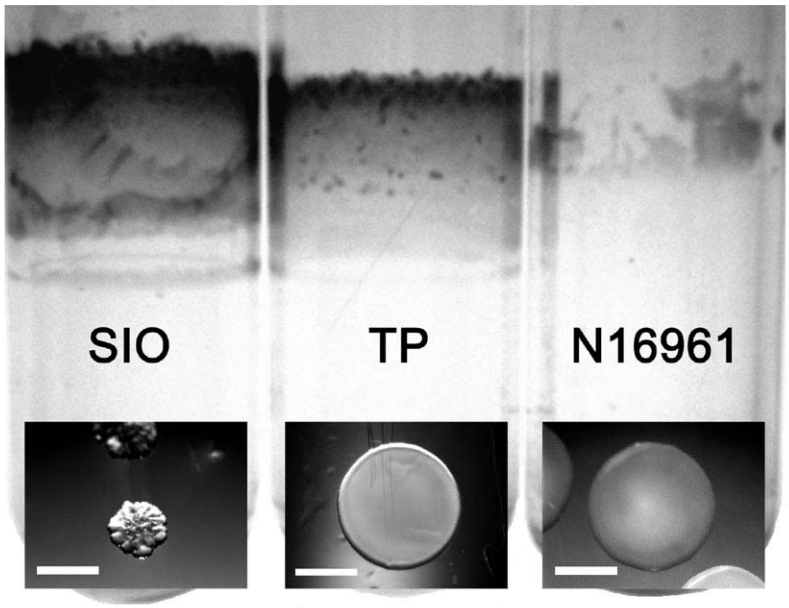

Figure 2.1. Crystal violet staining of the biofilms attached to borosilicate tubes of the two environmental *V. cholerae* strains, SIO and TP, and the clinical strain N16961. Insets document the colony morphology of each strain. Scale bars represent 1mm at 60X magnification.

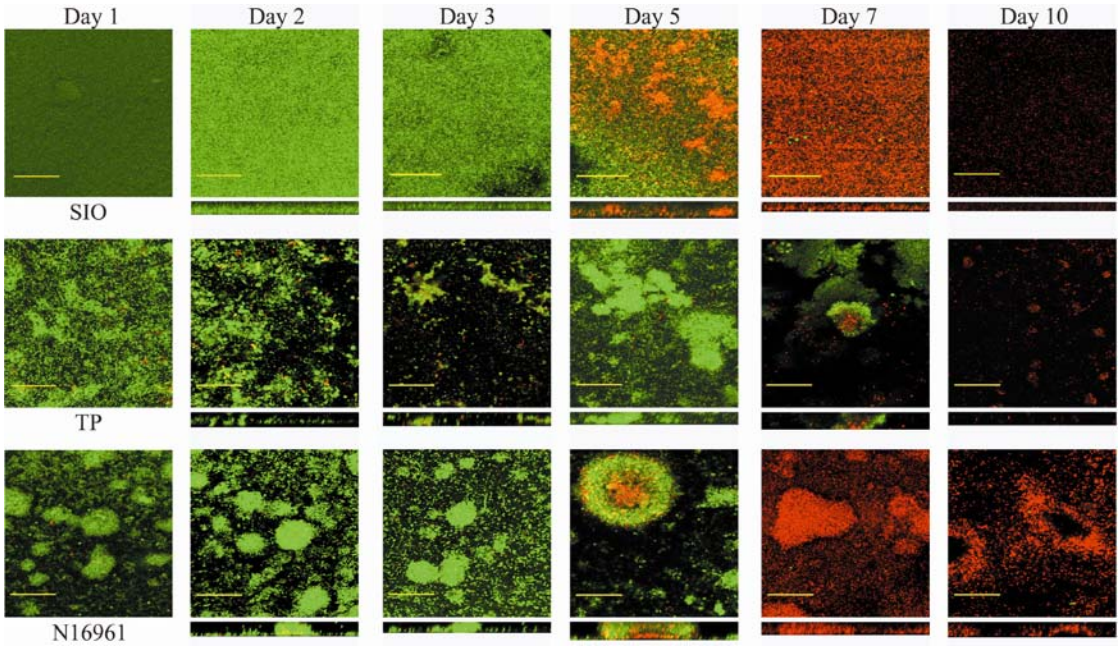

Figure 2.2. Steps in the development of *V. cholerae* SIO, TP and N16961 biofilms using the LIVE/DEAD® BacLight staining kit. By the first day, strain SIO rapidly colonizes the entire glass surface as a monolayer of cells (green stained cells). As the biofilm ages, strain SIO cells begin to die by day five (red stained cells), and completely detach from the glass substratum by day 10. The development of *V. cholerae* TP proceeds differently with discreet clusters of cells developing on the substratum between days 2-5. On day seven the centers of these clusters appear to die off and by day 10 no significant attachment can be seen to the glass. Strain N16961 begins forming distinct, rounded microcolonies (~100µm) immediately after attachment. Similar to strain TP the centers of the microcolonies begin to die off by day five with death of the entire biofilm occurring by day seven. This is followed by dispersal of the biofilm, leaving hollow colonies devoid of cells. Scale bars represent 50 µm.

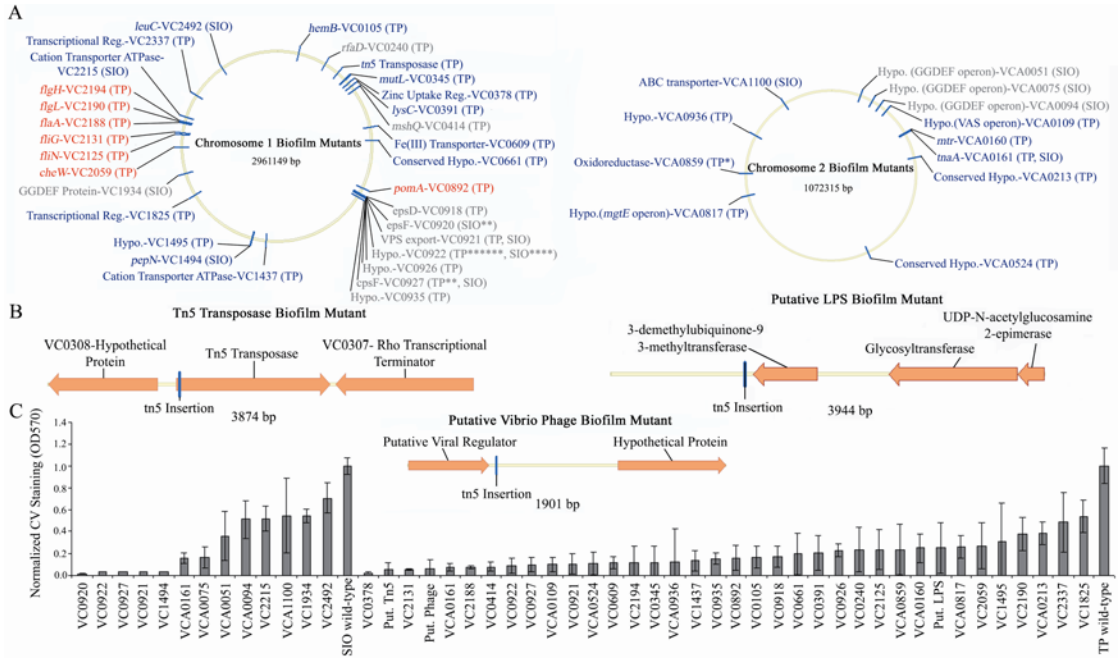

Figure 2.3. Biofilm mutants of *V. cholerae* SIO and TP. (A) Genomic map of the genes important for biofilm formation recovered in this study. Label includes TIGR VC number of the mutated locus, putative gene function and the parental *V. cholerae* strain. (* represents multiple mutations in the same locus.) (B) Loci of the three biofilm transposon insertions isolated in strain TP whose genome sequence is not present in the N16961 strain. (C) Biofilm formation measured by crystal violet staining of the biofilm mutants. Values are normalized to the wild-type biofilm formation. Error bars represent one standard deviation from the mean.

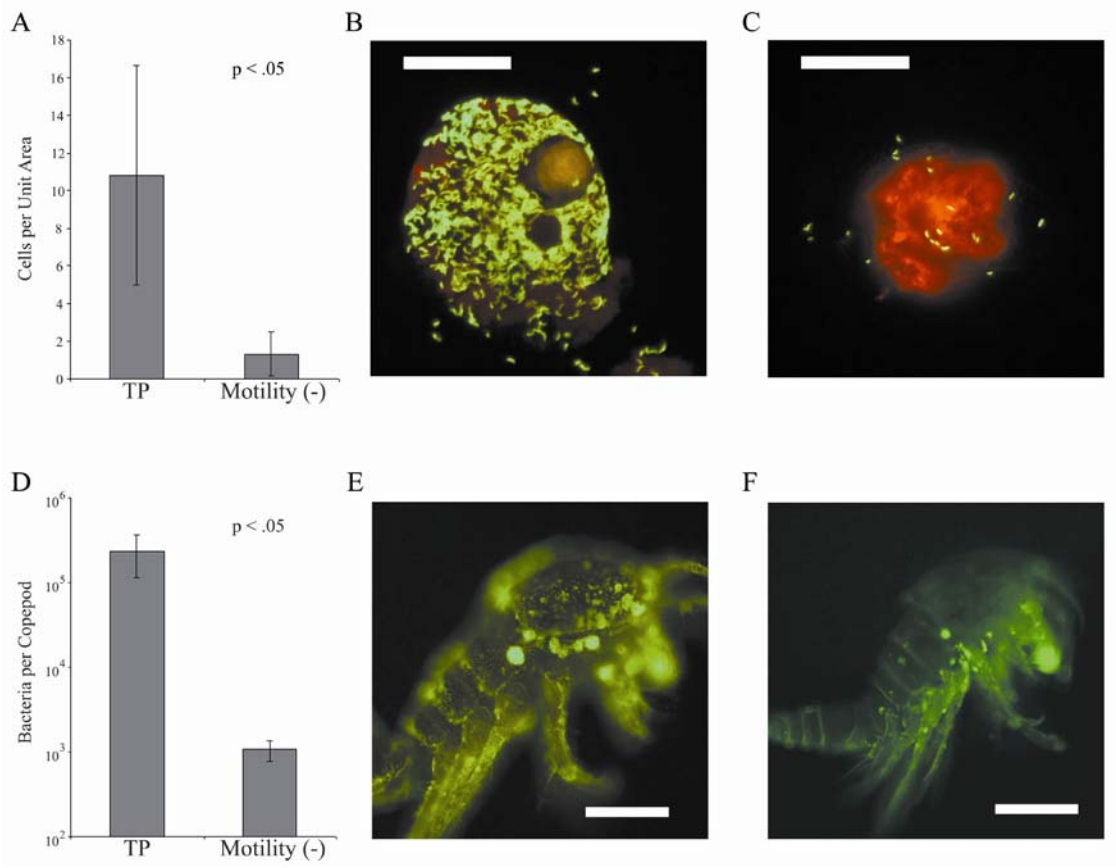

Figure 2.4. Comparison of the ability of *gfp*-tagged wild-type *V. cholerae* and motility mutants to colonize the dinoflagellate *L. polyedrum* and *T. californicus*. (A) Quantification of attached cells of the TP wild-type and a motility mutant of TP to the surface of the dinoflagellate. (B and C) Epifluorescent images of these strains to the surface of dead *L. polyedrum*. Pictures were taken at 1000X magnification and scale bars represent 20µm. (D) Quantification of attached cells of the TP wild-type and a motility mutant to the surface of copepods. (E and F) Epifluorescent images of TPf and T2123f to the surface of dead.*T. californicus*. Pictures were taken at 100X magnification and scale bars represent 200µm. (Error bars in each graph represent one standard deviation from the mean.)

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CHAPTER 3

Indole acts as an Extracellular Cue Regulating Gene Expression in *Vibrio cholerae*

Abstract

Indole has recently been proposed to act as an extracellular signal molecule influencing the biofilm formation in a range of bacteria. In this study the role of this molecule in *V. cholerae* biofilm formation has been examined, and it is shown that indole directly acts to up-regulate the genes involved in Vibrio Polysaccharide (VPS) production, which is essential for *V. cholerae* biofilm formation. Beyond these genes, it was also determined using microarrays that indole can influence the expression of many other genes, including those involved in motility, protozoan grazing resistance, iron utilization and ion transport. Further, a transposon mutagenesis screen has identified several components of the signal transduction system once the indole signal has been received. This regulatory cascade involves the DksA protein along with known regulators of VPS production, VpsR and CdgA with ultimate control of gene expression being coordinated through the cyclic di-guanylic acid (cdiGMP) second messenger system.

Introduction

Bacterial cells commonly use chemical signaling as a way to monitor the extracellular environment for changes in specific conditions and respond accordingly. A diverse catalog of intra- and extracellular signal molecules have recently been characterized in bacteria and include such compounds as acyl homoserine lactones used in quorum sensing systems, numerous oligopeptide autoinducers of gram positive bacteria and cyclic nucleotides used in intracellular signal cascades (reviewed in Camilli and Bassler 2006). As would be expected, the phenotypic response to these compounds generally involves traits, such as biofilm formation, virulence, motility, bioluminescence, sporulation and competence, which are beneficial under adverse conditions.

Indole is a relatively recent addition to the list of signaling molecules used by bacteria. It is produced as a by-product of the break down of tryptophan by the enzyme tryptophanase (TnaA, Evans et al. 1941). Since the expression of the *tnaA* gene is controlled by catabolite repression, it is only transcribed during carbon limitation (Yanofsky et al. 1991). As a result of this regulation, large quantities of indole are generally produced during the stationary phase of growth. Indole has long been known to act as a chemorepellent of *E. coli* (Tso and Adler 1974), but only recently has this molecule also been shown to control the expression of a wide assortment of genes and phenotypes unrelated to chemotaxis, in many different bacteria. For example, in *E. coli* indole controls the expression of genes involved in amino acid metabolism (Wang et al. 2001), plasmid maintenance (Chant and Summers 2007) and quorum sensing (Lee et al. 2007) among other functions (Collet et al. 2007). Additionally, indole may function as an inter-species signal contributing to biofilm formation in an assortment of different bacteria known to carry a copy of the tryptophanase gene (Martino et al. 2003).

It was previously shown that this effect also extends to the etiological agent of the pandemic disease cholera, *Vibrio cholerae* (see chapter 2). Transposon insertions in the tryptophanase gene contained within the genomes of two environmental strains of *V. cholerae* were shown to result in diminished biofilm formation by each mutant, and that

supplementation of indole within the growth medium was able to complement the biofilm defect of these strains (Mueller et al. 2007). Due to the reliance of these strains on the production of vibrio polysaccharide (VPS) for biofilm formation, it was theorized that indole was influencing the regulation of VPS secretion within these strains.

Genes encoding proteins for the production of VPS are contained within two operons within the *V. cholerae* genome (*vpsI* and *vpsII*), and their regulation involves multiple transcriptional activators and repressors. The main activator of VPS production appears to be the σ^{54}-dependent transcriptional activator VpsR, which is essential for *vps* gene expression (Yildiz et al. 2001). A secondary activator of these genes is VpsT. This protein is not essential for *vps* transcription, but acts synergistically with VpsR to activate expression (Casper-Lindley and Yildiz 2004). Antagonizing these activities is the master transcriptional regulator of quorum sensing in *V. cholerae*, HapR, which is translated as autoinducer molecules accumulate in the extracellular environment. Since HapR is a repressor of *vps* expression, it is thought that quorum sensing acts to down-regulate VPS production and biofilm formation once the cell density increases above a given threshold (Hammer and Bassler 2003). Superimposed on these regulatory mechanisms is the recent discovery that intracellular signal molecules also play a role in biofilm-related phenotypes, such as VPS production (Tischler and Camilli 2004). In this case, it appears that various proteins of *V. cholerae* with GGDEF and/or EAL domains can modulate intracellular levels of the second messenger cyclic di-guanylic acid (Lim et al. 2006). These concentration fluxes influence the regulators described above and ultimately many genes of *V. cholerae* involved with motility, chemotaxis, virulence and biofilm formation (Beyhan et al. 2006). Thus, the regulation of processes such as biofilm formation appears to be multi-faceted, depending on a variety of environmental and genetic determinants.

In this study it is shown that indole is one of these environmental variables, and can act as an extracellular signal in *V. cholerae* influencing the expression of many different types of genes, including those involved in transport, virulence, biofilm formation and motility.

Evidence is also provided showing that indole signal transduction involves previously characterized gene regulatory systems of *V. cholerae*, such as the VPS regulators VpsR and VpsT, the cdiGMP second messenger system and DksA a RNA polymerase regulatory protein.

Materials and Methods

Strains, plasmids and growth conditions. All strains and plasmids used in this study are listed in Table 3.1. Wild-type strains SIO and TP of *V. cholerae* were isolated from southern California coastal waters. All *Escherichia coli* and *V. cholerae* strains were grown in LB broth (Miller 1972) supplemented with appropriate antibiotics at 37°C, except when stated otherwise. Antibiotics used in this study were kanamycin (Km, 50μg/mL for *E. coli*; 200μg/mL for *V. cholerae*), chloramphenicol (Cm, 20μg/mL for E. coli; 5μg/mL for *V. cholerae*), gentamycin (Gm, 50μg/mL), and ampicillin (Ap) and rifampicin (Rif) at a concentration of 100μg/mL.

***V. cholerae* mating.** *E. coli* strain S17-1λ*pir* (Simon et al. 1983) was used as a donor for all conjugation experiments with strains of *V. cholerae*. All strains were grown overnight to stationary phase at 37°C. *V. cholerae* were subcultured at 1:100 dilution and grown in LB medium at 22°C until the mid-log phase of growth was reached (OD_{600nm} ~ 0.7). *E. coli* and *V. cholerae* (if necessary) were washed of antibiotics by centrifugation (2 minutes, 13,000 x g) and resuspended in equal amounts of fresh LB. One milliliter of washed *E. coli* was then added to 4 mL of *V. cholerae* and briefly vortexed. This mixture was then vacuum filtered onto a sterile membrane (0.45μm pore size, 47mm diameter), which was placed atop of a LB agar plate and left overnight at 37°C. Membranes were then transferred to tubes containing 10 mL of LB medium and cells were removed by vortexing. Dilutions were plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS, 89g/L, Difco) or LB agar, both supplemented with appropriate antibiotics, and grown at 37°C overnight.

DNA Manipulations. All PCR reactions were carried out with Expand High-Fidelity PCR kits (Roche) or Taq polymerase (Invitrogen) and all oligonucleotides (IDT DNA Technologies) used for PCR and DNA sequencing are listed in the Appendix II-Table S3.1. PCR purification was carried out using the MoBio PCR purification kit according to the manufacturer's specifications and DNA sequencing was performed by SeqXcel, Inc. (San Diego, CA). Qiaprep Spin Miniprep kits (Qiagen) were used for plasmid purifications and restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA).

The method of Gene Splicing by Overlap Extension (SOEing) PCR as developed by Horton (Horton 1997) was used to engineer in-frame deletions of specific genes within the *V. cholerae* genomes. PCR amplicons were designed and subcloned into pGPKm as previously described (Mueller et al. 2007). After sequence verification, each plasmid was electroporated into *E. coli* S17-1λ*pir* and conjugated into the desired *V. cholerae* strain (see above). Subsequent screening for plasmid integration events and in-frame deletion verification was then performed as explained in Mueller, et al (Mueller et al. 2007).

Transposon mutagenesis library generation, screening and mutant identification. Conjugations transferring plasmid pRL27 (Larsen et al. 2002) into *V. cholerae* strain S9149 were carried out as described above. Transposon mutant libraries were made by arraying recovered ex-conjugants from the LB agar plates containing Rif 100μg/mL and Km 200μg/mL onto petri dishes with LB Km 200μg/mL in 49-sample (7x7) grid format. After overnight incubation of these plates at 37°C, each cell patch was replica-plated onto one LB agar plate supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 100μg/mL) and one containing X-gal (100μg/mL) and indole (500μM). Each plate was grown at 37°C and the following day individual patches were qualitatively assessed for blue color production with and without indole. Putative mutants altered in *lacZ* expression were re-patched onto LB X-gal (100μg/mL) plates with or without indole (500μM). Mutants displaying phenotypes different from the parental S9149 strain were preserved at -80°C in LB containing 15% glycerol.

The sequences of flanking DNA surrounding transposon insertions within these mutant strains were then retrieved using an arbitrary PCR technique first described by O'toole (O'Toole et al. 1999). PCRs resulting in amplicons of approximately 500 base pairs were cleaned and sequenced using Sanger's dideoxy chain termination method (Sanger et al. 1977). Recovered sequence data was analyzed on 06/07 by performing BLAST analysis (Altschul et al. 1990) against the *Vibrio cholerae* strain N16961 genomic sequence (Heidelberg et al. 2000).

Crystal violet quantification of biofilm formation. Strains were initially streaked onto LB agar and allowed to grow overnight at 37°C. Biological replicates originating from three unique colonies for each strain were inoculated into 5 mL liquid cultures of LB medium and grown for ~16 hours. Five microliters of each culture was then inoculated into 5 mL of fresh LB and grown overnight at 37°C with moderate shaking. Biofilms formed by each strain were then quantified by a method presented by O'Toole (O'Toole et al. 1999). For each culture, 50 μL of crystal violet (.1% w/v) was then added to allow for staining of the adherent cells. The medium was then washed from each tube and replaced with 5 mL of 95% ethanol and the OD_{570nm} of individual replicates were recorded. Pair-wise comparisons of the data of each sample were then analyzed for significance using a student's t-test ($\alpha = .05$).

Auto-aggregation assay. Strains were grown as above to obtain three biological replicates. After overnight growth in 5 mL of LB medium, each sample was allowed to settle for at least one hour. Once the flocculent particles within each culture had settled to the bottom of each tube, 200 μL was removed from the top portion of each culture and the OD of this sample was read at 600nm using a Spectramax M2 microplate reader (Molecular Devices).

Miller assays for β-galactosidase activity. A protocol similar to that as described by Miller (Miller 1972) was used to assay for alterations in *lacZ* expression. In brief, three biological replicates of each strain were grown overnight in LB broth and subcultured 1:1000 in fresh LB medium (+/- indole 500μM from 1M stock in methanol) and grown for an additional 20

hours, at which point the OD_{600nm} was measured. One milliliter aliquots were then removed from each of these cultures and cells were centrifuged (2 minutes, 13,000 x g) and resuspended in 500 μL CPRG lysis buffer (250mM Tris pH= 7.4, 2.5mM EDTA, .25% Igepal). For samples where indole concentration was estimated, the supernatant of each was retained for later analysis. Cells were then lysed by freezing at -20°C for 30 minutes and subsequently thawing at room temperature. One hundred microliters of lysate was then added to 900 μL Z-buffer and β-galactosidase activity was assayed as outlined previously (Miller 1972). Control experiments performed with cells grown in the presence of LB supplemented with methanol (.05%), which is the indole solvent, demonstrated no appreciable change in *vpsL::lacZ* expression (data not shown).

For the co-culture experiment, three biological replicates of each strain were grown overnight in LB medium from single colonies and sub-cultured together in fresh LB at a 1:5000 dilution. Cultures were grown for 48 hours and ratios of each strain were monitored throughout using plating experiments. For all cell combinations, the miller units calculated were normalized to the percentage of S9149 cells in the total OD, as represented by the percentage of S9149 colony counts obtained for each two-strain culture. In this manner, the calculated β-galactosidase activity was not reflective of all of the cells harvested, but only those producing LacZ.

For the conditioned media experiment, biological replicates of S9149 ($indole^-$) and S9171 ($indole^+$) were grown overnight in 11 mL of LB medium at 37°C. One milliliter aliquots were removed from each tube for later use in inoculating new cultures. The remaining 10 mL of each culture were centrifuged to pellet the cells (10 minutes, 3,640 x g) and the resulting supernatants were filter sterilized through 0.22μm membranes. To 3.8 mL of each of these conditioned supernatants, 200 μL of 20X YT (Yeast extract = 100g/L; Tryptone = 200g/L) was added. At this point, each biological replicate of S9149 was inoculated at a 1:1000 dilution into fresh LB medium, $indole^-$ conditioned medium and $indole^+$ conditioned medium. After 24 hours growth, miller assays were performed as described above.

Indole concentration measurements. The concentration of indole in the supernatants of cell cultures was measured by mixing 250 μL of supernatant with 250 μL trichloroacetic acid (20% w/v). After incubation on ice for 15 minutes each sample was centrifuged to remove precipitated proteins (10 minutes, 13,000 x g). This supernatant was then added to 500 μL Kovac's reagent (Sigma-Aldrich Co.), vortexed and the OD_{571nm} was measured for 200 μL of the top layer. A standard curve of known indole concentrations was recorded and used to estimate the amount of indole in each sample.

RNA isolation transcription analysis using whole genome transcription profiling and semi-quantitative RT-PCR. *V. cholerae* strains were initially grown on LB agar plates at 37°C overnight. Individual colonies from each plate were inoculated into 5 mL of LB medium and grown overnight at 37°C. Each strain was then subcultured at a 1:1000 dilution in fresh LB (+/- indole 350μM) and grown for 20 hours at 37°C with moderate shaking. One milliliter samples from each culture were harvested, and RNA was isolated as described previously (Yildiz et al. 2004). RNA used for transcriptome profiling was processed and microarrays were analyzed as previously described (Beyhan et al. 2006). For meta-analysis of microarray experiments, a X^2-test was used to evaluate statistically significant differences between over- and under-expressed genes in previously published expression data sets and the results obtained in these current experiments. Data sets were downloaded from the supplemental tables of published reports (See table 3.3) and genes were grouped together if they demonstrated a significant up-regulation or significant down-regulation under the treatment conditions. The resulting gene sets from each report were then individually compared to the sets of differentially regulated genes found in the study to determine the overlap between the two data sets. From this, a 2x2 X^2-test was performed and significance was assessed as having a p-value ≤ 0.01.

The OneStep RT-PCR kit (Qiagen) was used to measure relative expression of a gene of interest versus a control transcript in whole RNA samples. Experiments were performed according to the manufacturer's specifications. Each reaction contained 500 ng of

RNA as template for a multiplex PCR amplifying both a control gene transcript (*tufB*) and the targeted gene of interest (e.g. *vpsL*). After 23, 26, 29, 32 and 35 amplification cycles 10 µL aliquots were removed from each reaction and visualized with standard agarose gel electrophoresis techniques. Scanned images were recorded using the Typhoon gel imaging system and PCR amplicon intensities were measured using ImageQuant software (GE Healthcare).

Motility Assays. Bacterial strains were grown at 37°C in LB medium to stationary phase from individual colonies, subcultured at a 1:100 dilution in fresh medium and grown to an OD_{600nm}= 0.4 at 37°C with moderate aeration. Two microliters of each culture was then added to the center of LB soft agar plates (0.3% agar). Each plate was incubated for up to 72 hours at 37°C and diameters of the zone of motility were measured periodically.

Membrane depolarization assays. The effect of indole on cytoplasmic membrane depolarization was assessed using 3,3′-dipropylthiacarbocyanine [diSC3(5)], a membrane potential-sensitive dye according to a method developed by Zhang, et al (Zhang et al. 2000). Three biological replicates of each strain to be tested were grown to an OD_{600nm}= 0.5. Cells were centrifuged (5 minutes, 13,000 x g), washed with 5mM HEPES buffer (pH 7.8) and resuspended in fresh buffer to an OD_{600nm}=0.125. Aliquots of 150 µL were then added to individual wells of a 96-well microplate containing $diSC_3(5)$ (final concentration=0.4 µM), and quenching of the fluorescent dye due to accumulation within the membrane was monitored continuously using a microplate reader ($\lambda_{excitation}$= 622 nm, $\lambda_{emission}$=670 nm). After eight minutes, a stock solution of KCl was added to each well to a final concentration of 100mM. Once equilibrated (t ~ 5 min.), indole or methanol (solvent control) was then added to each cell suspension at increasing concentrations and fluorescence readings were recorded continuously for 20 minutes. Methanol addition did not significantly differ from a control experiment were nothing was added and fluorescence was monitored (data not shown).

GFP tagging of *V. cholerae* strains and confocal laser scanning microscopy (CSLM). Tri-parental conjugations for inserting the green fluorescent protein (GFP) gene into

the *V. cholerae* chromosome were performed as described previously (Beyhan et al. 2006). Biofilm formation by GFP-expressing strains within coverglass chambers (Nalge Nunc International) was assessed using CSLM. Biological replicates for each strain were inoculated from stationary phase cultures into chambers containing fresh LB medium (+/- indole, 500μM) at a final concentration of 10^6 cells/mL. Static growth was allowed to proceed for 6 hours at 37°C, at which point all media was removed from the chambers and attached cells were washed twice with 100mM Phosphate Buffered Saline (PBS, pH=7.0). After resuspension in 100mM PBS, biofilms were visualized with a Nikon C1si microscope, and analysis of Z-stacks and three-dimensional rendering was performed with NIS Elements software (Nikon Instruments, Inc.).

Results

Indole controls biofilm associated phenotypes in *Vibrio cholerae*. A previous transposon mutagenesis screen for biofilm mutants in two environmental strains of *V. cholerae*, SIO and TP, identified the tryptophanase gene as being essential for proper biofilm formation (Mueller et al. 2007). When the biofilms of these *tnaA::*Tn5 mutants were examined with crystal violet staining it was noted that the SIO mutant (S1101) formed ~3.5X less biofilm than its parental wild-type, and the TP mutant (T1101) formed ~6X less biofilm than the wild-type (figure 3.1A). Since indole, a by-product of the tryptophanase reaction, had previously been identified as a factor controlling biofilm formation in other bacteria (Martino et al. 2003), the effect of exogenous indole addition on the biofilms of these *tnaA* mutants was tested. In this experiment, indole added exogenously at concentrations of 350μM was able to fully complement the biofilm formation of these mutants to wild-type levels (figure 3.1A).

The influence of indole on various properties of strain SIO was studied in more detail, due to its genetic tractability and the clear phenotype differences between mutant and wildtype strains (i.e. rugosity, as discussed in chapter 2). The biofilms formed by GFP-expressing SIO and S1101 (strains S4100 and S4101, respectively) were examined further using confocal

laser scanning microscopy (CSLM) to investigate the morphological effects of indole on these biofilms. As seen in figure 3.1B, the biofilms of the parental S4100 strain grown on glass coverslips for 6 hours in LB broth under static conditions were distinctly different than those of S4101, the tryptophanase mutant. Without the addition of indole to the biofilm chamber, the S4100 strain began to form regular microcolonies covering the glass substratum with an average height of 25.8 ± 5.0μm. In contrast, the microcolonies of S4101 biofilms were much smaller and exhibited sparse surface coverage. Additionally, these biofilms were thinner and only reached an average height of 15.5 ± 2.6μm. When indole was added back to each strain, an up-shift in biofilm formation was discovered. While strain S4100 produced a slight increase in surface coverage when exogenous indole was added, a clear difference was seen for S4101 under these conditions. The biofilms of S4101 with exogenous indole nearly doubled in thickness to an average height of 28.0 ± 4.4μm and the surface coverage increased considerably and was not significantly different from that of the parental strain with indole.

Another notable phenotypic difference among the SIO-derived strains was that a tryptophanase mutant did not exhibit the auto-aggregative phenotype of its parental strain. Under stationary growth conditions wild-type cultures form multi-cellular clumps that settled to the bottom of the tube within liquid media. When liquid growth of the tryptophanase mutant was examined a lack of aggregation within the medium was evident. Figure 3.1C, which shows the OD_{600nm} of the cultures of the SIO and S1101 strain with and without indole upon settling, demonstrates this effect. This figure also shows that when indole is added back to the *tnaA* mutant strain, the auto-aggregation phenotype is restored to near wild-type levels.

Indole regulates VPS gene expression in *Vibrio cholerae.* In addition to affecting biofilm formation and auto-aggregation, indole induces the smooth colonies of S1101 to revert to the rugose phenotype of the SIO parental wild-type when grown on LB agar plates (Mueller et al. 2007). It has previously been shown that all three of these phenotypes are linked to over-production of exopolysaccharides (Watnick and Kolter 1999, Aslim 2007, Yildiz 1999).

Therefore, it was hypothesized that indole is exerting its effects, at least in part, by regulating *vps* gene expression. To test this, a *lacZ* reporter fusion to the *vpsL* gene was constructed in both *tnaA*$^+$ and Δ*tnaA* SIO strains (S9171 and S9149, respectively). In these strains, β-galactosidase activity serves as a proxy for *vps* gene expression in the presence and absence of endogenously produced indole.

The initial experiment performed examined the simultaneous production of endogenous indole and β-galactosidase activity throughout the growth phases of S9171 grown in batch culture (Figure 3.2A). It was observed that indole production by the *tnaA*$^+$ strain mirrored *vpsL::lacZ* expression. During early log-phase growth both indole and β-galactosidase levels are low. However, in keeping with *tnaA* catabolite repression (Yanofsky et al. 1991), late log- and stationary-phase cultures produced increased amounts of indole, and concomitantly, increased amounts of β-galactosidase. While this experiment showed that extracellular levels of indole and *vpsL* induction correlate with one another, it did not address whether indole is responsible for *vpsL* induction.

To deal with this question, strain S9149 (Δ*tnaA*) was used to monitor *vpsL::lacZ* expression under conditions of exogenous indole addition. Similar to the biofilm results reported above, S9149 *vpsL::lacZ* expression was decreased approximately 10-fold compared to its indole-producing parental strain, S9171 (figure 3.2B). Further, increasing concentrations of exogenous indole (0-400μM) resulted in corresponding increases in *vpsL::lacZ* expression. Full complementation of *vpsL::lacZ* induction occurred with concentrations of exogenous indole as low as 100μM, which is well below the observed concentration of endogenous indole produced by S9171. These data illustrate that indole supplied exogenously can regulate VPS expression when supplied at physiologically relevant concentrations.

Indole acts as an extracellular signal regulating VPS production. To further address the hypothesis that endogenously produced indole acts as a signal controlling VPS in *V. cholerae*, experiments were performed to determine whether indole produced from an originating cell be taken up by a neighboring cell and elicit a response (i.e. *vpsL::lacZ* up-

regulation). First, co-culture experiments were performed with indole +/- donor strains (S2148-Δ*lacZ, tnaA*$^{+}$ and S2150-Δ*lacZ,* Δ*tnaA*, respectively) and an indole recipient strain (S9149-Δ*tnaA, vpsL::lacZ*). As shown in figure 3.3, the indole recipient strain S9149 produced very little β-galactosidase activity when grown in co-culture with S2150 (Δ*lacZ,* Δ*tnaA*). However, when strain S9149 (Δ*tnaA, vpsL::lacZ*) was grown with the indole-producing strain S2148 (Δ*lacZ, tnaA*$^{+}$) the β-galactosidase activity increases ~8-fold. This increase is strikingly similar to the activity achieved when indole is added exogenously to the initial co-culture conditions with strains S9149 and S2150, demonstrating that exogenously and endogenously produced indole have the same VPS regulatory effect.

An experiment was also performed in which strain S9149 was grown by itself using in conditioned or unconditioned media and VPS regulation was measured (figure 3.3). S9149 grown in fresh LB medium, resulted in very little *vpsL::lacZ* transcription. S9149 grown in a conditioned medium from an indole-negative strain produced a similar result. However, when S9149 was grown in conditioned medium derived from an indole producing strain, the level of β-galactosidase production rose significantly (>3-fold). Taken together, these results solidify the conclusion that the VPS regulatory response is produced specifically by indole and not another component of the supernatant.

Indole regulates biofilm formation and *vpsL* expression in clinical strains of *V. cholerae*. To investigate whether indole influences biofilm formation and VPS production in clinical strains of *V. cholerae* in addition to these non-O1/O139 environmental strains, in-frame deletions of the *tnaA* gene were made in the *V. cholerae* O1 El Tor strains: N16961 (smooth variant Heidelberg et al. 2000) and 92A1552 (smooth and rugose variants Yildiz and Schoolnik 1998) of *V. cholerae*. Unexpectedly, when the tryptophanase gene was deleted in the rugose variant of 92A1552, a shift to a smooth colony morphology was not observed. Additionally, no dramatic differences in the biofilm forming ability were recorded between any of the wild-type strains and their Δ*tnaA* derivatives (data not shown). However, indole addition

resulted in an increase that was significantly different in its biofilm formation from the smooth variant of the tryptophanase mutant of N16961 (figure 3.4A).

To determine whether indole was also influencing the expression of *vps* genes in this strain, the relative expression of the *vpsL* gene was measured using semi-quantitative RT-PCR (figure 3.4B). Here, a clear increase in the abundance of *vpsL* transcript was observed in the RNA prepared from cells grown with indole versus those without. These results suggest that production of endogenous indole may not influence VPS production and biofilm formation in wild-type clinical strains, but the effect of indole on these phenotypes can still be produced when indole is added exogenously. This underscores the observation that different strains of *V. cholerae* regulate biofilm formation through different regulatory mechanisms (Mueller et al. 2007).

Transcriptional regulation by TnaA activity and indole production extends beyond VPS genes. To explore whether indole regulation of gene expression extends beyond those involved in VPS biosynthesis, whole-genome expression profiles of the wild-type SIO strain and strain S1101 (*tnaA::*Tn5) grown to stationary phase in LB medium either in the presence or absence of exogenously added indole were obtained and compared. Significantly regulated genes were defined by having ≤1% false-positive discovery rate and ≥1.5-fold transcript abundance differences between each sample. Five sets of pair-wise comparisons were made from the resulting transcriptome profiles in order to assess the influence of indole and/or tryptophanase activity on gene expression. Table S3.2 of Appendix II provides the complete list of differentially regulated genes within each data set. The first comparison made was between the transcriptomes of SIO and S1101 to understand the effects of a tryptophanse mutation on gene expression (SIO/S1101). The second comparison examined the effects of indole alone by matching the expression data of the tryptophanse mutant, S1101, grown with or without exogenous indole (S1101(+I)/S1101). The next two comparisons evaluated the expression changes of SIO grown in the presence of exogenous indole to S1101 and SIO grown solely in LB medium (SIO(+I)/S1101 and SIO(+I)/SIO,

respectively). Here, the consequences of artificially high indole concentrations on gene expression were evaluated. Finally, the expression data from the SIO strain and S1101 grown with indole was compared to determine whether indole alone could complement the expression changes resulting from a *tnaA* mutation (SIO/S1101(+I)).

Table 3.2 displays the summarized results of these comparisons noting the number of differentially regulated genes in each pair. Due to the large amount of genes regulated and the disparity of content within each data set, a composite data set of indole-regulated genes was created. This data set was first composed of genes that demonstrated a relative change in expression when exogenous indole was added to SIO and S1101 (SIO(+I)/S1101 and SIO(+I)/SIO). Additionally, the data set of genes differentially regulated between S1101 and SIO was compared with the "indole complementation" data set (SIO/S1101(+I)). If a gene was shared between these two data sets and in the presence of indole its relative expression returned toward wild-type expression levels, it was considered to be indole regulated.

This composite data set consists of 418 total genes. Of these, 247 genes appear to be down-regulated by indole, while 171 genes are up-regulated (table 3.2). These genes were grouped according to their annotated functional roles, as defined by conserved hidden-markov models within their sequence, and a histogram containing the number of up- and down-regulated genes of each functional category was created (Figure 3.5). Not surprisingly, genes involved in cell envelope maintenance and production were strongly up-regulated. Many genes within this group are involved in the production of VPS, and, in total, 16 of the 18 genes contained within the two *vps* operons were up-regulated by indole when all gene profile comparisons were considered. The lone exceptions were VC0923, which is a homolog of VC0919 and is annotated as a hexapeptide domain-containing protein with transferase activity and VC0927 that encodes the UDP-N-acetyl-D-mannosamine transferase, *cpsF*. Up-regulated genes that are not known to function in VPS production, but are also found in this category, include: (i) a putative rare Lipoprotein A (VC0948), (ii) *ompU* (VC0633), a porin which aids in resistance to anti-microbial peptides (Mathur and Waldor 2004), and (iii) *kdtA*

(VC0233), a Kdo-transferase enzyme involved in lipopolysaccharide biosynthesis (Clementz and Raetz 1991).

Another notable functional category, which was differentially regulated by indole, is the group containing genes responsible for general cellular processes (Figure 3.5). Included within this functional class are many genes that have a role in chemotaxis (VC0098, VC1248, VC1298, VC1313, VC1399, VC1602, VC1868, VC2059, VC2062, VC2202, VCA0008, VCA0773, VCA0988, VCA1089, VCA1090, VCA1091, VCA1092, VCA1093, and VCA1096) and flagellar biosynthesis (VC1612, VC2135, VC2141, VC2142, VC2187, VC2188, VC2191, and VC2200). These include ten of the 26 annotated chemotaxis (Che) proteins and nine of the 43 methyl-accepting chemotaxis proteins (MCPs) of the *V. cholerae* genome (Heidelberg et al. 2000). While most of these MCPs have an unknown role in chemotaxis, it is interesting to note that none of the putative annotated MCPs of *V. cholerae* were found to be up-regulated in response to indole in any of the transcriptome comparisons.

A model for flagellar biosynthesis in *V. cholerae* has been proposed by Prouty, *et al* (Prouty et al. 2001). Here, four different classes of flagellar genes are controlled in a coordinated, step-wise manner by various transcription factors resulting in the production of a functional single, polar flagellum. In the current study, indole was found to down-regulate only genes in class III and IV, which are dependent on the alternative sigma factors σ^{54} and σ^{28} (FliC), and the σ^{54}-dependent transcriptional activator FlrC. To determine whether these transcriptional effects extend to phenotypic changes, the motility of SIO and S1101 on LB soft agar plates supplemented with or without exogenous indole was tested. After 72 hours growth, the SIO strain grown on LB-indole soft agar plates was reduced 16% relative to SIO on LB soft agar alone (data not shown). Further, S1101 demonstrated a 29% reduction in swimming motility when grown in the presence of indole (data not shown). While these results agree with previous reports showing the effect of indole or VPS over-expression on motility (Yildiz et al. 2004; Lee et al. 2007), it is not known whether the reduced swimming capacity of

these cells is due to reduced flagellar biosynthesis, a reduced capacity for chemotaxis or a combination of the two.

A set of genes that showed a relative increase in expression are annotated to fall within a group of genes with previously unknown functions (Figure 3.5). However, recent work has shown that the *hcp* (VC1415 and VCA0017), *vgrG* (VCA0018 and VCA0123) and *vasK* (VCA0120) genes of this group are involved in virulence-associated secretion (VAS), which have a role in infection and virulence towards eukaryotic cells. Specifically, it has been shown that these genes, and their homologues within other species, function in mediating: (i) effective infection and nitrogen fixation by *Rhizobium leguminosarum* within nodules of the pea plant (Bladergroen et al. 2003), (ii) disease in fish by the piscine pathogen *Edwardsiella tarda* (Srinivasa Rao et al. 2004), (iii) death of the phagocytic eukaryote *Dictyostelium discoideum* by *V. cholerae* (Pukatzki et al. 2006), (iv) and pellicle and biofilm formation in *Vibrio parahaemolyticus* and *Actinobacillus actinomycetemcomitans*, respectively (Kachlany et al. 2000; Enos-Berlage et al. 2005).

In addition to the above five genes, the array results indicate that the surrounding genes of the entire VAS operon (VCA0107-VCA0123; Appendix II -Table S3.2) are all indole-responsive. This operon includes many hypothetical proteins thought to be involved in protein transport, an AAA+ family protein involved in protein translocation through the membrane and a σ^{54}-dependent transcriptional regulator, which has been shown to function along with RpoN to activate expression of these genes (Mougous et al. 2006; Pukatzki et al. 2006). It was noticed, however, that these genes did not show a significant increase in transcript abundance in the pair-wise comparison between S1101 grown on LB supplemented with indole versus growth on LB medium alone. To validate this result, semi-quantitative RT-PCR was performed on S1101 grown in LB with and without indole to measure the abundance of VCA0108 transcript relative to the transcript of the house-keeping gene *tufB*. The VAS gene transcript was approximately 1.7-fold more abundant in cells grown in the presence of indole than without (data not shown). Therefore, it appears that in the S1101 background the VAS

operon can be up-regulated by indole; although, its detection may be below the limits of the microarray experiments performed.

It has been shown previously that the VAS operon is highly conserved within seven proteobacteria species that exhibit intimate host interactions (*E. coli*, *Y. pestis*, *S. Typhi*, *A. tumefaciens*, *R. leguminosarum*, *M. loti* and *P. aeruginosa*, Das 2003). The conservation of this operon was re-evaluated to determine whether these genes are found in more diverse organisms. Out of 375 completely sequenced and annotated genomes examined, 68 organisms were found to carry homologues for at least half of the genes within the VAS operon. Figure 3.6 shows the phylogenetic distribution of these organisms. All of the microbes belong to the proteobacteria. This reinforces the notion that the functionality of this operon appears to extend only within this group.

A final functional category of genes demonstrating a distinct bias in the expression profiles was the transport and binding protein group. Figure 3.5 shows that this class, which includes genes involved in amino acid transport, iron uptake and carbohydrate transport, is down-regulated by indole. The largest sub-group of genes within this category contained sequences coding for proteins that function as iron-transport systems and in siderophore production (VC0475, VC0771, VC0772, VC0774, VC0775, VC0776, VC0777, VC1264, VC1544, VC1545, VC1547, VC2210, VC2211, VCA0911, and VCA0915). When the pair-wise comparisons were examined to elucidate the conditions driving this repression, it was noticed that very few of these genes showed appreciable changes in expression under physiologically relevant indole concentrations. Instead, most of these genes were repressed only under artificially high indole conditions where gene expression of wild-type SIO grown in LB supplemented with exogenous indole was compared to SIO or S1101 grown solely in LB medium.

The iron-dependent repressor Fur and the small RNA RhyB stringently control the expression of many iron-regulated genes in *V. cholerae* (Davis et al. 2005; Mey et al. 2005). In addition to controlling genes involved in iron uptake these regulators also appear to direct

the expression of genes responsible for other functions, such as energy metabolism and oxidant detoxification. To determine whether indole controls the same genes that are regulated by Fur, the expression profiles of the indole regulated genes were compared with previously reported Fur regulated genes using a X^2 test. In principal, this test can calculate the expected shared number of genes between two data sets that should show similar (up/up or down/down) or dissimilar (up/down or down/up) regulation by random chance given the total number of up- and down-regulated genes within each of the two original data sets. The divergence from the expected random overlap is assessed using critical values and a corresponding level of significance is assigned (p-value). When comparisons between data sets demonstrate high significance ($p \geq 0.01$), the overlapping gene sets are considered to not be due to random events, and it may be hypothesized that similar regulation patterns are found in both expression profiles. As shown in table 3.3, there is very high correlation between the indole and Fur regulated genes. In fact, all but one of the shared genes exhibited repression by both indole and Fur. This high correlation leads to the conclusion that indole may stimulate Fur activity leading to the downstream repression of the Fur regulon.

In addition to the differential regulation of these various functional classes of genes, the expression of many interesting individual genes was also altered by tryptophanse activity and indole production. As Figure 3.7 shows, various activators and repressors that are known to influence exopolysaccharide production were differentially regulated in the transcriptome comparisons. Included within these are genes involved in: (i) direct transcriptional activation of *vps* (*vpsR* and *vpsT*), (ii) quorum sensing (*cqsA*, *luxQ*, *fis* and *hapR*), (iii) cdiGMP biosynthesis and degradation (18 GGDEF- and/or EAL-domain containing proteins; *mbaA* and *cdgA* shown in figure 3.7), and (iv) membrane stress sensing and response (*cpxPAR*, *rpoE* and *rseA*).

A X^2 test was used to compare the data sets with other previously published whole genome expression profiles in order to deduce similarities between expression patterns and predict possible regulators of the indole response. Several patterns emerge from the results

of these analyses, which are shown in table 3.3. The data sets with the most significant correlation to indole/tryptophanase expression profiles are from experiments performed under conditions of altered VPS production. These include transcriptome analyses performed with mutant strains of the *vpsT* and *vpsR* transcriptional activators, and with GGDEF protein mutants (*mbaA* and *cdgA*) or cells grown with artificially high levels of cdiGMP. These results are not completely unexpected, as all of these data sets show differential regulation of many of the genes within the *vpsI* and *II* operons. However, even when these 18 *vps* genes are excluded from the analysis there remains significant overlap between the data sets, indicating that the similarities extend beyond VPS production.

Other regulons, which appear to be similar to the indole-regulated gene set, are those controlled by the alternative sigma factor σ^{54} (RpoN) and the nucleic acid binding protein Hfq. Hfq has been shown previously to be involved in quorum sensing in *V. cholerae* (Lenz et al. 2004). Since, the Δ*hfq* mutant only significantly affects expression of one of the genes in the two *vps* operons, most of the shared gene expression patterns are a result of non-VPS genes. σ^{54} regulates motility and virulence and even other global regulators including HapR and σ^{38} (RpoS) (Yildiz et al. 2004; Pukatzki et al. 2006), many of which are also regulated in the presence of indole.

Another striking pattern, which emerges from these comparisons, is that the significant correlations for many tend to disappear when the SIO/S1101(+I) data set is used in comparison. In this data set the wildtype SIO strain is compared to the S1101 tryptophanase mutant, which has been grown in indole. If the indole is able to fully complement gene expression of the tryptophanase mutant there should be no differences in expression between the mutant and wildtype. This is clearly not the case, since many genes still show differential expression. However, since this suite of genes do not show significant correlation with the other data sets in this meta-analysis, they do not appear to be reflective of an indole effect and possibly represent effects due to the loss of the tryptophanase gene; thus, supporting the notion that indole can complement global gene expression.

Another result was that the expression profiles of many other transcriptional regulators that exhibit differential regulation by indole are not highly correlated with the indole data sets. For instance, the *hapR* gene is shown to be up-regulated by indole, but its regulon exhibits no significant similarity to the indole-responsive genes. Thus, although HapR is a repressor of VPS production, *hapR* up-regulation by indole does not lead to a repression of *vps* expression under the conditions tested. Similarly, the *rpoE* and *toxR* data sets do not correlate with the indole/tryptophanase expression profiles. There may exist strain variations in these regulons or indole induction is not sufficient for activation, or it is influencing multiple controlling factors of these regulons in a complex fashion. Whole genome hybridization microarray experiments indicate that strain SIO does not contain many of the genes (e.g. VPI-1 and CTX elements) found within the traditionally defined ToxR regulon of *V. cholerae* (M. Miller, personal communication, Bina et al. 2003), which may account for some of these results.

It was also noticed that the expression profile of a strain with a mutation in the membrane stress sensing protein RseA shows significant correlation with three of the data sets (Indole regulated, SIO(+I)/S1101 and SIO(+I)/SIO). All three of these conditions contain genes that may be regulated under conditions of artificially high indole concentrations found in the SIO(+I) condition where exogenous indole was added to the wildtype SIO strain. It is possible that the correlation with these data sets may be a result of high indole conditions causing membrane stress.

Indole does not cause membrane depolarization at physiological levels. Since the Cpx and RpoE membrane stress regulators demonstrated significant changes in expression in whole genome profiling experiments, it was investigated whether indole was able to cause appreciable membrane stress under the conditions employed for bacterial growth. For this, the ability of indole to depolarize the membrane of *V. cholerae* cells was tested by continuously tracking the fluorescence of the membrane soluble dye diSC3(5) using the method developed by Zhang, et al (Zhang et al. 2000). As shown in figure 3.8 the fluorescence of this molecule is quenched as it diffuses into the interior of the cell. Once the

interior and exterior dye concentrations had reached homeostasis (15 minutes; black arrow in fig. 3.8), indole or methanol alone (indole solvent) was added to the cells to track membrane depolarization effects. At this point samples containing high amounts of indole (1.0 mM) exhibited membrane depolarization, which is shown by the observed increase in fluorescence as the dye begins to leak out of the cell interior. However, samples with physiologically relevant concentrations of indole (0.5 mM) did not exhibit this increase and were not statistically different than samples treated with the methanol control. These data suggest that at high concentrations indole can affect *V. cholerae* membranes and correlates with previous findings demonstrating that high levels of indole (2mM) can induce a stress response in *E. coli* through the BaeSR two-component regulatory system (Nishino et al. 2005), which *V. cholerae* is not known to have. However, at the concentrations produced by strain SIO there appears to be no consequence on membrane polarity.

VPS production in response to indole is not controlled by known membrane stress regulators or quorum sensing circuits. Although indole does not appear to cause membrane depolarization at lower concentrations, this result does not rule out the possibility that RpoE or Cpx may have a role in the indole response. To directly test the role of these proteins on the indole response, in-frame deletions of *rpoE* and *cpxA* were made in the S9149 parental strain and *lacZ* expression was assessed in the presence and absence of indole. In addition to the single deletion mutant strains (S9185-Δ*rpoE* and S9190-Δ*cpxA*), a strain carrying deletions in both genes (S9209-Δ*rpoE*, Δ*cpxA*) was also constructed. In this manner, it was possible to assess the effect, if any, each system has on the indole response or if one system can compensate for the other in terms of indole regulation. When these strains were grown in conditions with or without exogenous indole they all exhibited the wild-type response and, thus, were not impaired with the up-regulation of the *vpsL::lacZ* fusion in the presence of indole (figure 3.9A). These results support the conclusion that indole is not causing a membrane stress that is recognized by Cpx or RpoE.

Among the many genes known to regulate VPS production, numerous genes involved in the known quorum sensing systems of *V. cholerae* are differentially regulated by indole (figure 3.7). These systems affect VPS regulation through modulating LuxO activity in response to fluxes in autoinducer concentrations, which subsequently leads to downstream regulation of HapR activity, a VPS repressor. To assess indole's influence on these quorum sensing mechanisms a similar knockout approach was taken as in the *rpoE* and *cpxA* analyses. In-frame deletions were introduced in the *fis* (S9211) and *luxO* (S9213) genes. Only single mutations were made since both of these genes act in concert with each other at the same position upstream of HapR in the quorum sensing circuit (see figure 3.7). Deletion of either of these genes had no significant effect on indole-regulated *vpsL* expression (figure 3.9B). Since there is no abolishment of an indole response, it is concluded that the indole sensing mechanism does not converge with LuxO-controlled quorum sensing.

Indole regulation of VPS proceeds through DksA, VpsR and GGDEF domain-containing proteins. In order to further develop the details of the indole-signaling circuit, a transposon mutagenesis screen was performed to look for mutants that display an indole non-responsive phenotype. Of the approximately 11,000 transposon mutants screened, four unique genes with transposon insertions were recovered which demonstrated a consistent low-level of *vpsL::lacZ* expression that did not change significantly upon the addition of exogenous indole (table 3.4). Interestingly, three of these four strains carried mutations in sequences coding for GGDEF-domain containing proteins (VCA0074 and VC1376) or in a hypothetical protein immediately upstream of one of these genes (VCA0074). Further, each of these genes were recovered multiple times from independent transposition events (VCA0075 - 5 independent mutations, VCA0074 and VC1376 - 2 independent mutations each). This demonstrates the importance of these genes in controlling the indole-induced VPS response and suggests a near-saturation of the genome by the transposon mutagenesis screen.

VCA0074, which has been named *cdgA*, has diguanylate cyclase activity and increases intracellular cdiGMP levels, which leads to the production of VPS and the formation

of biofilms in *V. cholerae* (Lim et al. 2006). On the *V. cholerae* genome, the *cdgA* gene resides within a two gene operon and is located immediately downstream of VCA0075. A transposon mutation in VCA0075 has previously been shown to also affect the biofilm formation of *V. cholerae* (Mueller et al. 2007); although, it is not clear whether this is a direct consequence of VCA0075 mutation by the transposon or an indirect result of polar effects on downstream *cdgA* expression.

Previous transcriptome experiments were reviewed to understand what genes potentially control the expression of the *cdgA* operon (see table 3.3 for references). These data show that the *cdgA* operon was only differentially regulated in response to mutations in known regulators of VPS expression (Δ*hapR*, Δ*vpsR*, Δ*vpsT*, Δ*rpoN*, and Δ*vpvC*) or to cells grown under conditions of artificially high intracellular cdiGMP concentration. The *cdgA* operon was always up-regulated by activators of VPS production and down-regulated by repressors. It did not exhibit any differential regulation in microarray experiments comparing *rpoE*, *hfq*, *toxR*, and *rhyB* mutants, which also do not alter the overall regulation of the VPS operons. Considering there are 41 genes encoding GGDEF-domain containing proteins in the *V. cholerae* genome it is striking that CdgA is consistently produced along with VPS and that it seems to be a key component allowing the cell to respond to indole.

Only one mutant was recovered which does not respond to indole and contains and insertion in a gene or operon whose product(s) are not known to affect the production of GGDEF proteins. This mutant contains an insertion in the *dnaK* suppressor protein gene, *dksA*. This gene is the third within an eight-gene operon and codes for a protein thought to interact directly with the RNA polymerase holoenzyme (E). Through this physical interaction, DksA promotes the dissociation of E from the housekeeping sigma factor σ^{70} allowing the RNA polymerase molecule to interact with alternative sigma factors such as σ^{54} (RpoN), (reviewed in Gourse et al. 2006). Interestingly, this gene was not differentially regulated in any of the whole genome profile comparisons made, suggesting that indole's effect may be post-translational.

A second class of mutants was found to produce no measurable β-galactosidase activity. Included in this group are two hypothetical proteins and two transporter genes. None of these genes have a known role in VPS production. A fifth mutant contained a transposon insertion in the *hmpA* gene, which encodes a soluble flavohemoglobin known to counteract nitrosative stress in *Salmonella enterica* (Bang et al. 2006). As VPS production has previously been shown to counteract oxidative stress (Yildiz and Schoolnik 1999), this gene may play a role as a sensor which can regulate VPS production in response to oxidative stress. The last mutant in this group contained a disruption in the *vpsR* gene, which is essential for VPS production (Yildiz et al. 2001). This key regulator of VPS production is known to combine with other regulators including VpsT and GGDEF-domain proteins to direct *vps* transcription (Beyhan et al. 2007). Therefore, it was not surprising that this mutant demonstrated no measurable β-galactosidase activity. These results indicate that indole-induction of VPS proceeds requires VpsR, cdiGMP, DksA and additional factors.

Discussion

The behavior patterns observed in bacteria are a direct result of their ability to constantly monitor gradients of specific chemicals within their environment and to respond accordingly through changes in gene expression and protein activity. One of the most well-known examples of this ability is the phenomenon of quorum sensing, whereby a bacterial cell assesses the levels of autoinducer molecules to coordinate its gene expression with that of the surrounding population (reviewed in Waters and Bassler 2005). Recently, it has been proposed that the molecule indole, which is a natural product of the breakdown of tryptophan by the enzyme tryptophanase, can act a stationary phase signal molecule in bacteria regulating gene expression and affecting biofilm formation (Wang et al. 2001; Martino et al. 2003). Previous work showed that a mutation in the tryptophanase gene of *V. cholerae* strains can influence biofilm formation and the ability to produce rugose colonies, and it was speculated that this was a result of reduced VPS production (Mueller et al. 2007). In this

current study, it has been shown that indole secreted by one cell can act as an extracellular signal that is sensed by others within a population, and that the perception of this signal leads to the coordinated up-regulation of *vps* gene expression, restoring the biofilm and rugose phenotypes of these cells.

These results correspond with those of Martino, *et al*. (Martino et al. 2003), who demonstrated that indole enhances the biofilm formation of many different organisms which carry the tryptophanase gene, including *E. coli* strain S17-1. However, they are in contrast to other recent findings, which have shown that indole appears to down-regulate biofilm formation in *E. coli* strain K-12 (Lee et al. 2007). Similarly, the biofilm formation of the rugose variant of *V. cholerae* strain 92A1552 is not affected by deletion of the *tnaA* gene, suggesting that indole's effects may be strain specific. While the underlying reason for this difference is unknown, it is interesting to point out that whole transcriptome expression patterns of the *tnaA* mutant of *V. cholerae* strain SIO used in this study exhibit a key similarity with those found for a *tnaA* mutant of *E. coli* K-12 (Lee et al. 2007), possibly accounting for these contradictory results. Both *V. cholerae* SIO and *E. coli* K-12 appear to down-regulate genes involved in cell motility and chemotaxis (27 genes in *V. cholerae* and seven in *E. coli*). This repression of motility has been proposed to be a strong mediating factor for the indole-driven down-regulation of *E. coli* K-12 biofilm formation, since increases in motility of *E. coli* strains positively influences biofilm development (Wood et al. 2006). In the case of *V. cholerae* strain SIO, however, motility is not a factor contributing to abiotic biofilm formation, since deletion of the *flaA* gene has no effect on this phenotype. Instead, the production of the exopolymer matrix, which is comprised of VPS, appears to be the main contributor to this strain's ability to form biofilms (Mueller et al. 2007).

Another set of genes that were differentially regulated by indole in the transcriptome data were those of the VAS operon. This operon is known to play and important role in avoidance of grazing by protozoan predators (Pukatzki et al. 2006), and was found to be to be conserved in many members of the proteobacteria (Das and Chaudhuri 2003; this study).

Although many organisms are either plant- or gut-associated bacteria, it is clear that a small group of marine bacteria contain this gene cluster (*i.e.* all of the sequenced *Vibrios* analyzed and *Shewanella frigidimarina*). The fact that these genes are found in marine bacteria suggests that these organisms may be utilizing the products of this operon during interactions with various eukaryotic hosts. Also, given that this operon is indole-regulated, it should be noted that many of these organisms are found in environments with high concentrations of indolic compounds. While many gut-bacteria are exposed to the high indole concentration in the human gastro-intestinal tract, the plant-associated bacteria are often exposed to indole-3-acetic acid (IAA), which is a major plant hormone and commonly referred to as auxin. Thus, there appears to be strong conservation of the types of organisms carrying this operon and of the environments where these microbes are found.

One emergent theme from the microarray experiments is that many of the indole-regulated genes appear to be controlled, directly or indirectly, by the alternative sigma factor, σ^{54} (RpoN). Included within this set are the aforementioned genes involved in flagellar biosynthesis, the *vpsI* and *II* operons that encode proteins that function in VPS manufacture and secretion, and genes of the VAS operon. In each case, the regulation of these genes depends not only on σ^{54}, but also on the specific σ^{54}-dependent transcriptional activators: FlrC that is involved in flagellar biosynthesis (Prouty et al. 2001), VpsR, that controls *vps* transcription (Yildiz et al. 2001; Yildiz et al. 2004) and VasH, a regulator of the VAS operon (Pukatzki et al. 2006). The indole effect on σ^{54}-mediated gene expression is not related to *rpoN* expression, but does appear to stem in part from its influence on σ^{54} transcriptional gene expression. Indole appears to down-regulates *flrC* and up-regulates *vasH*, consistent with the patterns of motility and VAS gene expression. In contrast, *vpsR* does not show a significant expression change in response to indole. It may be that indole-controlled VPS regulation stems from post-transcriptional effects on VpsR activity.

It is apparent that indole is controlling many of the same genes that are governed by regulators of VPS synthesis. In addition to VpsR this includes VpsT and multiple GGDEF-

domain containing proteins (e.g. CdgA and MbaA). However, not all regulators of VPS synthesis showed significant similarities with indole-controlled genes. The expression profile of genes controlled by HapR did not correlate with the microarray results in any of the comparisons performed. HapR is known to be the master transcriptional regulator of the convergent quorum sensing pathways of *V. cholerae*, and has been shown to regulate many other genes beyond those responsible for VPS production (Zhu et al. 2002). Although HapR affects the transcription of numerous known regulators of VPS such as *vpsT*, *vpsR* and *cdgA*, these regulators can also function independently of HapR to control VPS production (Beyhan et al. 2007). In addition, many strains of *V. cholerae* often carry mutations in the *hapR* gene rendering it non-functional and presumably inactivating the quorum sensing mechanisms of these strains (Heidelberg et al. 2000; Kovacikova and Skorupski 2002). The *hapR* gene of SIO has been sequenced and is known to contain a 10-bp deletion in the 5' end of the gene (data not shown). It is not known if this deletion affects quorum sensing in particular strain, but this result provides for that possibility. It is concluded that indole regulation is dependent on regulators such as σ^{54}, VpsR, VpsT and GGDEF proteins rather than those involved in quorum sensing.

Support for this conclusion was provided by additional genetic experiments. Inactivation of genes involved in quorum sensing (*fis* and *luxO*) had no effect on the up-regulation of VPS by indole. On the other hand, mutations in genes involved in other routes of VPS regulation (VpsR, and the GGDEF proteins *cdgA* and VC1376) abolished the indole-driven VPS response. Previous studies have shown that two of these genes are involved in a regulatory cascade controlling VPS production. In this cascade, VpsR up-regulates *cdgA* expression (Beyhan et al. 2007), whose protein product is proposed to increase the intracellular concentrations of the second messenger cdiGMP through its guanylate cyclase activity. Rising cdiGMP concentrations then appear to signal increased transcription of genes involved in VPS production and repression of those responsible for motility (Beyhan et al.

2006). These findings coincide seamlessly with the results of this work, and implicate indole as a mediator of this intracellular cdiGMP regulatory cascade.

A third gene that is thought to be a component of this signal cascade is *vpsT*, another known transcriptional activator of *vps* (Casper-Lindley and Yildiz 2004). This gene shows consistent up-regulation by indole in the transcriptome comparisons; therefore, it was hypothesized that it may also have a role in indole induction of VPS. To test this, multiple attempts to make mutations within the *vpsT* gene were performed, but the mutant was never recovered. These results were unexpected given that Δ*vpsT* derivatives of clinical strains of *V. cholerae* have previously been made. VpsT may have an essential role in the genetic background of the strains used in this study, and a role for this protein in the indole response can not be ruled out.

Another significant finding is that the gene *dksA* appears to be involved in the indole response. This is intriguing given this role of DksA in modulating global transcription within cells during environmental change. Bacterial transcription is highly dependent on the association of RNA polymerase (RNAP) with various sigma factors, which regulate specific sets of genes. One of these, σ^{70}, controls genes which are highly expressed under rapid growth (housekeeping genes), while alternative sigma factors control genes necessary for specific environmental conditions such as nitrogen assimilation (σ^{54}), membrane stress (σ^{E}) and the stationary-phase of growth (σ^{S}). As environmental conditions change, the association of RNAP with the various sigma factors also changes, resulting in global fluctuations in transcription patterns.

Recently, it has been shown that the protein DksA along with the intracellular alarmone ppGpp may enhance this process by binding directly to RNAP and indirectly modulating its associations with different sigma factors (reviewed in Gourse et al. 2006). This binding negatively affects transcription at σ^{70} promoters, which in effect, increases the available intracellular pool of RNAP able to interact with σ^{54}. As more RNAP holoenzyme-containing σ^{54} ($E\sigma^{54}$) is produced, increased interactions with σ^{54}-dependent transcriptional

activators takes place and transcription from σ^{54} promoters proceeds at higher levels. It has been proposed that DksA can act in concert with ppGpp to passively promote transcription from σ^{54} promoters (Bernardo et al. 2006). Although, no previous findings have demonstrated that indole can have either direct or indirect consequences on ppGpp production or DksA activity, it has been shown that various indole derivatives, such as indole-based antimicrobial compounds and indole-3-acetic acid, can influence RelA-based production of ppGpp and the response of cells to this alarmone (Sundar and Chang 1993; Takahashi et al. 2004).

Based on the data presented in this report, a model for indole regulation is shown in figure 3.10. In this scenario catabolite-repressed tryptophanase activity increases as carbon and energy supplies begin to dwindle, leading to a concomitant increase in indole production and excretion by individual cells. Once in the extracellular environment it is unclear how the cells receive the indole signal. Though, it has been suggested the Mtr transporter can actively transport indole (Yanofsky et al. 1991), or, due to its hydrophobic nature, indole can embed within the membrane and subsequently diffuse through (Gaede et al. 2005). It is possible that indole's ability to embed within the membrane can cause membrane stress. However, at the physiological concentrations of indole observed, this does not appear to be the case. It can not ruled out, though, that the reception of this signal may involve membrane stress systems beyond those tested here (i.e. Cpx and RpoE) or does not involve depolarization. More studies will be needed to clarify this issue of signal reception and determine how indole physically interacts with the bacterial cell.

Once the signal is received, though, it is proposed that DksA passively promotes RNAP and σ^{54} association, in possibly a ppGpp-dependent manner. Another possibility is that indole can interact directly with DksA to promote differences in RNAP availability. Despite this ambiguity, the microarray and meta-analysis results imply that indole influences many genes falling under the direct control of σ^{54}, including the VAS and VPS operons. Indirectly, though, it appears as though indole may be influencing the genes of the σ^{38} (RpoS) regulon also. In fact, a down-regulation of the *rpoS* gene is found in the microarray results, and this may be a

result of the fact that σ^{54} can act to negatively regulate the transcription of *rpoS* (Yildiz et al. 2004). Further, since the σ^{38} regulon is known to include genes involved in siderophore production and iron acquisition in *Vibrio vulnificus* (Lee et al. 2003), it is possible that the down-regulation of the iron acquisition machinery observed in the microarrays is due to σ^{54}-driven down-regulation of *rpoS* (figure 3.10).

As for the regulation of genes involved in motility and VPS production, it is believed that this is a result of the finding the indole response incorporates the previously described cdiGMP signal system of *V. cholerae* (Tischler and Camilli 2004; Beyhan et al. 2006). The results of the transposon mutagenesis screen support this model, since the majority of indole non-responsive mutations are in genes coding for GGDEF-domain containing proteins. It is proposed that once VpsR is activated post-translationally it can interact with $E\sigma^{54}$ to enhance the expression of CdgA and VpsT. (Both of which appear to have VpsR promoter binding sites (Yildiz et al. 2004) and are upregulated in the expression profiles.) This increase in CdgA leads to an increase in cdiGMP levels within the cell, which goes on to encourage expression of *vps* genes and repress those involved in motility, leading to the overall enhancement of biofilm formation in *V. cholerae* strain SIO.

Given the multiple roles of biofilms and VAS in stress protection, predator-prey interactions and virulence towards eukaryotic hosts, the tryptophanase enzyme must have an important role in environmental survival. Due to its regulation by catabolite repression, indole production through TnaA activity appears to be an efficient signaling system for adaptation to stressful environments.

Table 3.1 Strains and plasmids used in chapter 3.

Strain or plasmid	Relevant Genotype or Description	Source or reference
E. coli		
S17-1λπ	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	(Simon et al. 1983)
V. cholerae		
92A1552R	Wild-type 92A1552-Rugose variant	(Yildiz and Schoolnik 1998)
AR2101	92A1552R Δ*tnaA*	This Study
92A1552S	Wild-type 92A1552-Smooth variant	(Yildiz and Schoolnik 1998)
AS2101	92A1552S Δ*tnaA*	This Study
N16961	Wild-type N16961	(Heidelberg et al. 2000)
N2101	N16961 Δ*tnaA*	This Study
SIO	Wild-type SIO	(Purdy et al. 2005)
S1101	SIO *tnaA::*Tn5, Km^R	(Mueller et al. 2007)
S2101	SIO Δ*tnaA*	(Mueller et al. 2007)
S2148	SIO Δ*lacZ*	This study
S2150	SIO Δ*lacZ,* Δ*tnaA*	This study
S4100	SIO, *rRNA::gfp*, Gm^R	This study
S4101	SIO *tnaA::*Tn5, *rRNA::gfp* Km^R, Gm^R	This study
S9149	SIO Δ*lacZ,* Δ*tnaA,* Δ*vpsL::lacZ*	This study
S9170	SIO Δ*lacZ,* Δ*tnaA,* Δ*vpsR,* Δ*vpsL::lacZ*	This study
S9171	SIO Δ*lacZ,* Δ*vpsL::lacZ*	This study
S9185	SIO Δ*lacZ,* Δ*tnaA,* Δ*rpoE,* Δ*vpsL::lacZ*	This study
S9190	SIO Δ*lacZ,* Δ*tnaA,* Δ*cpxA,* Δ*vpsL::lacZ*	This study
S9209	SIO Δ*lacZ,* Δ*tnaA,* Δ*cpxA,* Δ*rpoE,* Δ*vpsL::lacZ*	This study
S9211	SIO Δ*lacZ,* Δ*tnaA,* Δ*fis,* Δ*vpsL::lacZ*	This study
S9213	SIO Δ*lacZ,* Δ*tnaA,* Δ*luxO,* Δ*vpsL::lacZ*	This study
S9216	SIO Δ*lacZ,* Δ*tnaA, VC1376::*Tn5, Δ*vpsL::lacZ*	This study
S9218	SIO Δ*lacZ,* Δ*tnaA, VC1673::*Tn5, Δ*vpsL::lacZ*	This study
S9219	SIO Δ*lacZ,* Δ*tnaA, cdgA::*Tn5, Δ*vpsL::lacZ*	This study
S9224	SIO Δ*lacZ,* Δ*tnaA, dksA::*Tn5, Δ*vpsL::lacZ*	This study
S9225	SIO Δ*lacZ,* Δ*tnaA, VCA0075::*Tn5, Δ*vpsL::lacZ*	This study
S9226	SIO Δ*lacZ,* Δ*tnaA, VC0338::*Tn5, Δ*vpsL::lacZ*	This study
S9227	SIO Δ*lacZ,* Δ*tnaA, hmpA::*Tn5, Δ*vpsL::lacZ*	This study
S9228	SIO Δ*lacZ,* Δ*tnaA, VC0143::*Tn5, Δ*vpsL::lacZ*	This study
S9229	SIO Δ*lacZ,* Δ*tnaA, VC1731::*Tn5, Δ*vpsL::lacZ*	This study

Table 3.1 Continued

Strain or plasmid	Relevant Genotype or Description	Source or reference
TP	Wild-type TP	(Purdy et al. 2005)
T1101	TP *tnaA::*Tn5, Km^R	(Mueller et al. 2007)
Plasmids		
pCC12	pRS415 vpsL promoter, Ap^r	(Casper-Lindley and Yildiz 2004)
pFLcm	pFL122L containing *cat* gene from pBSL181	(Mueller et al. 2007)
pFL*cdgA*	pFLcm, *cdgA* operon, Cm^r	This Study
pGPKm	pGP704sac28 containing Km^r gene	(Mueller et al. 2007)
pGP*cpxA*	Δ*cpxA* in pGPKm	This study
pGP*fis*	Δ*fis* in pGPKm	This study
pGP*lacZ*	Δ*lacZ* in pGPKm	This study
pGP*luxO*	Δ*luxO* in pGPKm	This study
pGP*rpoE*	Δ*rpoE* in pGPKm	This study
pGP*tnaA*	Δ*tnaA* in pGPKm	This study
pGP*vpsL::lacZ*	Δ*vpsL:lacZ* in pGPKm	This study
pGP*vpsR*	Δ*vpsR* in pGPKm	This study
pRL27	Tn5-Rl27 *ori*R6K Km^r	(Larsen et al. 2002)
pMCM11	pGP704::mTn*7*-GFP, Gm^r Ap^r	(Beyhan et al. 2006)
pUX-BF13	oriR6K helper plasmid, *mob/oriT*, provides the Tn*7* transposition function in *trans*; Ap^r	(Bao et al. 1991)

Table 3.2 Whole genome expression profiles of SIO and S1101.

Pairwise Comparison	Total Genes Regulated	Up-regulated	Down-regulated
SIO/S1101	572	263	309
S1101(+I)/S1101	74	52	22
SIO(+I)/S1101	642	352	290
SIO(+I)/SIO	259	95	164
SIO/S1101(+I)	284	159	125
Indole regulated	418	171	247

Table 3.3 X^2 analysis of the tryptophanase/indole expression profiles compared to published data sets. (Critical values ≥ 11.35 represent P ≤ .01 with three degrees of freedom and are shown in ***bold, italic*** font)

Expression profile	Indole regulated	SIO / S1101	S1101(+I) / S1101	SIO(+I) / S1101	SIO(+I) / SIO	SIO / S1101(+I)
Δ*fur*[1]	***146.19***	2.23	***14.26***	***46.20***	***216.50***	0.96
cdiGMP[2]	***82.16***	***80.34***	***26.67***	***82.16***	***106.40***	***25.34***
Δ*vpsR*[3]	***69.52***	***48.24***	***38.08***	***41.94***	***17.50***	7.17
Δ*vpsT*[3]	***67.38***	***37.45***	***24.60***	***39.44***	***123.39***	11.02
Δ*cdgA*[3]	***64.95***	***30.07***	***118.84***	***41.84***	***136.81***	***24.94***
Δ*hfq*[4]	***38.76***	***12.71***	***25.27***	***38.76***	***62.77***	***12.10***
Δ*mbaA*[5]	***37.14***	***15.25***	***71.22***	***12.21***	***74.88***	7.99
Δ*rpoN*[6]	***31.55***	***20.57***	***15.73***	***24.52***	***39.21***	6.91
Δ*rseA*[4]	***28.41***	10.58	7.24	***28.41***	***55.04***	1.35
Δ*hapR*[3]	4.27	6.51	3.37	7.03	9.01	10.22
Δ*rpoE*[4]	1.73	4.18	7.41	1.73	3.82	***12.53***
Δ*toxR*[7]	0.68	0.77	0.24	4.16	0.00	0.20

[1] composite data set of (Davis et al. 2005; Mey et al. 2005)
[2] from (Beyhan et al. 2006)
[3] from (Beyhan et al. 2007)
[4] from (Ding et al. 2004)
[5] from (Lim et al. 2007)
[6] from (Yildiz et al. 2004)
[7] from (Bina et al. 2003)

Table 3.4 Transposon mutants altered in *vpsL::lacZ* production (NT – Not Tested).

TIGR #	Strain	Altered Gene Name/ Conserved Domains	Predicted Function	β-Galactosidase Activity	
				LB	LB + Indole
Parental	S9149	Δ*tnaA*, Δ*lacZ*, Δ*vpsL::lacZ*		15.57 ± 3.11	30.86 ± 4.86
Indole Non-responsive mutants					
VC0596	S9224	*dksA* - *dnaK* suppressor protein	Regulatory functions	10.31 ± 2.28	14.27 ± 3.25
VC1376	S9216	GGDEF family protein	Cell Signalling	16.06 ± 2.82	21.06 ± 1.81
VCA0074	S9218	*cdgA*- GGDEF family protein	Cell Signalling	4.65 ± 0.74	5.19 ± 2.21
VCA0075	S9225	Hypothetical protein	Unknown	NT	NT
VPS(-) mutants					
VC0338	S9226	Sodium symporter	Ion transport	0.00	0.00
VC1673	S9219	AcrB-D-F Transporter	Ion transport	0.00	0.00
VCA0183	S9227	*hmpA*- Flavohemoglobin	Electron transport	0.00	0.00
VC0665	S9170	*vpsR*- σ^{54} dependent transcriptional regulator	Regulatory functions	0.00	0.00
VC0143	S9228	Hypothetical protein	Unknown	0.00	0.00
VC1731	S9229	Conserved hypothetical protein	Unknown	0.00	0.00

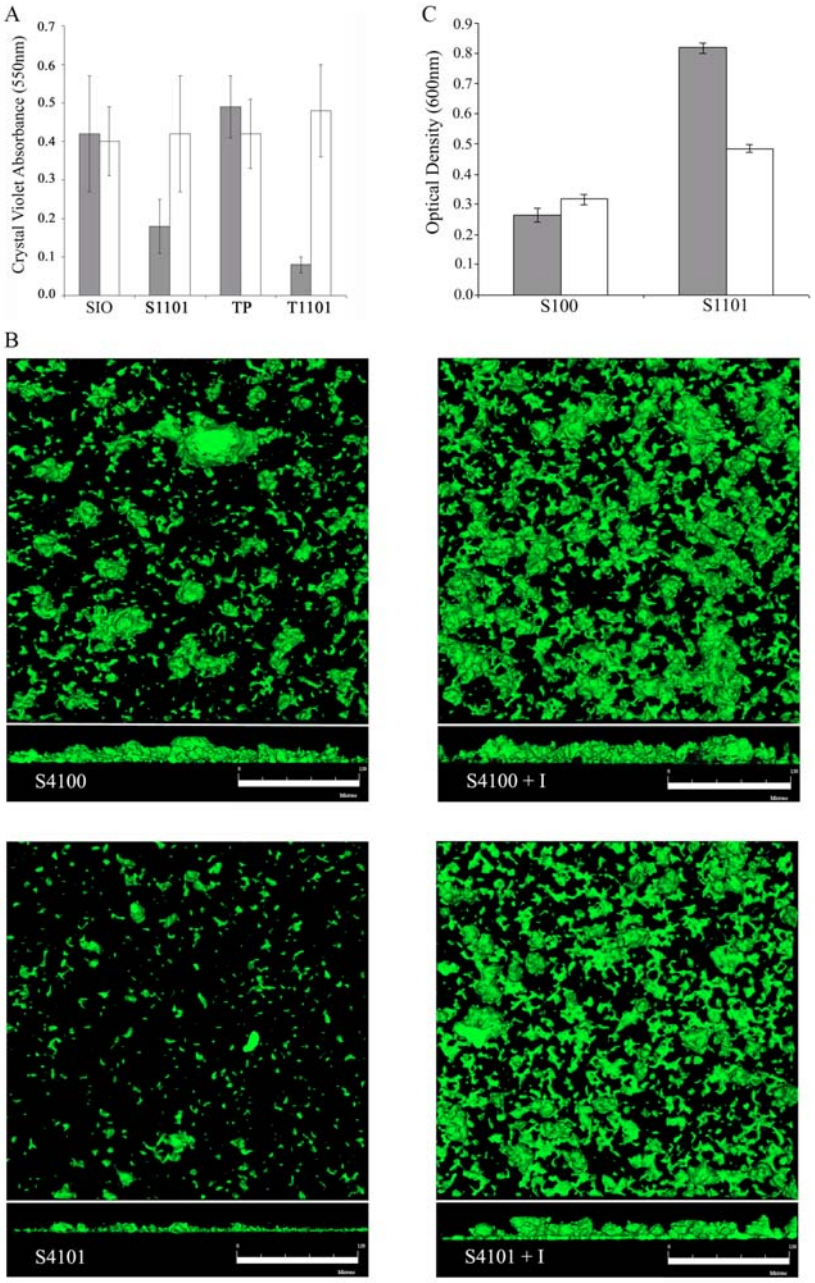

Figure 3.1 Phenotypes of *V. cholerae* tryptophanase mutants. A. Biofilm accumulation of wild-type SIO and TP strains and their corresponding *tnaA ::*Tn5 mutants S1101 and T1101 grown in LB (grey bars) and LB supplemented with indole (350μM, white bars). B. Confocal micrographs of the biofilms of S4100 and S4101 grown in LB (+/- Indole 500μM) for six hours under static conditions. Top picture is XY-axis and bottom picture is Z-axis. C. Measure of the autoaggregation phenotype of SIO and S1101 grown in LB with Indole 500μM (white bars) or without (grey bars). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

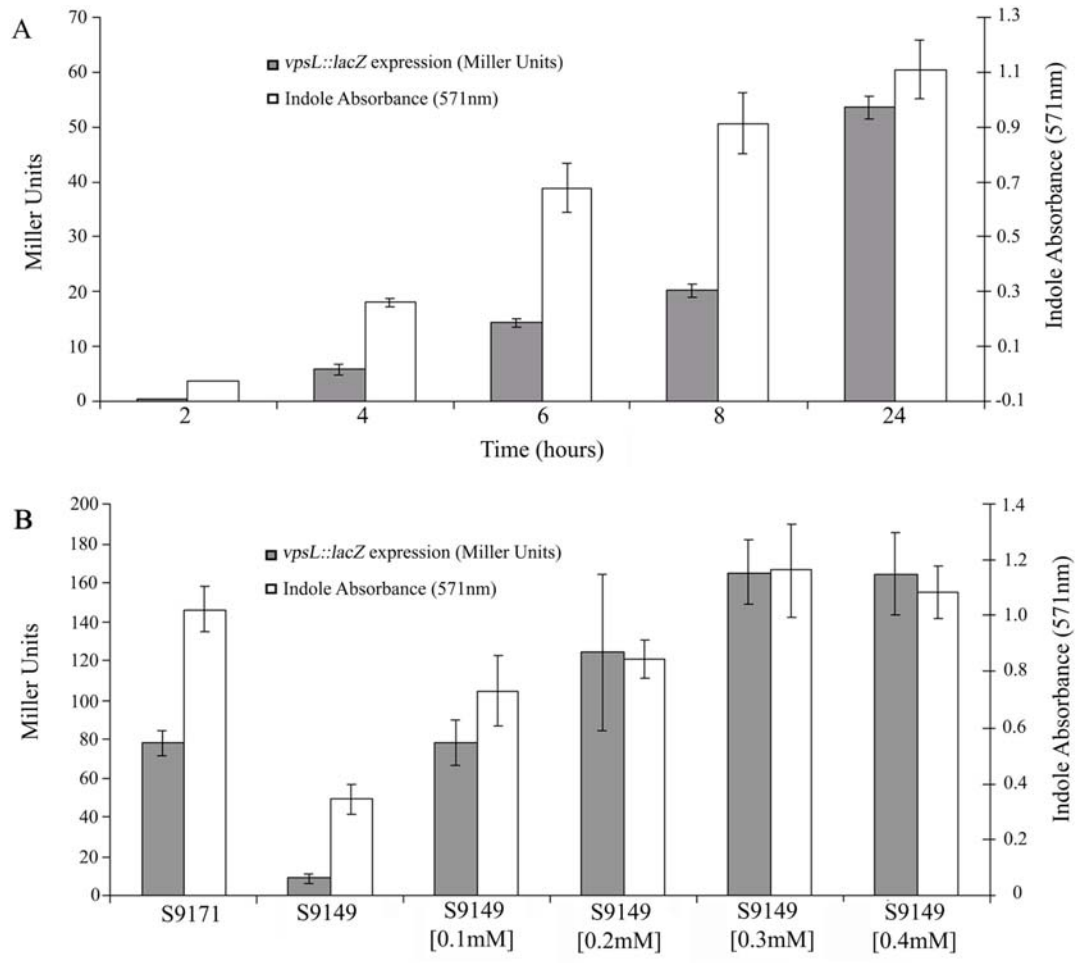

Figure 3.2 Indole production correlates to increased *vpsL::lacZ* transcription. A. Measured endogenous indole (white bars) and β-galactosidase (grey bars) production by strain S9171 (Δ*lacZ*, *vpsL ::lacZ*) over a 24-hour period. B. Comparison of the β-galactosidase production (grey bars) in response to endogenous indole production (white bars) in strain S9171 and exogenous addition of various concentrations of indole (0-0.4mM; white bars) to cultures of strain S9149 (Δ*lacZ*, Δ*tnaA vpsL ::lacZ*). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

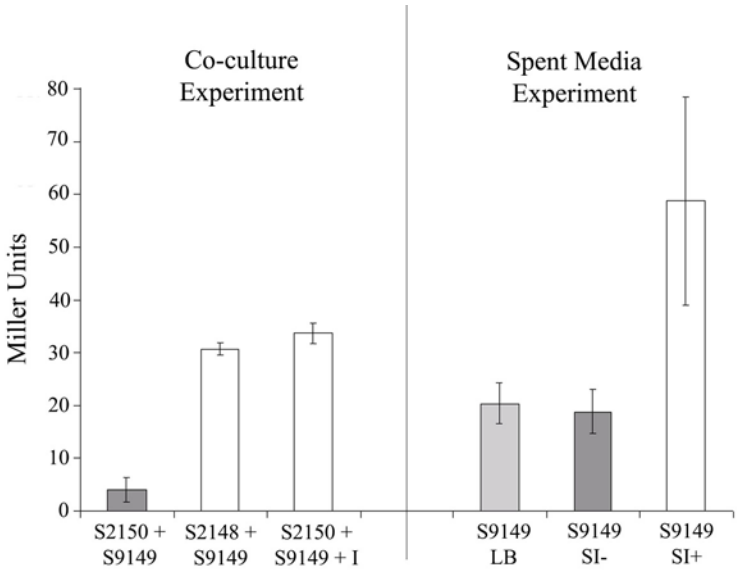

Figure 3.3 Endogenously produced indole acts as a signal to stimulate the expression of *vpsL* in recipient cells. In the co-culture experiment, an indole-negative strain S2150 ((Δ*lacZ*, Δ*tnaA*) does not up-regulate *vpsL::lacZ* transcription of the indole-recipient S9149 (Δ*lacZ*, Δ*tnaA vpsL ::lacZ*) (grey bar), while the indole-donor strain (S2148 - Δ*lacZ*) can complement *vpsL::lacZ* transcription to similar levels as when 0.5mM indole is added exogenously (S2150 + S9149 + I; white bars). The same up-regulation by indole is seen when S9149 is grown in conditioned media grown from indole-positive (S9149 SI+; white bar) and indole-negative (S9149 SI-; dark grey bar) conditions. Growth in LB alone also did not stimulate *vpsL::lacZ* up-regulation (S9149 LB; light grey bar). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

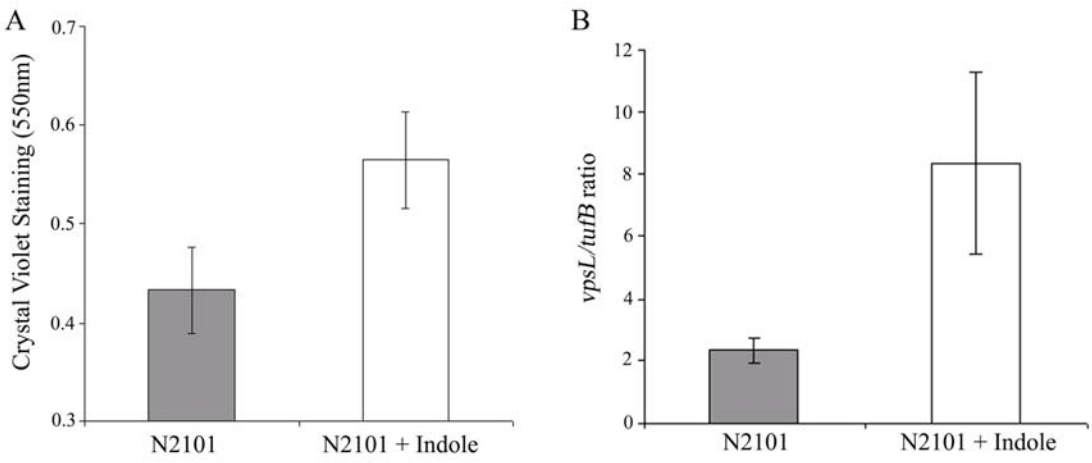

Figure 3.4 The Δ*tnaA* mutant the O1 El Tor *V. cholerae* strain N16961 (N2101) up-regulates biofilm formation and *vpsL* transcription in response to indole. A. The biofilm formation of N2101 grown in LB (grey bar) and LB plus indole 0.5mM (white bar). B. Relative transcription levels of the *vpsL* gene to the constitutively expressed *tufB* housekeeping gene in strain N2101 grown overnight in LB (grey bar) and LB plus indole 0.5mM (white bar). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

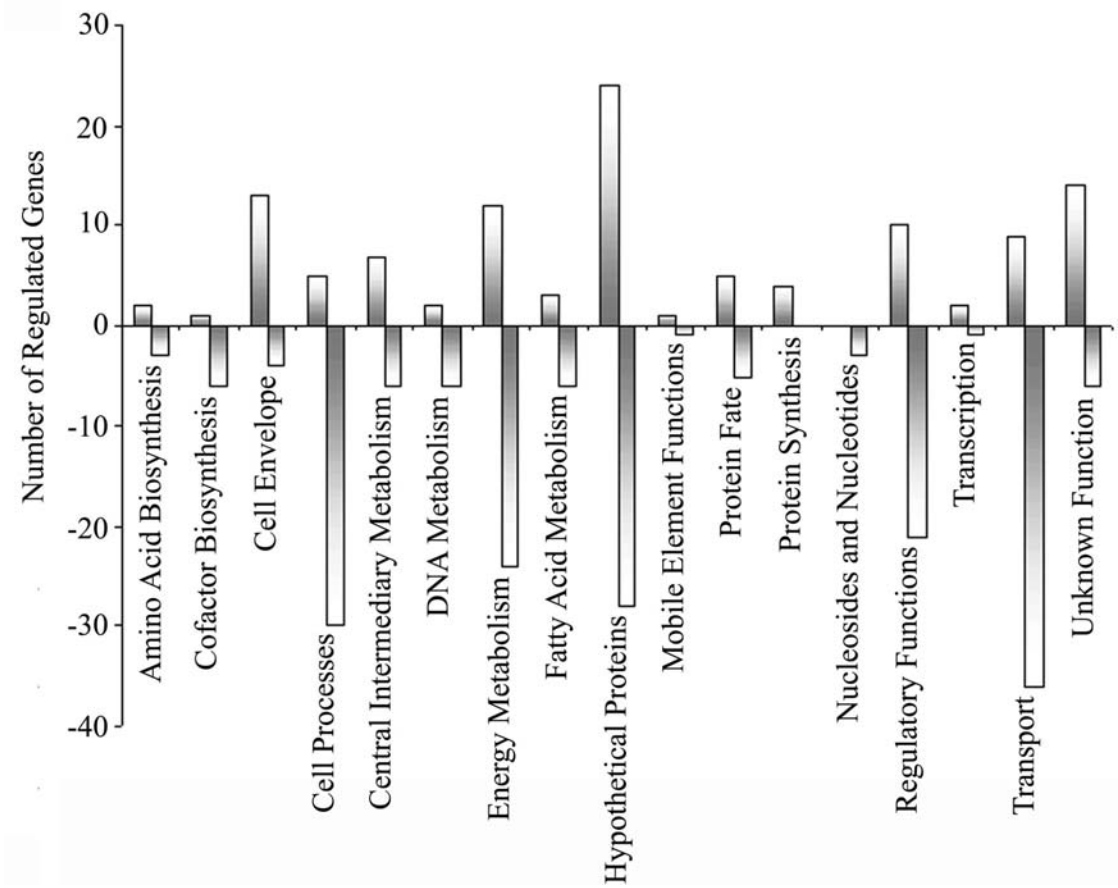

Figure 3.5 Histogram of the role categories of over/under expressed indole-regulated genes. Genes were designated as indole-regulated based on their expression patterns from each microarray experiment performed. Each gene was assigned a role according to hidden markov models within their sequences. Positive and negative bars represent the numbers of genes up- and down-regulated by indole respectively.

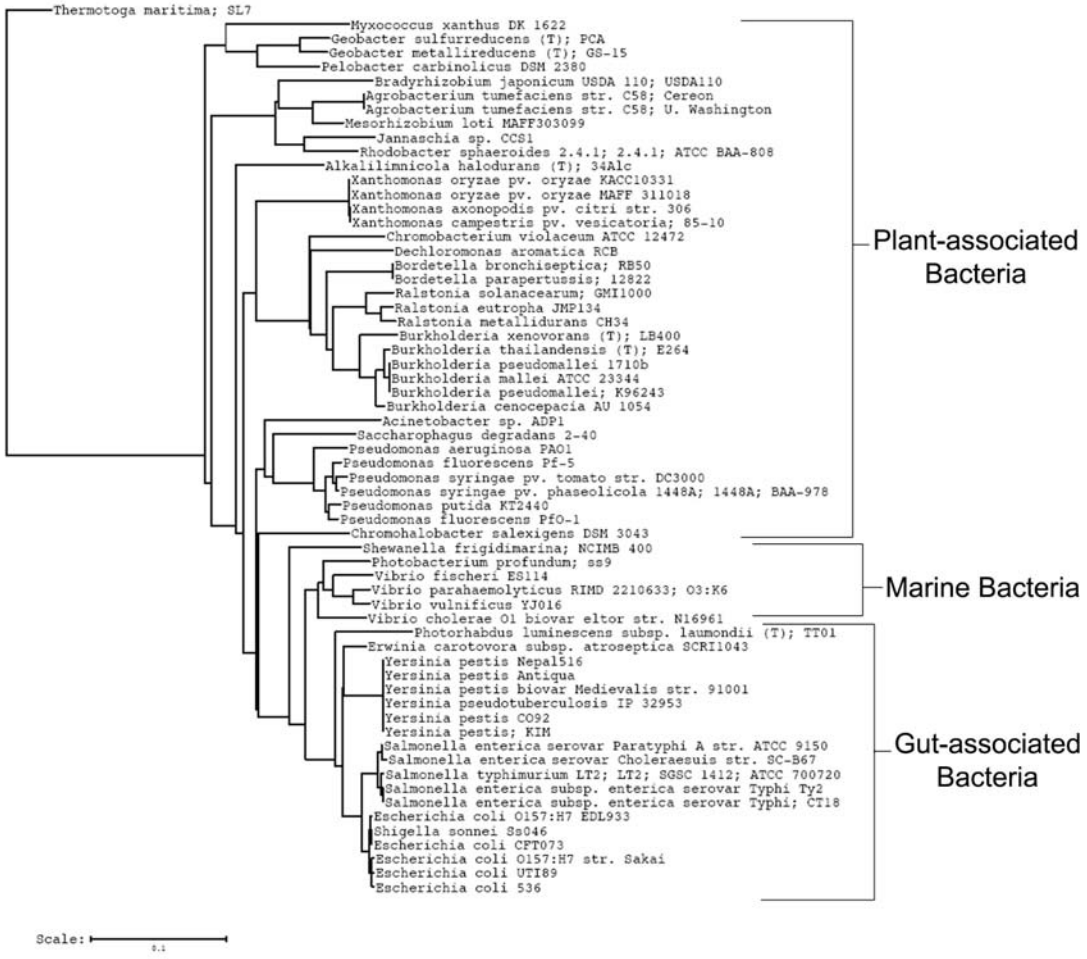

Figure 3.6 Phylogenetic analysis of organisms whose fully sequenced genomes contain conserved VAS operons. Phylogeny is based on 16s rRNA sequences and was constructed as a neighbor-joining tree using the Treebuilder program of the Ribosomal Database Project II v. 9.0 (Cole et al. 2007). Conservation of the VAS operon was assessed as containing at least nine of the 17 annotated genes of the *V. cholerae* N16961 VAS operon.

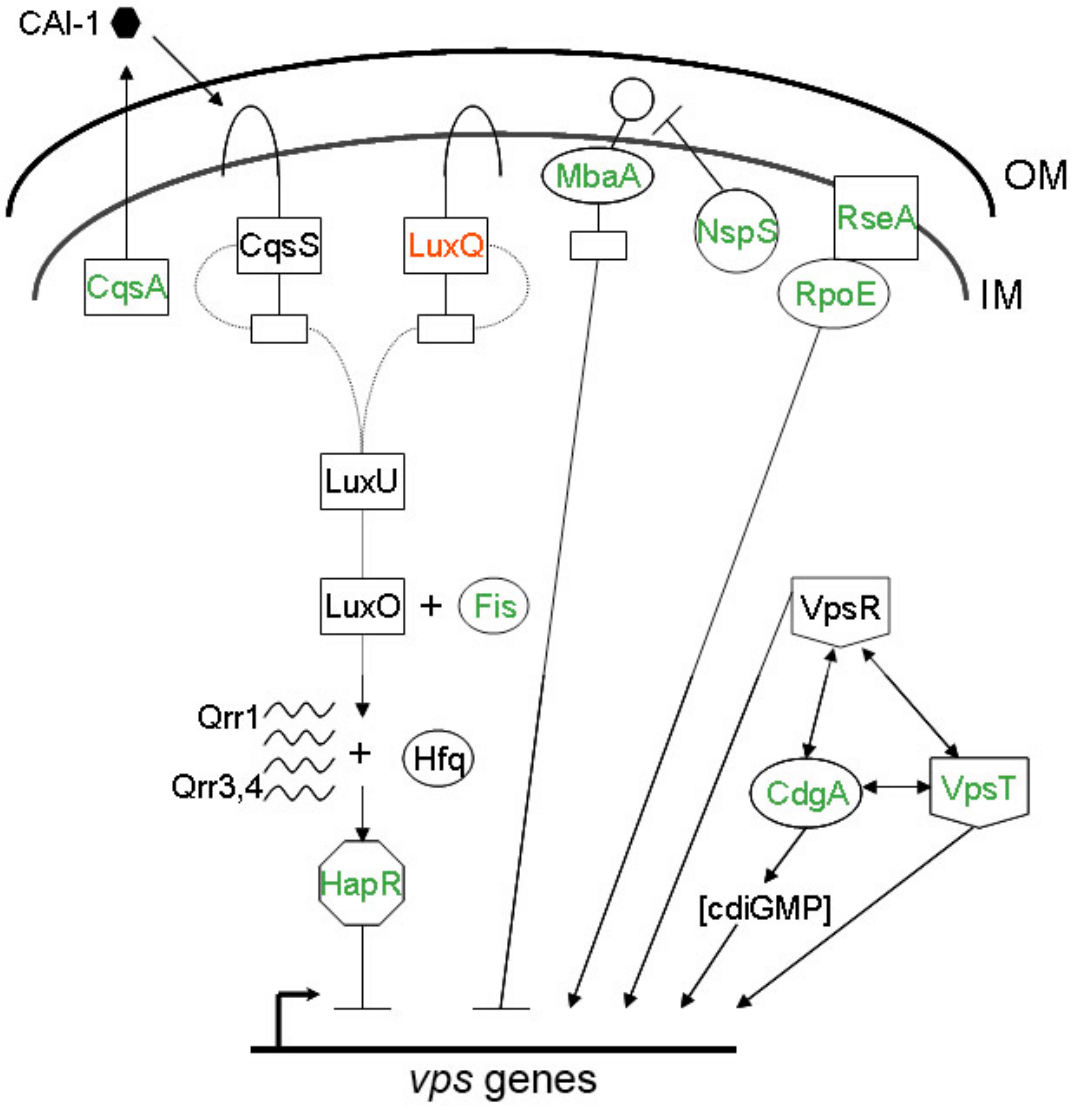

Figure 3.7 Known regulators of exopolysaccharide production are differentially expressed in indole/tryptophanase microarray experiments. Genes that are up-regulated by indole are shown in green and those down-regulated by indole are shown in red. Arrowheads designate transcriptional activation by up-stream component and flat lines represent transcriptional repression by the upstream factor.

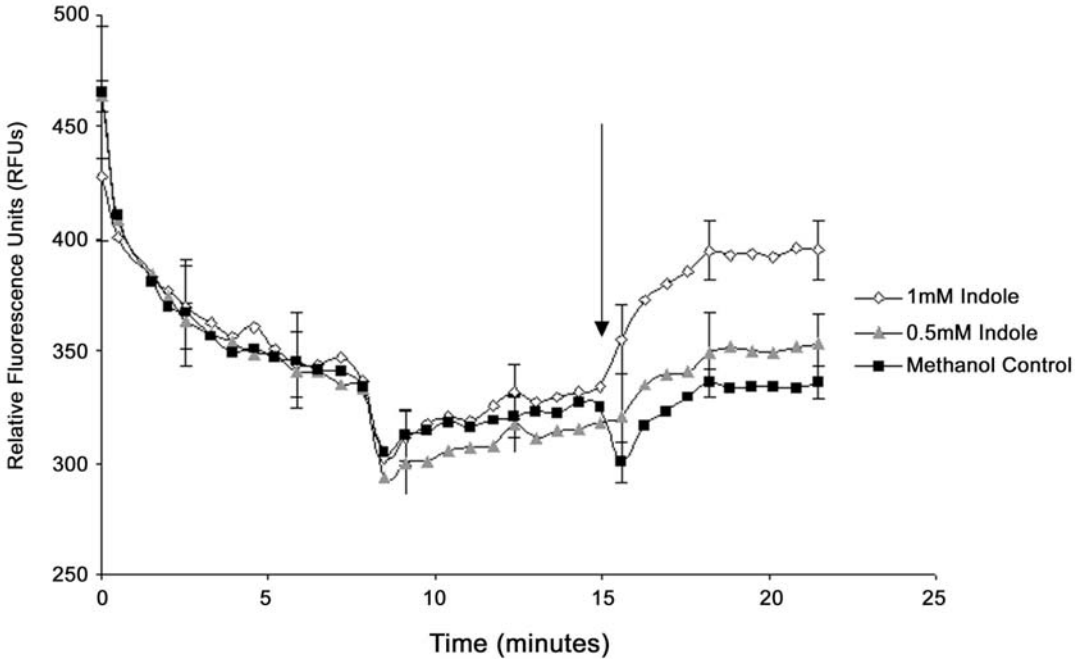

Figure 3.8 Indole does not cause membrane depolarization at physiologically relevant levels. Indole addition (black arrow) to cells of *V. cholerae* at a concentration of 1mM (white diamonds) induces membrane depolarization. However, lower concentrations of 0.5mM (grey triangles), which mimic physiological production levels, do not induce a significant change in membrane depolarization from the control conditions (black squares). Error bars represent one standard deviation from the mean of three biological replicates.

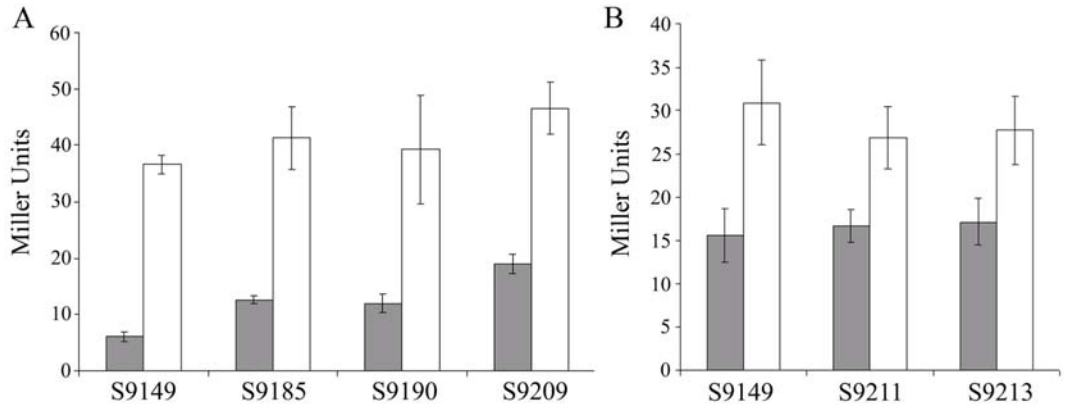

Figure 3.9 Indole induction of *vpsL::lacZ* does not proceed through known membrane stress regulators or quorum sensing. A. Strains carrying mutations in known membrane stress regulators (S9185-Δ*rpoE,* Δ*lacZ,* Δ*tnaA*, *vpsL::lacZ*, S9190-Δ*cpxA*, Δ*lacZ*, Δ*tnaA*, *vpsL::lacZ*, S9209-Δ*rpoE,* Δ*cpxA*, Δ*lacZ*, Δ*tnaA*, *vpsL::lacZ)* show a similar up-regulation of β-galactosidase activity in response to growth under in LB supplemented with indole 0.5mM (white bars) versus LB alone (grey bars) as compared with the parental S9149 strain (Δ*lacZ,* Δ*tnaA*, *vpsL::lacZ*). B. A similar pattern was also seen for quorum sensing mutant derivatives S9211 (Δ*fis,* Δ*lacZ,* Δ*tnaA*, *vpsL::lacZ*) and S9213 (Δ*luxO,* Δ*lacZ,* Δ*tnaA*, *vpsL::lacZ*).

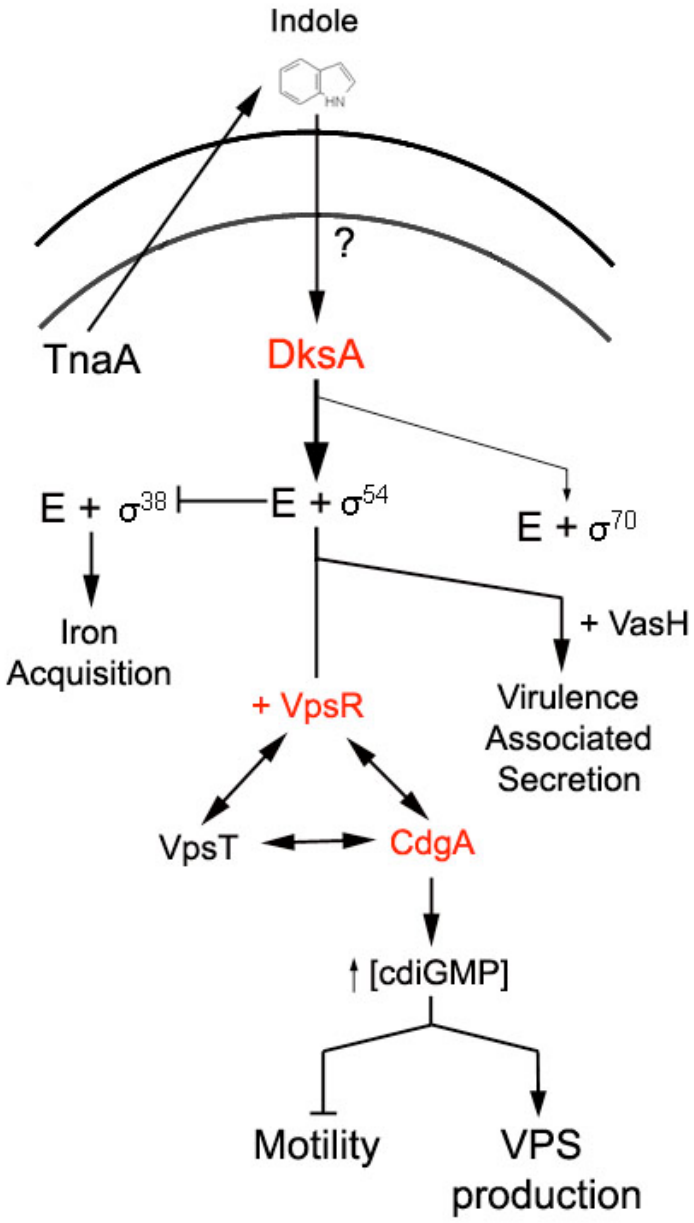

Figure 3.10 Model of indole-regulated gene expression. Indole affects DksA activity and downstream associations between RNA polymerase holoenzyme and σ^{54}. This transcriptional complex can: (i) repress σ^{38} transcription and its activation of iron acquisition mechanisms, (ii) couple with VasH to enhance VAS operon transcription, and (iii) combine with VpsR to activate expression of CdgA, VpsT and itself. Up-regulation of CdgA leads to increases in cdiGMP levels and subsequent repression of motility and chemotaxis genes and activation of *vps* genes. The corresponding protein products of genes that are non-indole responsive and were recovered in the transposon mutagenesis are shown in red font.

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CHAPTER 4

Conclusions and Future Directions

Vibrio cholerae, the waterborne pathogen responsible for the severe diarrheal disease cholera, is an active member of many coastal environments throughout the world's temperate and tropical regions. It is commonly isolated from cholera-endemic regions, such as Bangladesh and India, but can also be found in regions where cases of cholera are never reported, such as the La Jolla coastline. Why is this? As it turns out, the more we discover about this intriguing bacterium, the more complex this answer becomes. In a simple sense, at least, we must look into the water to answer this question. For, it is how this organism spends its time outside of the human body that dictates how we ultimately meet with it again. Essentially, we must develop a greater understanding of the environmental factors constraining its growth in aquatic habitats and the adaptations that this organism employs in order to circumvent these external limitations. In this vein, the focus of this dissertation was to examine the underlying molecular mechanisms behind the ability of *V. cholerae* to survive in aquatic habitats, specifically, through the formation of biofilms.

As a first step, the variety of biofilms that different strains of *V. cholerae* form was examined and characterized in a gross morphological sense. Here it was found, quite simply, that not all biofilms look alike. First, it was discovered that different strains of *V. cholerae* can form very different biofilms from each other although the genes involved in this process are more or less conserved between strains. For example, aside from three loci in TP, all of the biofilm mutations recovered in the transposon mutagenesis screen performed in chapter 2 were in previously sequenced *V. cholerae* genes (from N16961 published genome; Heidelberg et al. 2000). This suggests that while many of the genes responsible for biofilm formation exist in different strains, different regulation of these genes could lead to the observed phenotypic differences.

In addition, it was noted that even the same strain can form different biofilms based on environmental conditions. For instance, strain SIO forms a distinct biofilm from strain TP when grown on a glass surface. While both form three-dimensional biofilms attached to the glass substratum, differences in the ability to form microcolonies and temporal progression of

growth stages ultimately lead to very distinct biofilms. However, when these same strains are grown on biotic surfaces, such as copepods and dinoflagellates, the biofilms they produce are similar to each other, but very different than the ones formed on glass, as they apparently lack the three-dimensional structure of the latter.

It should be mentioned, though, that further analysis of these biotic biofilms has been preformed using CSLM, and while these biofilms are not like the three-dimensional biofilms observed on glass surfaces with mushroom-like structures growing out into the surrounding liquid medium, they are three-dimensional, nonetheless. The key difference is that the biofilms on biotic surfaces grow downward into the biotic surface, instead of outward (figure 4.1). Therefore, it would appear that in both cases, the biofilms of *V. cholerae* are simply growing towards the prevailing nutrient source. However, just because the logic behind the biofilm formation is the same in both cases, this does not mean that the proteins responsible for biofilm formation in each case are the same. As was shown in chapter 2, many genes are involved in biofilm formation on abiotic surfaces, but only genes involved in motility have been discovered to be responsible for biofilm formation on biotic surfaces, thus far. Since *V. cholerae* is known to associate with biotic surfaces in aquatic environments (Huq et al. 1983; Gil et al. 2004) and since most *V. cholerae* biofilm research is focused on the ability to colonize abiotic surfaces, it would be beneficial to study biofilms on biotic surfaces and the mechanisms behind their formation in further detail. Initially, developing an understanding of the nature of the motility defect on biofilm formation would be productive (i.e. does a flagellum provide any additional aid to colonization aside from the propulsive force required to reach a surface?). Then, subsequent research focusing on additional genes involved in this process (e.g. chitinases) would also help to bring a greater understanding of how *V. cholerae* performs this process.

Another interesting aspect of this research could focus on defining the role of this microenvironment in contributing to: (i) the emergence of toxigenic strains within environmental sources and (ii) promoting an infectious dose through ingestion. With regard to

the former, it would be interesting to examine the role of these biofilms in the transfer of the CTXϕ to non-toxigenic strains, thereby providing evidence for the transfer of this phage in an environmental setting where *V. cholerae* is commonly found. As to whether these biofilms are related to infection, a simple experiment examining the infectivity of biofilms on biotic surfaces versus equal numbers of planktonic cells would be informative. As biofilms are thought to combat acid stress, which is a major limitation to infection by *V. cholerae* (Cash et al. 1974; Nalin et al. 1979), it could be that biofilms of toxigenic strains of *V. cholerae* formed on plankton would be better suited to survive stomach passage and cause disease. These experiments would help to redefine the potential hazards for contracting cholera from a more ecological sense.

One of the genes found in this work to influence biofilm formation was the tryptophanase gene. Further analysis of the nature of this mutation led to a major component of the research performed. Specifically, it was found that the indole by-product of the tryptophanase reaction acts as an extracellular signal, which can cue widespread changes in gene expression leading to the production of biofilms and virulence factors and the repression of motility and ion transport. This response is particularly intriguing given that this molecule and its derivatives are found in high concentrations in various environments (e.g. human gut and the rhizosphere of plants), and that it is commonly produced by many different bacteria species as carbon supplies begin to diminish due to catabolite repression of the *tnaA* gene. Therefore, cell-to-cell signaling using indole could be important in a variety of settings. For example, one could foresee a scenario where a *V. cholerae* cell within a phagosome of a eukaryote becomes limited for carbon and starts to produce indole. In this environment, indole could concentrate very quickly and trigger a gene expression response within the bacterial cell, which includes the production of a VPS coat to protect against oxidative stress (Yildiz and Schoolnik 1999), and virulence factors such as Hcp and VgrG to kill the eukaryote (Pukatzki et al. 2006). It will be important to define the environmental context of this signaling mechanism in the future and to determine its role in the survival of organisms, which employ it.

In line with the above example, experiments have been undertaken to test the question of whether indole does have a role in resistance to protozoan grazing by promoting the secretion of virulence factors. While preliminary findings of this work show that a tryptophanase mutant of *V. cholerae* is susceptible to grazing and indole aids in resistance (S. Saini, personal communication), more work will needed to determine the exact mechanism of this indole-mediated resistance. One of the major obstacles will be separating the role of indole from the role of the tryptophanase gene. A previous study has found that the tryptophanase gene is important for the killing of *C. elegans* by *E. coli*, but when indole was added alone to a *tnaA* mutant it could not fully complement its virulence (Anyanful et al. 2005). One of the reasons for this may be is that exogenously supplied indole is not continually available in an environment such as a phagosome. Therefore, an experiment could be performed where the grazing resistance of a *tnaA*+ strain carrying a plasmid with an inducible toluene o-monooxygenase gene (TOM, which converts indole to an insoluble indigoid) could be compared to the *tnaA*+ strain with no plasmid. In this scenario, the strain carrying the TOM gene will no longer be resistant, due to the conversion of the indole signal.

In addition to defining indole's role in environmental survival, it will also be important to determine how this molecule is exported, recognized by the receiving cell, and to define all of the components of the resulting signal cascade. The results of the transposon mutagenesis screen in chapter 3 elucidated some of the components of this signal cascade (e.g. GGDEF proteins, VpsR and DksA), but it is still unclear how this molecule is initially perceived (i.e. at physiologically relevant concentrations does indole trigger a membrane stress response or is it bound to a specific receptor?). Along these lines, it is interesting to note that VpsR, a major component of the indole signal system, is a member of the DmpR transcriptional regulator family, which are activated through the binding aromatic compounds (O'Neill et al. 1998). While it is not known if indole directly associates with any *V. cholerae* proteins including VpsR, it would be an interesting idea to pursue. Another future experiment that is in preparation is to test the effect of indole on the intracellular levels of cdiGMP. This experiment will verify the

role of GGDEF proteins, such as CdgA, in the indole-signaling cascade. If indole is influencing cdiGMP levels, it will provide an intriguing parallel to a system in plants where Indole 3-acetic acid influences the levels of cyclic GMP (cGMP) resulting in adventitious root development (Pagnussat et al. 2003).

In addition to the indole non-responsive genes found by the transposon mutagenesis screen in chapter 3, mutant strains which also showed altered up-regulation of VPS were investigated to determine what genes might also repress VPS production and mutant strains which could not grown on high indole conditions. These mutants are listed in table 4.1. Although, these mutants have not been thoroughly characterized it is interesting to note that many of the mutations, which up-regulate VPS, are found in genes involved in central metabolism and electron transport (e.g. cytochrome c oxidase genes, pyruvate dehydrogenase genes, etc.) These results imply that the metabolic state of the cell may influence whether VPS is produced and lend support to the idea that exopolysaccharide production can be a way for a bacterial cell to maintain stored energy reserves (Jefferson et al. 2004). It was also found that only mutations in genes involved in ubiquinone biosynthesis led to indole sensitivity, although the reason for the interdependence between these molecules is not known.

The research contained within this dissertation has attempted to bridge the gap between the lab and the real-world environments by using molecular genetic approaches to discover some of the processes employed by *V. cholerae*, which are influential in environmental setting. This work provides insight into some of the mechanisms *V. cholerae* may use for survival and lays the groundwork for the application of this knowledge for future more ecologically relevant work. By no means has the book been written (quite literally!) on the mechanisms *V. cholerae* uses to survive in environments outside of the human gut, though, and much more work remains to be done.

Table 4.1 VPS activation and indole sensitive mutants recovered by transposon mutagenesis screen from chapter 3**.**

TIGR #	Name	Common Name/Conserved Domains	Predicted Function
VPS up-regulated			
VCA1096	*cheY*	Chemotaxis protein	Chemotaxis
VC0002	*mioC*	Cell division protein	Electron transport
VC0575	*petC*	Ubiquinol-cytochrome c reductase	Electron transport
VC1440	*ccoQ*	Cytochrome c oxidase, subunit	Electron transport
VC1442	*ccoN*	Cytochrome c oxidase, subunit	Electron transport
VC2051	*dsbE*	Thiol:disulfide interchange protein	Electron transport
VC2053	*ccmE*	Cytochrome c-type biogenesis protein	Electron transport
VC2293	*ngrC*	NADH:ubiquinone oxidoreductase	Electron transport
VC2414	*aceE*	Pyruvate dehydrogenase complex	Energy metabolism
VC2415	*pdhR*	Pyruvate dehydrogenase repressor	Energy metabolism
VC2437		Sugar kinase, pfkB family	Energy metabolism
VC2616	*aruD*	Succinylglutamate 5-semialdehyde dehydrogenase	Energy metabolism
VC2544	*fbp*	Fructose-1,6-bisphosphatase	Glycolysis
VC2738	*pckA*	Phosphoenolpyruvate carboxykinase	Glycolysis
VC0055		Coproporphyrinogen III oxidase	Heme biosynthesis
VC0240	*rfaD*	ADP-L-glycero-D-mannoheptose-6-epimerase	LPS biosynthesis
VC1920	*lon*	ATP-dependent protease LA	Protein fate
VC2153		VanY family protein	Protein fate
VC0768	*guaA*	GMP synthase	Purine biosynthesis
VC1130	*vicH*	DNA binding protein	Regulatory functions
VC1213		LuxR family transcriptional regulator	Regulatory functions
VC2453		Sensor histidine kinase-response regulator	Regulatory functions
VC2465	*rseB*	Sigma-E regulatory protein	Regulatory functions
VC2614	*crp*	Cyclic AMP receptor protein	Regulatory functions
VCA0132	*rbsR*	Ribose operon repressor	Regulatory functions
VC0395	*galU*	UTP--glucose-1-phosphate uridylyltransferase	Sugar-nucleotide biosynthesis
VC2087	*sucA*	2-oxoglutarate dehydrogenase	TCA cycle
VC2775	*gidA*	Glucose inhibited division protein A	tRNA modification
VC0165		Conserved hypothetical protein	Unknown
VC0234		Conserved hypothetical protein	Unknown
VC0758		Conserved hypothetical protein	Unknown
VC2098		Hypothetical protein	Unknown
Indole Sensitive			
VC2475	*ubiH*	Ubiquinone hydroxylase	Quinone biosynthesis
VC2476		Conserved hypothetical protein	Unknown

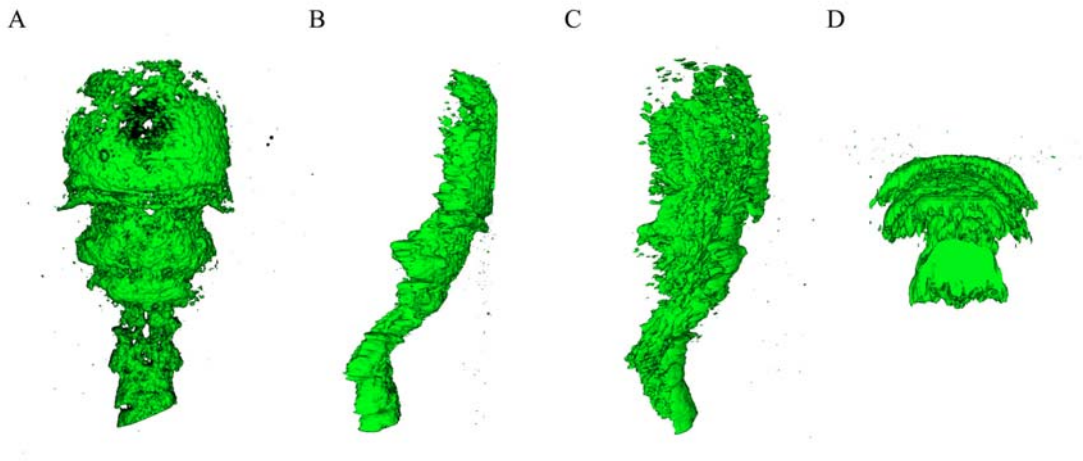

Figure 4.1 Three-dimensional confocal micrograph of a copepod carapace colonization by *gfp*-tagged *V. cholerae* strain 92A1552 (smooth variant). *V. cholerae* cells are shown with green fluorescence and copepod carapace exhibits no background fluorescence. A. Dorsal view of copepod carapace. B. View A rotated vertical axis by 90°. C. View A rotated around vertical axis by 135°. D. View A rotated around horizontal axis by 90°.

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Appendix I: Chapter 2 Supplemental Information

Table S2.1. PCR Primers used in this study (Listed 5′ – 3′)

Primers for arbitrary PCR	
pRL27Extdx	CCAGAAAGTGAGGGAGCCA
pRL27Extsx	GACAACAAGCCAGGGATG
pRL27Intdx1	GAGTCGACCTGCAGGCATGC
pRL27Intsx	CGCACTGAGAAGCCCTTAGAGC
Arb1	GACCACGAGACGCCACACTNNNNNNNNNNCATGC
Arb2	GACCACGAGACGCCACACTNNNNNNNNNNACTAG
Arb3	GACCACGAGACGCCACACT
Arb7	GACCACGAGACGCCACACTNNNNNNNNNNGTTGC
Arb8	GACCACGAGACGCCACACTNNNNNNNNNNGATAT
Primers for panhandle PCR	
pRL27DxPan	TTCCGTGGCAAAGCAAAAGTTCAA
pRL27SxPan	CCGAGCTCGAATTCATCGATGAA
AP1	GGATCCTAATACGACTCACTATAGGGC
AP2	AATAAGGCTCGAGCGGC
pGPKm plasmid based primers for insert verification of subclones	
SacBF	CACCGTACGTCTCGAGGAAT
SacBR	GTGCCACCCTTGACTAGAGG
SOEing PCR Primers (VC# designates the deleted gene)	
VC0378a	CAATCTAGAAGGAGAAAGCCTAACCATGG
VC0378b	AATATGTGCAGCGGAGAAACACGGTTTTAC
VC0378c	GTTTCTCCGCTGCACATATTTCTTCAATTT
VC0378d	CAAGAGCTCTTGATCATCAATACCGATAC
VC0378v1	GCATTGCCAAGAGCAGTCGA
VC0378v2	CCTTTTTCCGCAACTGTGGC
VC0408a	CAAGAGCTCCCGTACGTTACGTAAGTGAG
VC0408b2	TAATTTTCATTCTCTTTCATGTGAATACGCAGCA
VC0408c2	ATGAAAGAGAATGAAAATTATAGGTAAAGGTT
VC0408d	CAATCTAGATATTGATGCAGACTTGGCGA
VC0408v1	GCCGTACGTTACGTAAGTGAGTTGA
VC0408v2	CCTTCGGTATTGATGCAGACTTG
VC0892a	CAACAGAGCTCTTACTCTGCTACATCGCTAA
VC0892b	CTTGAATCGCAACGAAAGCCATGCCACCAA
VC0892c	GGCTTTCGTTGCGATTCAAGATGGGCAGAA
VC0892d	CAACATCTAGATCTGACATCGCTTGTGCTTG
VC0892v1	TCGGTTGTTCTGGCTGTGGA
VC0892v2	CTGCTGCCCCAAGTTTTCTA
VC0934a	CAAGAGCTCACCTGCCGCGGTTGGTTCAGTGATTGA
VC0934b	CAAGAGTAGCCCTAGGCTAGACGCTCCTAACCGATGTAAG
VC0934c	GAGCGTCTAGCCTAGGGCTACTCTTGAATAAATATCGATT
VC0934d	CAATCTAGAAGTCTTCCTCTAAACGCGTTAATGC
VC0934v1	ATGCAATTGGCGGCGGCGTA
VC0934v2	CAAAGGTCAGCCAACGGCGA
VC1437a	CAACAGAGCTCCCACCGACAATGCCGCCAATTAAGG
VC1437b	CGGCACAAATGCTTGGTCGTTGGGTTACAACTTAC
VC1437c	ACGACCAAGCATTTGTGCCGGCAGGTACATCC

Table S2.1 Continued

SOEing PCR Primers (VC# designates the deleted gene)	
VC1437d	CAACATCTAGAGCCTTGGTTCCTGATCATAC
VC1437v1	TGAACATCAATGAGCTCTGTCAGTG
VC1437v2	AAGCCTTGGTATAAACAATTTTGGC
VC1494a	CAACAGAGCTCTCCAGAATTTGATTCTTATT
VC1494b	GGCAGGGAGTTTAAAAGCGATGGATAACCTAGCG
VC1494c	TCGCTTTTAAACTCCCTGCCACTTTTGCTTTT
VC1494d	CAACATCTAGACTCGATTGATATCCAAAAGA
VC1494v1	TGGTCGACTCCAGAATTTGATTCT
VC1494v2	TCGGACTACCATTTCTCGATTGATA
VC2188a	CAACATCTAGATGATTGAGTTCAACGAGAAC
VC2188b	AAGAATTGAATCATTTGCGAAGCGGTCAGC
VC2188c	TCGCAAATGATTCAATTCTTGCCCAAGCGA
VC2188d	CAACATCTAGAGCATCTTGAGAGATATAACA
VC2188v1	CGCGAAATCCAAAAGTACCT
VC2188v2	TGCCCTAAACCCTCTGAAAT
VC2215a	CAACAGAGCTCAGCATGCGAAATTT
VC2215b	GATTGGCATTCGCCACTAGCGCTTTTACTTGCTC
VC2215c	GCTAGTGGCGAATGCCAATCGCCTGCGCTG
VC2215d	CAACATCTAGATCAGTTCCCTTGATAAGAGC
VC2215v1	CCGTCATTTGAATTACCTTTGTGAG
VC2215v2	TGTCTAGCCCTTCATTCTCTTCGAT
VC2337a	CAACAGAGCTCAATACACTCTCTTCTCCATC
VC2337b	TGGGCGAAAAGATGACGCGTGAAACCGTTG
VC2337c	ACGCGTCATCTTTTCGCCCACCATCGTTCG
VC2337d	CAACATCTAGAGGTTAACGCTCGCATTACCA
VC2337v1	GCGATAATCCGTTGTGGGGT
VC2337v2	TCTGATTTACCGCCGCTGCC
VCA0161a	TTCCTGCGTAATCCCTTCTC
VCA0161b	TTTCCATGCTGGGTATTCCT
VCA0161c	AAGCCAATGCTCGTAACGTC
VCA0161d	CCAAACGAAAGCGGAAAATA
VCA0161v1	TGTGATTCACCACCCACTGTTTTAT
VCA0161v2	CATTGATCGCTAATCCTGAAACACC
VCA0859a	CAACAGAGCTCCTAACTTTTCATCACCTTGT
VCA0859b	GCAACAAGGGGCAGCCTTGTCGTTAGAGCTTTC
VCA0859c	ACAAGGCTGCCCCTTGTTGCGCCATGGTCA
VCA0859d	CAACATCTAGAGCTAATGGTCAAGGTGTAGTCGCCC
VCA0859v1	TTTTCTAAATCGGCGTACAAGGG
VCA0859v2	TGAATTTGATCTCTTGTTCGGGG

Table S2.2. Flanking sequence of transposons located within DNA not found within the N16961 published sequence.

>T1138 Putative_LPS_locus_Vibrio_cholerae_strain_TP
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ccg

Table S2.2. Continued

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>T1143 Putative_Tn5_transposase_Vibrio_cholerae_strain_TP
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Table S2.2. Continued

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Appendix II: Chapter 3 Supplemental Information

Table S3.1. PCR Primers used in chapter 3 (Listed 5′ – 3′)

Primers for arbitrary PCR	
pRL27Extdx	CCAGAAAGTGAGGGAGCCA
pRL27Extsx	GACAACAAGCCAGGGATG
pRL27Intdx1	GAGTCGACCTGCAGGCATGC
pRL27Intsx	CGCACTGAGAAGCCCTTAGAGC
Arb1	GACCACGAGACGCCACACTNNNNNNNNNNCATGC
Arb2	GACCACGAGACGCCACACTNNNNNNNNNNACTAG
Arb3	GACCACGAGACGCCACACT
Arb7	GACCACGAGACGCCACACTNNNNNNNNNNGTTGC
Arb8	GACCACGAGACGCCACACTNNNNNNNNNNGATAT
pGPKm plasmid based primers for insert verification of subclones	
SacBF	CACCGTACGTCTCGAGGAAT
SacBR	GTGCCACCCTTGACTAGAGG
SOEing PCR Primers (VC# designates the deleted gene)	
VCA0161a	TTCCTGCGTAATCCCTTCTC
VCA0161b	TTTCCATGCTGGGTATTCCT
VCA0161c	AAGCCAATGCTCGTAACGTC
VCA0161d	CCAAACGAAAGCGGAAAATA
VCA0161v1	TGTGATTCACCACCCACTGTTTTAT
VCA0161v2	CATTGATCGCTAATCCTGAAACACC
LacZ_F	CAACACTGCAGTGAGGGATGTACGCCGTAGAGCAAA
LacZ_R	CAACACTCGAGATTTAGCAACCGATTTTCTCATCCG
VC0290a	CAAGAGCTCTAAGACCATCGACTTGATAC
VC0290b	TATGTTCGAATACGGCATGAACTAATCTTC
VC0290c	TCATGCCGTATTCGAACATATTCGGTCTAG
VC0290d	CAATCTAGATGCTCAATTAGCCGAAGACT
VC0290v1	TTGAGTGTGATGACTTTCGGTGAGAAT
VC0290v2	CGGGTTGGGATACAGACAATAAAA
VC0665a	CAACAGAGCTCAAAGAGTCTGGTGATGATGA
VC0665b	ACATGGTTGCCACAACAAGAGAGCCAGGTA
VC0665c	TCTTGTTGTGGCAACCATGTATCGCTTACT
VC0665d	CAACATCTAGAAAAGCAATGATGCAGATACG
VC0665v1	TGAACGATGCTGAAGACCAA
VC0665v2	GGAGTGCAGTTTGGGTTGAA
VC0934lacA	CAAGAGCTCACCTGCCGCGGTTGGTTCAGTGATTGA
VC0934lacB	CAAGAGTAGCCCTAGGCTAGACGCTCCTAACCGATGTAAG
VC0934lacC	GAGCGTCTAGCCTAGGGCTACTCTTGAATAAATATCGATT
VC0934lacD	CAATCTAGAAGTCTTCCTCTAAACGCGTTAATGC
VC0934v1	ATGCAATTGGCGGCGGCGTA
VC0934v2	CAAAGGTCAGCCAACGGCGA
VC1021a	CAAGAGCTCTTAGAGTTTGAGTTAGCGGC
VC1021b	TTTGCAGCTTCAGCGGTGTGAGGTAAGAAC
VC1021c	CACACCGCTGAAGCTGCAAACTTGGAATGA
VC1021d	CAAGGTCTCTCTAGAAAATTGGAATGGTGTGAACGC
VC1021v1	GCCGCAATGTATCAGCATGC
VC1021v2	CCTGCAGGACGAATGAGCTTAG

Table S3.1 Continued

SOEing PCR Primers (VC# designates the deleted gene)	
VC2338a	CAACAGAGCTCAAGGAAAACATTTGCTGATC
VC2338b	TGCTTCGCGTTTTTTGCTCACTCAGCCGCA
VC2338c	TGAGCAAAAAACGCGAAGCAACAAGCCAAT
VC2338d	CAACATCTAGACACATAACCCTGCAGTAAGG
VC2338v1	AAAGCCGTCGACACTGTCGC
VC2338v2	TCGCAACCGCAGTCAGAACA
VC2467a	CAAGAGCTCTAAATTCCTCAAAGAGTTGT
VC2467b	CGGAAAATTCCCAAAAGGTTAAATGCTTGCTT
VC2467c	AACCTTTTGGGAATTTTCCGCGCTCGTGAG
VC2467d	CAATCTAGAGTTTGCAGCACAGGCAGTTC
VC2467v1	TCCCAAACTGTAGCGAGTCA
VC2467v2	ACTCACTGGCTCAGCGCTTC
VC2693a	CAAGAGCTCGGAGCGGATGATTA
VC2693b	CAAAGATGCGCCCATACAGG
VC2693c	CGCATCTTTGGAGGCTTACAAGTTCGCTTTACTTT
VC2693d	CAATCTAGAAAATTGACGTTCGATCTCTG
VC2693v1	AAGAGATCGATCGGGTGATC
VC2693v2	AGGCTTCTTTGAACTGCGCA

Table S3.2. Whole-genome expression profiles listing differential expression of individual genes of the *V. cholerae* N16961 genome for each pair-wise comparison performed.

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0006	1.78				
VC0009	0.54				
VC0022	1.64				
VC0029			0.61		0.66
VC0034	1.62				
VC0035			1.84		
VC0039			1.65		
VC0045	0.36		0.30	2.83	0.09
VC0049			1.55		
VC0054			0.59		
VC0055	0.64				
VC0059	2.51		5.45	2.76	1.98
VC0066			1.57		
VC0068			1.53		
VC0089	0.27	0.25			
VC0091			0.51	0.56	
VC0097	2.43		2.22		2.02
VC0098	0.54		0.26	0.40	0.64
VC0100		1.84			
VC0104	0.18		0.62		0.53
VC0105			0.61	0.66	
VC0106	0.55		0.47		0.63
VC0110	1.55				
VC0115	0.39		0.57		0.46
VC0122			0.64		
VC0127			0.62		
VC0128			0.64		
VC0130	1.52		1.91		1.50
VC0135			0.66	0.64	
VC0136	1.75		1.89		
VC0137	1.53		1.56		
VC0139			0.59	0.63	
VC0145			1.53		
VC0154	0.46		0.66		
VC0160	5.37		3.84		2.94
VC0166	0.56	0.63			
VC0170					1.52
VC0173	0.50		0.47		
VC0174			1.73		
VC0178	3.99		4.52		4.60
VC0190			1.52		
VC0191	1.77				
VC0192	0.61				0.59
VC0193			0.47		

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0194	0.22				
VC0200				1.54	
VC0204	0.42				
VC0214	1.54				1.54
VC0221	0.65				
VC0230	0.59				0.65
VC0233	1.96		1.88		1.72
VC0242			1.63		
VC0244	0.56				
VC0249	0.36		0.60		0.59
VC0254	1.61				
VC0255	0.31				
VC0269			1.61		
VC0272	0.09		0.51		0.49
VC0274	1.84				
VC0278	1.85		1.60		
VC0279			1.87		
VC0288	0.63				
VC0289	1.52				
VC0290	2.32	1.60	1.76		
VC0291	1.61		1.62		
VC0295				0.59	
VC0297	0.59		0.35		0.53
VC0298	0.39		0.50		0.53
VC0299	0.33	0.66	0.42		0.50
VC0300	0.35		0.55		0.60
VC0304	0.55				
VC0306			0.63		
VC0316	0.56				
VC0319	1.66				1.52
VC0324	1.71				
VC0326	1.96		1.72		1.58
VC0328			0.49	0.63	
VC0341	1.57				
VC0343	1.77				
VC0344	1.77		1.82		1.94
VC0345	1.50				
VC0360	1.70		2.24		1.62
VC0361	1.91		2.09		1.62
VC0365			0.61		
VC0366	1.65				
VC0368	1.54				
VC0382			0.56	0.50	
VC0396			1.60		
VC0413	0.55				0.64

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0423			0.56	0.40	
VC0432	0.62				
VC0446	0.27		0.22		0.09
VC0450	0.47				
VC0454				0.52	
VC0455	0.55				0.66
VC0464	0.36		0.56		0.48
VC0465			0.56		
VC0466	0.58		0.50		0.66
VC0471	0.36				
VC0474				0.66	
VC0475			0.36	0.35	
VC0480			1.55		
VC0484			0.38	0.41	
VC0486	1.92	2.10			
VC0487	3.34	2.43			
VC0488		1.96	0.31		0.37
VC0490	0.53				
VC0497	0.53				
VC0506			1.51		
VC0507			1.57		
VC0510	0.55			1.54	
VC0516	0.26				
VC0519	1.57		1.96		
VC0520			1.55		
VC0533			0.46	0.59	
VC0534			0.48	0.47	
VC0544				1.50	
VC0547				0.65	
VC0555			0.04		
VC0563			1.59		
VC0564	1.55		1.83		
VC0570	2.07		1.87		1.62
VC0571	1.65		1.74		
VC0578	1.53				
VC0583	1.79		1.75		1.56
VC0589	0.63				
VC0599	0.63				
VC0606	0.48				
VC0607	2.48		2.85		2.96
VC0611			1.63		
VC0615			1.50		
VC0616	0.53				
VC0630	2.47		1.72		2.32
VC0633	2.08		2.31		1.77

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0638	0.62		0.61		
VC0643	0.22				
VC0649		1.53			
VC0655			1.58		
VC0658			0.60	0.57	
VC0663			1.51		
VC0666			1.56		
VC0668			0.57	0.66	
VC0676			0.65		0.59
VC0681	1.51				
VC0687			0.54		
VC0697			0.54		
VC0699	2.21		2.67		
VC0701	3.33		3.37		2.58
VC0702			1.53		
VC0703			1.92	1.70	
VC0704	4.92	2.52	3.01	1.54	1.96
VC0706	1.74				
VC0707	0.62				
VC0712	2.24		3.11	2.35	1.57
VC0717	1.51				
VC0728	0.60				
VC0736	1.57				
VC0743	1.80				1.53
VC0745	1.64				
VC0747	1.76	2.06			
VC0748	2.50	2.30			
VC0749	1.85	2.12			
VC0750	1.79	2.04			
VC0752		1.67			
VC0753		1.63			
VC0754		1.73			
VC0763	0.57		0.60		
VC0771				0.65	
VC0772				0.57	
VC0774			0.36	0.39	
VC0775				0.62	
VC0776			0.62	0.56	
VC0777			0.63	0.57	
VC0782	0.49				
VC0797	0.54				
VC0798					0.65
VC0799			1.62		
VC0801			0.14		0.07
VC0802	0.47				0.62

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0805	0.34		0.56		0.58
VC0806	1.58				
VC0808			0.32		
VC0809	0.56				
VC0831	0.37				0.56
VC0842	4.06		4.18		2.62
VC0851	1.88				
VC0857	1.57		1.86		1.62
VC0865	0.64				
VC0886	0.48				0.61
VC0887	0.60				
VC0895		0.60	1.73		
VC0897	1.63				
VC0898			0.66		
VC0899	0.64		0.60		
VC0905	0.66				
VC0907	0.63				
VC0912	0.54				
VC0913			0.67		0.64
VC0916	11.91	4.80	5.44	2.19	2.48
VC0917	2.39		1.87	1.54	
VC0918	3.00	1.93	3.05	1.96	1.55
VC0919	3.05	1.65	2.47	1.75	1.58
VC0920	0.53		0.61		0.52
VC0921			1.51		
VC0922	1.88		1.52		
VC0924			1.62	1.52	
VC0925	1.73		1.68		
VC0926	1.55		1.72		
VC0928	14.14	3.35	14.32	3.40	4.21
VC0930	13.49	4.60	7.59	2.59	2.93
VC0931	2.34		2.41	1.89	
VC0932	10.66	3.52	4.38	2.49	2.18
VC0933	10.90	2.88	5.57	2.76	2.63
VC0934			1.90		
VC0935	9.37	2.29	7.23	3.64	2.88
VC0936	2.36		5.40	2.86	1.89
VC0937	1.81		2.69	2.45	
VC0938				1.74	
VC0939	1.89		2.14		
VC0945	1.73				
VC0948	1.64		1.57		1.59
VC0950			1.93		
VC0954			1.60		
VC0956			0.53		

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC0984			2.02		
VC0995	0.44			1.57	0.43
VC1009				0.62	
VC1020			0.32		
VC1033			4.16	3.47	
VC1037	1.57				
VC1042			0.53		0.56
VC1046	1.69	1.56			
VC1047	1.52	1.56			
VC1053			0.63		
VC1061			0.64		
VC1068	0.60				
VC1069	0.48				0.58
VC1072				0.58	
VC1073	0.44				
VC1077	0.65				
VC1078			0.53	0.52	
VC1080	0.66		0.42		0.65
VC1081			0.48	0.43	
VC1083			1.71		
VC1086	0.27				
VC1087			0.63	0.59	
VC1088	0.63				
VC1095					1.59
VC1116					0.63
VC1120	0.65				0.61
VC1125			0.57		
VC1130	1.77		1.54		
VC1133	0.64				
VC1142	0.50		0.65		0.61
VC1144	0.62				
VC1147	0.59				
VC1149			0.61		
VC1153			0.58		
VC1155	8.43				
VC1165	0.29		0.58		0.60
VC1167	0.39				0.10
VC1176	5.22		12.22		4.66
VC1184		0.57			1.52
VC1185			0.49		
VC1191	1.69		1.64		
VC1194			0.50		
VC1203	0.26		0.43	0.60	
VC1204			0.53	0.57	
VC1205	0.49		0.44	0.64	0.66

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1212			0.35	0.52	
VC1217			1.56		
VC1224	1.51		1.62		
VC1225	1.62				
VC1230	0.06				
VC1240			0.57		
VC1242			0.59		
VC1247	0.59				
VC1248			0.42	0.42	
VC1250	0.64		0.55		
VC1251			0.53	0.63	
VC1253					1.88
VC1256			0.45	0.60	
VC1259	2.15	3.02			
VC1260	1.61				
VC1261	0.65				
VC1262	0.54			0.64	
VC1264				0.62	
VC1273	0.21				
VC1282	1.56				1.53
VC1283	0.50				0.63
VC1285			0.12		
VC1291			0.59		
VC1292	0.33				0.55
VC1295			1.63		1.59
VC1296	1.75		1.61		
VC1298			0.41	0.36	
VC1312			0.62		
VC1313				0.63	
VC1317			0.47		
VC1318			0.51	0.61	
VC1324	0.61				
VC1327	0.56				0.65
VC1332	0.43				
VC1334	0.48		0.58		0.62
VC1337	0.64				
VC1338	0.44				0.51
VC1344			0.43	0.50	
VC1345	0.66		0.55	0.59	
VC1346	0.58	0.60	0.65		
VC1347	0.64		0.53	0.57	
VC1348			0.32	0.48	
VC1349	0.58				0.64
VC1350	1.53	1.81			
VC1352	2.37		3.47		2.35

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1353				1.53	
VC1354	1.62				
VC1355			0.59		
VC1359				0.65	
VC1368		1.55			0.59
VC1369	0.63		0.53		
VC1370			0.50	0.56	
VC1371	1.64		1.54		
VC1375	5.35				
VC1378			0.65		
VC1379			0.58		
VC1383			0.36		0.54
VC1384	2.46				
VC1385			0.55		0.47
VC1389	0.61		0.44	0.56	
VC1399	0.51			0.66	
VC1406	0.24				
VC1407	1.59				
VC1412	0.40				0.51
VC1415			3.46	4.26	
VC1418	0.20				
VC1420	1.54		1.93	1.71	
VC1424			0.57		
VC1425				0.63	
VC1427	1.65				
VC1435	0.63				0.56
VC1438			0.64		
VC1443			0.61		
VC1444			1.63		
VC1446			1.51		
VC1449	1.74		1.55		
VC1450	2.05		1.89		
VC1451			1.76		
VC1462	0.54			1.56	
VC1463	0.44				0.52
VC1467	0.38				
VC1468			2.16	1.65	
VC1479			0.59		0.59
VC1480			0.67		
VC1481			0.63		
VC1490	1.55		1.60		
VC1493			0.65		
VC1494			0.64		
VC1495			0.53		
VC1504			0.64		

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1506		1.58			
VC1507			1.65		
VC1509	0.60		0.60		0.64
VC1510			1.79		
VC1511			1.67	1.56	
VC1512			2.09	1.61	
VC1513			1.66	1.54	
VC1514			2.08	1.74	
VC1515			2.07	1.80	
VC1516			2.01	1.77	
VC1517	1.55				1.51
VC1518	1.76		1.90		
VC1519	1.92				
VC1522	1.84		2.53		1.98
VC1531	1.78		1.54		1.57
VC1538			0.62		
VC1539			2.48		
VC1543				0.64	
VC1544				0.61	
VC1545				0.66	
VC1547			0.63	0.55	
VC1548			0.64	0.57	
VC1550			0.46		0.48
VC1554			0.62		0.66
VC1557	0.54		0.66		0.66
VC1564	0.26		0.39		0.40
VC1565			0.51		
VC1571			1.90		
VC1572			0.53	0.55	
VC1573			0.59	0.50	
VC1578			0.60		
VC1585	2.65		7.94	5.99	2.01
VC1589	0.45				
VC1590	0.51				
VC1591	0.48				0.64
VC1595	0.48		0.29		0.26
VC1596	0.23		0.32		0.44
VC1598			0.36		
VC1599	2.00				1.85
VC1602			0.58	0.64	
VC1603			0.61		
VC1606	0.63		0.50		
VC1608	0.62		0.49	0.67	
VC1611	0.51		0.38	0.58	0.66
VC1612			0.41	0.50	

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1614			1.51		
VC1615			0.57		0.55
VC1619	0.66				
VC1622	1.55				
VC1627	0.51		0.60		
VC1630	1.62	1.58			
VC1636				1.53	
VC1640	1.62				
VC1641				0.66	
VC1642			0.21		
VC1643			0.47		
VC1644	0.43		0.39		0.36
VC1645			0.63		
VC1646	0.31	0.53			0.57
VC1647	0.61				
VC1648					0.58
VC1650			0.14		0.11
VC1653	0.65		0.59		
VC1654			0.66		
VC1657	0.33				
VC1660	0.59				0.66
VC1662			0.56		
VC1664			0.59		
VC1665			0.54	0.63	
VC1667	0.17		0.53		
VC1669	1.52				
VC1670			2.09	1.56	
VC1672			2.21	1.83	
VC1674	0.63		0.59		
VC1675	0.58		0.56	0.66	
VC1679			0.64		
VC1681			0.66		
VC1683	0.46				0.60
VC1684	0.46				
VC1685	0.44		0.34		0.48
VC1687	0.08		0.47		0.32
VC1688			0.59	0.62	
VC1689	0.62				0.66
VC1699			0.58		
VC1701	2.14		2.87	1.98	
VC1704		0.64		0.48	
VC1707	0.62		0.50		
VC1709			0.31	0.57	
VC1710	0.61		0.29	0.33	
VC1713			0.59	0.57	

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1722	1.78		2.73	1.94	
VC1723			0.64		
VC1734			0.48		0.36
VC1736	0.59		0.37	0.61	0.60
VC1740		2.04		1.85	
VC1741	6.94	2.53	1.65		2.10
VC1751	0.43		0.60		0.52
VC1766			0.38		0.28
VC1768	0.18				
VC1770	0.17				
VC1777			0.65		
VC1782			0.32		0.34
VC1803	0.54				
VC1809					0.65
VC1810	0.62				
VC1812	2.56		2.18		1.82
VC1815	2.28				
VC1820	0.14				
VC1822	0.45				0.59
VC1823	0.37		0.44		0.58
VC1824			0.08	0.61	0.09
VC1825	0.47				
VC1826	0.24		0.38		
VC1827	0.16				
VC1828	1.88	1.99			
VC1831			0.26	0.50	0.42
VC1832			0.51		0.65
VC1834	2.05		1.63		1.66
VC1844	0.57				
VC1847	0.55		0.53		0.50
VC1850	0.61				
VC1851			0.23	0.39	0.60
VC1853	0.43				
VC1860			0.50		0.66
VC1861	1.57				1.69
VC1863	1.81		1.60		1.83
VC1864	2.05		1.84		1.79
VC1868			0.59	0.59	
VC1870			0.59		
VC1872			0.56	0.58	
VC1873	0.65		0.45	0.63	
VC1874			0.54	0.57	
VC1875	0.51				
VC1877	1.56				1.67
VC1880	2.40		1.93		2.05

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC1888	5.01	2.07	9.13	3.77	2.42
VC1890			1.51		
VC1898					1.57
VC1900	1.63				
VC1904			1.51		
VC1911			0.61		
VC1912			1.77		1.87
VC1914			0.58		
VC1915	1.62		1.96		1.63
VC1916	1.65				
VC1926			0.63		
VC1928			2.05		
VC1929	3.66	1.55	2.95	1.71	1.94
VC1931			0.57		
VC1933			0.35	0.45	
VC1938	1.53	1.55			
VC1939	0.61				
VC1940	0.60				
VC1950	0.39	0.46			
VC1951	0.36	0.46			
VC1952		2.77	0.41		0.46
VC1953	0.53				
VC1964	1.68				
VC1970	0.62				
VC1972					0.62
VC1976			0.48	0.63	
VC1983	0.17				
VC1984			0.65		
VC1985	2.15	1.63			
VC1988	0.58				
VC1991	0.50				
VC1997	0.26		0.40		0.40
VC1998	1.89		1.81		
VC1999	1.56				
VC2001	0.52				
VC2004			0.56	0.53	
VC2008	0.40				0.57
VC2013				0.63	
VC2017	0.47				0.59
VC2018			0.47		
VC2025			1.98		
VC2026	1.73		2.27		1.64
VC2029			0.08		0.05
VC2038			0.55	0.61	
VC2042			0.37		

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC2044	1.74	1.58			
VC2045				1.65	
VC2058				0.66	
VC2059			0.66	0.64	
VC2062			0.64	0.62	
VC2063	0.59		0.56		
VC2071			0.44		
VC2075	0.50				0.66
VC2076	0.64	0.64			
VC2080	0.66		0.66		
VC2081	0.54			2.84	0.26
VC2084			0.64	0.66	
VC2086			0.63	0.65	
VC2090			0.66		
VC2092			0.62		
VC2099			0.61		
VC2112	0.40		0.57		0.50
VC2114			0.20		0.28
VC2117			0.23		
VC2121	1.59		1.70		
VC2122	1.62				
VC2135	0.60		0.66		0.64
VC2139			0.54		
VC2141			0.55	0.60	
VC2142			0.55	0.51	
VC2144			0.62		
VC2149	0.59	0.58			
VC2161	0.40				
VC2168			0.11		
VC2177	1.84		2.06		2.09
VC2178			0.62	0.58	
VC2184	1.65				
VC2187			0.52	0.57	
VC2188				0.63	
VC2189				0.57	
VC2191			0.63	0.65	
VC2192			0.64		
VC2194			0.62		
VC2196			0.63		
VC2199			0.61		
VC2200			0.61	0.63	
VC2201			0.64		
VC2202			0.65	0.65	
VC2209	0.66				
VC2210			0.54	0.44	

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC2211			0.57	0.59	
VC2212			0.49		
VC2216	0.57		0.63	1.58	0.40
VC2226	1.62				
VC2227	1.72				
VC2231	1.92	1.63			
VC2247	0.61				
VC2264			0.59		
VC2280				0.66	
VC2281	0.55		0.59		
VC2282				0.65	
VC2303			1.65		
VC2314	0.62				
VC2315	0.49				0.66
VC2316			2.18		
VC2318	1.92		1.73		
VC2319	0.66				
VC2328			0.09		
VC2330	0.63				
VC2331			1.52		
VC2337			0.66		
VC2339			1.83		
VC2340	0.58		0.46	0.57	
VC2341			0.56	0.60	
VC2343			1.68		
VC2353			2.27		1.71
VC2367	1.57		1.82		
VC2374	0.65				0.36
VC2376					0.55
VC2379	1.66				
VC2385	0.51		0.61		0.59
VC2388	1.63				
VC2389			1.62		
VC2393			1.79		1.60
VC2396	0.66				
VC2402			1.60		
VC2403	0.57				
VC2414			0.58		
VC2416	0.51				
VC2422			0.62		
VC2424			0.59		
VC2425			1.55		
VC2427			0.63		
VC2434			0.62		
VC2454	0.63				0.64

Table S3.2 Continued

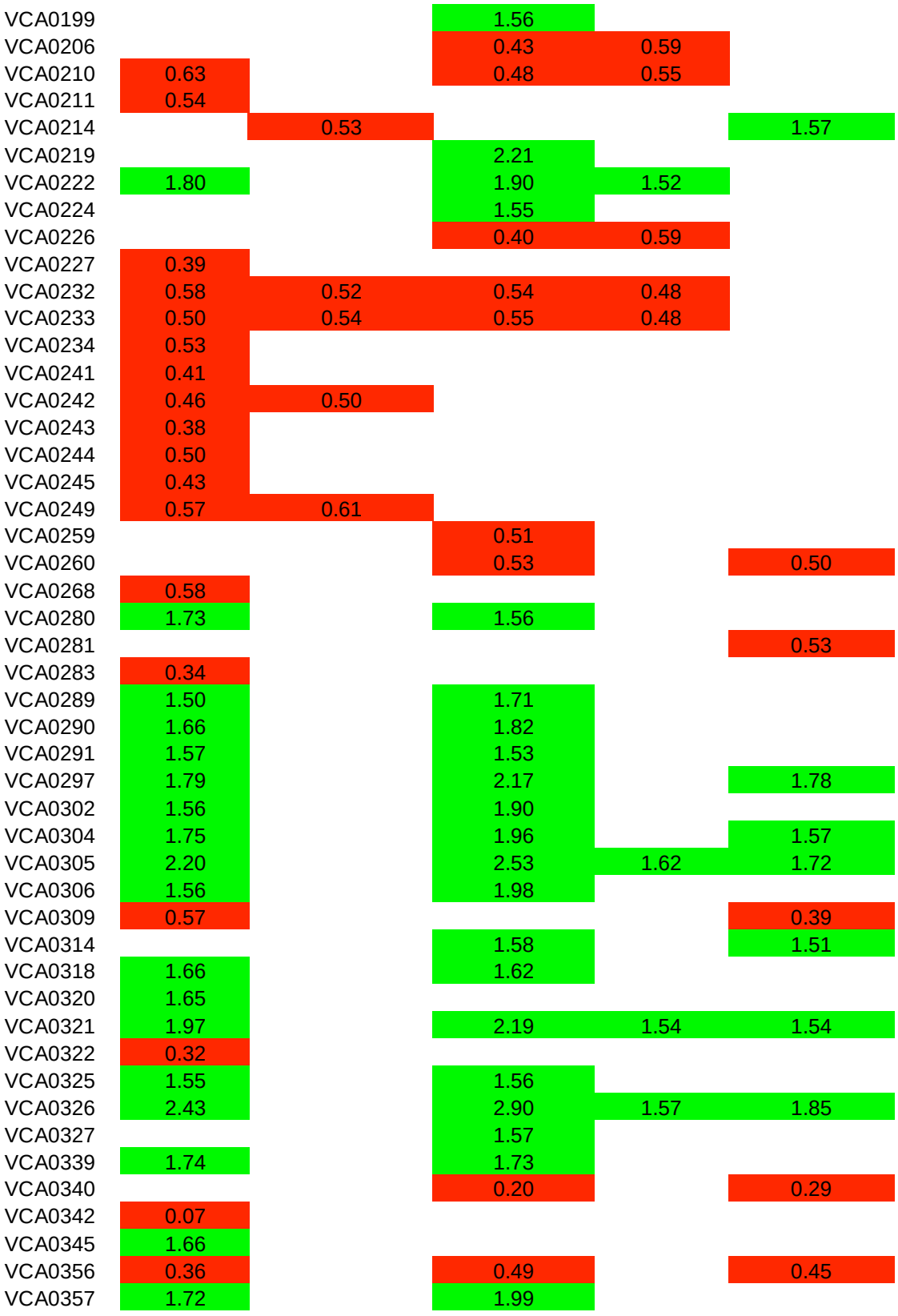
VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC2462			0.64		
VC2466	1.75				
VC2467	1.78	1.62			
VC2487	0.58		0.65		
VC2497			0.51		
VC2501			0.62		
VC2508			4.13		
VC2509			2.02		
VC2510			1.71		
VC2511			1.76		
VC2520	0.58				0.66
VC2521			0.26		
VC2532			0.63		
VC2533			0.65		
VC2548	1.57		1.57		
VC2552			0.61		0.63
VC2553			0.50		0.52
VC2561	0.51		0.54		0.56
VC2562	0.61		0.54		0.61
VC2569	1.60				
VC2570			1.74		
VC2575			1.68		
VC2576	2.13		2.13		1.70
VC2577	1.55		1.85		
VC2579			1.61		
VC2581			1.56		
VC2582	1.50				
VC2583	1.52				
VC2606			1.51		
VC2612			1.69		
VC2615			0.40	0.24	
VC2616			0.49	0.32	
VC2617			0.37	0.27	
VC2619					1.53
VC2629			1.51		1.67
VC2633				0.52	
VC2641			1.73		
VC2642	0.37		1.94	2.83	
VC2643			1.81		
VC2644			1.78		
VC2647	1.73		1.71		
VC2663	0.37		0.65		0.58
VC2664				0.64	
VC2665			0.65	0.65	
VC2667			2.06	1.67	

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VC2668			1.55		
VC2676			1.62		1.53
VC2691	0.56		0.07		0.05
VC2692	0.61		0.40		0.43
VC2693	0.57		0.48		0.54
VC2694			0.51	0.51	
VC2700	0.61				
VC2704	0.34		0.48		0.63
VC2705	0.38		0.53		
VC2711	1.72		1.59		1.53
VC2717	1.58				
VC2718			0.57		
VC2720		1.51			
VC2728	0.64				
VC2730	1.52				
VC2737	0.66				
VC2747	2.70	1.92	1.57		
VC2749			1.56		
VC2753	2.31		4.46	2.70	1.65
VC2761	0.59				0.63
VC2765	2.15		1.99		1.76
VC2766	1.52		1.57		
VC2767	0.31		0.23		0.22
VC2768	0.55				0.63
VC2769			1.54		
VCA0002			1.58		
VCA0003	0.66				
VCA0005	2.00		1.52		
VCA0008	0.55		0.35	0.43	
VCA0017	1.73		4.08	4.29	
VCA0018			2.07	2.14	
VCA0019	1.53		1.67		
VCA0020			1.83	1.97	
VCA0021				0.48	
VCA0023	1.91		2.18		2.01
VCA0029	1.61		1.73		
VCA0030	1.70		2.01	1.63	
VCA0031	0.52		0.23		0.32
VCA0042				0.58	
VCA0046	0.26		0.55		0.50
VCA0055	1.93		3.03		
VCA0056			1.69		1.50
VCA0063	0.40				0.60
VCA0065	0.57				
VCA0066	0.57				

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0068					0.33
VCA0070					0.42
VCA0072	0.47	0.40			
VCA0074	1.74				
VCA0075	2.33	1.55	1.69		
VCA0080			0.52	0.50	
VCA0089	0.55				
VCA0095	0.61				
VCA0096	0.54			1.60	
VCA0098	0.63				
VCA0099		0.56			
VCA0105				1.70	
VCA0107	1.74		3.91	3.25	
VCA0108	3.68		5.39	3.89	1.95
VCA0109	6.44		15.20	3.60	4.22
VCA0110	3.00		3.86	2.73	1.77
VCA0111	1.82		2.20		1.71
VCA0112	2.87		4.89	3.38	1.94
VCA0113			2.03	1.96	
VCA0114	3.29		4.01	3.15	1.63
VCA0115	1.85		1.71		1.51
VCA0116	2.08		4.27	3.18	1.73
VCA0117	2.32		2.85	1.80	1.59
VCA0118	1.72		2.21	1.91	
VCA0119	1.75		3.02	1.84	1.64
VCA0120	2.62		2.80	2.13	
VCA0121	2.46		4.99	2.63	1.89
VCA0122				1.78	
VCA0123				1.64	
VCA0139	0.27	2.61	0.11		0.11
VCA0158	1.74				
VCA0159	1.57				
VCA0160	3.62			0.50	2.07
VCA0161	44.50		6.84	0.30	14.38
VCA0162	0.24		0.31		0.16
VCA0163			0.44		
VCA0169			0.60		
VCA0170			0.16	0.43	
VCA0171			0.32		
VCA0182	0.64				
VCA0185	0.03		0.26		0.18
VCA0187	0.62				0.64
VCA0188	0.62				0.56
VCA0192	1.56				
VCA0193	0.63				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VCA0206 0.43 0.59
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VCA0211 0.54
VCA0214 0.53 1.57
VCA0219 2.21
VCA0222 1.80 1.90 1.52
VCA0224 1.55
VCA0226 0.40 0.59
VCA0227 0.39
VCA0232 0.58 0.52 0.54 0.48
VCA0233 0.50 0.54 0.55 0.48
VCA0234 0.53
VCA0241 0.41
VCA0242 0.46 0.50
VCA0243 0.38
VCA0244 0.50
VCA0245 0.43
VCA0249 0.57 0.61
VCA0259 0.51
VCA0260 0.53 0.50
VCA0268 0.58
VCA0280 1.73 1.56
VCA0281 0.53
VCA0283 0.34
VCA0289 1.50 1.71
VCA0290 1.66 1.82
VCA0291 1.57 1.53
VCA0297 1.79 2.17 1.78
VCA0302 1.56 1.90
VCA0304 1.75 1.96 1.57
VCA0305 2.20 2.53 1.62 1.72
VCA0306 1.56 1.98
VCA0309 0.57 0.39
VCA0314 1.58 1.51
VCA0318 1.66 1.62
VCA0320 1.65
VCA0321 1.97 2.19 1.54 1.54
VCA0322 0.32
VCA0325 1.55 1.56
VCA0326 2.43 2.90 1.57 1.85
VCA0327 1.57
VCA0339 1.74 1.73
VCA0340 0.20 0.29
VCA0342 0.07
VCA0345 1.66
VCA0356 0.36 0.49 0.45
VCA0357 1.72 1.99

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0360		1.56			
VCA0374	0.66				
VCA0375	1.76		1.87		
VCA0377			1.61		
VCA0378	1.82		2.40	1.53	1.71
VCA0379	0.33				
VCA0381			2.37		1.58
VCA0383	3.15		3.75	1.67	2.52
VCA0384	1.92		2.54		1.80
VCA0389	1.53		1.87	1.58	
VCA0390	1.71		2.17	1.57	
VCA0394	2.07		2.45		1.95
VCA0398				1.62	
VCA0404	3.35		4.31	1.88	2.29
VCA0407	1.56		1.89		
VCA0412	1.54		1.68		
VCA0418			1.65		
VCA0420	0.65				
VCA0429	1.53		1.94		
VCA0430	1.86		2.27	1.54	1.61
VCA0434	1.81		2.20		1.50
VCA0435	1.85		2.65	1.62	1.81
VCA0441				0.38	
VCA0448	1.93		2.07		1.91
VCA0449	0.39				
VCA0453	1.89		1.89		
VCA0455	0.06				
VCA0456	1.96		2.90	1.93	1.73
VCA0457	0.58				
VCA0462	1.78		2.15		1.64
VCA0471	0.55				
VCA0478	1.61				
VCA0480	1.63		1.93		
VCA0483			1.57		
VCA0486	2.27		2.12		1.80
VCA0487	1.50				
VCA0492	1.63				
VCA0493			0.31		0.24
VCA0494	1.59		1.62		
VCA0495	0.21				
VCA0497	0.58				
VCA0502			1.52		
VCA0505	1.53		1.61		
VCA0509	0.50				
VCA0510			1.78		1.52

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0512	0.62				
VCA0517					0.61
VCA0518	0.65				
VCA0519			1.67		
VCA0523	1.50				
VCA0524			1.56		
VCA0527			0.63		
VCA0529	0.55				
VCA0538		1.64	0.55		0.53
VCA0539			0.57		
VCA0541	0.65				
VCA0547	0.53	0.38			
VCA0550	1.67				
VCA0556				1.97	
VCA0557	0.52				
VCA0568			1.69		
VCA0577			0.65	0.53	
VCA0583			0.57	0.59	
VCA0584					1.54
VCA0593			0.42	0.54	
VCA0606	1.54				
VCA0622			0.62	0.65	
VCA0623			0.65		
VCA0629	1.89		1.68		1.71
VCA0636	0.52				0.61
VCA0640			0.62	0.57	
VCA0641	0.64		0.63		
VCA0642			0.53	0.64	
VCA0643			0.59		
VCA0644			0.43	0.53	
VCA0645			0.55		
VCA0650	0.42				
VCA0656	0.64				
VCA0664					0.63
VCA0688			0.46	0.40	
VCA0689			0.56	0.54	
VCA0690				0.53	
VCA0691			0.38	0.46	
VCA0697			0.51	0.53	
VCA0698			0.27	0.46	
VCA0704	0.57				0.64
VCA0705	0.21				
VCA0706	0.36				
VCA0709			1.52		
VCA0713			0.55		

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0716			1.97		
VCA0721	1.84		2.48	2.17	
VCA0722			0.64		
VCA0723	0.57				
VCA0725			0.60		
VCA0726	0.66				
VCA0731	0.62		0.41	0.64	0.61
VCA0736				0.62	
VCA0738	0.58				
VCA0746	0.25		0.65		0.66
VCA0747				1.58	
VCA0748			0.64		0.64
VCA0749	0.54				0.56
VCA0752			0.64		
VCA0754			1.64		1.54
VCA0756	0.05			1.86	0.46
VCA0758		0.65	1.52		
VCA0760			1.81		
VCA0763	1.58				1.72
VCA0769	0.63				
VCA0773	0.37		0.42		0.58
VCA0775	0.28		0.57		
VCA0784			1.78		
VCA0785			1.62	1.54	
VCA0787			0.66	0.62	
VCA0788			0.59		
VCA0793	0.06				
VCA0795	0.54		0.64		0.47
VCA0796			0.51	0.46	
VCA0803	0.58		0.41	0.62	
VCA0809	1.56				
VCA0823			1.63		1.74
VCA0825	0.64				
VCA0826	1.81		1.62		1.51
VCA0827	0.56		0.44	0.53	
VCA0828			0.36	0.51	0.61
VCA0829	0.42		0.57		
VCA0830	0.45		0.54	0.61	
VCA0834			1.81		
VCA0838			0.65		
VCA0849	1.63	1.73	2.89	1.91	1.51
VCA0854	1.53				1.54
VCA0857			1.59		
VCA0858	0.47				0.67
VCA0860			0.30		0.29

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0861	0.26				
VCA0862	1.59				
VCA0864	0.64				
VCA0866	0.51				0.63
VCA0867	0.29	0.27			
VCA0868				0.64	
VCA0875	0.64				
VCA0878			1.53		1.51
VCA0880			1.55		
VCA0882	0.62				
VCA0887			1.91		
VCA0888			1.72		
VCA0890			0.59		
VCA0891					1.54
VCA0901			0.49	0.64	
VCA0902	0.56				
VCA0905			0.51		
VCA0908			0.59		
VCA0911				0.63	
VCA0915			0.54	0.60	
VCA0916	2.21		2.78		2.19
VCA0917	1.62		1.52		
VCA0922			1.92		2.08
VCA0925				0.58	
VCA0926			1.61		
VCA0928	0.11		0.66		0.59
VCA0931			0.52	0.55	
VCA0933	2.02				
VCA0936					1.51
VCA0946			1.50		
VCA0949			1.51		
VCA0950	1.59				
VCA0952	2.78	2.14	2.44	1.88	
VCA0956			0.58		
VCA0957	1.55	2.23			0.66
VCA0958	1.64				
VCA0966			1.78	1.53	
VCA0967	1.54		1.61		
VCA0974	0.61				
VCA0976				0.64	
VCA0977			0.64	0.60	
VCA0978			0.56	0.50	
VCA0980	0.61				
VCA0981			0.54		
VCA0988			0.48	0.50	

Table S3.2 Continued

VC#	SIO/S1101	S1101(+I)/S1101	SIO(+I)/S1101	SIO(+I)/SIO	TnaA(+)/SIO(-)
VCA0990			0.55		0.55
VCA0991			0.37		
VCA0994	3.44		1.96		2.31
VCA0998	0.39		0.45		0.52
VCA1012				1.54	
VCA1017	0.56				0.62
VCA1024			0.54	0.59	
VCA1031	0.61		0.64		
VCA1032	0.36	2.66	0.21		0.09
VCA1033			0.59		
VCA1049					0.61
VCA1053			1.61		
VCA1054	0.58				
VCA1055	2.60		1.84		1.91
VCA1064	0.49				0.63
VCA1065	0.63				
VCA1070	1.51				
VCA1071	2.75	1.55	2.29		2.04
VCA1072	3.67		3.10	1.67	2.07
VCA1073	4.44	1.79	3.37		2.47
VCA1075					0.63
VCA1077	0.23				
VCA1085			0.57	0.46	
VCA1086			0.39	0.54	
VCA1087			0.24	0.27	
VCA1089			0.50	0.52	
VCA1090			0.34	0.46	
VCA1091			0.27	0.30	
VCA1092			0.19	0.27	
VCA1093	0.56		0.34	0.43	
VCA1095	0.62				
VCA1096			0.20	0.28	
VCA1097	0.62		0.22	0.31	0.65
VCA1100			0.42		
VCA1102	1.82		1.67		1.78
VCA1103			0.30		0.23
VCA1104	0.12				
VCA1106	0.58			1.55	0.56
VCA1113			0.64		