THE ROLE OF OXYGEN ON THE *IN VITRO* AND *IN SITU* ECOPHYSIOLOGY OF MARINE *VIBRIO* SPECIES

by

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A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biological Sciences

Charlotte

2018

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ABSTRACT

BRITNEY LEE PHIPPEN. THE ROLE OF OXYGEN ON THE *IN VITRO* AND *IN SITU* ECOPHYSIOLOGY OF MARINE *VIBRIO* SPECIES. (Under the direction of JAMES D. OLIVER)

Hypoxia is an emerging concern in coastal ecosystems worldwide, and the number of areas experiencing hypoxia has increased exponentially in recent years. It is most prevalent in summer months when elevated temperatures lead to increased oxygen consumption by resident biota, reduced oxygen solubility in sea water, and limited ventilation of these waters. In the face of a warming climate, there is uncertainty in how hypoxia will influence estuarine microbiota, especially in regards to opportunistic bacterial pathogens, and their impact on host species. The frequency at which they experience hypoxia, combined with the lack of understanding as to which factors are required for virulence, make *Vibrio* species an ideal model for studying hypoxia. The goals of this dissertation were to explore both the ecophysiology of the opportunistic human pathogen, *V. vulnificus*, and the host-pathogen interactions of *V. coralliilyticus* and the Eastern oyster *Crassostrea virginica.* Utilizing a variety of phenotypic, molecular, and next-generation sequencing techniques these studies have revealed a distinct response to both *in vitro* and *in situ* hypoxia. Furthermore, these studies allowed us to characterize the different responses of clinical and environmental genotypes of *V. vulnificus*, which has revealed important mechanisms for environmental maintenance and virulence. Additionally, these studies allowed us to describe the mechanisms that facilitated the rapid mortality in oysters exposed to hypoxia and *V. coralliilyticus*.

Collectively, this dissertation contributes greatly to our understanding of hypoxia on representative *Vibrio* species, which has implications for both human and ocean health.

DEDICATION

My parents: Michael S. Phippen and Heather L. Hendrickson
My fiancé: William H. Nichols
My Grandmothers: Carol M. Phippen and Mary Sue Parker

This dissertation is specifically dedicated to you.

First and foremost, I would like to thank my parents for their continued support of all my dreams, including the completion of my doctoral degree. I was always told that I could do anything I put my mind to, which has encouraged me to step outside of my comfort zone and question everything, making me the scientist I am today. I could not have gotten through all the tests and milestones without my mom's mantra "I know everything there is to know about _____, it is coming to me effortlessly and easily," which I have recited countless times throughout my academic career. To my dad, always telling me how proud he is and to remember to make time to play my guitar, I appreciate these little reminders. Although I am dedicating, or offering this dissertation as a symbol of love, dedication can also be defined as devoting wholly and earnestly, which is what Billy has done for almost a decade. He has helped guide me through happy, sad, tired, and hard days proving that loving a doctoral student is not the easiest job, but he passed with flying colors. Thank you from the bottom of my heart for all the cups of coffee and encouraging words, I could *not* have done this without you. Finally, I would like to dedicate this to both of my grandmothers, who taught me how to be an empowered yet compassionate woman. Thank you both for always being honest, showing me how special life is, and reminding me to seize every day.

ACKNOWLEDGMENTS

I would first like to acknowledge the immense support I have received the past 6 years from my advisor Dr. James D. Oliver. The passion that Dr. Oliver has for both research and teaching has inspired me to keep reaching for my goals even when I wanted to give up. I would also like to thank my committee members Dr. Adam M. Reitzel, Dr. Rachel T. Noble, Dr. Craig Baker-Austin, and Dr. Jerry M. Troutman for their invaluable advice, time, and encouragement. I also acknowledge the support from both the Department of Biological Sciences and the Graduate School at UNC Charlotte. Specifically, I thank the Thomas L. Reynolds Graduate Student Research Award and the Lucille P. and Edward C. Giles Dissertation Year Fellowship for funding portions of my research and stipend, respectively. Furthermore, I acknowledge my friend, colleague, and role model Dr. Tiffany C. Williams, who contributed both intellectually and emotionally to my scientific journey. I thank you for the courage to push me just a little bit further than I would have gone on my own, and your gracious time in the critical editing of manuscripts and training were priceless. I would also like to thank all the other postdoctoral fellows, graduate and undergraduates that have assisted, inspired, and challenged me throughout my graduate program.

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1.1 *Vibrio vulnificus*

The gram-negative halophilic bacterium, *Vibrio vulnificus*, is the leading cause of seafood-related deaths in the United States (1). Ubiquitous in estuarine and coastal environments worldwide, this bacterium has been isolated from a multitude of sources, including water, sediment, fish and shellfish. Its association with shellfish, primarily oysters, is of particular concern as eating raw or undercooked seafood can cause septicemia carrying a 50% mortality rate (2, 3). Along with septicemia, this bacterium is also responsible for severe wound infections, which may result in amputation of the infected area (4). As global temperatures continue to increase giving rise to new and changing environments, the number of infections caused by *V. vulnificus* could continue to increase, as well as its geographical range.

1.2 Taxonomy

1.2.1 Biotypes

Vibrio vulnificus is an extremely diverse species comprised of three distinct biotypes based on specific host ranges and phenotypic differences (5, 6). All three biotypes can cause disease in a susceptible host either after either ingestion of raw or undercooked seafood (biotype 1), or through an open wound (biotypes 1-3). Human septicemia infections are generally caused by biotype 1 strains, whereas wound infections are known to be caused by all three biotypes, although biotype 2 is rarely associated with human infection (5).

Biochemically we can separate these biotypes using a multitude of phenotypic tests, however the diversity within these biotypes does yield some strains that do not phenotypically match and thus molecular methods are utilized (7). Generally, biotype 1

strains are indole positive, have variable serotypes, and are associated with both septicemia and wound infections (8). Biotype 2 strains primarily infect eels and are indole negative, and have a homogeneous lipopolysaccharide O-antigen (9, 10). Interestingly, biotype 3 strains are a hybrid of biotypes 1 and 2 and possesses phenotypic characteristics of both biotypes, although primarily indole positive with variable serotypes (11). Another interesting feature of biotype 3 strains is that they seem to be geographically isolated to tilapia farms in Israel, whereas biotypes 1 and 2 are globally distributed (8).

As phenotypic and biochemical tests alone are not sufficient for biotype identification of all strains of *V. vulnificus,* there are multiple molecular tools that allow for more precise classification. Multilocus sequence typing (MLST) has proven to be the most common method to distinguish between biotypes, however, additional phylogenetic tools have become popular in more recent years (11-13). A comprehensive study by Bisharat et al. (2005) investigated ten loci, five from each of *V. vulnificus'* two chromosomes in a diverse set of 159 *V. vulnificus* strains. Their results indicated two populations, A and B, with biotype 1 being distributed in both populations and biotype 2 only present in population A. Interestingly, population A was saturated with human disease isolates whereas population B was largely representative of environmental isolates. Even more interesting is the fact that the biotype 3 strains were not associated with either population and further phylogenetic analysis indicated that biotype 3 was a hybrid, containing genes that originated from each population due to recombination. It has been hypothesized that this hybrid biotype arose from the introduction of *Tilapia* spp. from multiple locations around the world (11).

1.2.2 Genotypes

Although all three biotypes have been associated with human pathogenicity, biotype 1 is the major cause of primary septicemia in humans, and for this reason I will focus on those strains for the remainder of this introduction. We can further divide biotype 1 strains of *V. vulnificus* into two disparate genotypes, clinical (C-) and environmental (E-), based on isolation source and molecular markers (14). We can rapidly differentiate between these genotypes by endpoint polymerase chain reaction (PCR), amplifying the "virulence-correlated gene" (*vcg*). with 90% of clinical strains have the *vcgC* allele and 87% of environmental isolates have the *vcgE* allele (6, 12, 15). Furthermore, MLST analyses have shown that biotype 1 strains separate into two distinct lineages, which correlate with this C- and E-genotyping system (12). Although less predictive, we can also group these strains based on 16S rRNA, with 66.7% of C-genotypes correlating with type B 16S rRNA and 80% of E-genotypes correlating with type A (13). Although these genotypes correlate with disease it is not necessarily predictive, as studies have shown that certain E-genotype strains are highly virulent in an iron-overloaded mouse model (16).

1.3 Ecology of Biotype 1 Strains

1.3.1 Distribution of genotypes

Even though there is a large population of individuals that are potentially exposed to *V. vulnificus* through either the ingestion of raw or undercooked seafood or through a wound, there are very few clinical cases each year. Multiple host factors play a role in the pathogenicity of *V. vulnificus*, which is discussed below, but there is still a large proportion of at risk individuals that do not become infected (17). Subsequently, there

have been multiple studies that have surveyed the occurrence of biotype 1 strains in the environment, and with few exceptions the E-genotypes are the dominant genotype found (18-20). In a recent survey off the coast of North Carolina Williams et al (2017) found that 89.7% and 86.2% of strains sampled from water and shellfish were E-genotypes, respectively. This coincides with other studies covering a wide range of locations along the Atlantic and Gulf Coasts of the United States that have shown E-genotypes consistently represent about 85% of total strains in the environment, at least in water, sediment, and shellfish (12, 21-24). Since most (85%) clinical cases are caused by C-genotype strains, it has been hypothesized that the low abundance of this genotype in the environment might be why there are not more human infections.

1.3.2 Salinity and temperature

Numerous studies have shown the importance of salinity and temperature on the distribution and abundance of *V. vulnificus* in the environment (23, 25-29). There is a distinct seasonality that correlates with the decreased temperatures occurring throughout cooler months, as this bacterium prefers ambient water temperatures greater than 10-15˚C (28). Although water temperature is an important predictor of *V. vulnificus* abundance, in some cases being responsible for greater than 50% of variability in detection, it is not the only factor. Salinity is another important driver of its distribution. However, the fact that *V. vulnificus* has a wide optimal salinity range (5-20‰) has given rise to conflicting reports on its impact on abundance (25, 27, 30). As these factors contribute greatly to abundance of *V. vulnificus* and other pathogenic *Vibrio* species, they have been integrated into a real-time risk assessment map for their presence in waters worldwide based on

modeling done by Baker-Austin et al (31). The extent of climate change on shaping these and other abiotic factors influencing their abundance is discussed below.

1.3.3 Viable but nonculturable (VBNC) state

Many studies have focused on understanding the seasonality and/or the apparent "loss" of *V. vulnificus* and other *vibrio* species during months in which estuarine water temperatures fall below 10˚C (32). This led to the description of a dormancy, or viable but nonculturable (VBNC) state, in which cells are no longer culturable on routine media despite retaining viability and potential for causing disease (33). Over 100 bacterial species have been described as entering this state, including both pathogenic and non-pathogenic species (34). Although ATP production remains high, VBNC cells are described as having decreased macromolecule synthesis, respiration rates, nutrient transport, and having a rounded or dwarfed appearance (32, 35). Both *in vitro* and *in situ* studies of *V. vulnificus* during the VBNC state show the continued expression of multiple putative virulence factors, as well as genes encoding capsular polysaccharides (CPS) and stress response regulators (32, 36, 37). Interestingly, studies investigating differential gene expression between C- and E-genotypes have shown that certain factors, such as CPS, were continuously expressed in E-genotypes during VBNC but were undetectable by day 14 in the C-genotype (37). These results further support the hypothesis that these genotypes respond differently to environmental factors, such as decreased temperature, and may shape population dynamics.

Entry into the VBNC state is an extremely effective way for bacteria to survive environmental stressors, but only if they can successfully regain culturability, termed "resuscitation" (32, 38). In *V. vulnificus,* we can resuscitate cells from a temperature-

induced VBNC state by simply placing the cells at room temperature overnight, which has been shown *in vitro, in situ,* and *in vivo* (39). Additionally, Ayrapetyan et al. (2014) have shown that the addition of cell-free supernatant into natural seawater and oysters can resuscitate those bacteria from the VBNC state. They went on to describe that this was due to the ability of cells to respond to extracellular signals, specifically autoinducer 2 (AI-2), which is involved in the interspecific quorum sensing phenomenon (40). These results provided a potential explanation for the mechanism by which cells resuscitate, which has implications for the seasonality of *V. vulnificus* as well as aquaculture and public health.

1.3.4 Biofilms and association with chitin

Having the ability to inhabit dynamic niches in a planktonic or a sessile state is an important factor for the survival of *V. vulnificus* in estuarine environments. These lifestyles are driven by numerous factors, including but not limited to nutrient status, environmental conditions, and chemical signals (41-44). Most microbial attachment involves the formation of complex biofilms, or conglomerations of cells that are encased in an exopolymer matrix, which are key for environmental survival (43, 45). The biofilm lifestyle provides easier access to nutrients, increased propensity for horizontal gene transfer, and protection from grazing and antimicrobial compounds (46). To understand these advantages one must first understand the process of biofilm formation, which follows this general pattern; i) initial attachment to the surface via type IV pili, ii) production of extracellular polysaccharides such as EPS and VPS, iii) maturation, which includes replication and production of more matrix components, and iv) dispersal (regulated by QS and increased motility) (46-48). These steps are all tightly regulated as

they control the production of various polysaccharides, attachment/motility mechanisms, and expression of other machinery within the biofilms (41, 49).

V. vulnificus regularly associates and forms biofilms on chitin, the most abundant polymer in marine ecosystems and a major constituent of marine aggregates (50). Our lab has previously shown an inherent ability for E-genotypes to attach to chitin more efficiently than C-genotypes, suggesting a role for persistence in the environment (51). However, once attached, the C-genotypes show higher resistance to physiologically-relevant stressors than E-genotypes, providing evidence for attachment as a protective state. The up-regulation of genes involved in type IV pilin production (*pilA, pilD* and *mshA*) in E-genotypes was shown to be involved in this phenomenon (51). Additionally, studies have shown that attachment to chitin induces competence in *V. vulnificus* and *V. cholerae*, which contributes to overall fitness enhancement (52, 53). Understanding the dynamics and regulation of biofilm formation, dissolution, and what abiotic factors lead to these in both genotypes will greatly contribute to understanding the ecology of *V. vulnificus.*

1.3.5 Association with *Crassostrea virginica*

Naturally occurring throughout the Atlantic Ocean and Gulf of Mexico, the Eastern Oyster, *C. virginica,* is an important reservoir for *V. vulnificus* (23, 50, 54). Although the concentrations of *V. vulnificus* in water might be quite low, there have been studies showing upwards of 6×10^4 CFUs (colony forming units) of *V. vulnificus* per gram of oyster tissue (55). *V. vulnificus* associates not only with the oyster tissue surfaces but is also distributed throughout the tissue, primarily in the digestive gland. Oysters preferentially eat particles ranging from 5-7 μm in diameter, and smaller particles, like

bacterial cells, quickly pass through the gills and are excreted (56). Thus, to quantify uptake of *V. vulnificus* Froelich et al. utilized marine aggregates in which both genotypes of *V. vulnificus* were incorporated. Interestingly, they found that E-genotypes incorporated significantly more than C-genotypes into marine aggregates and thus were incorporated into oyster tissue significantly more, which may again help to unveil the genotypic disparity we see in the environment (57).

1.4 Human disease and pathogenesis

1.4.1 Clinical manifestations

Biotype 1 strains of *V. vulnificus* are not only responsible for causing primary septicemia, but are also responsible for causing both wound infections and gastroenteritis (1). Septicemia caused by this bacterium carries up to a 75% mortality rate in susceptible hosts, with most of those infections being caused by C-genotypes (21). Cases of septicemia are rarely seen in healthy individuals, whereas those with underlying immune disorders such as HIV/AIDS, liver disease, or diabetes are extremely vulnerable (58). Most often, patients who have septicemia ate raw or undercooked shellfish, primarily oysters, which harbor high loads of this bacterium (59). Wound infections are generally caused by a subset of E-genotypes that we and others have designated as E-wound strains and, although less fatal, still carry a 25-50% mortality and often results in amputation of the infected area (4). Wound infections occur when the bacterium enters a previously acquired wound/sore or upon injury in waters where *V. vulnificus* is abundant (60, 61). The onset of both septicemia and wound infections is quite rapid with deaths occurring as early as 24-hours post infection. Initial symptoms include fever, hypotension, chills, and the appearance of skin lesions. The skin lesions are generally confined to the

subcutaneous regions of the patient's extremities, and the area is extremely swollen and painful (62).

1.4.2 Capsule production

To effectively cause disease, *V. vulnificus* must be able to evade host immune responses, which they do in part by the production of capsular polysaccharides (CPS) (63). Encapsulated cells are better able to withstand opsonization by serum complement and thus are not recognized and phagocytosed by host macrophages (64). *V. vulnificus* has multiple CPS types, of which two operons contain the highly conserved genes *wza, wzb* and *wzc*, which are involved in transport of polymers across the outer membrane of the cell (65-68). Studies investigating mutants in *wzb* in *V. vulnificus,* which lack CPS and are translucent, show decreased ability to survive human serum when compared to the encapsulated wild type strains (69). Furthermore, Williams et al. (2014) found that this was not a genotype-specific phenomenon, as both C- and E-genotypes lacking CPS will rapidly succumb to the bacteriocidal effects of serum. Although CPS is clearly important for survival in host serum, it is not constitutively expressed, as it has been shown to be inhibited during both stationary phase growth and when cells are grown at 37˚C relative to 30˚C (70, 71). Understanding other abiotic factors that contribute to CPS production, such as anoxia/hypoxia, might help us further understand the importance of capsule as a virulence factor in *V. vulnificus*.

1.4.3 Attachment and motility

There have been numerous studies investigating the role of various attachment and motility mechanisms and their role in virulence of *V. vulnificus*. There are a multitude of systems utilized by this bacterium to attach to substrata, such as pili and

flagella, which both play a role in their ability to cause disease (72, 73). Pili are appendages with which *V. vulnificus* can attach to surfaces such as host epithelial cells. Mutants in the type IV pilin genes *pilA* and *pilD,* encoding a pilin protein subunit and prepilin peptidase respectively, are unable to attach to host epithelial cells as effectively as the respective wild-type cells (73-75). Furthermore, these mutants had a higher LD_{50} in mouse infection models, and were also reported to decrease secretion of various cytotoxic molecules (76). Another type IV pilin protein, mannose-sensitive hemagglutinin (MSHA), has been shown to be important in attachment to chitinous surfaces in a variety of *Vibrio* species, including *V. vulnificus*, but its role in pathogenicity is less clear (51, 73).

The flagellum of *V. vulnificus* is important in motility, biofilm formation, lethality, and cytotoxicity. Like other *Vibrio* species, *V. vulnificus* has a single polar flagellum, and there are approximately 60 genes that have been identified to be part of this molecular machine (72, 77). Mutations in a flagellar basal body protein, FlgC, showed significant decreases in their cytotoxicity to HeLa cells, and the LD_{50} in suckling mice was increased over 10,000-fold. Additionally, mutations in *flgE,* which encodes a flagellar hook protein, yielded non-motile cells that both increased LD_{50} and decreased their ability to form biofilms (72, 77). Another interesting mutation was identified in a flagellar biosynthesis protein (FliP), which again was non-motile and attenuated for virulence, but could cause severe skin infections in the murine model but not septicemia (78). These and other studies have shown that without the ability for *V. vulnificus* to adhere to host cells, they cannot successfully cause disease, so this mechanism is extremely important in the virulence of this, and another *Vibrio* species.

1.4.4 Cytotoxicity

To date, there has not been a distinct virulence factor identified in *V. vulnificus*, but rather a plethora of factors that work together to cause cellular damage and cytotoxicity. Although this bacterium produces numerous toxins and exoenzymes, a few that have shown to be important are the cytolysin (VvhA), metalloprotease (VvpE), and the repeats-in-toxin (RTX) RtxA1 (79-84). The purified VvhA cytolysin causes severe cellular damage leading to apoptosis in host cells due to the formation of pores in the host's cell membrane, which leads to extreme vascular permeability. Despite its potency, there has been some speculation on the role of VvhA on the virulence of *V. vulnificus* as mutants do not impact the $LD_{50,}$ and produce the same amount of tissue damage as wild-type cells (79, 85). It has been suggested that rather than being important in pathogenicity, this cytolysin may play a role in environmental survival (79, 86). Similarly, purified VvpE created necrotic lesions as well as vascular permeability leading to edema, but mutational analysis revealed it was not required for virulence (87, 88). Additional studies have also shown that these putative virulence factors were not up-regulated in human serum relative to artificial sea water, which again supports the hypothesis that these may not be as important in virulence (86). Finally, RtxA1, which is homologous to the protein encoded by the *rtxA* toxin gene in *V. cholerae,* can form pores in cellular membranes of host cells (82, 83). Although additional RTX toxins have been identified in *V. vulnificus* they have not shown to be important in virulence (78). However, *rtxA1* mutants are attenuated for cytotoxicity and cannot disrupt tight junctions of host cells. Additionally, the mutant was unable to successfully travel to the liver of a murine model, which suggests an inability to cause septicemia (82, 83, 89). More elegant

mutational studies have supported the role of *rtxA1* in the cytotoxicity of *V. vulnificus* and the accessory roles of other putative virulence factors (76).

1.4.5 Virulence regulation: quorum sensing

QS can be described as a bacterial communication system in which chemical signals (autoinducers) are produced and accumulate extracellularly, followed by their binding to the corresponding membrane bound receptors, which leads to coordinated gene expression responses (90, 91). These systems rely on the sensing of specific autoinducers, which are chemically different for each system. In the genome of *V. vulnificus* there are two out of the three currently described quorum sensing (QS) systems in Gram-negative bacteria present, autoinducer-2 (AI-2) and autoinducer-3 (AI-3) (86, 92). Although the genes for the interspecies QS system AI-3 (*qseBC*) are in the genome, there have been no studies investigating its specific role in virulence of *V. vulnificus.* However, the interspecific AI-2 system, LuxS/LuxR (SmcR), where *luxS* is responsible for the production of the AI-2 molecule and *smcR* is the corresponding transcriptional regulator, shows a clear role in the virulence of this bacterium (93-96).

Specifically, mutants in both *luxS* and *smcR* showed decreased cytotoxicity and increased LD_{50}, however, *luxS* was shown to specifically influence the expression of both *vvhA* and *vvpE* (93, 97). There are conflicting reports on the cytoxicity of QS mutants in this and other *Vibrio* species so there is a need for more in-depth investigations regarding their relationship to virulence. Another important factor is the role that QS has on CPS production, biofilm formation, and motility, as these are all important for virulence as described above. There have been multiple studies showing a negative relationship with QS and biofilm formation in *V. vulnificus,* however, Lee et al. (2013) described the

mechanism in more detail. They showed that CPS was controlled by QS such that when the cells reached a density where QS signaling was activated, the cells turned on the production of CPS, which limited and controlled the size and maturation of the biofilms. When they mutated *smcR,* the resultant colonies were translucent, and formed robust biofilms compared to wild-type cells (98). Additional studies have shown that *smcR* also regulates flagellar motility and biogenesis, which has further implications for both virulence and environmental maintenance (99).

2.1 *Vibrio coralliilyticus*

2.2 Ecology

2.2.1 Identification and characterization

In 1999, seven kilometers east of the main island of Zanzibar on Mawi Island in the Indian Ocean, healthy and diseased corals were removed and brought back to the laboratory and bacteria were isolated (100). Strains were isolated from the mucus of the diseased coral *Pocillopora damicornis* and grown on both marine and Thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Initially, the strains isolated from the diseased corals were identified as Vibrionaceae whereas healthy corals did not yield many from this family (100, 101). As with other *Vibrio* spp. this bacterium was found to be Gram-negative curved rod with a single polar flagellum that grew cream colored colonies. Furthermore, the biochemical tests, Biolog and Api-20 NE, were unsuccessful in its identification although it was said to have been closely related to a variety of *Vibrio* species. Finally, 16S rDNA sequencing revealed the closest relative to be *V. vulnificus* (97%).However, to be the same species they need to be >99% similar and thus it was determined to be a new species, *V. coralliilyticus* (100, 101). Confirmation of this new species was performed by

additional 16S rDNA sequencing, DNA–DNA hybridization data, fluorescent amplified fragment length polymorphism (AFLP), and phenotypic properties. Its closest phylogenetic neighbors were determined to be *V. tubiashii, V. nereis,* and *V. shiloii*, and this led to the reclassification of strains that were once thought to be *V. tubiashii,* which are now known as *V. coralliilyticus* (101).

2.2.2 Distribution and association with hosts

Like other *Vibrio* species, because of its global distribution, *V. coralliilyticus* has been isolated from a variety organisms. It has been isolated from a wide range of coral species from the Atlantic, Pacific, and Indian Oceans as well as the Mediterranean and Red Seas, including *P. damicornis*, *Phyllogorgia dillatata, Mussismilia braziliensis, Mussismilia hispida,* and the octocoral *Pseudopterogorgia Americana* (101-105). Along with corals it has been shown to cause fatal disease in a variety of species including oyster larva, shrimp, rainbow trout, unicellular algae, and can kill flies in the laboratory (102, 106-108). One of the interesting questions surrounding *V. coralliilyticus* is whether or not this is an opportunistic pathogen or a frank pathogen in a variety of species, specifically corals. There is some discrepancy due to the fact that this bacterium is rarely identified in healthy animals, and if this is the case, where might the natural reservoir for *V. coralliilyticus* be?

2.3 Virulence

2.3.1 Temperature dependent virulence

Many studies on *V. coralliilyticus* have shown a clear relationship between temperature and the ability to cause disease in corals and their algal symbionts (109). Specifically, when *V. coralliilyticus* was first identified, it was found to only cause tissue

necrosis at temperatures above 27˚C, whereas the symbiotic algae were killed between 24-26.5˚C and is completely avirulent at temperatures below 24˚C (101). However, isolates from the Great Barrier Reef showed an optimal infectivity between 28-31˚C, which is slightly higher than that for the originally isolated strain (104). This is a huge problem in the face of climate change, especially in areas which the sea surface temperatures are rising at abnormally high rates, which caused two mass coral mortalities in the Caribbean (110). There is an urgent need for understanding how temperature and other abiotic factors regulate virulence in this and other marine pathogens as the infected species in some cases are important ecosystem engineers (111). Although the initial investigations attributed this temperature-dependent virulence to a single virulence factor, the zinc metalloprotease VcpA, there are now studies describing a large virulence program that is regulated by temperature, which are discussed below.

2.3.2 Flagellar motility

For *V. coralliilyticus* to successfully cause disease in corals it must first be able to locate the coral mucous via chemotaxis. Studies utilizing coral mucous as an attractant in capillary assays show that mutants in *flhA,* encoding a motor switch protein*,* are unable to locate and enter the capillaries when compared to the wild-type or complemented strains. Additionally, the same mutant was reduced up to 1,000-fold in its ability to attach to the corals, and no bleaching occurred (112). When grown at 24˚C this bacterium appears to be non-motile, with no lateral flagella detected by electron microscopy, whereas at 27˚C the bacterium is fully motile (113). In addition, at the increased temperature there is a dramatic increase in the expression of a multitude of chemotaxis genes, with approximately 80% being upregulated. These and other studies have supported the

hypothesis that flagellar motility and chemotaxis are important for virulence in *V. coralliilyticus* (106, 112, 113).

2.3.2 Secretion systems

Of the seven described secretion systems in bacteria (T1-T7SS), *V. coralliilyticus* possesses all but T5SS and T7SS, as well as multiple versions of type 4 pilus accessory systems (106). These systems work to bring macromolecules into the cells whereas others have been shown to be important in direct killing of neighboring cells, both prokaryotic and eukaryotic. The general systems, T1 and T2SS, are important for the secretion of various virulence factors, including proteases and hemolysins, and are required by most *Vibrio* species for effective host colonization (114). T6SS is involved in contact-dependent killing, and *V. coralliilyticus* appears to have two clusters that are like *V. vulnificus,* which may be important in direct lysis of both host and symbiont cells in the corals (106, 115). At 27˚C, this bacterium increases the expression of T1SS, T2SS, MSHA and other type 4 pilus mechanisms, many genes involved in both T6SS clusters. Although it is unclear which genes are necessary for the formation of the T6SS machinery, transmission electron micrographs suggest a structure present at the increased temperature that is similar to this system (106, 113). The vast repertoire of secretion systems, and their clear increase in elevated temperatures, provides evidence that they are important in pathogenicity though more investigation is needed.

2.3.3 Proteases

Within the genome of *V. coralliilyticus* there are 17 identified metalloproteases, zinc-proteases, and putative proteases. Of those, nine are described as secreted proteases, whereas the others were designated as membrane bound or cytoplasmic (106, 113).

Genes of the M4 peptidase family, *vcpA*, *vcpB*, and *vchA*, were identified as well as other members of various families homologous to virulence factors in related species. All the M4 peptidase proteases have significant homology with other, previously described virulence factors in *V. cholerae* and *V. tubiashii* (116, 117). Mutants were created of *vcpA,* which has been described as a key virulence factor, but was not significantly different in its ability to cause disease in coral, *Artemia*, *Symbiodinium* Clade C1, and *Drosophila* when compared to the wild-type strain. However, distinct enzymatic and secretomic differences were found in both the mutant and the wild-type strain. The *vcpA* mutant displayed higher hemolytic activity and secreted 18 unique proteins, and the wild-type had increased caseinase, gelatinase and phospholipase activities (106). These results suggested that, with the vast set of proteases and hemolysins present, the mutation in *vcpA* led to an increase in other redundant factors, which is why the mutant was still able to cause disease. As there are so many proteases produced in *V. coralliilyticus*, and the number and type varies from strain to strain, it is hard to determine the exact role of temperature on their regulation. There are some that are increased at temperatures >27˚C but without mutational analyses it will be difficult to determine if these increases are meaningful with regards to virulence (101, 113).

2.3.4 Quorum sensing (QS)

In addition to the QS systems described previously, there are two additional systems present in certain strains of *V. coralliilyticus,* AI-1/LuxMN and CAI-1/CqsAS (113). The former is an intraspecific QS pathway that responds to acyl-homoserine lactones (AHLs) that are produced by the bacterium, and the CAI-1/CqsAS system (Cholera auto-inducer system) is similar to the AI-2 system with its own histidine kinase

receptor and distinct autoinducer (118). The mechanisms for how virulence in *V. coralliilyticus* might be controlled by QS is pure speculation at this point as no previous studies, apart from the work presented in chapter 5 of this dissertation, have investigated this phenomenon. There is, however, evidence that this system may be influenced by increased temperatures on the proteome of two strains of *V. coralliilyticus* as well as increased bioluminescence in the reporter strain assays (113).

3.1 Environmental hypoxia

3.1.1 Eutrophication, dead zones, and oxygen minimum zones

Aquatic hypoxia, defined as < 30% DO saturation (< 2 mg O_2 L^{-1}), is a major environmental stressor in coastal ecosystems worldwide, and the number of areas which experience hypoxia and anoxia has increased exponentially in the past three decades (119-121). Major sources of hypoxia in these systems are weather patterns such as El Niño and La Niña, coastal upwelling, and eutrophication (122-124). Coastal boundary systems (CBS) and semi-enclosed seas (SES) include the Gulf of Mexico and the Baltic Sea, respectively, and are two areas which are likely to experience increased hypoxia due to global climate change projections (125). The Intergovernmental Panel on Climate Change (IPCC) [2014] predicts a 4.37°C increase in the surface waters of the Baltic Sea from 2010-2099, whereas there has been a 0.31°C increase from 1982-2006 in the Gulf of Mexico. This warming, combined with natural and anthropogenic eutrophication, increased microbial activity, and increased thermal stratification will likely exacerbate chronic and seasonal hypoxic events.

Natural and anthropogenic eutrophication has long been recognized as an important factor influencing water quality in estuaries worldwide, and the increased

nutrients in these coastal systems has led to many problems such as algal blooms, fish kills, habitat constraint, microbial community shifts, and oxygen deficiency (124, 126, 127). These areas undergoing such hypoxic events, are termed dead zones, and typically occur in summer when elevated temperatures lead to high rates of oxygen consumption by the resident biota, reduced oxygen solubility in seawater, and stratification of coastal waters. There is no area that is immune to this phenomenon and as global temperatures continue to rise, dead zones have become problematic, with frequencies, durations, and locations expanding annually (122, 124). Along with dead zones, naturally occurring oxygen minimum zones (OMZs) are also expanding, impacting the resident macro/microbiota in these changing environments. The OMZs are expected to expand both at the upper (shallow) boundary and the lower boundary as a result of global climate change, which may impact the host/pathogen dynamics in these areas (128).

3.1.3 Impact on *Vibrio* species

As facultative anaerobes, *Vibrio* species are able to metabolize in both aerobic and anaerobic conditions. Such phenotypic plasticity allows these bacteria to rapidly adapt to changing environments, which is an important factor in the colonization and growth of a wide variety of dynamic niches (129). In the environment, there are many instances in which vibrios will experience hypoxia and anoxia, not only in estuarine waters, but also in mollusks during low tide, the GI tracts of certain marine animals, in the sediment, and even within biofilms (discussed in section 1.3.4). The frequency at which they experience hypoxia, combined with the lack of understanding as to which factors are required for virulence, make *Vibrio* species an ideal model for studying both *in situ* and *in vitro* hypoxia.

There are two perspectives when discussing hypoxia and how that relates to *Vibrio* species, 1) in the context of human health, and 2) how it influences health of marine organisms. The former is highly concerned with the inevitable spread of areas experiencing frequent vibrio infections. Recently, infections caused by such pathogenic *Vibrio* species as *V. vulnificus, V. parahaemolyticus,* and *V. cholerae* are being reported in areas where such infections were never seen before, as far as 65°N, during unseasonably warm heat wave events (130-133). Although the aforementioned increases were not specifically investigated in the context of hypoxia, it has been shown that hypoxia is positively correlated with temperature and is at its peak during summer months worldwide, corresponding to high levels of *Vibrio* spp. in similar environments (134). Although numerous cases of *Vibrio* infections have been reported in coastal areas experiencing frequent hypoxic/anoxic events, or from seafood originating from these areas, its impact on abundance, and virulence potential has been largely overlooked.

The second perspective, the impact on marine organisms, is an area that spans many fields, probably the most dynamic of which are those that include host-pathogen interactions. Many *Vibrio* species are part of the normal flora of marine organisms such as fish, shellfish, and corals, however, many of these can also become pathogenic if the animal is stressed or if the environmental conditions increase their virulence (23, 111, 129). A classic example is that of *V. coralliilyticus* and the coral *Pocillopora damicornis,* in which the bacterium only causes disease when water temperatures rise above 26˚C. This interaction is driven by the bacterium, which shows a significant increase in multiple virulence factors at the increased temperature (100). Other examples such as *V. tapetis* and the clam, *Ruditapes philippinarum,* show a direct correlation between temperature

and disease, however, this interaction is largely due to the immune function of the clams (135). Much work has been done to study the effect of certain abiotic stressors on the virulence of bacteria, however, investigating this in the context of hypoxia and how that can shape host-pathogen interactions is understudied.

3.1.4 Clinical hypoxia

Within the mammalian gastrointestinal tract, rather than being anaerobic, there is a gradient of oxygen present. This has been characterized by He et al. (1999) in the intestines of mice, which showed that from the mid stomach, through the mid duodenum, mid colon/small intestine, and finally the sigmoid colon–rectal junction there is a decrease in oxygen tension (136). There have been multiple studies showing the effect of oxygen on *V. cholerae* within the rat ileal loop model, however, its effect on additional *Vibrio* species is unclear (137-139). Importantly, to cause septicemia, orally ingested *V. vulnificus* must travel through these oxygen gradients, attach to intestinal epithelial cells, and penetrate the bloodstream. The importance of understanding hypoxia *in vitro* should provide insight into the virulence potential of *V. vulnificus*, a pathogen in which no singular virulence factor has been described.

CHAPTER 1

ROLE OF ANAEROBIOSIS IN CAPSULE PRODUCTION AND BIOFILM FORMATION IN *VIBRIO VULNIFICUS*

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Citation

Phippen BL, Oliver JD. 2015. Role of anaerobiosis in capsule production and biofilm formation in *Vibrio vulnificus*. Infection and Immunity **83:**551-559.

Abstract

Vibrio vulnificus, a pervasive human pathogen, can cause potentially fatal septicemia after consumption of undercooked seafood. Biotype 1 strains of *V. vulnificus* are most commonly associated with human infection and are separated into two genotypes, clinical (C) and environmental (E), based on the virulence-correlated gene. For ingestion-based vibriosis to occur, this bacterium must be able to withstand multiple conditions as it traverses the gastrointestinal tract and ultimately gains entry into the bloodstream. One such condition, anoxia, has yet to be extensively researched in *V. vulnificus*. We investigated the effect of oxygen availability on capsular polysaccharide (CPS) production and biofilm formation in this bacterium, both of which are thought to be important for disease progression. We found that lack of oxygen elicits a reduction in both CPS and biofilm formation in both genotypes. This is further supported by the finding that *pilA*, *pilD*, and *mshA* genes, all of which encode type IV pilin proteins that aid in attachment to surfaces, were downregulated during anaerobiosis. Surprisingly, E-genotypes exhibited distinct differences in gene expression levels of capsule and attachment genes compared to C-genotypes, both aerobically and anaerobically. The

importance of understanding these disparities may give insight into the observed differences in environmental occurrence and virulence potential between these two genotypes of *V. vulnificus*.

Introduction

Vibrio vulnificus, an opportunistic human pathogen, is the leading cause of seafood-related deaths in the United States (1). Ubiquitous in estuarine and coastal waters, *V. vulnificus* has been isolated from a multitude of sources, including sediment, oysters, water, shrimp, clams, and fish (2, 3). Consumption of raw or undercooked seafood, particularly oysters, causes rapid septicemia, carrying a 50% mortality rate (1).

V. vulnificus is an extremely diverse species comprised of three distinct biotypes based on specific host ranges and phenotypic differences (4, 5). Although all three biotypes have been associated with human pathogenicity, biotype 1 is the major cause of primary septicemia in humans. Biotype 1 isolates can be further subdivided into two distinct genotypes, clinical (C) and environmental (E) (6). These genotypes can be easily and rapidly determined by utilizing endpoint PCR to amplify the species-specific virulence- correlated gene (*vcg*), specifically targeting polymorphisms within this allele (4). Of *V. vulnificus* strains isolated from clinical cases, 90% have the *vcgC* allele (C-genotype), while 87% of environmental isolates have the *vcgE* (E-genotype) allele (4, 7). Although genotype correlates with virulence, it is not necessarily predictive, as shown by Thiaville et al. They found that 3 of the 9 most virulent strains, when inoculated subcutaneously in iron dextran-treated mice, possessed the *vcgE* allele, which confirms that not only those strains with the *vcgC* allele can cause disease (8).

Multilocus sequence typing of six highly conserved housekeeping genes also supports the division of biotype 1 strains of *V. vulnificus* into two distinct lineages, which correlates with the C- and E-genotyping system (9, 10). Additionally, 16S rRNA typing has shown that C-genotypes correlate highly (66.7%) with B-type 16S rRNA, whereas E-genotypes correlate (80%) with A type 16S rRNA (11, 12). Despite having molecular markers for differentiating between strains associated with disease and those typically considered less virulent, the mechanisms for how *V. vulnificus* survives and colonizes the host still needs more investigation.

The human gut is described as being anaerobic; however, it has been shown that there is in fact a gradient of oxygen present in mammalian hosts (13, 14). He et al. described an oxygen gradient present in the intestines of mice which showed a decrease in oxygen tension from the midstomach, midduodenum, midcolon/small intestine, and finally the sigmoid colon-rectal junction (13). Orally ingested *V. vulnificus* must travel through these oxygen gradients, attach to intestinal epithelial cells, and be able to penetrate the bloodstream for primary sepsis to occur. Although anaerobiosis and subsequent genotypic changes have not been characterized in *V. vulnificus*, there have been multiple studies showing the effect of oxygen on *V. cholerae* (15–18). Various proteins have been differentially expressed in proteomic evaluations of *V. cholerae* incubated with and without oxygen (19). Six proteins highly expressed during anaerobiosis were located on the *Vibrio* pathogenicity island (20), and five of these were involved in intestinal colonization (19). Other notable proteins involved in iron acquisition, metabolism, exopolysaccharide production, and biofilm maturation were enhanced by anaerobiosis (19). Understanding the differences between C- and E-

genotypes in response to anaerobiosis may help elucidate their specific virulence potentials.

Capsule production in *V. vulnificus* is an important virulence determinant and has been shown to significantly enhance pathogenicity (21–23). Encapsulated (opaque colonies on solid media) strains can undergo a reversible phase transition to the nonencapsulated (translucent colony) phenotype (24). Translucency negatively correlates with virulence, as shown by decreased serum resistance, lowered lethality in mice, and susceptibility to phagocytosis (23, 25–27). *V. vulnificus* has given rise to multiple CPS types (28–30), two of which show significant homology with the group I and group IV CPS operon in *Escherichia coli* (31–33). Encoded in these operons are three highly conserved genes, *wza wzb* and *wzc*, involved in transport of polymers across the outer membrane of the cell (31, 33, 34). Environmental conditions have been previously shown to initiate differential regulation of group 1 CPS genes and play a role in phase variation in this bacterium as well as other human pathogens (29, 32, 35, 36). It is important to understand the regulation of CPS genes in *V. vulnificus* during anaerobiosis, a condition it encounters in its natural environment and in the human host (14, 15, 37).

Polysaccharide production has been previously implicated in the ability for multiple *Vibrio* species to form biofilms, i.e., surface-associated bacterial communities enclosed by a polysaccharide matrix (38–43). A correlation between polysaccharide production and biofilm formation has been demonstrated in *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *V. fischeri* under aerobic conditions; however, this relationship has yet to be investigated anaerobically (40, 43–46). The genome of *V. vulnificus* contains multiple highly conserved polysaccharide loci, including those that produce

exopolysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (CPS) (31, 33, 34, 36, 47–49). EPS has been shown to correlate positively with biofilm formation in *V. vulnificus* (50), but previously a negative relationship between CPS and biofilm formation aerobically in this bacterium, as well as other *Vibrio* species, has been described (43, 44, 51). However, the effect of oxygen availability on biofilm formation has not been examined in *V. vulnificus*.

Additionally, type IV pili have been previously shown to contribute to attachment to both abiotic and biotic surfaces, as well as having a role in the initial stages of biofilm formation in other *Vibrio* species (40, 41, 43, 44, 52). Specifically, *pilA*, *pilD*, and *mshA*, encoding a pilin protein subunit, prepilin peptidase, and mannose-sensitive hemagglutinin, respectively, have been shown to be important for biofilm formation as well as attachment to chitinous substrates in *V. vulnificus* (39, 53, 54). Again, the role of oxygen on expression of type IV pili and the subsequent effects on biofilm formation in *V. vulnificus* are poorly understood.

Along with type IV pili, flagella have exhibited a role in the formation of biofilms in multiple human pathogens (40, 55). The role of flagella in *V. cholerae* varies between strains, but lack of functional flagella has been associated with an overall decrease in attachment (40, 42). *V. parahaemolyticus* also exhibits decreased biofilm formation when genes for multiple proteins involved in flagellum production are mutated (43). As with other *Vibrio* species, *V. vulnificus* utilizes a single polar flagellum that aids in motility and attachment. Aerobically, nonflagellated mutants of *V. vulnificus* exhibit an inability to attach to polystyrene and glass wool (56), and we further examined its role in attachment during anaerobiosis.

As a facultative anaerobe, *V. vulnificus* has the capability to metabolize under both aerobic and anaerobic conditions. Such phenotypic plasticity allows *V. vulnificus* to rapidly adapt to changing environments and is an important factor in the colonization and growth in these various niches. The primary goals of this study were to understand the relationship between CPS and biofilm formation in *V. vulnificus* during anaerobiosis and to uncover the molecular underpinnings that are likely responsible during these processes.

Materials and Methods

Bacterial strains and culture conditions.

V. vulnificus strains utilized in this study are listed in Table 1 and were stored at 80°C in Bacto Luria-Bertani broth (LB; BD Difco, NJ) containing 20% glycerol. These strains were chosen because they have been well characterized by genome sequencing and multilocus sequence typing (MLST) analysis, as well as virulence potential analysis based on serum survival (8-11, 48, 49, 57-60). All strains were grown in Bacto heart infusion (HI) broth (BD Difco, NJ) overnight (ON) at 30°C with aeration in a rotary incubator. For type IV pilin mutants, media were supplemented with both 25 µg/ml and 50 µg/ml of streptomycin (S9137; Sigma) and spectinomycin (S4014; Sigma), respectively. ON cultures of all strains were inoculated at a 1:100 ratio into HI broth containing 0.5 mg/liter resazurin (Applied Chemical), an indicator of redox potential. This reversible indicator, which is pink at a redox potential above -51 mV and is colorless at -110 mV, was used to indicate when culture media were aerobic or anaerobic, respectively (61). Cultures were incubated for 24 h at 37°C with and without oxygen. All samples were cultured in glass borosilicate tubes with loose-fitting caps to allow oxygen

to freely enter the tubes as well as efficient removal of oxygen in the anaerobe chamber. Additionally, anaerobic samples were grown in anaerobe chambers utilizing the GasPak EZ anaerobe container system with BBL GasPak anaerobic indicators.

RNA harvesting

Anaerobic cultures were removed using a 3-ml sterile syringe that was prefilled with a 2:1 ratio of RNAprotect (Qiagen) to cell culture, following the manufacturer's protocol. The use of the syringe allowed for rapid uptake of culture into the RNAprotect immediately following the opening of the anaerobe chamber, thereby minimizing exposure to oxygen. Aerobic cultures were removed and RNA protected by using a similar method, omitting the use of the syringe, as the cultures were already freely exposed to oxygen. Cells were stored at -80°C until lysis and extraction of RNA. RNA extraction was performed as described by Williams et al. (54). Briefly, RNA-protected cell pellets were lysed by gently vortexing with 10 mg/ml lysozyme in Tris-EDTA (TE) buffer at pH 8.0 for 30 min. RNA extraction was performed on lysed cells using the RNeasy minikit (Qiagen) following the manufacturer's instructions, with the addition of the optional on-column DNase I treatment. RNA was eluted twice with nuclease-free water, and a final postextraction DNase treatment was performed using the "rigorous" treatment with Turbo DNA-free (Ambion), according to the manufacturer's protocol. The total amount and quality of final RNA were determined using a NanoDrop spectrophotometer (Thermo), and samples having an $A260/A280$ ratio of >1.7 were stored at -80°C.

PCR to detect DNA contamination

RNA was analyzed for DNA contamination by using endpoint PCR to amplify *vvhA*, a species-specific gene target (4). Primers for *vvhA* were used with Promega Go-*Taq* DNA polymerase, 5X Green GoTaq reaction buffer, and 10 mM deoxynucleoside triphosphate (dNTP) mix. An annealing temperature of 53.1°C and 40 cycles of amplification were used, following the manufacturer's instructions. Contamination was indicated by any amplification of the *vvhA* gene, and such samples were not utilized for further experiments.

Primer design

Primers for quantitative reverse transcription-PCR (qRT-PCR) were designed using the NCBI Primer-BLAST software against three sequenced C-genotype strains of *V. vulnificus* (CMCP6, YJ016, and MO6-24) and three sequenced E-genotype strains (JY1701, JY1305, and E64MW). Primers optimized for this study are listed in Table 2. The IDT OligoAnalyzer 3.1 software was used to evaluate primer quality, and specificity was determined using *in silico* PCR. Primer pair efficiency was further estimated by using an *in silico* PCR estimation tool, and those yielding efficiencies of >1.5 were purchased from Sigma-Aldrich. These underwent further validation by utilizing endpoint PCR for the genes of interest in each strain.

Relative qRT-PCR

An optimized qRT-PCR protocol, described by Williams et al. (54), was used in this study with slight modifications. Briefly, 1 µg of total RNA was reverse transcribed with qScript cDNA supermix (Quanta Biosciences) to measure relative expression of target genes. cDNA was then diluted to 50 ng/µl for relative qRT-PCR. PerfeCTa SYBR

green FastMix, low ROX (Quanta Biosciences) was utilized for expression with at least two biological and three technical replicates for each strain. This resulted in at least six threshold cycle (*CT*) values per target gene. To correct for sampling error, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an endogenous control, was used for normalization of target genes. Fold change in expression levels was measured in two ways: (i) in anaerobically grown relative to aerobically grown cells within the same genotype, and (ii) in E-genotype strains relative to C-genotype strains. Fold change in gene expression was calculated using the Pfaffl equation (62), and previously described PCR efficiency analysis was utilized to account for difference in the primer sets (63).

Biofilm quantification

Biofilms were quantified using previously described assays with slight modifications (64). Briefly, all strains were grown as described above and inoculated at a 1:100 ratio into 2 ml autoinducer bioassay (AB) medium supplemented with 1% fumarate as a sole carbon source (65, 66). Borosilicate tubes were used for all biofilm experiments, with cells incubated for 24 h at 37°C under aerobic and anaerobic conditions. After 24 h, supernatants from these tubes were removed, followed by quantification of the CFU/ml. CFU/ml were quantified by serial dilutions, followed by plating of 100 µl of supernatant onto HI plates for both aerobic and anaerobic cultures. The remaining attached biofilm was washed 3 times by the addition of 10 ml of phosphate-buffered saline (PBS) with a serological pipette, subsequent removal, and repeated with fresh PBS. Washed tubes were then stained with 1% crystal violet for 15 min and then washed 3 times as described above with PBS to remove excess stain, and the dye was eluted with 95% ethanol. Eluted crystal violet was quantified by spectrophotometry at 550 nm. Absorbance values were

normalized by dividing the optical density at 550 nm (OD550) for each sample by its corresponding log CFU/ml.

Capsule production

For phenotypic evaluation of *V. vulnificus* CPS variants, all strains were grown in HI broth overnight at 30°C with aeration in a rotary incubator. These cultures were then aseptically streaked on HI plates supplemented with 1.5% agar. Plates were then incubated for 24 h at 37°C under either aerobic or anaerobic conditions and then examined for the presence or absence of capsule by qualitative visualization of colony opacity (22).

Statistical analysis

All data were analyzed by using Prism (version 5.0; GraphPad Software Inc.). Two-way analysis of variance (ANOVA) was utilized for biofilm formation analysis, followed by Sidak's *post hoc* test for multiple comparisons. Gene expression results were analyzed in the following two ways: (i) for anaerobically grown cultures relative to aerobic cultures, and (ii) for E-genotypes (JY1701 and JY1305) relative to C-genotypes (CMCP6 and MO6-24). Significant differences between target transcripts were evaluated using Mann-Whitney (nonparametric) rank-sum tests with adjusted *P* values calculated using the Bonferroni method.

Results and Discussion

Role of anoxia on CPS production

Aerobically, *V. vulnificus* strains grown on solid media had opaque colony morphologies. However, all strains grown for 24 h on solid media without oxygen

resulted only in colonies exhibiting the translucent phenotype, indicating a reduction in CPS biosynthesis. Additionally, all strains remained translucent on solid media with a prolonged anaerobic incubation of up to 72 h. However, when anaerobic plates were removed from the anaerobe jar and exposed to oxygen, the colonies regained opacity after approximately 24 h, suggesting that these colonies regained their ability to produce capsule when oxygen was available. When grown anaerobically for 24 h in liquid culture, the medium was only slightly turbid compared to that of the aerobic cultures. This corresponded to a significant difference ($P < 0.001$) between the OD_{610} of aerobic cultures compared to anaerobic cultures (Table 3), which normally is indicative of less growth. However, we determined that after 24 h under either condition, anaerobic CFU/ml were not significantly different (C-ge- notypes, $P = 0.20$; E-genotypes, $P = 0.23$) than in aerobic cultures when plated on solid media for both genotypes (Table 3). This disparity suggests that although translucency by definition is described by growth on solid media, anaerobically grown cells might in fact be translucent in liquid culture as well. Another possibility is that these cells are smaller, which may also produce similar results when comparing OD_{610} to CFU/ml. This was important, in that it required all normalizations to be based solely on plate counts rather than optical density readings, which did not reflect anaerobic CFU/ml accurately. Further investigation into the biological function of translucency in *V. vulnificus* is needed, as its implication is not clear in response to oxygen availability in the environment or the human host.

To investigate the relationship between anoxia and loss of CPS, we examined expression of several of the genes responsible for capsule production, *wza*, *wzb*, and *wzc*, in *V. vulnificus* under aerobic and anaerobic environments. In *E. coli* as well as *V.*

vulnificus, *wza* is involved in transporting the polymers to the outer membrane of the cell by forming a multimeric structure composed of outer membrane lipoprotein (34, 55, 67). In *E. coli*, the coordinated function of both a cognate phosphatase (*wzb*) and a tyrosine kinase (*wzc*) is also required for the formation of the CPS outside the cell (21, 31, 32, 51), but the specific functions of these genes have not been characterized for *V. vulnificus*. Previous studies have shown that a mutation in any one of these genes results in a translucent phenotype. This results in an accumulation of these polymers inside the periplasmic space, leading to cells lacking external CPS (24).

We found that, relative to aerobically grown cultures, all three genes were significantly downregulated ($P < 0.001$) under anaerobic conditions for *V. vulnificus*, regardless of genotype (Fig. 1). Although both C- and E-genotypes significantly downregulated genes responsible for CPS production anaerobically, E-genotypes exhibited higher expression levels than C-genotypes under both conditions (Fig. 2). Upon entry into the anaerobic environment of the small intestines within the human host, the bacterium must evade a number of physiological threats. Considering the essential role of capsule in pathogenicity, the finding that CPS production was greatly reduced during anaerobiosis is an interesting result. It may be that the presence of capsule is not advantageous at the early stages of colonization in the human host but may be essential for later stages of disease progression, i.e., evasion of phagocytosis in the bloodstream.

Role of CPS in biofilm formation

Studies have shown a negative relationship between CPS and biofilm formation in *V. vulnificus* and that translucent strains form more robust biofilms (51). Thus, we hypothesized that anaerobiosis would lead to an increase in biofilm formation due to the

reduction in CPS exhibited under this condition. To determine whether translucency plays a role in biofilm formation under anaerobiosis, we allowed all strains listed in Table 1 to form biofilms for 24 h under aerobic and anaerobic conditions. While both C- and E-genotypes were able to form biofilms under these conditions, there was a significant ($P < 0.05$) decrease in biofilm formation under anaerobic conditions (Fig. 3). Our findings suggest that the reduction in CPS during anaerobiosis may play a role in the decreased ability of *V. vulnificus* to form biofilms under this condition, differing from what has been shown previously under aerobic conditions (51). Lack of available oxygen may play a larger role in the formation and/or dispersal of these biofilms, which may override any effect the opacity of the cells may have on biofilm formation.

To examine the specific role of CPS in biofilm formation, we utilized a transposon mutant, CVD737, which is unable to synthesize CPS because of a disruption in the *wza* gene (21). *V. vulnificus* C-genotype strain MO6-24 is the parental strain from which CVD737 was derived (21). Aerobically, our results supported previous findings (51), in which the complete absence of CPS was found to significantly ($P < 0.0001$) enhance biofilm formation (Fig. 4). However, we found that during anaerobiosis, both CVD737 and the parent strain formed significantly less biofilm than did cells grown aerobically. Interestingly, there was not a significant difference ($P > 0.05$) between the two strains in their ability to form biofilm anaerobically. Thus, the downregulation of CPS genes anaerobically in the wild-type parent led to a very similar phenotype as in the mutant strain, resulting in similar levels of biofilm formation. This further suggests that decreased capsule production, during anaerobiosis, may not be the only determinant in the ability for *V. vulnificus* to form robust biofilms.

Role of anoxia on surface attachment proteins

Since *V. vulnificus* formed significantly less biofilm anaerobically than under aerobic conditions (Fig. 3), we investigated expression of genes responsible for the production of cell surface proteins implicated in biofilm formation. Anaerobically, expression of both *pilA* and *pilD* was significantly downregulated ($P < 0.0001$) in both C- and E-genotypes relative to expression levels in aerobically grown cultures (Fig. 5). Additionally, mutants lacking type IV pili in *V. vulnificus* have previously shown an inability to form biofilm efficiently (39, 52). We found that, although under both conditions mutants formed significantly less biofilm than the parental strain (Fig. 6), there was still an overall decrease in formation during anaerobiosis. Anaerobically, this decrease was consistent with lower expression of *pilA* and *pilD* in the wild-type strain, which led to poor biofilm formation (Fig. 3).

We also found that lack of oxygen significantly decreased ($P < 0.0001$) expression of *mshA* in both genotypes (Fig. 5). This suggests an inability for both genotypes to make functional type IV pili anaerobically, possibly indicating that they are not required in this environment. Interestingly, during *in vivo* growth of *V. cholerae*, transcriptome analysis revealed that *mshA* was highly expressed compared to levels during *in vitro* growth (16). However, others have shown that MshA is not required for *V. cholerae* to colonize the gastrointestinal tract but that it aids in effective attachment to zooplankton and other biotic surfaces in the environment (54, 68, 69).

We examined *flgE*, a hook protein subunit, as a determinant for flagellum production, since *flgE* mutants do not have a functional flagellum (56, 70). Additionally, *flgE* mutants of *V. vulnificus* have previously shown an inability to form robust biofilms

compared to their wild-type parental strains (35). Since the flagella in *V. vulnificus* are important in initial attachment to a multitude of surfaces (35) and we found that the biofilm is impaired anaerobically (Fig. 3), we hypothesized that genes involved in its production would also be decreased anaerobically. Interestingly, during anaerobiosis, *V. vulnificus* significantly increased ($P < 0.001$) expression of *flgE* (Fig. 5) and was significantly more motile in sloppy agar (data not shown). Rather than aiding in initial biofilm formation, this anaerobic upregulation of *flgE* implies another role for the flagellum, such as chemotaxis, in this environment.

Our lab has previously described that E-genotypes intrinsically express greater amounts of *pilA*, *pilD*, and *mshA* when incubated aerobically in artificial seawater (ASW) than do C-genotypes (54). In that study, the increase in E-genotype expression relative to C-genotype was implicated in the differential ability of *V. vulnificus* to attach to chitin and was suggested to be an important determinant for environmental persistence (54). We found the expression levels of all four genes were significantly higher ($P < 0.01$) in E-genotypes than in C-genotypes, aerobically as well as anaerobically (Fig. 7). Higher expression levels aerobically (Fig. 7A) coincided with an increase in biofilm formation (Fig. 3), and this may indicate an important role for type IV pili in the formation of biofilms under this condition. However, we did not find a similar relationship for cells grown anaerobically. This may be due to the diversity of these two genotypes, and the function of these surface proteins may be more important in the environment, as described for other *Vibrio* species (68, 71). As with our previous expression results, *flgE* expression was also significantly increased ($P < 0.01$) in the E-genotypes (relative to C-genotypes) under both conditions (Fig. 7).

Conclusions

Pseudomonas aeruginosa biofilm dispersal has previously been shown to be induced by lack of available oxygen, which triggers a change in expression of genes involved in sessile versus planktonic lifestyles (72). We propose that when oysters harboring *V. vulnificus* are ingested, they traverse to the gut, which subsequently results in exposure to anaerobiosis. Presumably in an attached state, *V. vulnificus* would respond to the anaerobic conditions and possibly switch to a planktonic lifestyle. Our results support this switch by the downregulation type IV pili and the upregulation of flagella, which is indicative of nonsessile living and why biofilm formation is impaired anaerobically. Although the gut is anaerobic, oxygen is present in various amounts in the tissues surrounding the lumen (14). The upregulation of *flgE* we described under this condition may aid in such an ability of *V. vulnificus* to move toward a more favorable environment where the cells may then attach and subsequently penetrate through the gut lining and into the bloodstream. As we observed a reversion to an encapsulated phenotype after removal from anaerobic conditions, we suggest that once in the oxygenated bloodstream, genes for CPS are upregulated. This encapsulated phenotype can then evade host immune responses, replicate, and cause potentially fatal septicemia, as the ability to transition from opaque to translucent phenotypes and vice versa is an important virulence determinant (21, 23, 24, 73).

Additionally, this bacterium is part of the normal flora in oysters and is frequently exposed to various levels of oxygen during low tide and seasonal drought. This exposure to anaerobiosis in the environment, and the prevalence of E-genotypes in oysters as well as the water column, may result in the differences between C- and E-genotype numbers

of *V. vulnificus* (74). Our lab has previously shown an innate difference in expression levels of type IV pili aerobically (54), and these results further validate those findings as well as expound upon this under conditions lacking oxygen.

There are still many unanswered questions about how *V. vulnificus* is able to successfully cause disease in the human host and why some genotypes correlate more highly with pathogenesis. Responses to environmental parameters encountered in the host are important indicators of virulence potential, and this study reports for the first time the role of anaerobiosis for *V. vulnificus*.

More investigation is needed before we can elucidate if cellular physiology during anaerobiosis has a role in predicting pathogenic potential in both C- and E-genotypes. We are currently studying the molecular mechanisms involved in metabolic regulation, exoenzyme production, and stress response during anaerobiosis. Such studies will help determine if this condition enhances pathogenicity in one genotype over the other. If we can understand the role of oxygen on *V. vulnificus* and uncover its impact on global gene regulation, this could potentially improve current methods for effective prevention, harvesting, and storage of potentially harmful oysters.

Acknowledgements

We kindly thank Anita Wright at the University of Florida (Gainesville, FL) and Rohinee Paranjpye of the NOAA Northwest Fisheries Science Center (Seattle, WA) for providing the CPS mutant and the type IV pilin mutants, respectively. We also thank Tiffany Williams and Mesrop Ayrapetyan for their suggestions in the preparation of the manuscript. These studies were supported by the U.S. Department of Agriculture, award

number 2009-03571. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Agriculture.

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Table 1. *V. vulnificus* strains used in this study

Strain	Isolation Source	Genotype[a]	6S rRNA[f]
CMCP6	Clinical, Blood	C	B
MO6-24[b]	Clinical, Blood	C	B
JY1701	Environment, Oyster	E	A
JY1305	Environment, Oyster	E	A
CVD737[c]	Translucent mutant	C	B
C7184[d]	Clinical, Blood	C	B
C7184ΩA[e]	*pilA* mutant	C	B
C7184ΩD[e]	*pilD* mutant	C	B

[a] Genotypes determined by identification of the virulence-correlated gene (4)

[b] Parental wild-type strain of translucent CPS transposon mutant.

[c] Translucent transposon mutant that can not synthesize CPS (15)

[d] Parental wild-type strain of type IV pilus mutants

[e] Type IV pilin mutants (34, 46)

[f] Typing according to polymorphisms in the 16s rRNA gene (9, 12)

Table 2. Primers designed for qRT-PCR

Gene	Primer target	5'-3' Sequence[a]	Product size (bp)	Strains Amplified
Glyceraldehyde phosphate dehydrogenase	*gapdh*	F: TGAAGGCGGTAACCTAATCG R: TACGTCAACACCGATTGCAT	97	All
Outer membrane lipoprotein	*wza*	F: TTGCCGGTTGTACTGTACCC R: TCTTGGCTGCTATCGTTCAC	79	All
Cognate phosphatase	*wzb*	F: GTAAGCCAGCCGATGCAATG R: GCTACACAGCGCAGAGGTAA	98	All
Tyrosine autokinase	*wzc*	F: GCTGCTTGGCATTCTTTTGGA R: GTTGAAAGTAGCGCAACCGC	93	All
Type IV pilin protein subunit[C]	*pilA*	F: TCATTGGTGTGTTAGCCGCA R: GCTGAGGCAGCTTCTGACTT	73	JY1305 only
Type IV pilin protein subunit[C]	*pilA*	F: TTGCTAAAAGTGAAGCCGCA R: CCAGCTGCACTAGTAGGGTT	169	JY1701 only
Type IV pilin protein subunit[C]	*pilA*	F: GCACAGCTCCAACCAGTAGT R: TTGGCGGCACTTCAACAATG	57	CMCP 6 MO6-24
Type IV pilin protein prepilin[C] peptidase	*pilD*	F: GGCTTACTGGTAGGCAGCTT R: GGTTTCTGTCGGCGGTGATA	126	JY1701 JY1305
Type IV pilin protein prepilin[C] peptidase	*pilD*	F: TTGGCTTACTGGTAGGCAGC R:GGTTTCTGTCGGTGGTGTGA	128	CMCP 6 MO6-24
Mannitol-sensitive hemagglutinin	*mshA*	F: CAAGGCGGTTTCACCCTGAT R:CAGATTTAGAAAACGCGGAGCC	90	All
Flagellar monomeric hook subunit	flgE	F: TCTAACTGAGCTGCGGACAA R: TACTGCTCAATCTGGCTGGC	145	All

[a] F: Forward primer target, R: Reverse primer target

[b] Products amplified between sequenced strains *in silico*

[C] Primers designed for this gene were different between strains/genotypes

Table 3. Quantification of *V. vulnificus* cells in liquid culture after 24 hour incubation in differential conditions

Genotype[a]	Condition	OD_{610}[b]	Log CFUs/ml[b]
C	Aerobic	0.40±0.02*	8.48±0.08
C	Anaerobic	0.14±0.02*	8.05±0.17
E	Aerobic	0.491±0.02*	8.91±0.16
E	Anaerobic	0.193±0.01*	8.50±0.12

[a] C-genotypes; CMCP6 and MO6-42, E-genotypes; JY1701 and JY1305

[b] Mean ± standard error

* Asterisks indicate a significant ($p<0.05$) difference (Mann-Whitney nonparametric rank-sum tests) between OD_{610} of aerobic compared to anaerobically grown cultures within the same genotype.

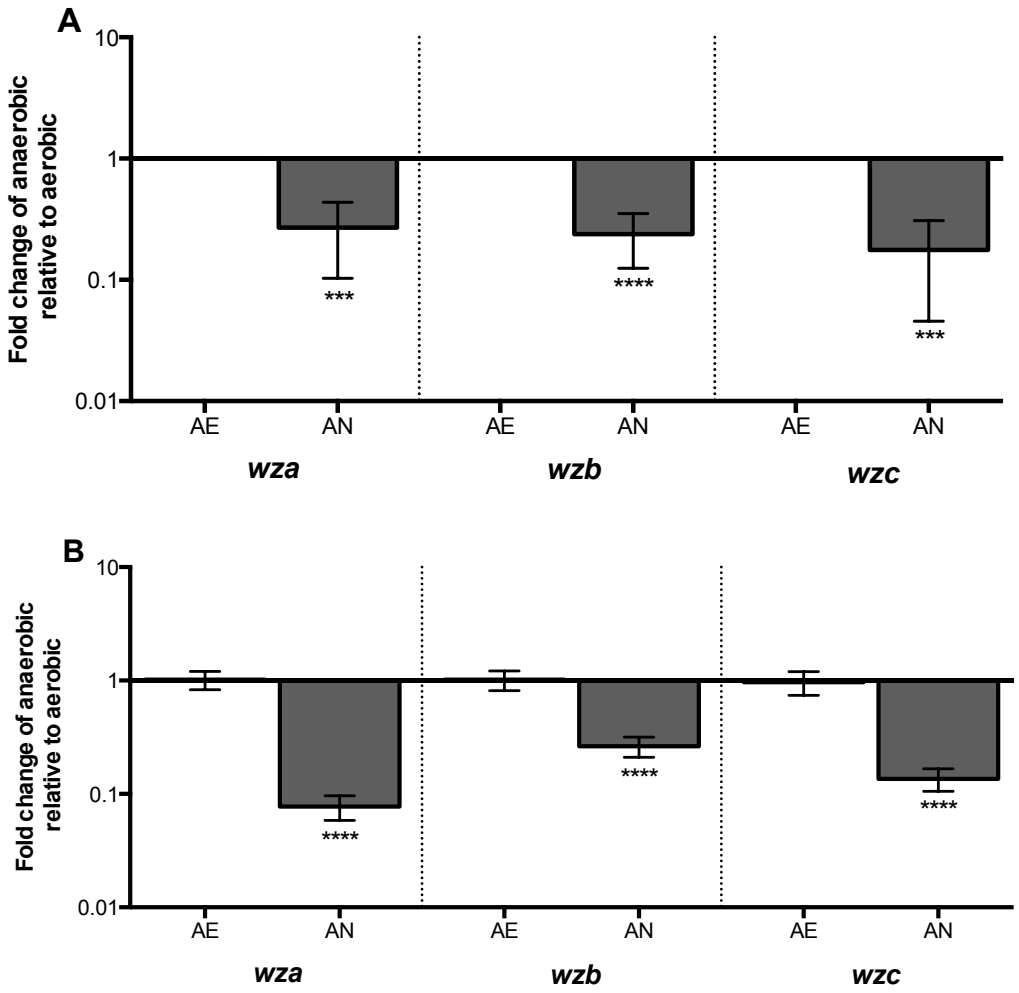

Figure 1. Effect of anaerobiosis on capsular polysaccharide (CPS) gene expression in both C-genotypes (A; CMCP6 and MO6-24) and E-genotypes (B; JY1701 and JY1305) of *V.vulnificus.* Each graph represents expression levels of genes involved in CPS production (*wza-wzb-wzc)* anaerobically relative to aerobic. Error bars represent the standard error of five biological replicates, comprising of three technical replicates averaged for each strain (two of each genotype). Asterisks represent statistically significant differences between aerobic and anaerobic expression of each gene transcript using non-parametric Mann-Whitney test with corrected p-value utilizing Bonferonni calculation. Both C-(A) and E-genotypes (B) significantly (*** correspond to $p<0.001$, and **** to $p<0.0001$) down-regulate *wza, wzb* and *wzb* in response to anaerobiosis.

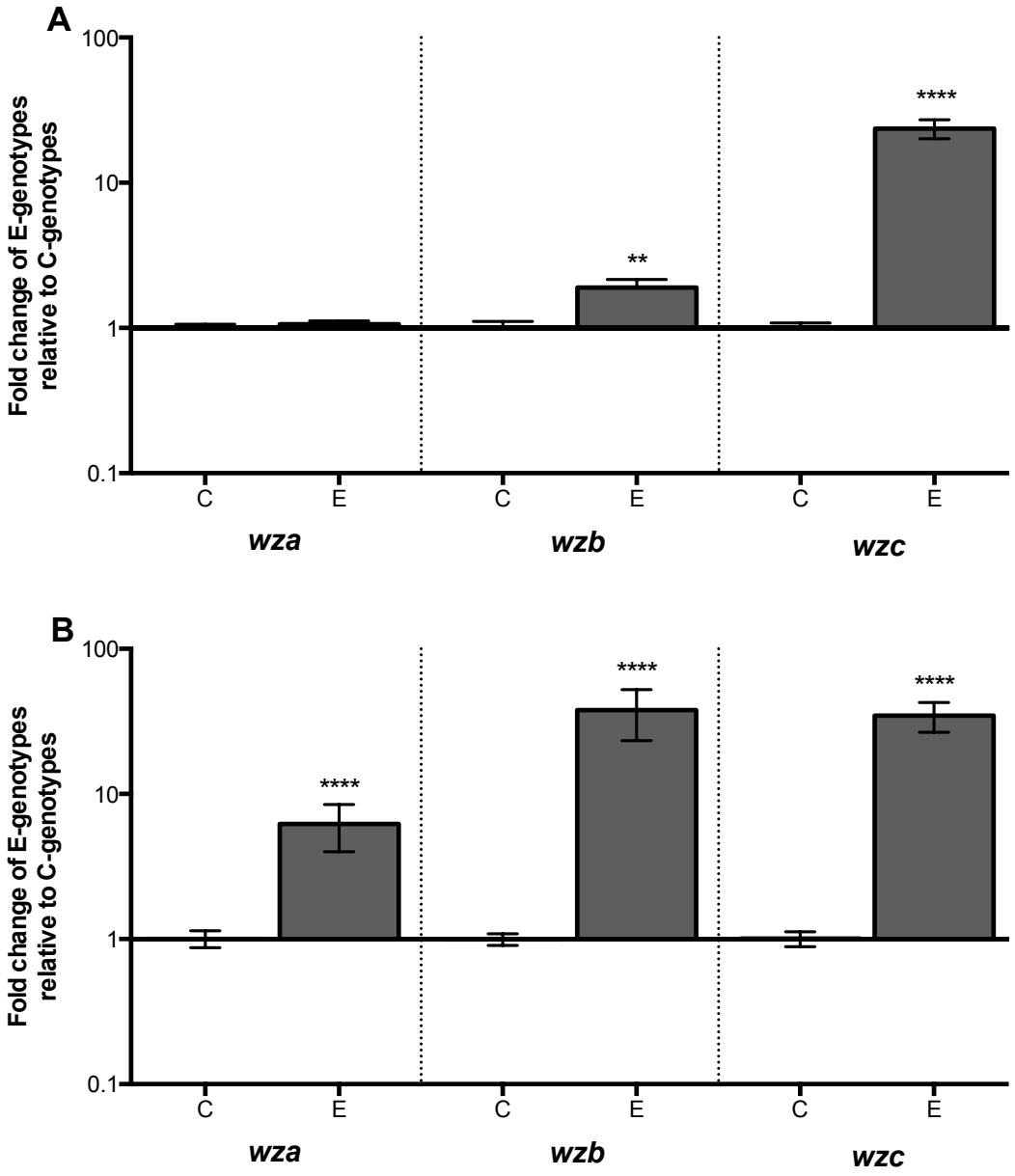

Figure 2. Expression of genes involved in capsular polysaccharide (CPS) production by two E-genotypes (E; JY1701 and JY1305) relative to two C-genotypes (C; CMCP6 and MO6-24) of *V. vulnificus* in aerobic (A) and anaerobic (B) conditions. Error bars represent the standard error of five biological replicates, comprising of three technical replicates averaged for each strain (two of each genotype). Asterisks represent statistically significant differences (**correspond to $p<0.01$, and **** to $p<0.0001$) between E-genotypes relative to C-genotypes of each gene transcript using non-parametric Mann-Whitney test with corrected p-value utilizing Bonferonni calculation. Anaerobically, E-genotypes express significantly more *wza-wzb-wzc* than C-genotypes, compared to only *wzb* and *wzc* aerobically.

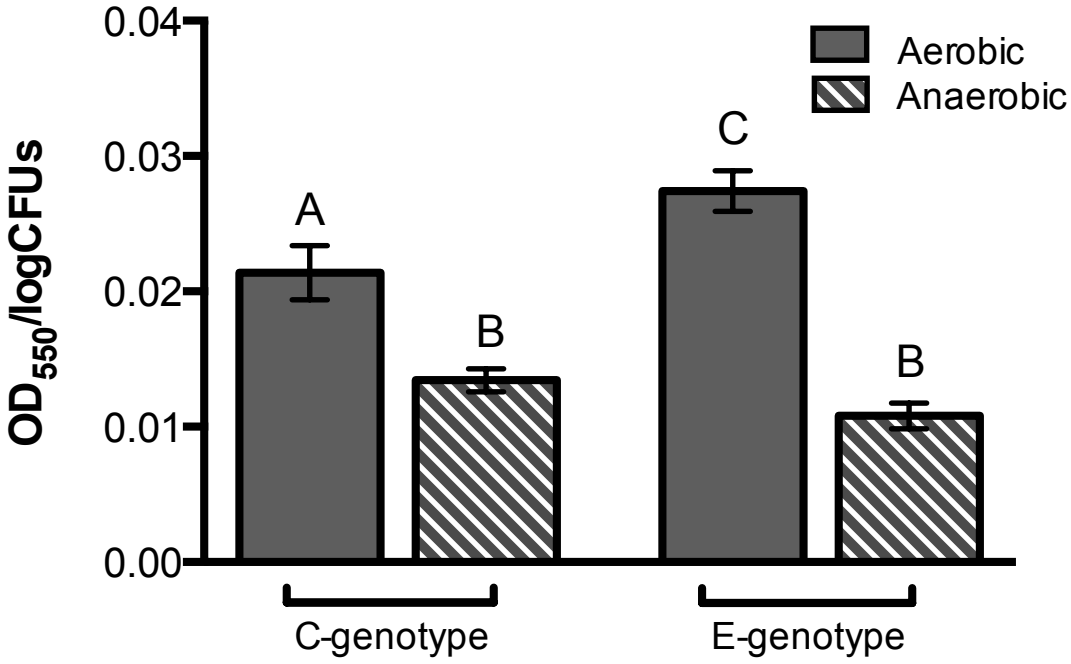

Figure 3. Biofilm formation of clinical (CMCP6 and MO6-24) and environmental (JY1701 and JY1305) genotypes of *V. vulnificus* after 24 hour incubation in both aerobic (grey bars) and anaerobic (checkered bars) conditions. Different letters indicate significance ($p<0.001$) between genotypes and culture conditions (N=26). Two-way ANOVA showed that oxygen has a role in limiting biofilm formation in both C and E-genotypes, and that E-genotypes form significantly more biofilm aerobically than C-genotypes.

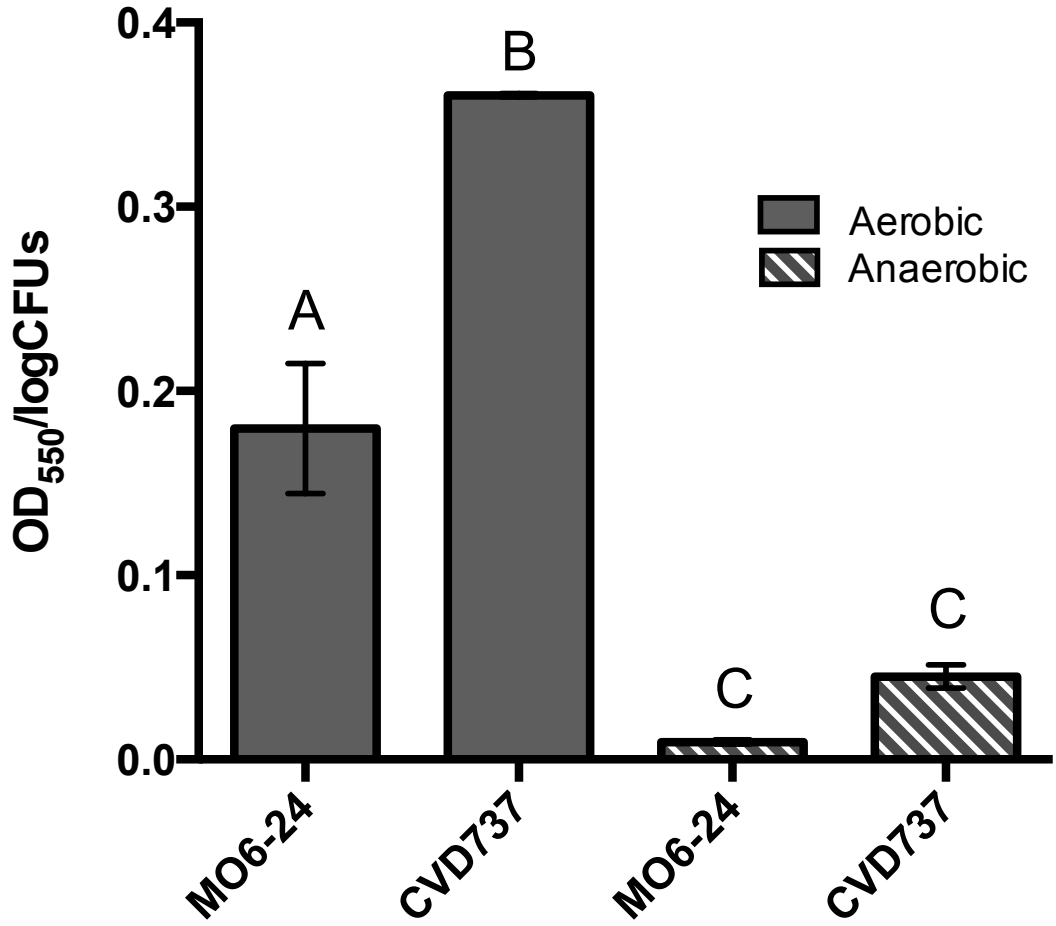

Figure 4. Biofilm formation of a transposon mutant of *V. vulnificus* (CVD737) lacking functional CPS biosynthesis compared to the wild-type parental strain (MO6-24). Quantification of biofilm after 24 hour incubation in both aerobic (grey bars) and anaerobic (slashed bars) conditions was used for all replicates. Different letters indicate significance between the parent and mutant strain when incubated in aerobic and anaerobic conditions (N=18). Two-way ANOVA showed that capsule has a negative role in aerobic biofilm formation, but no significant role anaerobically.

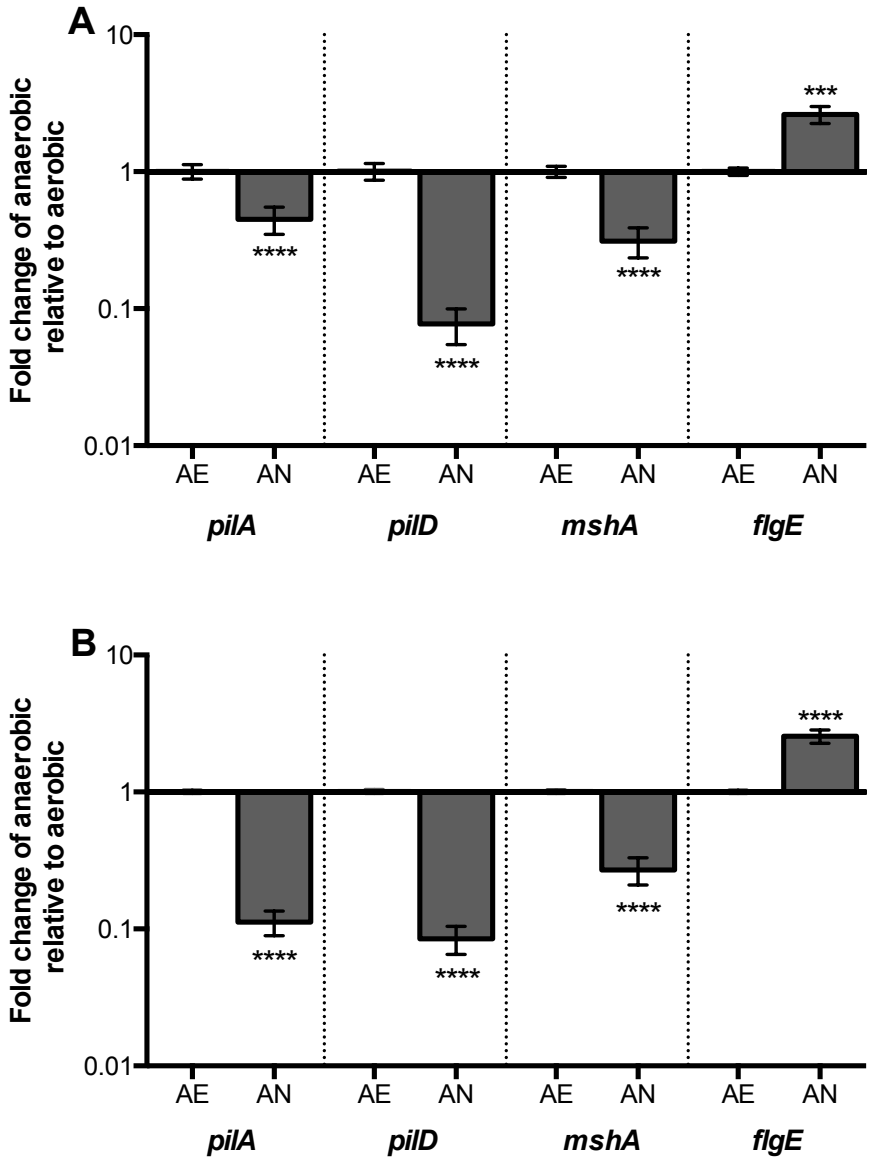

Figure 5. Effect of anoxia on expression of multiple surface attachment genes involved in biofilm formation in *V. vulnificus* C-(A; CMCP6 and MO6-24), and E-genotypes (B; JY1701 and JY1305). Error bars represent standard error of five (two strains in each group) biological replicates with three technical replicates. Asterisks represent a significant decrease in anaerobic (AN) expression of *pilA, pilD* and *mshA* (type IV pili) when compared to aerobic (AE) expression in both genotypes, and a significant increase in *flgE* (flagellar hook protein). Asterisks represent statistically significant differences between aerobic and anaerobic expression of each gene transcript using non-parametric Mann-Whitney test with corrected p-value utilizing Bonferonni calculation. Both C-(A) and E-genotypes (B) significantly (*** correspond to $p<0.001$, and **** to $p<0.0001$) effect of anaerobiosis on type IV pili and flagella expression in both genotypes.

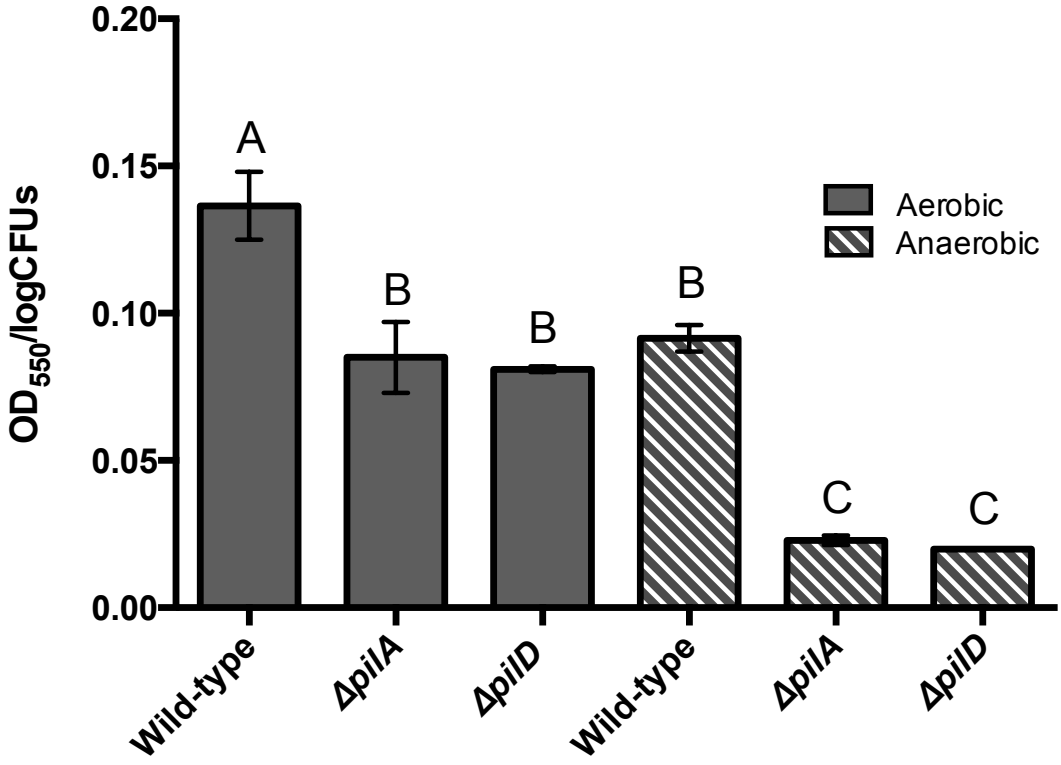

Figure 6. Biofilm formation of a type IV pilin A (pilA) (C7184ΩA) mutant and prepilin peptidase (*pilD*) (C7184ΩD) mutant of *V. vulnificus* compared to parental wild-type strain (C7184). Quantification of biofilm after 24 hour incubation in both aerobic (grey bars) and anaerobic (slashed bars) conditions was used for all replicates (n=12). Different letters represent statistical significance ($p<0.001$) between mutant and wild-type in both conditions. Two-way ANOVA showed that both the wild-type and the mutant strains produced significantly less biofilm anaerobically.

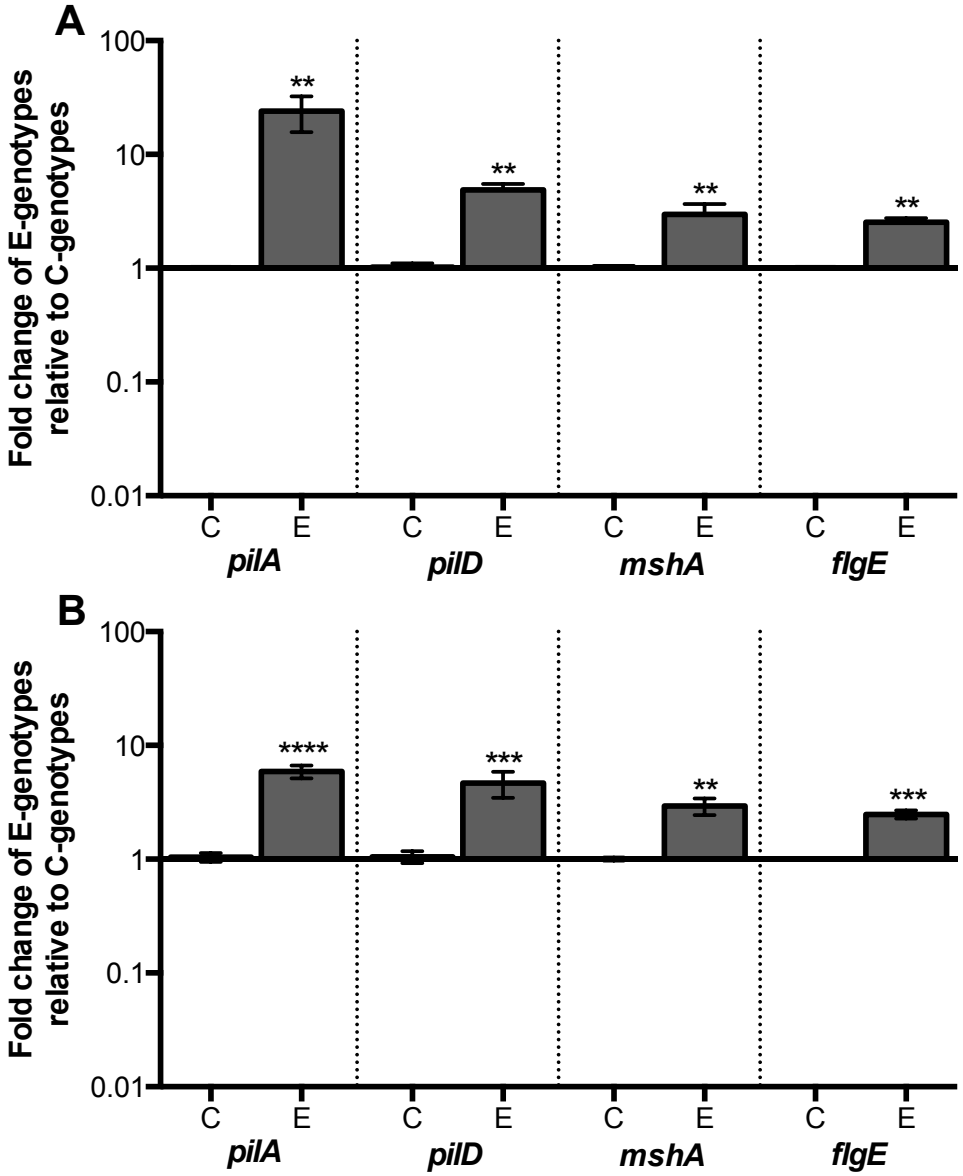

Figure 7**.** Expression of genes involved in biofilm production by two E-genotypes (E; JY1701 and JY1305) relative to two C-genotypes (C; CMCP6 and MO6-24) of *V. vulnificus* in aerobic (A) and anaerobic (B) conditions. E-genotypes expressed significantly more type IV pili (*pilA, pilD and mshA*) as well as flagella hook protein (*flgE*) in both aerobic and anaerobic conditions. Error bars represent the standard error of five biological replicates, comprising of three technical replicates averaged for each strain (two of each genotype). Asterisks represent statistically significant differences (**correspond to $p<0.01$, *** to $p<0.001$ and **** to $p<0.0001$) between E-genotypes relative to C-genotypes of each gene transcript using non-parametric Mann-Whitney test with corrected p-value utilizing Bonferonni calculation.

CHAPTER 2

CLINICAL AND ENVIRONMENTAL GENOTYPES OF *VIBRIO VULNIFICUS* DISPLAY DISTINCT, QUORUM-SENSING-MEDIATED, CHITIN DETACHMENT DYNAMICS

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Citation

Phippen BL, Oliver JD. 2015. Clinical and environmental genotypes of *Vibrio vulnificus* display distinct, quorum-sensing-mediated, chitin detachment dynamics. Pathog. Dis. **73:**ftv072.

Abstract

The ability for bacteria to attach to and detach from various substrata is important for colonization, survival, and transitioning to new environments. An opportunistic human pathogen, *Vibrio vulnificus* can cause potentially fatal septicemia after ingestion of undercooked seafood. Based on genetic polymorphisms, strains of this species are subtyped into clinical (C) and environmental (E) genotypes. *V. vulnificus* readily associates with chitin, thus we investigated chitin detachment dynamics in these disparate genotypes. We found that C-genotypes detach significantly more than E-genotypes after 24 hours in aerobic as well as anaerobic conditions. Furthermore, expression of genes involved in type IV pilin production was significantly down-regulated in C-genotypes compared to E-genotypes, suggesting an importance in detachment. Interestingly, *gbpA*, a gene that has been shown to be important in host colonization in *V. cholerae*, was up-regulated in the C-genotypes during detachment. Additionally, we found that C-genotypes detached to a greater extent, and produced more quorum sensing (QS)

autoinducer-2 molecules relative to E-genotypes, which suggests a role for QS in detachment. These findings suggest that for *V. vulnificus,* QS mediated detachment may be a potential mechanism for transitioning into a human host for C-genotypes, while facilitating E-genotype maintenance in the estuarine environment.

Introduction

Abundant in estuarine environments, *Vibrio vulnificus* is a highly effective opportunistic human pathogen (1). This bacterium is isolated from a multitude of sources in the marine environment, however its association with shellfish is of particular concern (2-5). Consumption of raw or undercooked shellfish, especially oysters, can cause fatal septicemia that carries a 50% mortality rate. As a result of this high mortality rate, this bacterium is responsible for 95% of all seafood related deaths in the United States, and infections are currently on the rise (6). Along with septicemia, this bacterium is also responsible for potentially fatal necrotizing wound infections (1, 7, 8).

Presently, *V. vulnificus* can be separated into three distinct biotypes, each disparate in host range and phenotypic markers (9-13). Biotype 2 strains are most frequently associated with infections in eels, whereas biotype 3 strains are geographically isolated to tilapia farms in Israel, and have been described as being a hybrid of biotype 1 following the acquisition of genes from other species (13-15). Although all three biotypes can cause human infection, biotype 1 strains are those most associated with fatal septicemia (16). These strains are further differentiated by nucleotide polymorphisms in the virulence-correlated gene (*vcg)* yielding two distinct genotypes (9, 17, 18). Clinical (C) genotypes carry the *vcgC* allele*,* whereas environmental (E) genotypes have the *vcgE*

allele. In addition, although both genotypes are capable of causing disease, they highly correlate with isolation source, suggesting that the C-genotypes may be more effective at host colonization (18). Furthermore, C-genotypes have been shown to survive and grow in human serum, while its bactericidal effects rapidly kill E-genotypes (19). Elucidating what specific factors contribute to the differential ability to cause disease between genotypes is vital for prevention strategies.

Ubiquitous in marine and estuarine environments, *Vibrio* spp. regularly associate with chitinous substrates (20-23). Chitin is the most abundant polymer in these ecosystems and a major constituent of marine aggregates (24, 25). Association with chitin has been previously shown to be an important environmental reservoir for persistence, protection from stress, predatory protection, fostering horizontal gene transfer, and serving as a nutritional source for a multitude of *Vibrio* spp. (21, 22, 26-30). Our lab has previously shown an inherent ability for E-genotypes to attach to chitin more efficiently than C-genotypes, suggesting a role for persistence in the environment (23). The up-regulation of genes involved in type IV pilin production (*pilA, pilD* and *mshA)* in E-genotypes was shown to be involved in this phenomenon (23). However, once attached, the C-genotypes show higher resistance to physiologically-relevant stressors than E-genotypes, providing evidence for attachment as a protective state (23). Additionally, studies have shown that attachment to chitin induces competence in *V. vulnificus* and *V. cholerae*, which contributes to overall fitness enhancement (20, 21, 29, 30). A specific goal of the present study was to examine the detachment differences between both C and E-genotypes of *V. vulnificus* when attached to chitin, as detachment is not frequently studied and is likely important in the ability of cells to initiate infection.

Most microbial attachment involves the formation of complex biofilms, or conglomerations of cells that are encased in an exopolymer matrix (31). Previously shown to be triggered by various stimuli, detachment is the last step in the complex biofilm process (31-36). While this phenomenon has been examined in other bacteria, detachment is not well understood in *Vibrio* spp.. There are a myriad of inducers involved in the dispersal of cells from these biofilms such as cell cycle control, nitric oxide production, oxygen concentrations, iron availability, nutrient increases, and various signaling molecules (32, 37-43). As *V. vulnificus* is exposed to varying conditions from the estuary to the human host, better understanding of this complex process is necessary.

Various quorum-sensing (QS) systems have been described in *Vibrio* spp., and are defined as gene regulatory cascades utilizing various signaling molecules for cell-to-cell communication (44-48). Interestingly, certain species have numerous parallel converging systems, which coordinate both inter/intraspecific behaviors, including the ability to form and/or disperse from biofilms. There are two primary QS systems that rely on the concentration of either N-acylhomoserine lactone (AHL) or autoinducer-2 (AI-2) molecules to activate or inhibit the phosphorelay activity of their corresponding activators. The *V. cholerae* AI-2 system, an interspecific QS signaling pathway, has been shown to aid in dispersal by a decrease in exopolysaccharide (EPS) production (36, 43). This system specifically relies on the production of the CAI-1 molecule, which interacts with LuxO, the AI-2 system activator (36). *V. vulnificus* does not posses an AHL pathway, however, a homologous AI-2 pathway has been shown to limit the size of mature biofilms in *V. vulnificus* by the up-regulation of capsular polysaccharide (CPS) genes (49), through the production of AI-2. This tight regulation of biofilm formation

with QS has been proposed to be integral in niche expansion and persistence in the environment, as well as in host colonization. As QS is a key factor in the regulation of *V. vulnificus* biofilms, our study also investigated the relationship between interspecies cell signaling and dispersal.

Several nutritional stressors have also been shown to lead to increased dispersal rate in multiple bacterial species (35, 50-52). Notably, growth in oxygen-limited environments in *Pseudomonas aeruginosa* leads to detachment from biofilms. This detachment is attributed to the regulation of motility, production of EPS and rhamnolipids (glycolipidic biosurfactants) via cyclic-di-GMP signaling (34). We have previously shown that oxygen limitation plays a negative role in the ability for both C/E-genotypes of *V. vulnificus* to form biofilms, and thus may also contribute to subsequent detachment of this species (53). Here we have elucidated the role that anaerobiosis plays on the ability for *V. vulnificus* to detach from chitinous substrates, and the underlying mechanisms responsible for this dispersal.

Materials and Methods

Bacterial strains and culture conditions

V. vulnificus strains utilized in this study are described in Table 1, and were chosen as previously characterized representative strains of both C and E-genotypes. Strains were stored at -80°C in Bacto Luria-Bertani broth (LB) (BD Difco, NJ) with 20% glycerol. All strains were grown overnight (ON) in Bacto heart infusion (HI) broth (BD Difco, NJ), with the exception of BB170, which was grown in autoinducer (AI) broth (54). All strains were grown at 30°C with aeration in a rotary incubator. For JDO1, a *V.*

vulnificus luxS mutant, the medium was supplemented with 2µg/ml chloramphenicol (55). For anaerobic culturing, all strains were incubated in anaerobe chambers utilizing the GasPak™ EZ Anaerobe Container System with BBL™ GasPak Anaerobic indicators.

Chitin Detachment

A graphical representation of the chitin detachment assay is shown in Figure 1. More specifically, ON strains of *V. vulnificus* were centrifuged for 10 min at 5,000xg, HI was removed, and the cells resuspended in 15‰ artificial sea water (ASW) with no additional carbon source. The washing process was repeated three times until all residual nutrients were removed. Washed cultures were then serially diluted to a final concentration of $5x10^7$cells/ml in preparation for the detachment assay. A chitin magnetic bead (New England BioLabs) assay was utilized for initial attachment at 20°C for 1 hour on a rotisserie at 8 rpm as previously described by Williams et al. (23). For subsequent detachment, the attached cells were separated from the unattached cells utilizing a 1.5-ml microcentrifuge magnetic stand (Life Technologies). The cells were then washed 3x with 15‰ ASW and resuspended in 1 ml 15‰ ASW. The attached cells were then incubated for 24 hours in both aerobic and anaerobic conditions and the detached portion was quantified as described below.

Initial attachment was quantified as previously described by Williams et al. (23) with slight modifications. Briefly, vigorous bead bashing was performed on representative cultures from each replicate after being washed 3x with 15‰ ASW. The washed chitin beads were resuspended in 1 ml 15‰ ASW and 0.2 g of 0.5-mm ZR Bashing-Beads was added. These cultures were then vortexed at high speed for 1 minute to detach any bacteria that were previously attached to the chitin beads. A magnetic stand

was utilized to separate the supernatant which contained the previously attached bacteria. Serial dilutions of the supernatant were plated onto HI agar and incubated ON at 30°C, with the quantified bacteria referred to as the initial input (23). The additional tubes from each attachment experiment were washed as described above, resuspended in 1 ml 15‰ ASW and incubated for 24 hours in aerobic and anaerobic conditions. Separation and quantification of the chitin beads from the supernatant was performed as stated above, with the resultant cells referred to as the final output. The attached portion was then washed and bead-bashed as described above, and quantified to account for potential growth on the chitin beads.

Quorum Sensing Reporter Assay

QS activity was quantified utilizing the *V. harveyi* reporter assay described by Bassler et al. (56). *V. harveyi* strain BB170, which is bioluminescent in the presence of AI-2 molecules (56), was used as an indicator of AI-2 production in *V. vulnificus.* Specifically, ON cultures of *V. vulnificus* were grown as described above, and were re-inoculated to a concentration of 5 x 10^7cells/ml in 15‰ ASW with 100µl chitin magnetic beads. Cell-free supernatants (CFS) were collected at 24 hours from both aerobic and anaerobic conditions (illustrated by * in Figure 1) by filter sterilization through 0.22-µm syringe-driven filter units (Millipore, Tullagreen, Ireland) after magnetic stand separation and stored at -80°C until used. For detection of AI-2, BB170 was grown ON at 30°C on AI plates containing 1.5% agar. Colonies were removed by a sterile cotton tipped applicator and transferred into 10ml AI broth and centrifuged for 10 minutes at 5,000xg. Supernatants were removed and cells resuspended in fresh AI broth. This resuspension was diluted to an OD_{610} of 0.7 to 0.8 then incubated with shaking at 30°C until the OD_{610}

reached 1.1. A 1:500 dilution of the *V. harveyi* culture was created, which was referred to as the "working solution". A 1:10 ratio of CFS to working solution, with a final volume of 1.5ml, was created and incubated for 4 hours at 30°C with shaking. Bioluminescence was measured in a GloMax 96 microplate luminometer (Promega, Madison, WI) with 200µl of sample in each well. All replicates were performed in triplicate. Control samples containing 15‰ ASW and the working solution of BB170 at a 1:10 (vol/vol) ratio, for a total of 1.5 ml, were used to control for background bioluminescence by BB170. Additionally, all bioluminescence values were normalized to cell density of each replicate to control for differential growth between strains and conditions.

RNA harvesting

RNA was harvested after 24 hour incubation of the cells in 15‰ ASW at 20°C in both aerobic and anaerobic conditions (illustrated by * in Fig. 1). As previously described (53), both aerobic and anaerobic RNA samples were protected with a 2:1 ratio of RNAprotect (Qiagen) to cell culture, following the manufacturer's protocol. A previously optimized RNA extraction assay was performed as described by Williams et al. (23). Using a NanoDrop spectrophotometer (Thermo), RNA quality and quantity was examined and those samples having a 260/280 nm ratio of ≥1.7 and a concentration of ≥75ng/µl were stored at -80°C. Assessment of DNA contamination was performed as previously described (23, 53), utilizing a species-specific gene target, *vvhA*, to identify DNA contamination by end-point PCR amplification. Any amplification of the *vvhA* gene indicated DNA contamination, and such samples were not used for subsequent experiments.

Primer design

Primers used for this study are listed in Supplemental Table 1. Three sequenced C-genotype strains of *V. vulnificus* (CMCP6, YJ016, and MO6-24) and three sequenced E-genotype strains (JY1701, JY1305, and E64MW) were used as reference strains for qRT-PCR primer design, utilizing the NCBI Primer-BLAST software (23). Primer quality was assessed as previously described (23, 53) utilizing both the IDT OligoAnalyzer 3.1 software, and *in silico* PCR for specificity. Efficiencies of each primer pair were additionally predicted by using an *in silico* PCR estimation tool and those yielding efficiencies of ≥1.5 were used in this study. Primers underwent further validation utilizing end-point PCR for the genes of interest in each strain, and were purchased from Sigma Aldrich (23).

Relative qRT-PCR

Slight modifications to previously described relative quantitative reverse transcription-PCR (qRT-PCR) protocols were utilized for all expression studies (23, 53). Relative expression of target genes in this study was performed using 50ng/µl cDNA per reaction following the reverse transcription of 1µg of total RNA utilizing qScript cDNA Super Mix (Quanta Biosciences). Expression of three biological and three technical replicates for two strains of each genotype, yielding 27 threshold cycles (C_T) per target gene, was examined using PerfeCTa SYBR green FastMix, Low ROX (Quanta Biosciences). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous control, was used for normalization of target genes in order to correct for sampling error. Calculations of fold change in gene expression used the Pfaffl equation (57), and to

account for difference in the primer sets a previously described PCR efficiency analysis was used (58).

Addition of synthetic AI-2 to *V. vulnificus luxS* mutant

The AI-2 precursor, (s)-4,5-dihydroxy-2,3-pentanedione (DPD) (OMM Scientific, Dallas, TX), was added to some experiments involving the luxS mutant, JDO1, to mimic the wild-type phenotype of the parent strain (C7184). AI-2 is able to freely form in a liquid suspension containing 10-fold molar excess of boric acid and DPD, yielding a 50μM DPD stock solution (59). To each experimental system, a final volume of 250nM DPD per replicate was added, and performed as described above.

Statistical analysis

Unpaired Student's *t* tests or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons were utilized for detachment and bioluminescence analysis. Gene expression results were analyzed comparing i) E-genotypes (JY1701 and JY1305) relative to C-genotypes (CMCP6 and MO6-24) and ii) Δ*luxS* (JDO1) or *ΔluxS* + AI-2 relative to parent strain C7184. Mann-Whitney (nonparametric) rank-sum tests were used to determine significance between target transcripts with adjusted p-values calculated using the Bonferonni method. All data were analyzed using GraphPad Prism (version 5.0; GraphPad Software Inc.).

Results and Discussion

Aerobic Detachment Dynamics

As *V. vulnificus* strains have been shown to be genotypically diverse (1, 17, 18, 52), we utilized multiple strains of well-characterized C and E-genotypes to reveal

detachment dynamics. Initial attachment to chitin was quantified as described above and detachment was calculated as the number of cells in the supernatant (output) divided by the initial attached portion (input). The portion that remained attached was also quantified to verify that growth was not a confounding factor (data not shown). Importantly, we found a similar differential ability to attach between genotypes, as previously described by Williams et al., in which E-genotypes attach more efficiently (23). Thus, we hypothesized that these genotypes would also have a differential ability to detach after prolonged incubation with chitin. We found that, once attached, C-genotypes subsequently detached from chitin significantly more ($P = 0.022$) than E-genotypes after 24-hour of aerobic incubation (Fig. 2). This was an interesting finding as the switch from a sessile to a planktonic lifestyle could be linked to increased success when leaving one environment and transitioning to a new one, such as into the human host, and supports our original hypothesis.

To understand what mechanisms might be underpinning this detachment disparity, we examined gene expression of both genotypes at 24 hours in the same conditions. We first collected RNA from cells incubated with and without chitin beads, with differential expression between them revealing no significant expression differences (data not shown). This result allowed us to perform all further expression studies in 15‰ ASW at 24 hours without chitin beads.

In *V. vulnificus,* expression of type IV pilin proteins (*pilA, pilD and mshA*) have been shown to play a role in differential attachment efficiency, not only to chitin but also polystyrene and glass substrates (23, 53, 60, 61). Additionally, our lab has previously shown that E-genotypes express more type IV attachment genes under a variety of

conditions relative to C-genotypes. *PilA* and *pilD*, which encode a type IV pilin protein subunit and prepilin peptidase, respectively, are required for proper type IV pilin production (61, 62). Mutants lacking type IV pili have been shown to be severely impaired in their ability to attach, which may essentially "lock" these cells in a planktonic state (61). Mannitol-sensitive hemagglutinin (MSHA) is another type IV pilin protein that has been implicated in environmental persistence of multiple *vibrio* species, specifically in the ability to attach to chitinous substrates (63). As type IV pili are important in the attachment phenomenon, we speculated that dispersal would also be effected by expression of these genes. Here we show that E-genotypes expresseed significantly ($p<0.001$) more *pilA, pilD, and mshA* than C-genotypes (Fig. 3) at 24 hours in 15‰ ASW. A notable increase was seen in expression levels of *pilA,* which showed more than a 100-fold increase in E-genotypes when compared to C-genotypes. These findings suggest that an increase in type IV pilin expression in E-genotypes relative to C-genotypes under aerobic conditions (Fig. 3) may be an important factor for prolonged attachment (Fig. 2) of these genotypes.

Conversely, we found that *gbpA, flgE* and *cheA* were all down-regulated in E-genotypes relative to C-genotypes (Fig. 3) when cells were incubated aerobically. N-acetylglucosamine binding protein A (*gbpA*) is suggested to be a key factor in the attachment of *V. cholerae* to not only chitinous substrates but also to the mucosa of the intestinal epithelium (64). This down-regulation could be important, as E-genotypes are not commonly isolated from septicemia patients, and could explain in part why C-genotypes go on to cause disease. The down-regulation of both *flgE* and *cheA* (Fig. 3), flagellar hook subunit E and chemotaxis protein A, respectively, suggests that these cells

are more sessile (65-67). As both of these proteins are involved in motility and in sensing nutrients, it is plausible that they are not needed while cells are still attached, as they do not need to move toward or away from stimuli. However, more investigation is needed before this hypothesis can be fully explained.

Anaerobic Detachment Dynamics

As nutrient limitation has been shown to affect the dispersion of cells from an attached state to a free-living state, we explored anaerobiosis as a potential dispersion factor (32, 34, 51, 52). This condition has not been studied in *V. vulnificus,* although it has implications in both the environment and upon entry into the human gastrointestinal (GI) tract. Previously, we have shown there is a decreased propensity for both genotypes to attach and form biofilms when incubated for 24 hours in anaerobic conditions (53). We conjectured that this condition might play a greater role in detachment of both genotypes from substrata. Our results show that approximately 27% of C-genotype cells detach in response to 24 hours without oxygen whereas significantly less (2%) of E-genotype cells detach ($p<0.01$) under these conditions (Fig. 4). This is a similar trend as was seen in aerobic conditions, except that both C- and E-genotype cells were detached more efficiently with oxygen present, 36% compared to 19%, respectively (Fig. 2). The almost complete lack of detachment of the E-genotypes during anaerobiosis may provide insight into their distinct lifestyle preferences when confronted with this stress.

We further investigated under anaerobic conditions the expression of attachment, motility and chemotaxis genes which we examined during aerobic conditions, and which we found to be differentially regulated between these genotypes. We found similar trends showing that E-genotypes significantly ($p<0.001$) up-regulated genes involved in type IV

pilin production (*pilA*. *pilD* and *mshA*), and significantly ($p<0.001$) down-regulated *gbpA* and *flgE*, when compared to C-genotypes (Fig. 5). An intriguing difference we found was that the level of increase or decrease was dramatically impacted by anaerobiosis. Especially notable was that of *pilA* expression, which was over 200-fold increased in E-genotypes compared to C-genotypes, while both *pilD* and *mshA* increased approximately 6-fold and 8-fold, respectively (Fig. 3 and 5). In addition, *gbpA* and *flgE* expression was also decreased in the anaerobic cultures, although not significantly. One contrasting finding was that *cheA* was significantly ($p<0.0001$) up-regulated in response to anaerobiosis relative to C-genotypes, whereas in the aerobic culture an opposite but no significant trend was present (Fig. 3 and 5); this may be due to the complex roles that chemotaxis proteins play within the cell (67). Furthermore, there is not always a direct correlation between flagella production and chemotaxis protein production, and regulation of these genes may undergo post-translational control (68). More investigation is needed to understand the complex role that chemotaxis proteins may play on detachment, as there are multiple genes encoding these proteins in *V. vulnificus.*

Role of QS in Detachment

The impact of interspecific QS signaling pathways on a plethora of cell functions has been well studied in *V. vulnificus* (36, 47, 48, 69). Having an important role in virulence potential, as well as biofilm formation, makes this a logical regulatory system to examine during detachment (36, 48). In addition, studies on *V. cholerae* have shown their ‘dual lifestyle’ to be tightly regulated by QS, triggering attachment and subsequent detachment when alternating between the environment and the human host (70). As QS has been shown to differentially regulate genes involved in detachment of *V. cholerae*

biofilms, such as decreased EPS production (43), we speculated that it might also have a role in detachment of *V. vulnificus*. We first examined bioluminescence, using a *V. harveyi* reporter strain assay, in both C- and E-genotypes. We found that in both aerobic and anaerobic conditions, E-genotypes produced significantly less ($p<0.0001$) bioluminescence, indicating a decrease in AI-2 production (Fig 6). As E-genotypes also detached significantly less than C-genotypes (Fig. 2 and 4) in both conditions, this decreased production of AI-2 may be involved in their lack of dispersal.

To determine whether AI-2 production was required for a more robust detachment response, we utilized a LuxS mutant of *V. vulnificus*. This mutant (JDO1) is unable to produce an important AI-2 molecule precursor, 4,5-dihydroxy-2,3-pentanedione, which is encoded by *luxS* (55). This was confirmed in the bioluminescence assay, which showed that the Δ*luxS* strain produced significantly ($p<0.0001$) less AI-2 than both C and E-genotypes, regardless of the condition (data not shown). When we added exogenous synthetic AI-2 (DPD) to the Δ*luxS* strain bioluminescence was induced significantly ($p<0.0001$) more than by the mutant strain without this addition (Fig 6). We demonstrated that the percent detachment of the wild-type (wt) *V. vulnificus* was significantly higher ($p<0.001$) than the Δ*luxS* mutant strain (Fig. 7A). This result was consistent regardless of incubation with or without oxygen, however the Δ*luxS* strain was significantly less capable of detaching during anaerobiosis (Fig. 7A). In fact, the percent detachment in the mutant was very similar to that seen in the E-genotypes, which further supports our hypothesis that QS negatively impacts detachment in these genotypes. The addition of synthetic AI-2 to the Δ*luxS* mutant resulted in a significant ($p<0.0001$) increase in detachment compared to the Δ*luxS* mutant strain suggesting that increased QS

does in fact increase detachment (Fig 7A), however, the exact mechanism by which detachment is enhanced is unclear.

Finally, we wanted to explore expression patterns of the Δ*luxS* mutant relative to the parent strain during both exposure conditions in regards to attachment. This revealed that when compared to the wild type, Δ*luxS* was differentially regulating genes for attachment (Fig. 7 B and C). More precisely, the QS mutant significantly ($p<0.05$) up-regulated those genes involved in type IV pilin production in both aerobic and anaerobic conditions (Fig. 7 B and C). Interestingly, although there was a significant decrease in detachment in the Δ*luxS* strain aerobically and anaerobically (Fig. 7 A), we did not see a dramatic increase of these genes between conditions (Fig. 7 B and C). This may be due to the fact that detachment is multifaceted and cannot be completely understood without a more in depth investigation into other detachment factors. We also found that expression of *gbpA* was decreased almost 100-fold in the mutant strain (Fig. 7 B and C). This finding corroborates our previous finding, where strains with decreased detachment also showed a decrease in *gbpA* expression in both conditions (Fig. 3 and 5). Importantly, we found that when synthetic AI-2 was added to Δ*luxS,* there were no significant expression differences when compared to the wild-type parental strain, except for *pilD* (Fig 7 B and C) in both conditions. This result substantiates the importance of QS in the production of these attachment genes in *V. vulnificus*. In *V. cholerae, hapR*, which is the key regulatory component of the QS network, has been shown to regulate *gbpA* by targeted degradation (71). Although Δ*luxS* still has a functional *luxR* (hapR homologue), we can infer that QS in *V. vulnificus* might directly regulate the expression of *gbpA,* due to its decreased expression in the mutant strain (Fig. 7 B and C) as well as in the E-genotypes (Fig. 3 and

5) which are limited in their QS abilities (Fig. 6). However, the specific mechanisms of this pathway need to be further invested to understand this network more comprehensively.

Conclusions

This study is the first to examine detachment dynamics of both clinical and environmental strains of *V. vulnificus* under aerobic and anaerobic conditions, and to investigate potential molecular mechanisms that facilitate this detachment phenomenon. We found two distinct lifestyle preferences during our study, as described in the proposed illustration (Fig. 8). The finding that E-genotypes show decreased ability to detach from chitin, with only 2% detachment in anaerobic environments, suggests a role for its maintenance in the environment, and increased propensity for sessile living. Interestingly, oysters preferentially take up *V. vulnificus* when attached to marine snow (25), of which chitin is a major constituent, and others have shown that E-genotypes attach more efficiently to chitin (23). This points to E-genotypes predominating in oysters, which intuitively might lead to an increased consumption of these genotypes, but they are not typically associated with septicemia. In contrast, C-genotypes readily detached in both conditions compared to E-genotypes, which suggests an enhanced ability to switch from an attached state to a planktonic state. This ability is noteworthy, as most septicemia cases are caused by C-genotypes, and the mechanisms regulating this disparity are largely unknown. We propose that even though the C-genotypes might not predominate in this system, they have an increased ability to detach, which will allow for their reattachment to the intestinal epithelium. We also have shown that QS has a specific role in the

dispersal of *V. vulnificus* from chitin, which has important implications for both clinical and environmental survival of these diverse ecotypes. Further investigation into the impact of QS inhibitors on detachment is needed, as this may effectively limit the ability for C-genotypes to attach to the intestinal epithelium and cause potentially fatal septicemia. Additionally, as detachment is highly dynamic, more investigation into dispersal factors will be important for understanding this phenomenon.

Acknowledgements

We kindly thank Rohinee Paranjpye and Bonnie L. Bassler for providing the *V. vulnificus* type IV pilin mutants, and the *V. harveyi* BB170 strain, respectively. Additionally, we thank Matthew W. Parrow for providing the synthetic AI-2 used in this study, and Leigh Robertson for enhancing the graphical representations. This work was supported by the United States Department of Agriculture [2009-03571]. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Agriculture.

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Table 1 Characterization of *Vibrio vulnificus* strains utilized in this study

Strain	Genotype[a]	Isolation Source	16S rRNA type[b]	Lineage[c]	Biotype
CMCP6	C	Blood	B	II	1
C7184	C	Blood	B	II	1
MO6-24	C	Blood	B	II	1
YJO16	C	Blood	B	I	1
JY1701	E	Oyster	A	I	1
JY1305	E	Oyster	A	I	1
ENV1	E	Oyster	A	I	1
JDO1	C	C7184 *luxS* mutant	B	II	1

[a] Genotypes determined by end-point PCR of the species specific virulence correlated gene (9,18)

[b] Polymorphisms found in 16S rRNA delineated between 16S rRNA type A and type B

[c] Analysis of six housekeeping genes by multi-locus sequence analysis was used to separate *V. vulnificus* into two distinct lineages

Table S1. Primers designed for qRT-PCR

Gene	Primer target	5'-3' Sequence[a]	Product size (bp)	Strains Amplified
Glyceraldehyde phosphate dehydrogenase[b]	*gapdh*	F: TGAAGGCGGTAACCTAATCG R: TACGTCAACACCGATTGCAT	97	All
Type IV pilin protein subunit[bc]	*pilA*	F: TCATTGGTGTGTTAGCCGCA R: GCTGAGGCAGCTTCTGACTT	73	JY1305 only
Type IV pilin protein subunit[bc]	*pilA*	F: TTGCTAAAAGTGAAGCCGCA R: CCAGCTGCACTAGTAGGGTT	169	JY1701 only
Type IV pilin protein subunit[bc]	*pilA*	F: GCACAGCTCCAACCAGTAGT R: TTGGCGGCACTTCAACAATG	57	CMCP6 C7184 YJO16 JDO1
Type IV pilin protein prepilin peptidase[bc]	*pilD*	F: GGCTTACTGGTAGGCAGCTT R: GGTTTCTGTCGGCGGTGATA	126	JY1701 JY1305
Type IV pilin protein prepilin peptidase[bc]	*pilD*	F: TTGGCTTACTGGTAGGCAGC R:GGTTTCTGTCGGTGGTGTGA	128	CMCP6 C7184 YJO16 JDO1
Mannitol-sensitive hemagglutinin[b]	*mshA*	F: CAAGGCGGTTTCACCCTGAT R:CAGATTTAGAAAACGCGGAGCC	90	All
N-Acetylglucosamine binding protein[b]	*gbpA*	F: TTGAGTGGACCTTTACCGCC R:CGGGCAAGTGGTTGATTTGG	91	All
Flagellar monomeric hook subunit	*flgE*	F: TCTAACTGAGCTGCGGACAA R: TACTGCTCAATCTGGCTGGC	145	All
Chemotaxis protein A	*cheA*	F: GTGCTTTTGCCGCTGGTTTA R: CAGTGAGCGCATCGGTAGAT	150	All

[a] F, forward primer target; R, reverse primer target.

[b] Primers previously described by Williams et al. 2014

[c] Strains/genotypes required different sequences.

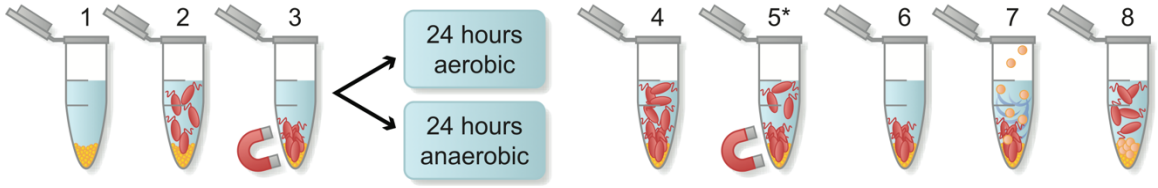

Figure 1. Graphical representation of chitin detachment methodology. Chitin magnetic beads are added to 15‰ ASW with no additional carbon source (1), and *V. vulnificus* is added and incubated for 1 hr at 20°C for initial attachment (2). The initial attached (input) portion from one representative sample of each genotype was quantified by magnetic separation and plate counts (3). The remaining samples were resuspended in fresh 15‰ ASW, and incubated for 24 hours at 20°C in both conditions. Tubes were removed from each condition (4) and magnetic separation was employed to quantify detached (output) portion (5). Additionally, as illustrated by the asterisk, cells were RNA protected for gene expression, and cell-free supernatant was collected for quorum sensing assays. The remaining attached portion was resuspended in fresh 15‰ ASW and the supernatant was quantified following vigorous bead bashing (6-8).

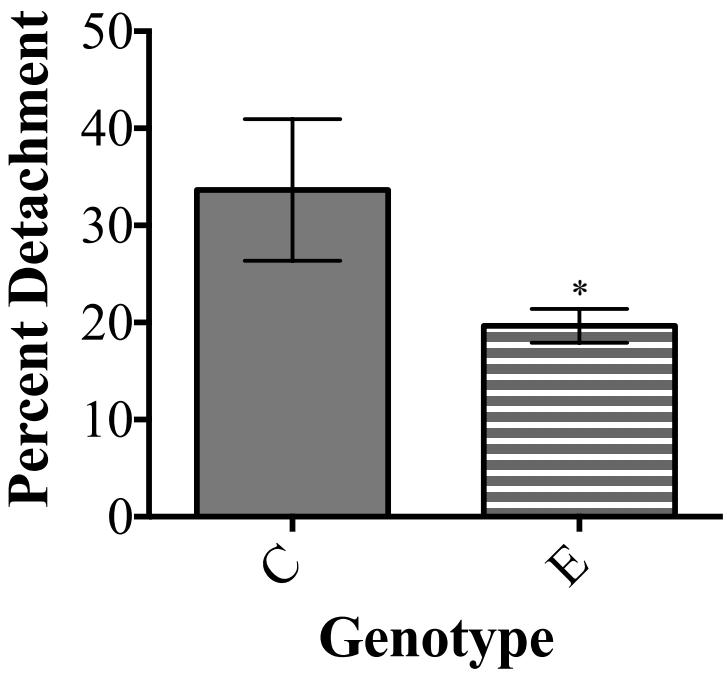

Figure 2. The differential ability for C-genotypes (Grey bars: CMCP6, MO6-24, and YJ016) and E-genotypes (Striped bars: JY1701, JY1305 and ENV1) of *V. vulnificus* to detach from chitin after a 24-hour aerobic incubation. C-genotypes detached significantly more than E-genotypes as represented by the asterisk (unpaired *t* test; $P = 0.022$) under oxygenated conditions. Error bars represent the standard deviation of 10 biological replicates, each comprising four technical replicates averaged for each strain.

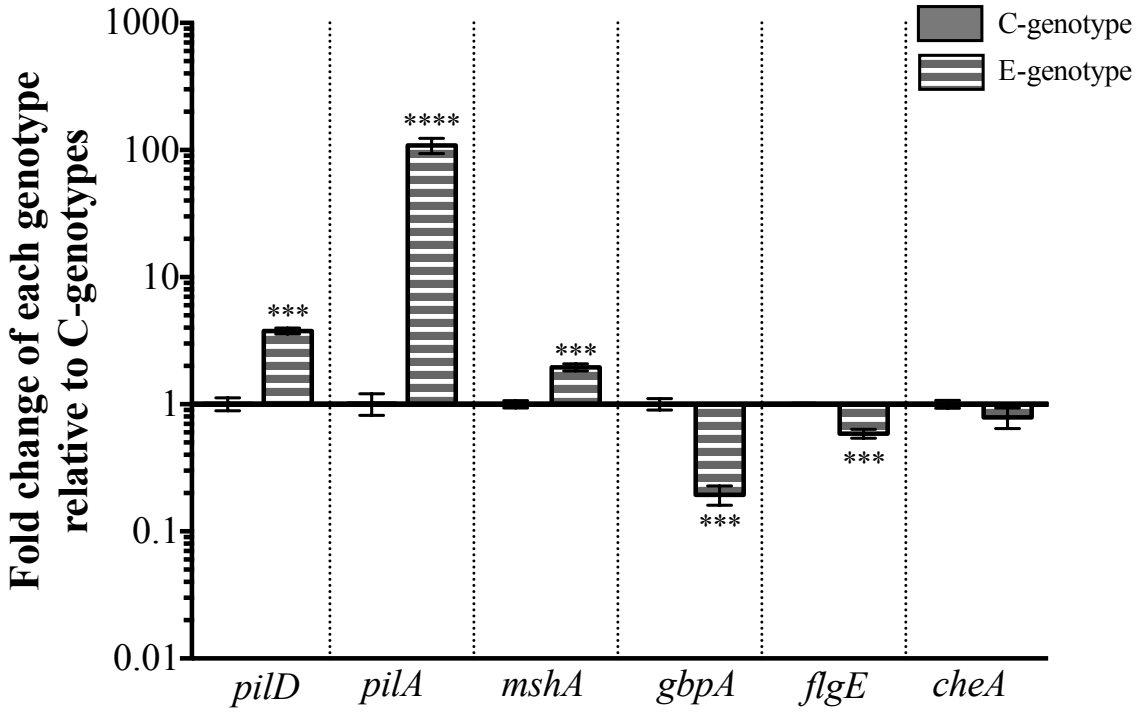

Figure 3. Relative gene expression of various genes involved in attachment and motility under aerobic conditions in *V. vulnificus*. E-genotypes expressed significantly more *pilA, pilD,* and *mshA,* than C-genotypes, but significantly down-regulated expression of *gbpA* and *flgE*. Error bars represent standard deviation of four biological replicates, each comprising three technical replicates averaged for each strain (Grey bars: CMCP6 and MO6-24) and E-genotypes (Striped bars: JY1701 and JY1305). Asterisks represent statistically significant differences when E-genotypes were analyzed using a non-parametric Mann-Whitney test relative to C-genotype expression of each gene transcript (***, $P < 0.001$; ****, $P < 0.0001$). Corrections in p-value were assessed using the Bonferonni calculation.

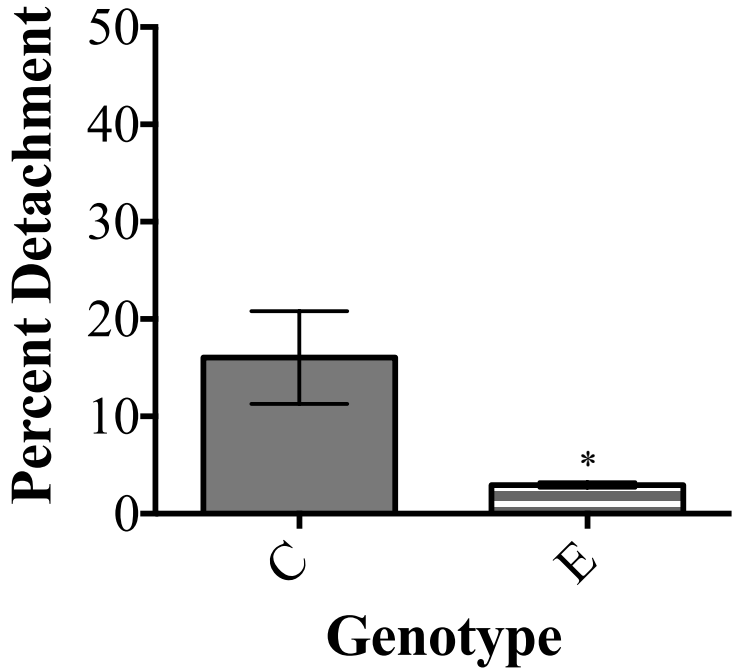

Figure 4. Role of anaerobiosis in detachment of both C-genotypes (Grey bars: CMCP6, MO6-24, and YJO16) and E-genotypes (Striped bars: JY1701, JY1305 and ENV1). E-genotypes detached significantly less than C-genotypes as represented by the asterisk (unpaired *t* test; $P = 0.028$) under oxygenated conditions. Error bars represent the standard deviation of 10 biological replicates, each composed of four technical replicates averaged for each strain.

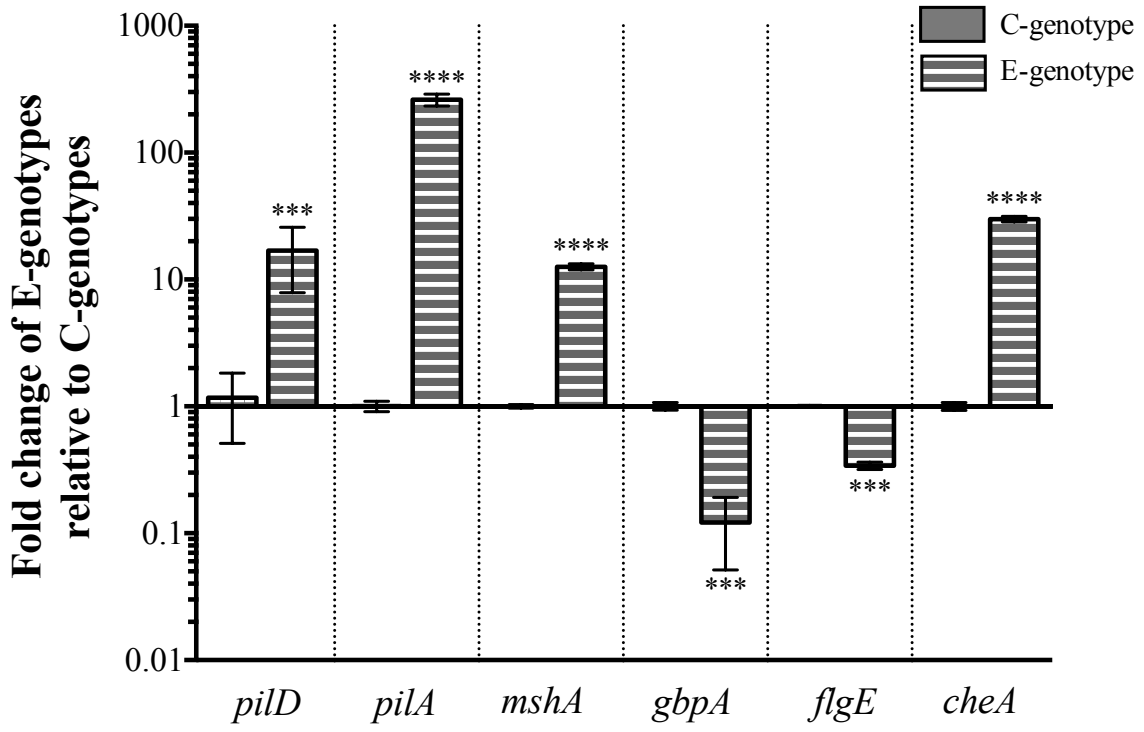

Figure 5. Relative expression of genes involved in attachment and motility under anaerobic conditions in *V. vulnificus*. E-genotypes expressed significantly more *pilA, pilD, mshA* and *cheA* relative to C-genotypes, but down-regulated expression of *gbpA,* and *flgE.* Error bars representative of the standard deviation of four biological replicates, composed of three technical replicates averaged for each strain (Grey bars: CMCP6 and MO6-24) and E-genotypes (Striped bars: JY1701 and JY1305). A non-parametric Mann-Whitney test was used to assess significance in E-genotype relative to C-genotype expression of each gene transcript, and asterisks represent statistically significant differences between genotypes (***, $P < 0.001$; ****, $P < 0.0001$). Corrections in p-value were assessed using the Bonferonni calculation.

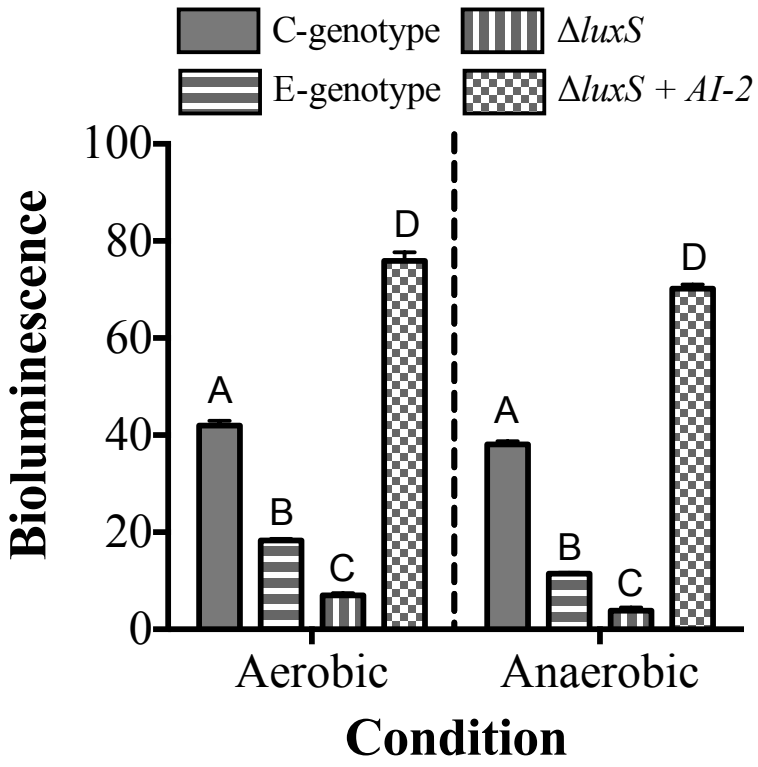

Figure 6. Quantification of quorum sensing (QS) in both C-genotypes (Grey bars), E-genotypes (horizontally striped bars), *ΔluxS* (vertically striped bars), and *ΔluxS* + AI-2 (checkered bars) in both aerobic and anaerobic conditions. In both conditions, E-genotypes produced significantly less AI-2 ($p<0.0001$) than C-genotypes, bioluminescence is calculated as described in methods. Additionally, the *ΔluxS* strain is significantly ($p<0.0001$) impaired in its ability to produce AI-2 in both conditions when compared to the wild-type and complemented strain. Individual one-way ANOVAs were used to analyze significance (represented by different letters) between strains in both conditions separately, and corrections in p-value were assessed using the Bonferonni calculation. Error bars represent the standard deviation of 12 biological replicates, each composed of five technical replicates averaged for each strain.

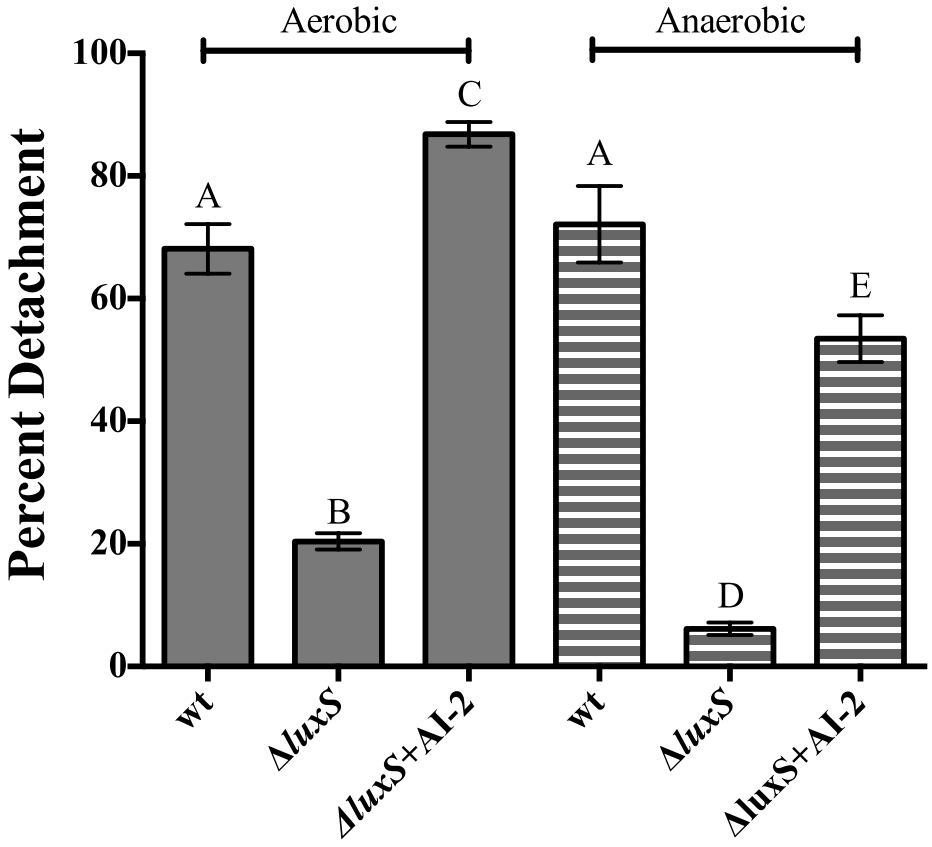

Figure 7a. Role of QS in detachment, utilizing a V. vulnificus mutant incapable of producing AI-2 (ΔluxS) compared to the wildtype parent (C7184), and *ΔluxS* with exogenous AI-2 added. A) ΔluxS showed a significant decrease in detachment when exposed to both conditions. Additionally, ΔluxS + AI-2 restored the wild-type phenotype, but anaerobically it detached significantly more than the wild-type strain. Differential detachment was analyzed using a two-way ANOVA with Tukey's post-hoc tests for multiple comparisons between aerobic (grey bars) and anaerobic (striped bars). Significance is shown by different letters comprising from analysis of 10 biological replicates with three technical replicates averaged for each strain, and error bars representative of their standard deviation around the mean.

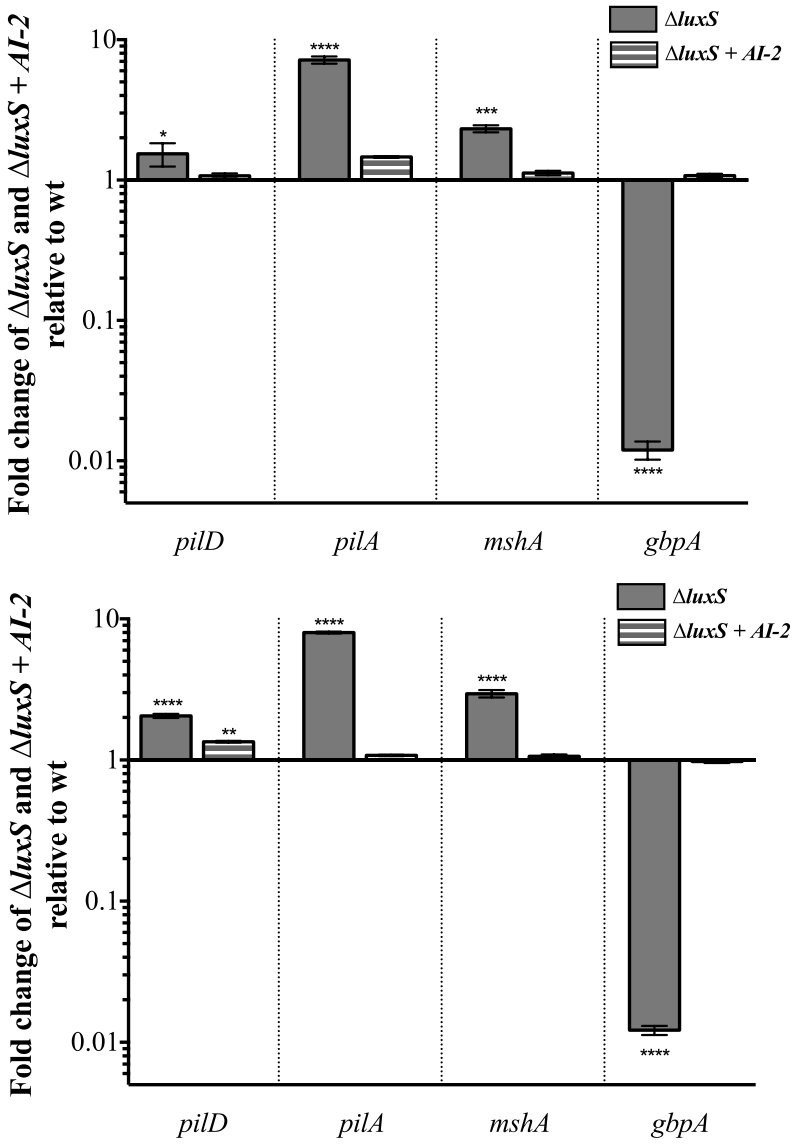

Figure 7bc. Role of QS on aerobic (B) and anaerobic (C) attachment gene expression in *ΔluxS* (grey bars) and *ΔluxS* + AI-2 (striped bars) when compared to its parental strain. Both aerobic and anaerobic conditions exhibited similar results in which ΔluxS expressed significantly more *pilA, pilD* and *mshA*, but significantly down-regulated *gbpA*, when compared to the parental strain. When the *ΔluxS* + AI-2 is compared to the wild-type strain there is only a significant difference in the expression of *pilD,* and no significant differences in the other genes examined. A non-parametric Mann-Whitney test was used to assess significance in ΔluxS and *ΔluxS* + AI-2 relative to its parental strains expression of each gene transcript. Asterisks represent statistically significant differences between these comparisons in both conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Error bars represent the standard deviation of 3 biological replicates, each with 3 technical replicates, and corrections in p-value were assessed using the Bonferonni calculation.

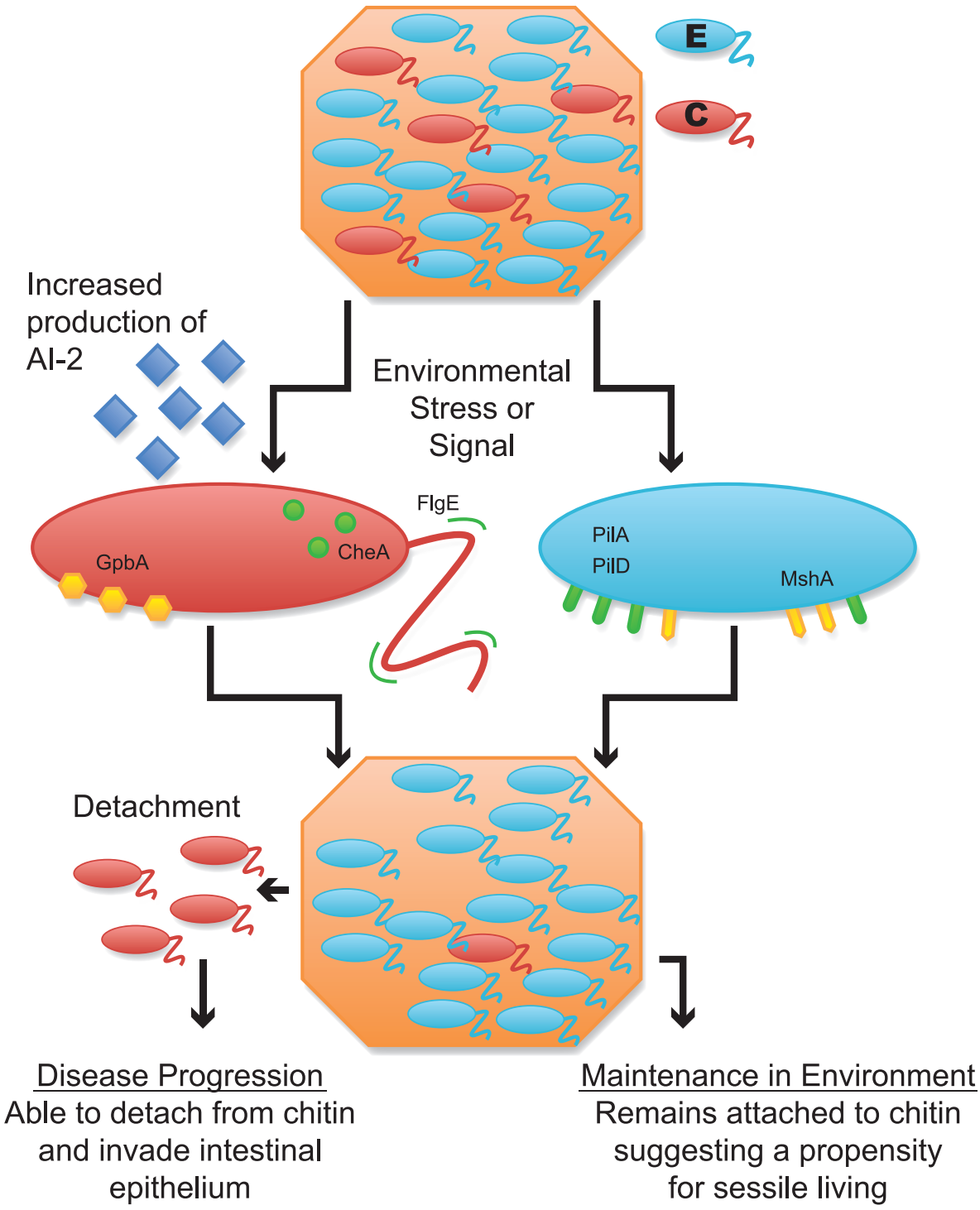

Figure 8. Schematic of molecular mechanisms unveiled in this study and the proposed impact on clinical manifestations and maintenance in the environment. This figure represents the differences between E-genotypes when compared to C-genotypes.

CHAPTER 3

IMPACT OF HYPOXIA ON GENE EXPRESSION PATTERNS BY THE HUMAN PATHOGEN, *VIBRIO VULNIFICUS*, AND BACTERIAL COMMUNITY COMPOSITION IN A NORTH CAROLINA ESTUARY

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Citation

Phippen BL, Oliver JD. 2017. Impact of hypoxia on gene expression patterns by the human pathogen, *Vibrio vulnificus*, and bacterial community composition in a North Carolina estuary. GeoHealth **1:**37-50.

Abstract

Estuarine environments are continuously being shaped by both natural and anthropogenic sources which directly/indirectly influence the organisms that inhabit these important niches on both individual and community levels. Human infections caused by pathogenic *Vibrio* species are continuing to rise, and factors associated with global climate change have been suggested to be impacting their abundance and geographical range. Along with temperature, hypoxia has also increased dramatically in the last 40 years, which has led to persistent dead zones worldwide in areas where these infections are increasing. Thus, utilizing membrane diffusion chambers we investigated the impact of *in situ* hypoxia on the gene expression of one such bacterium, *Vibrio vulnificus*, which is an inhabitant of these vulnerable areas worldwide. By coupling these data with multiple abiotic factors, we were able to demonstrate that genes involved in numerous functions, including those involved in virulence, environmental persistence, and stressosome production, were negatively correlated with dissolved oxygen. Furthermore,

comparing 16S ribosomal RNA, we found similar overall community compositions during both hypoxia and normoxia. However, unweighted beta diversity analyses revealed that, although certain classes of bacteria dominate in both low and high oxygen environments, there is the potential for quantitative shifts in lower abundant species, which may be important for effective risk assessment in areas that are becoming increasingly more hypoxic. This study emphasizes the importance of investigating hypoxia as a trigger for gene expression changes by marine *Vibrio* species, and highlights the need for more in depth community analyses during estuarine hypoxia.

Introduction

Aquatic hypoxia, defined by < 30% DO saturation (< 2 mg O_2 L^{-1}), in marine systems has been increasing in frequency, severity, and duration by both natural and anthropogenic sources, which is being shaped by global climate change (1-4). Primary sources of hypoxia are weather patterns such as El Niño and La Niña, coastal upwelling, and anthropogenic eutrophication (5, 6). Since the 1970s cases of reported hypoxic zones have increased dramatically with some of the more recent cases being documented in the Baltic Sea, an area undergoing rapid sea-surface temperature warming (7, 8). Recently, infections caused by such pathogenic *Vibrio* species as *V. vulnificus, V. parahaemolyticus,* and *V. cholerae* are being reported in areas where such infections were never seen before, as far as 65°N, during unseasonably warm heat wave phenomena (9-11). Although the aforementioned increases were not specifically investigated in the context of hypoxia, it has been shown that hypoxia is positively correlated with temperature and is at its peak during summer months worldwide (12), corresponding to

high levels of *Vibrio* spp. in similar environments (13-15). Although numerous cases of *Vibrio* infections have been reported in coastal areas experiencing frequent hypoxic/anoxic events, or from seafood originating from these areas, its impact on virulence potential has been largely overlooked.

A natural inhabitant of estuarine waters worldwide, *V. vulnificus* is a potentially fatal human pathogen. This bacterium is responsible for causing septicemia and/or wound infections carrying 50% and 25% mortality rates, respectively (16). *V. vulnificus* is commonly isolated from a variety of sources, including shellfish, fish, water, and sediment in estuaries worldwide (17). A remarkable feature of this bacterium is its genotypic variability, having not only three distinct biotypes, but multiple genotypes (18-20). Within biotype one strains of *V. vulnificus* there are two genotypes, clinical (C) and environmental (E), which correlate to both isolation source and specific polymorphisms in the virulence correlated gene (*vcg*) (21, 22). C-genotype strains are those most frequently causing primary septicemia after the consumption of raw or undercooked seafood, predominantly oysters, whereas E-genotypes predominate in estuarine environments but appear less virulent (22, 23). However, there is a subset of E-genotype strains capable of causing severe wound infections, which generally result from entry into a previously acquired wound or during an injury in estuarine waters harboring *V. vulnificus* (24, 25). Interestingly, the Gulf Coast is home to the largest hypoxic "dead" zone in the US (26, 27), and 21% of total *Vibrio* cases in the Gulf Coast states were confirmed to be caused by *V. vulnificus*. In contrast, non-Gulf Coast states had only 7% of such cases, as reported by the most recent "Cholera and Other *Vibrio* Illness Surveillance" (COVIS) Annual Report in 2014.

Oxygen deprivation is experienced by *V. vulnificus* not only in estuarine waters, but also in mollusks during low tide, during post-harvesting treatments, and in the gastrointestinal tract of human hosts (28-30). It is known that oxygen availability can influence the behavior of bacteria, from rapid changes in metabolic function to the production of virulence factors. Invasion from the GI tract, production of lipopolysaccharide and flagella, and up-regulation of key colonization factors are known to be facilitated by exposure to anoxia *in vivo* by *Salmonella*, *Escherichia coli*, and *V. cholerae*, respectively (31-34). We have previously shown that anoxia leads to a translucent colony phenotype and decreased biofilm formation by *V. vulnificus*, due to the down-regulation of genes involved in capsule production and type IV pilus formation *in vitro* (35, 36). *In situ* gene expression by *V. vulnificus* has been investigated thoroughly in response to both temperature and salinity, and during entry into the viable but nonculturable (VBNC) state (37-39). Such studies revealed that both temperature and salinity impacted the expression of various genes involved in stress response and putative virulence factor production, however, additional biotic and abiotic factors during the sampling events were not considered (37-39). The frequency at which *V. vulnificus* experiences hypoxia, combined with the lack of understanding which factors are required for virulence, makes it an ideal model for studying both *in situ* and *in vitro* hypoxia.

Along with understanding the role of oxygen on gene expression patterns by individual species, it is also important to understand how bacterial communities are impacted. Environmental conditions, such as temperature, salinity, pH, nutrient availability, and oxygen saturation, have been documented to have large impacts on prokaryotic community structure in marine systems. Oxygen availability is a key factor in

shaping both fresh and salt water bacterial community composition, especially when combined with spatial sampling (40, 41). However few studies have described these communities as they experience daily estuarine hypoxia, but rather typically focus only on oxygen minimum zones (42). Studies examining temporal open-ocean prokaryotic communities have shown that, among other factors, oxygen shows predictability in their structure, suggesting important ecological roles for these species (43). Additionally, Spietz et al. [2015] found that bacterial communities can be impacted at concentrations of DO higher than the definition for hypoxia, which proceeds visible effects on macroorganisms and suggests that these changes occur even in areas experiencing oxygen stress but not hypoxia. This heightened sensitivity to changing oxygen concentrations could potentially explain why certain bacterial species dominate in one condition over another. Furthermore, as global climate change is linked to increasing hypoxic events (6, 44-47), which may impact the distribution of pathogenic species in the environment, the need to understand these communities as a function of environmental change is paramount (48-50).

Here we explore the hypothesis that estuarine hypoxia results in distinct gene expression patterns by *V. vulnificus* and drives bacterial community composition at a moderately eutrophic site (as designated by NOAA, 1996) in the Bogue Sound of North Carolina. We addressed this hypothesis by 1) monitoring multiple abiotic factors over a three-day period to determine what parameters were most variable, 2) identifying expression levels of multiple gene families during high and low oxygen sampling events, and 3) determining bacterial community compositions during both hypoxia and normoxia.

Materials and Methods

Strains and Culture Conditions

A clinical isolate of *V. vulnificus*, CMCP6, was utilized for this study and was stored at -80°C in Bacto Luria-Bertani broth (LB) (BD Difco, Franklin Lakes, NJ, USA) with 20% glycerol. This strain was regularly grown overnight (ON) in Bacto heart infusion (HI) broth (BD Difco, Franklin Lakes, NJ, USA), at 30°C with aeration in a rotary incubator. For use in chamber deployment, ON cultures were grown and washed 3x in sterile 20‰ artificial sea water (ASW: Instant Ocean; Aquarium Systems, Mentor, OH, USA) and resuspended in 250 mL 20‰ ASW.

Site Description and Monitoring of Environmental Parameters

All experiments were conducted in mid-August at Hoop Pole Creek, which is part of the Hoop Pole Reserve on the eastern North Carolina coast, USA. This site is located near a storm drain input and is frequently exposed to increased nutrients due to rain water runoff. To monitor abiotic parameters a Hach Hydrolab minisonde 4a datalogger (OTT Hydromet, Germany) was utilized for the entirety of the exposure period. Temperature, salinity, pH, and DO were recorded every 30 minutes for five days during the sampling time. The datalogger was deployed at morning low tide and was secured to permanent poles positioned in the middle of the Hoop Pole Creek site. Additionally, the datalogger was checked twice daily to remove any debris or mud which might impact measurements.

Membrane Diffusion Chamber Preparation and Deployment

V. vulnificus was grown and prepared as described above and deployed into large volume membrane diffusion chambers. These were modified from the chambers originally designed by McFeters and Stuart (1972). These modified chambers consist of

two 76 mm, 0.2µm hydrophilic polycarbonate filters (Midland Scientific Inc., Omaha, NE, USA) positioned between two doughnut shaped sections of Plexiglas with a 2-inch piece of polyvinyl chloride (PVC) piping between them (Figure S1) (51). Before assembly, PVC pipes were sterilized by a 10% bleach bath for 20 min followed by rinsing in a sterile deionized water bath. Membranes were autoclaved, attached to the PVC pipe by aquarium grade silicon, and each side was checked for leaks by submerging the chamber in water and flowing air through the tube to make sure no bubbles were produced. Bacterial culture (250 ml) at a final cell concentration of approximately 10^6 CFU/mL was injected into each chamber using sterile syringes. Once filled, chambers were wrapped in black plastic hex fencing, placed in coolers, and covered with 20‰ ASW for transport to the field site. Chambers were attached securely to the pole harboring the datalogger during late afternoon low tide.

In situ RNA Harvesting

Approximately every 12 hours (at each low tide) for three consecutive days duplicate samples were removed from each of three chambers using sterile syringes pre-filled with RNAprotect (Qiagen, Valencia, CA) at a 2:1 ratio of RNAprotect to cell culture, following the manufacturer's protocol. A previously optimized RNA extraction assay was then performed as described by Williams et al. [2014]. RNA quality and quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and samples having a 260/280 nm ratio of ≥1.7 and a concentration of ≥75ng/µl were stored at -80°C. Using end-point PCR, determination of DNA contamination was performed as previously described (35, 52) utilizing the species-specific gene target, *vvhA*.

Primer design

Sequenced C- and E-genotype strains of *V. vulnificus* were used as reference strains for qRT-PCR primer design, employing NCBI Primer-BLAST software. Assessment of primer quality and specificity was performed as previously described employing *in silico* PCR, and the IDT OligoAnalyzer 3.1 software (35, 52). Primer pair efficiencies were predicted by *in silico* PCR estimation tools, and primers with efficiencies of ≥1.5 were selected and purchased from Sigma Aldrich. Primers optimized for this study are listed in Table S3.

Relative qRT-PCR

A previously described qRT-PCR protocol was adapted for all *in situ* expression studies (52). Gene expression of *V. vulnificus* strain CMCP6, was examined, in triplicate, for each sampling time using PerfeCTa SYBR green FastMix, Low ROX (Quanta Biosciences, Beverly, MA). Normalization of target genes was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous control, to correct for sampling error. The Pfaffl equation was used to calculate fold change in gene expression, accounting for differences in the primer pairs, utilizing a previously described PCR efficiency analysis (53). Gene expression results were analyzed comparing each sample time to the previous sample time, where t=0 was immediately upon chamber deployment; for example, x=12 represents fold change of 12 hr to 0 hr, x=24 represents fold change of 24 hr relative to 12 hr, and so on. Nonparametric Mann-Whitney rank-sum tests were used to determine significance between target transcripts with adjusted p-values calculated using the Bonferonni method. All gene expression data were analyzed using GraphPad Prism (version 5.0; GraphPad Software Inc.). Pearson's product-moment

correlational analysis was performed on gene expression and environmental parameters in SPSS Statistics software (version 24, IBM Corp., Armonk, NY, USA) and output visualized using GraphPad Prism.

Water Sampling and DNA Extraction

Water samples were taken at each RNA harvesting event for determination of bacterial community composition. Briefly, 1L of sea water was removed, stored in sterile 1L bottles, and placed in a cooler to be transferred to the lab for processing. Water was sampled approximately 3m from chamber location and sampled at mid-water depth. Triplicate water 250 mL samples were passed through a 0.2 µm pore size polyethersulfone filter using vacuum filtration, and the filters kept at -80°C until extraction. DNA from filters was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturers protocol.

16S rRNA Library Preparation and Analysis

Bacterial sequences were amplified using primers for the V3 and V4 region (Forward: 5' CTACGGGNGGCWGCAG, Rev: 5'GACTACHVGGGTATCTAATCC) as previously described (54) and triplicates were combined into one sample. Cleanup was performed using the Axygen AxyPrep Kit (Thermo Scientific, Wilmington, DE, USA), followed by index PCR using the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA), and a second PCR cleanup. Fluorometric quantitation was performed after each cleanup step by Qubit (Life Technologies, Carlsbad, CA, USA), and libraries were validated by identification of a ~630 bp product using a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA). Illumina MiSeq sequencing was performed at the David H. Murdock Research Institute, Kannapolis, NC, USA.

Sequences were assembled and edited using PEAR (55) and Trimmomatic 2 (56), and screened for singletons. Using the Qiime pipeline (57), chimeric sequences were removed using both ChimeraSlayer (58), and USEARCH 6.1 (59) and not used for subsequent analysis of samples. Operational taxonomic units (OTUs) were assigned using MOTHUR (60), aligned using PyNAST (61), and assigned to taxonomies using RDP classifier (62) and the Greengenes database (63). Beta diversity was calculated using both weighted and unweighted UniFrac (64) and visualized using Emperor (65). Sequences were made available through the NCBI BioSample database (Accession numbers: SAMN05771570, SAMN05771571, SAMN05771572, SAMN05771573, SAMN05771574, SAMN05771575).

Results and Discussion

Environmental profiles

In order to determine which environmental parameters might be driving the gene expression patterns of *V. vulnificus* we measured DO, temperature, salinity, and pH every 30 minutes during *in situ* chamber deployment. Measurements from the datasondes revealed distinct temporal abiotic profiles (Figure 1A). Peaks were designated as "High DO" and valleys as "Low DO", corresponding to trends in DO throughout our sampling periods. As expected, temperature was negatively correlated with DO (Pearson r = -0.917) and ranged from 26.19°C -31.69°C, whereas pH (7.6-8.5) and salinity (27.9‰-31.7‰) did not correlate with DO (r = 0.684, and r = 0.533 respectively). To determine which of these parameters varied the most we normalized change from one sampling time to the next while removing the unit of measurement, which was key in that each

parameter had a different unit and thus could not be statistically compared. Figure 1B shows a significant (one-way ANOVA, $p<0.0001$) difference in changing oxygen concentrations when compared to change in temperature, salinity, and pH. However, the latter were not statistically significant from one another, further illustrating the highly variable nature of oxygen availability at this site. Average DO saturation during high DO sampling times was 88.7% while low DO sampling events fell to an average of 20.9% (Table S1). Interestingly, apart from DO saturation, all parameters were more variable during the low DO sampling times but overall remained relatively constant (Table S1).

Hoop Poll Creek has been described as a highly dynamic site experiencing large daily fluctuations in numerous abiotic factors, especially oxygen (66). This is consistent with what we found for DO, however the other factors measured did not vary significantly (Figure 1B). Furthermore, this site is potentially eutrophic as a storm drain located at the head of the estuary sporadically adds excess nutrients to the surrounding water, which could exacerbate hypoxic intervals. The combination of the excess nutrients and warm summer temperatures may have resulted in increased photosynthetic activity during the day, resulting in increased DO, and rapid utilization and depletion of this DO overnight (67). There was no precipitation during our three-day sampling, which may explain why salinity remained high and relatively consistent. Additionally, there was little variability in daily high temperatures, which was 29.2°C ± 1.2°C. Our results provide further evidence that this site may be used for analyzing the effect of cyclical hypoxia and eutrophication on marine microorganisms.

In situ gene expression profiles

To characterize the response of *V. vulnificus* to these cyclical periods of hypoxia and normoxia, duplicate samples were removed from each of the three chambers over three days for relative qRT-PCR analysis during both low and high DO intervals. Exact environmental parameters measured at each sampling interval are summarized in Table S2. We investigated a variety of genes involved in multiple categories, including capsular polysaccharide (CPS)/exopolysaccharide (EPS) production, motility and attachment, global gene regulation, putative virulence factor production, stressosome formation, and general metabolism (Figure 2). Many of these genes have been previously shown to be differentially regulated by *V. vulnificus* in response to environmental and host stressors and/or those that have been implicated in virulence (35, 36, 68, 69).

Polysaccharide Production, Attachment and Motility

V. vulnificus produces multiple types of polysaccharides, notably CPS and EPS, which have implications in evading host phagocytosis and environmental persistence by facilitating biofilm formation (52, 69, 70). We investigated expression of two genes involved in CPS production, *wzb* and *cpsB,* which encode a cognate phosphatase and mannose-1-phosphate guanyltransferase, respectively (71, 72). We also analyzed expression of a polysaccharide export periplasmic protein, SypC, which is homologous to a gene for EPS production in the "*syp* locus" of *V. fischerii (73)*. Twelve hours after entry into the estuary, *wzb* was down-regulated whereas both *sypC* and *cpsB* were highly up-regulated (Figure 2A). In fact, *cpsB* was below the level of detection at t=0 (indicated by the arrow on the graph) with the level of expression set at a 100-fold increase. Generally,

there was a decrease in expression of all three genes in response to changing oxygen conditions, however, this was not consistent through our sampling intervals.

Capsule production in the human host is an absolute virulence determinant, as cells lacking CPS succumb to the bactericidal effects of human serum, but its role in the environment is unknown (69). As production of these polysaccharides is metabolically expensive, there must be a key ecological role for their production in the estuarine environment, especially under hypoxic stress. Consistent with our previous *in vitro* results, the expression of *wzb* was decreased during conditions where oxygen was limited, however the second CPS type and EPS production did not follow that trend (35).

Having the ability to inhabit dynamic niches in a planktonic or a sessile state is an important factor for bacterial survival in estuarine environments. These lifestyles are driven by numerous factors, including but not limited to nutrient status, environmental conditions, and chemical signals. We found that the expression of *pilA,* a type IV pilin protein subunit which is required for type IV pilus formation (74), was not driven by cyclical hypoxia *in situ* (Figure 2B). There was no significant expression change until 36 hours *in situ* when its expression was decreased, followed by a slight increase, and the lowest level of expression was seen at 72 hours (Figure 2B). Persistence of *Vibrio* species in the environment has been linked to the formation of another type IV pilin, mannitol-sensitive hemagglutinin (MSHA) (75), which we found to be upregulated up to 24 hours and subsequently down-regulated for the duration of our study (Fig 2B). Following the same pattern*, flgE*, coding for the monomeric hook subunit of the flagellum (76), was also not influenced by hypoxic intervals (Fig 2B). Again, the decreased expression of both the type IV pili as well as flagellar synthesis after 24 hours might suggest that *V.*

vulnificus has already committed to an attached phenotype, which would also explain the initial increase in expression of *mshA* and *flgE.*

Quorum Sensing (QS) and Stress Response

V. vulnificus utilizes density-dependent interspecific QS as a mechanism for impacting downstream regulation of genes involved in biofilm formation/dispersal, virulence, and *in vivo* survival (77). Additionally, QS has been shown to be integral in the dual life-cycle of *V. cholerae,* driving both environmental survival and host invasion lifestyles (78). We investigated the autoinducer-2 (AI-2) system of QS in *V. vulnificus,* which is currently the only known functioning system in this species (79), by measuring expression of the *luxS* gene responsible for production of the signaling molecule (AI-2). We found its expression was increased during hypoxic sampling events (Figure 2C) with over a 10-fold increase at the first hypoxic time point. We also found that expression of *rpoS*, the alternate sigma factor involved in general stress response (80), was increased during hypoxia (Figure 2C). Furthermore, *relA*, responsible for the production of (p)ppGpp alarmones (80), was also upregulated in a cyclical hypoxic manner. It has been proposed that the expression of *relA* leads to the accumulation of RpoS, leading to increased protection from subsequent exposure to stress (81). What is especially interesting was our observation that *relA* was below the limit of detection at t=0 and then highly expressed at the initial hypoxic exposure, which could be why we did not see a robust change in *rpoS* at later time points (Figure 2C). The increased expression of multiple gene regulatory cascades suggests that hypoxia requires global and coordinated expression of many downstream products, which occur at regular intervals.

Virulence Factor Production

V. vulnificus has a large repertoire of putative virulence factors that contribute to survival in the host and subsequent colonization (82). Homologous to the multifunctional autoprocessing RTX (MARTX) toxin of *V. cholerae,* the RtxA1 toxin of *V. vulnificus* has a different mode of action, causing lysis of host cell membranes (83). This toxin is transported out of the cell by a set of three genes (*rtxB-rtxD-rtxE*), and we utilized *rtxB* as a proxy for effective transport and function of the toxin (83). Another regulator of virulence, the *toxRS* system, has been shown to increase the production of another putative virulence factor, *vvhA*, which encodes a potent hemolysin (84). It has also been shown to regulate additional key virulence factors in *V. cholerae* and *V. parahaemolyticus*, and we examined *toxR* in this study (84, 85). Finally, we analyzed expression of a gene encoding a putative metalloprotease (*metp)*, which our lab has found to be differentially expressed in human serum when compared to ASW (86). All three genes were found to be cyclically expressed (Figure 2D), with the highest differences occurring again at the first low DO sampling event. We also saw a large decrease (>100 fold) in metalloprotease production at 48 hours *in situ*, and overall the expression of *toxR* varied significantly between low and high DO concentrations, whereas both *rtxB* and *metp* did not show significant changes at all intervals (Figure 2D). Although these factors are typically investigated in host pathogenesis, the high expression at times *in situ*, especially during hypoxia, leads us to hypothesize that they have distinct ecological functions.

Stressosome, Metabolism

We then examined the expression of the newly characterized stressosome module in *V. vulnificus,* and metabolic gene representatives for both anaerobic and aerobic

pathways. Recent analysis showed that *V. vulnificus* possesses genes homologous to the core of the stressosome, *rsbR/S/T*, which has generally been described as a gram-positive mechanism (87). In *Bacillus subtilis* the stressosome senses environmental stress and transmits it to the alternate sigma factor σ^B, which in turn regulates the expression of nearly 150 gene targets (88). However, σ^B is not present in the genome of *V. vulnificus* (86). Expression of all three genes, *rsbR/S/T*, were significantly upregulated in response to environmental hypoxia, as shown in Figure 2E. At each interval, there was differential expression upwards of 10-fold, suggesting that this module is influenced by environmental oxygen availability. Recently, Jia et al. [2016] described that the stressosome module in *V. brasiliensis* is activated by oxygen depletion and becomes inactive when oxygen is replenished, which is driven be ligand binding dynamics (89). The current study, in combination with the findings by Williams et al. [2014d] showing increased expression in ASW compared to human serum and by Jia et al. [2016] further validates the role of oxygen in the activation of the stressosome module in *V. vulnificus.* Lastly, we found that genes involved in both anaerobic (*fnr*) and aerobic (*ilvC* and *purH*) metabolism were upregulated in hypoxia (Figure 2F). The upregulation of *fnr* in low DO was anticipated as it is a main regulatory protein for the recognition of changing O_2 levels and controls regulation of anaerobiosis (90). In addition, although both *ilvC* and *purH* are primarily used during aerobic respiration (91), they can also be utilized during anaerobiosis, which may explain their upregulated expression during hypoxia.

Correlation of Environmental Parameters and Gene Expression

To directly understand the relationship between environmental parameters, primarily DO, and expression of various genes *in situ*, we performed Pearson's

correlational analysis on all expression results. A heat map of these correlations (Fig. 3) revealed that there was a significant ($p < 0.05$) negative correlation between the expression of many of the genes investigated with respect to changing DO. Although we found no significant correlation of those genes involved in type IV pilin formation, flagella, CPS production (*cpsB*), alarmone synthesis, or two of the metabolic genes (*ilvC* and *purH*), they generally trended in a negative direction. Temperature (column 2 in Figure 3) was significantly correlated with *flgE, fnr, wzb, sypC,* and two of the three stressosome genes, however, these genes were all positively correlated, as temperature was negatively correlated with DO. Both salinity and pH displayed very few significant correlations, except for *toxR* (pH and salinity), *rsbR* (salinity), and *wzb* (pH), all three of which were negatively correlated (columns 3-4 in Figure 3). These results suggest that the primary driving condition for gene expression change was DO, although temperature did show many significant correlations.

Bacterial community composition

We collected water samples at each high and low DO sampling event in order to assess bacterial communities, resulting in three high DO profiles and three low DO profiles. Figure 4A shows the relative abundance of the top twenty class-level taxonomic categories identified, with the top seven categories representing approximately 75% of the total OTUs identified. Our analysis identified between 53-58 categories of classified OTUs at the class level, and those unclassified comprised less than 3.5% of all sequence data.

In all samples, *Synechococcophycideae* accounted for ca. 26% ± 5% of all sequences, with the first sample (8/18/15 “Low DO”) having the lowest abundance at

16.7%, followed by *Alphaproteobacteria* (14% ± 1%), *Oscillatoriophycideae* (13% ± 3%), and *Flavobacteriia* (10% ± 1%). The high abundance of *Synechococcophycideae* is not surprising as it is a highly abundant picocyanobacteria inhabiting estuaries worldwide (92). This group is responsible for a significant portion of estuarine primary productivity and its abundance could in part explain why there was such a fluctuation in oxygen availability at this site (93). Additionally, previous studies have shown its abundance to be regulated most frequently by temperature and salinity, both of which remained relatively constant during our study (94). Furthermore, the top seven classifications are all regular estuarine inhabitants, which all fall under either subclasses of cyanobacteria, algal-associated bacteria, or those commonly found in eutrophic sites.

We then analyzed β-diversity from one sampling event to the next over the cyclical hypoxic intervals. For each time point, we calculated community dissimilarity between that sampling event and the next using the Bray-Curtis dissimilarity measure, with 1=perfect dissimilarity and 0=perfect similarity. We found that the communities were highly similar, both temporally and in response to changing oxygen conditions, with only the first time point compared to the second being the quite dissimilar (Figure 4B).

Finally, we compared the community structures as a measure of both the relatedness of members in the community and what members were present. We calculated this based on weighted UniFrac β-diversity measures after jackknifing the bacterial sequences. Visualization of the resultant principle coordinate analysis (PCoA) was performed in Emperor (65). The weighted (Figure 4C) UniFrac analyses showed grouping of samples at low (purple and orange) and high (yellow and blue) oxygen concentrations, although there was one outlier of each group (red and green). It was

interesting that the unweighted analysis (Figure 4D) showed low DO samples clustering together, whereas the high DO samples did not cluster with each other, possibly due to the sensitivity of rare sample abundance in unweighted analyses. This leads us to hypothesize that during normoxia there is a diverse community comprising many species, including those present at low abundance, and that diversity is subsequently lost as oxygen becomes limited. This lack of diversity during hypoxia should be further analyzed by deeper sequencing methods in order to better understand the specific differences between these conditions.

Conclusions

To our knowledge, this is the only comprehensive study on the role of *in situ* hypoxia on gene expression profiles of any *Vibrio* species. We observed the largely cyclical nature of these profiles to be negatively correlated with DO at this site (Figures 2, 3). We also examined bacterial community compositions at both high and low DO sampling events, finding that they were similar, although our unweighted β-diversity suggested that rare sample occurrences might result in a more diverse communities during normoxia (Figure 4). Importantly, this site experiences frequent nutrient inputs from a storm drain and represents a eutrophic estuarine environment, which should be explored further. These highly dynamic environments house a variety of opportunistic pathogens, including *V. vulnificus, V. parahaemolyticus,* and *V. cholerae*, all whose native ranges are expanding (49). This study highlights the importance of studying not only temperature and salinity as it relates to the abundance and ecophysiology of *Vibrio*

species *in situ*, but that of oxygen availability as hypoxia will only increase as global temperatures continue to rise.

Acknowledgments

This work was supported by the National Marine Fisheries Service (NA14NMF4270041, 2009-03571) and a University of North Carolina Research Opportunities Initiative award. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of and the NMFS/or the University of North Carolina system. We kindly thank Adam Reitzel, Tyler Carrier, and Karen Lopez for their help with the 16s rRNA sequencing, and Kevin Lambirth for assistance with analysis. We would also like to thank Amy Ringwood for allowing us to use her data loggers for field monitoring. Supporting data are included as one figure and two tables in an SI file, and all sequence data is available through the NCBI BioSample database (Accession numbers: SAMN05771570, SAMN05771571, SAMN05771572, SAMN05771573, SAMN05771574, SAMN05771575).

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Table 1. Environmental parameters measured at each sampling event

	Parameter	8/18/15	8/19/15	8/20/15
High DO				
	DO (%)	83.4	86.4	96.3
	Temperature (°C)	30.05	31.69	30.21
	Salinity (ppt)	31.69	30.21	29.79
	pH	8.13	8.49	8.26
Low DO				
	DO (%)	21	18.7	22.1
	Temperature (°C)	26.19	27.75	28.39
	Salinity (ppt)	31.68	29.77	27.54
	pH	8.13	7.98	7.59

Table S1. Descriptive Statistics of Environmental Parameters Measured

	Parameter	Mean[b]	Median[b]	SD[b]
High DO				
	DO (%)	88.7	86.4	6.751
	Temperature (°C)	30.7	30.21	0.904
	Salinity (ppt)	30.6	30.21	0.998
	pH	8.3	8.26	0.182
Low DO				
	DO (%)	20.9	21.8	1.882
	Temperature (°C)	27.4	27.75	1.132
	Salinity (ppt)	29.7	29.76	2.077
	pH	7.9	7.98	0.279

[a] Measurements recorded with Hach Hydrolab minisonde 4a data loggers

[b] All statistics run on measurements taken ±1.5 hours around sampling time

TABLE S2 Primers designed for qRT-PCR

Gene	Primer target	5'-3' Sequence[a]	Product size (bp)
Glyceraldehyde phosphate dehydrogenase	*gapdh*	F: TGAAGGCGGTAACCTAATCG R: TACGTCAACACCGATTGCAT	97
Cognate phosphatase	*wzb*	F: GTAAGCCAGCCGATGCAATG R: GCTACACAGCGCAGAGGTAA	98
Mannose-1-phosphate guanyltransferase	*cpsB*	F: TTGCTAAAAGTGAAGCCGCA R: CCAGCTGCACTAGTAGGGTT	109
Polysaccharide export periplasmic protein	*sypC*	F: ATGCAGTTAAAACGGGGCAG R: ACTCATCGTTGCCAGAGTCG	73
Type IV pilin protein subunit	*pilA*	F: GCACAGCTCCAACCAGTAGT R: TTGGCGGCACTTCAACAATG	57
Mannitol-sensitive hemagglutinin	*mshA*	F: CAAGGCGGTTTCACCCTGAT R:CAGATTTAGAAAACGCGGAGCC	90
Flagellar monomeric hook subunit	*flgE*	F: TCTAACTGAGCTGCGGACAA R: TACTGCTCAATCTGGCTGGC	145
S-ribosylhomocysteinase	*luxS*	F: GGGTTGTCGTACCGGTTTCT R: ATCTTCCATCGAAGCCGTCC	88
Sigma factor sigma-38	*rpoS*	F: TTCTCCACTATTAACCGCAG R: GACCGCGATTACTGTAACG	140
(p)ppGpp synthetase	*relA*	F: AAGTGATGTTGTCGGCTCGT R: CCAGCATGGTGGTGATGTCT	89
Fumarate and Nitrate Reductase	*fnr*	F: TGGCCTTTTTGGATCGGCTT R: TCAGGTGGTTGTGCGATTCA	131
Ketol-acid reductoisomerase	*ilvC*	F: TGGTTATCTCAGACACAGCGG R: CATCTGTCCCTACCGAAGGC	99
Purine nucleotide biosynthesis	*purH*	F: CGTCAAAGCCCGTTGTCTTC R: AGAAACGGCCACCGCTATT	150
RTX toxin transporter	*rtxB*	F:GCTCAACCTGCCAGTAGAACAAC R:TCACCCGCTCGTATGTCCAATG	144
Transmembrane regulatory protein	*toxR*	F: AACAACGTGAATGACGCAGC R: CAAGACAACGCAAAGTGGCA	213
Metalloprotease	*metp*	F: CTTACGGAGATGGAGACGGC R: GGATCGCCATCTTCTCCTGG	268
Anti-anti-sigma regulatory factor	*rsbR*	F: GGCTCAGAAACACCCCTGAA R: CACGGGCATGAATCTCTCCA	162
Negative regulator of sigma B	*rsbS*	F: GACCAGAGCAAAAGGGGTCA R: TTCACGACATCGAGTAGGCG	87
Anti-sigma B regulatory factor	*rsbT*	F: GTACGAGATAAAGGGCCGG R: GCAACAATCGTGGTGCCTTT	158

[a] F: Forward primer target, R: Reverse primer target

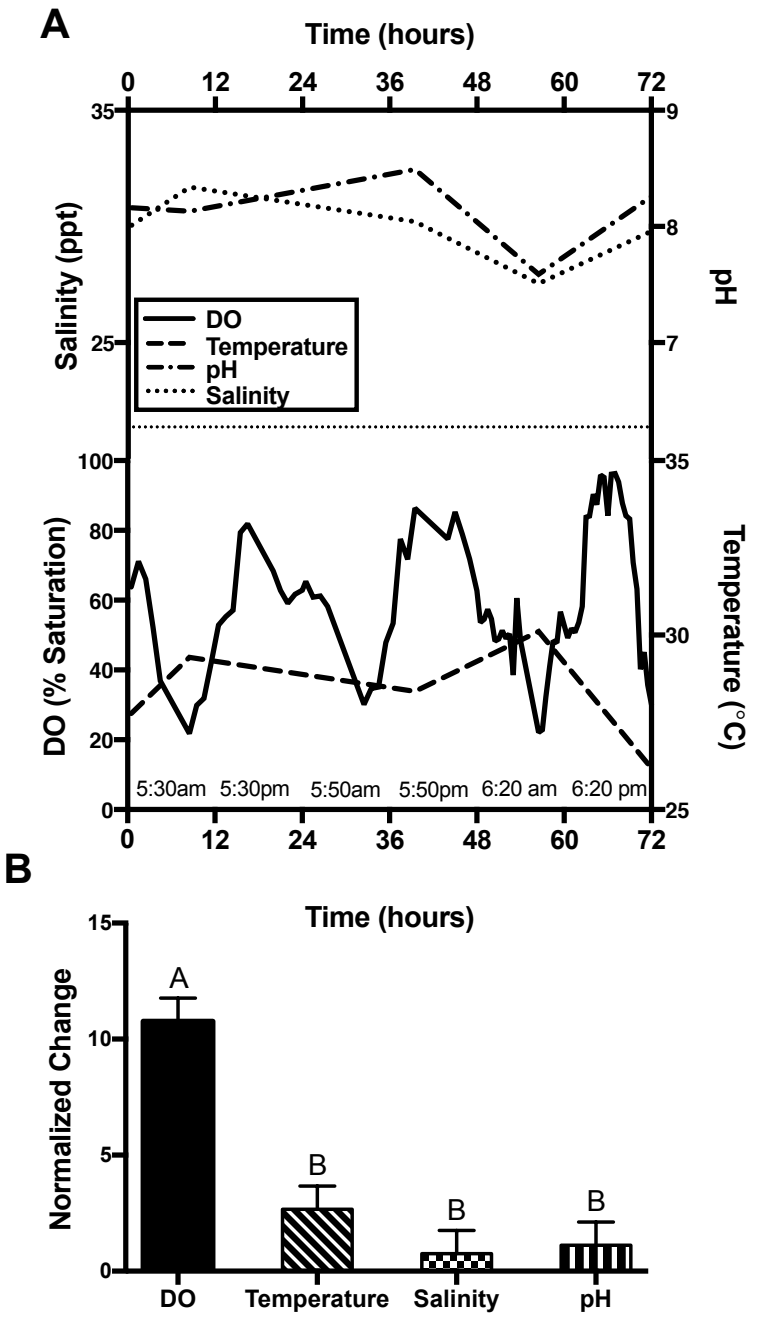

Figure 1. Temporal environmental parameters as measured every 30 minutes for three days (A), and normalized change in DO, temperature, salinity, and pH over time (B). Normalized change was calculated by calculating change from one sampling event to the next followed by subtracting the mean from each measured change and dividing by its SD, values used fell within ±1.5 hours around high and low DO sampling times. One-way ANOVA revealed a significant ($p<0.0001$) difference in DO normalized change when compared to all other parameters.

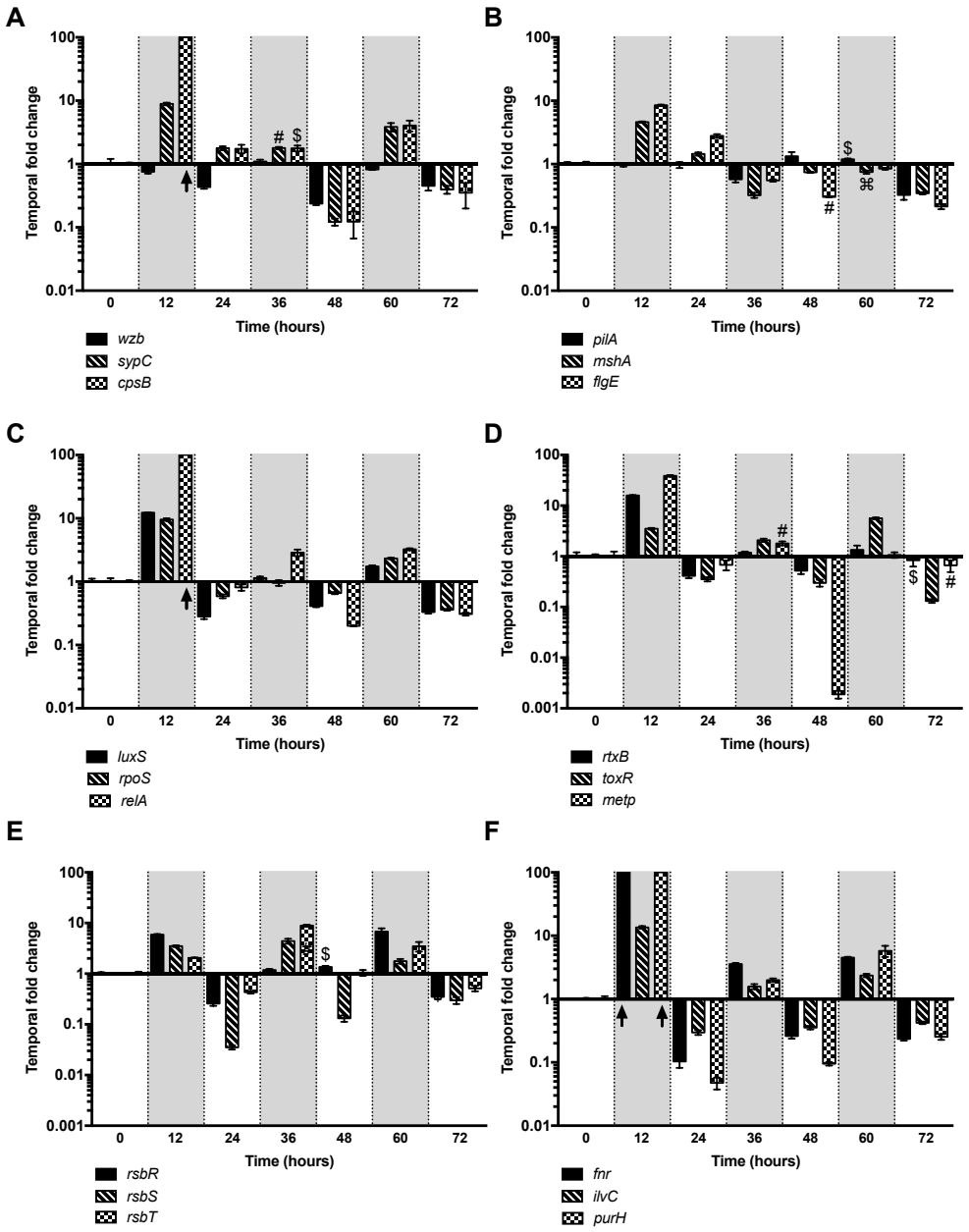

Figure 2. Relative temporal expression of representative genes involved in (A) CPS/EPS production, (B) attachment and motility, (C) global gene regulation, (D) putative virulence factor production, (E) stressosome formation, and (F) metabolic function. Temporal fold change represents expression at corresponding x-axis time relative to previous sample. Error bars represent SD of three biological replicates (n=3), comprising two technical replicates averaged for strain CMCP6. Grey shaded areas indicate hypoxic sampling events. Symbols (#/$/⌘) represent values that were *not* statistically different from the previous time point (one-way ANOVA, $p>0.05$). Arrows indicate that expression was outside the limit of detection at T=0 in which case the fold-changes were set to an arbitrary 100-fold increase.

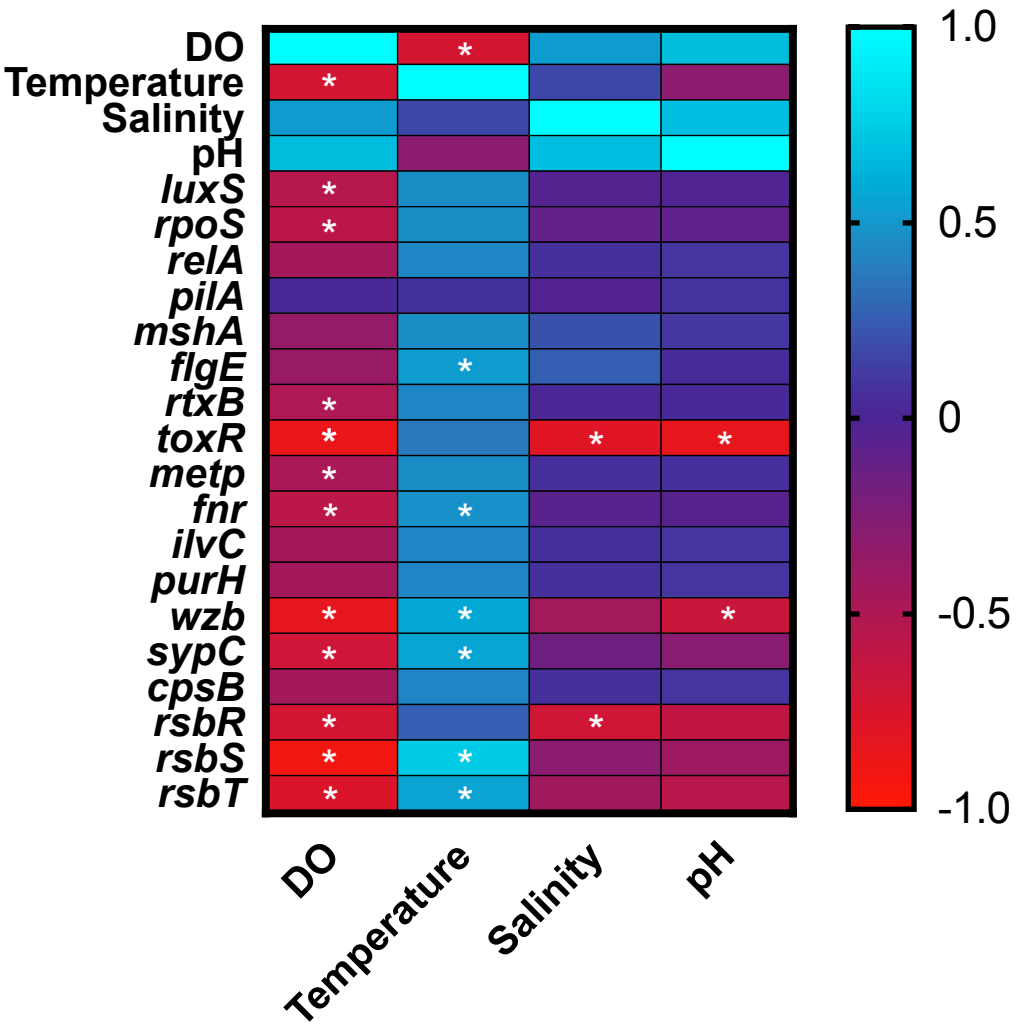

Figure 3. Heat map of correlations between environmental conditions and gene expression by *V. vulnificus*. The correlation coefficients are indicated by color and range from -1 (red, negatively correlated) to 1 (blue, positively correlated) with 0 (purple) indicating no correlation between variables. Environmental values used were taken within ±1.5 hours of the high and low DO sampling times, and were averaged at each sampling event. Averages of 2 biological replicates with 3 technical replicates each were used for expression values. Asterisks represent significant correlations ($p<0.05$) between environmental parameters and gene expression (Pearson Correlation).

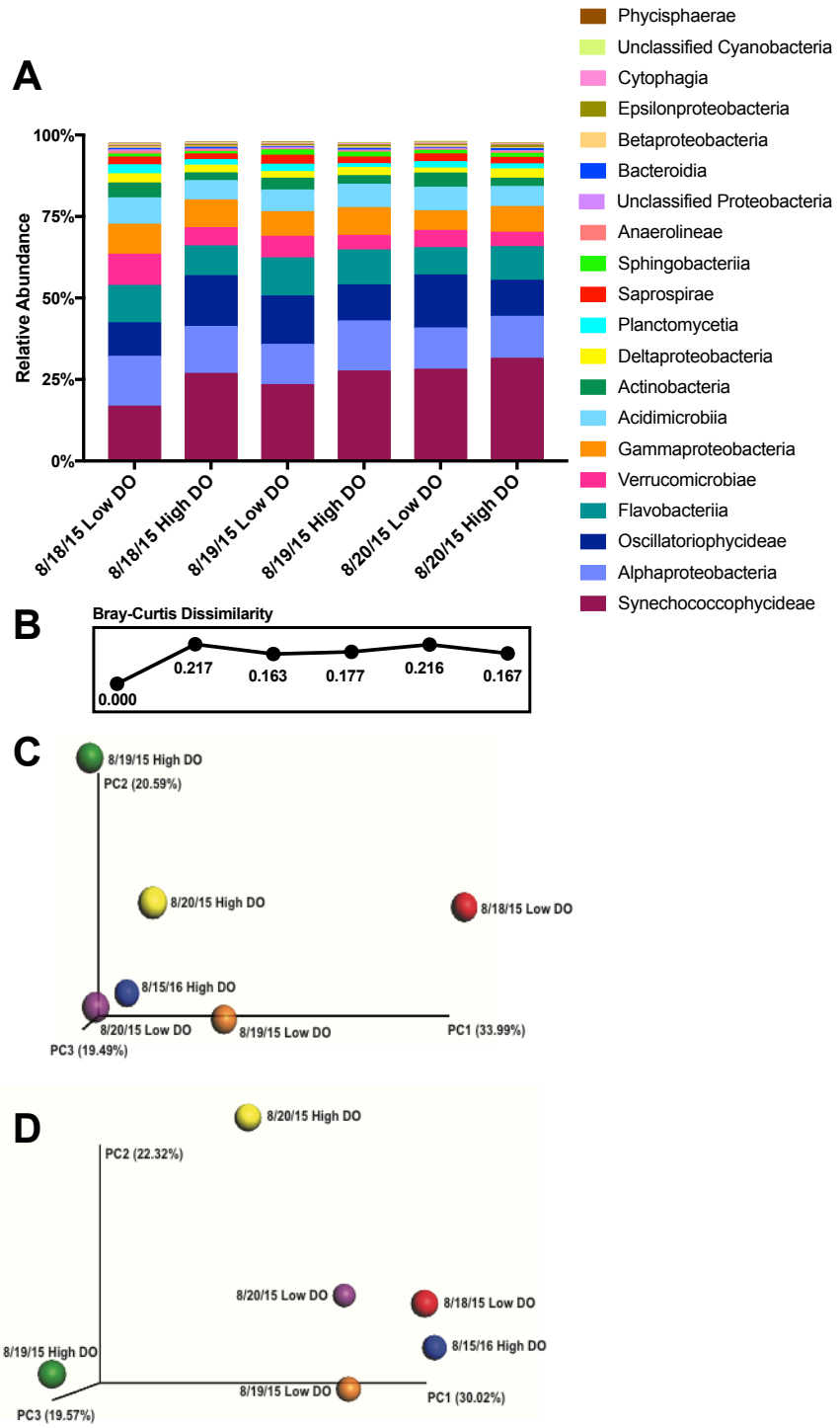

Figure 4. Relative abundance of 16S rRNA sample libraries (A), Bray-Curtis measure of dissimilarity (B), and beta-diversity PCoA plots (C-D) of bacterial communities collected at Hoop Pole Creek over a three-day sampling event. Abundances represent the top 20 OTUs present in the samples. Bray-Curtis represents relative abundances of all OTUs at one time-point compared to the next time point; 0=perfect similarity and 1=complete dissimilarity. Beta diversity of both weighted (C) and unweighted (D) UniFrac distances is shown with colors corresponding to sampling date and DO profile.

CHAPTER 4

TRANSCRIPTOME PROFILES BY CLINICAL AND ENVIRONMENTAL GENOTYPES OF THE OPPORTUNISTIC PATHOGEN *VIBRIO VULNIFICUS* EXPERIENCING OXYGEN LIMITATION

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Abstract

Vibrio vulnificus resides in estuaries worldwide and is isolated from a variety of sources including shellfish, fish, water, and sediment. Genetically diverse, it is divided into three biotypes, of which biotype 1 strains are most often associated with human disease. We divide those strains into environmental (E) and clinical (C) genotypes, with the latter being primarily associated with septicemia. Interestingly, C-genotypes are responsible for 85% of septicemia cases whereas only 15% are caused by E-genotypes, despite there being a predominance of E-genotypes in the environment. Although there are molecular markers for differentiating between strains associated with disease and those typically considered less virulent, the mechanisms by which *V. vulnificus* survives and colonizes during abiotic stress still need more investigation. *V. vulnificus* has the capability to metabolize in both anaerobic and aerobic conditions, and this phenotypic plasticity allows *V. vulnificus* to rapidly adapt to changing environments. Thus, we investigated the genetic mechanisms that facilitate the metabolic flexibility for this pathogen to successfully respond to anoxia, a prevailing environmental and physiological stressor. Utilizing an RNA sequencing approach, we investigated genotype-specific responses by *V. vulnificus* during both normoxia and anoxia, and found a variety of significantly differentially expressed genes (DEGs) in the E-genotype relative to the C-genotype. Our analysis revealed unique responses in both aerobic and anaerobic

conditions as well as many DEGs in both conditions, suggesting that certain loci are differentially expressed but not induced by anoxia. We found that the E-genotype significantly up-regulated the type six secretion system 2 in both conditions, which gave the E-genotypes a competitive advantage in nutrient limited conditions. Together this is the first study to investigate global gene expression differences in both genotypes of *V. vulnificus* both aerobically and anaerobically, providing a foundation for candidate genes/loci to be investigated. We hypothesize that E-genotypes are "environmental specialists" as suggested by the enrichment of genes involved in such processes as nutrient scavenging, quorum sensing, chemotaxis, oyster colonization, and biofilm formation. These findings, in combination with their competitive advantage in conditions similar to estuarine environments, begin to unveil the underlying mechanisms behind the imbalance of strains in the environment.

Introduction

Found in estuaries worldwide the opportunistic human pathogen, *Vibrio vulnificus*, has been isolated from a variety of sources including fish, shellfish, water and sediment (1). This bacterium has the ability to cause septicemia, carrying a 50% mortality rate, after consumption of raw or undercooked seafood. Furthermore, there are certain strains that can cause necrotizing wound infections, which often result in amputation of the infected area and carry a 25% mortality rate (2, 3). Despite the severity of these two clinical presentations there is little evidence explaining why some strains cause disease and others are considered less virulent.

An interesting feature of *V. vulnificus* is its extreme genetic diversity giving rise to three distinct biotypes. Biotype 1 is primarily an opportunistic human pathogen, biotype 2 is pathogenic to eels, and biotype 3 has been shown to be a hybrid of biotypes 1 and 2 and is geographically isolated to tilapia farms in Israel (4, 5). These biotypes all have the ability to cause human disease, however, biotype 1 strains are those most commonly associated with primary septicemia (2). Another layer of differentiation between biotype 1 strains has been described and thus we further separate these strains into clinical (C) and environmental (E) genotypes (6, 7). The former possesses the *vcgC* allele whereas the latter possesses the *vcgE* allele of the "virulence correlated gene", and is utilized as a rapid PCR based identification method of these strains when isolated (6, 7). Further evidence for this distinction has been made through both phylogenetic and multilocus sequence typing (MLST) methods investigating both conserved loci and putative virulence genes (4, 5, 8, 9). Recently, a comparative genomics approach identified a large core genome between these genotypes, however there were 278 and 167 unique genes in the C- and E-genotypes, respectively, as well as unique strain-specific genes within these categories (10).

Even though there is a large population of individuals that are potentially exposed to *V. vulnificus* through either the ingestion of raw or undercooked seafood or through a wound, there are relatively few clinical cases each year (11). This conundrum has led to extensive studies surveying the occurrence of biotype 1 strains in the environment where E-genotypes are typically the dominant genotype (12-14). Recently, Williams et al (2017) found that 89.7% and 86.2% of strains sampled from water and shellfish, respectively, off the coast of North Carolina were E-genotypes. This corresponds with other studies along

the Atlantic and Gulf Coasts of the United States that have shown E-genotypes consistently represent about 85% of total strains in the environment, at least in water, sediment, and shellfish (9, 15-18). It has been hypothesized that the low abundance of C-genotypes in the environment might be why there are not more human infections as they are responsible for approximately 85% of such infections. Nonetheless, there are still many unanswered questions regarding what contributes to their unequal abundances in both host and estuarine environments.

We and others have also hypothesized that these genotypic disparities may be due to the differential response of each genotype to a certain stressor. Previous studies have shown that C-genotypes are significantly more resistant to the bacteriocidal effects of human serum when compared to E-genotypes, although there is a subset of E-genotypes that are also highly resistant (19). Conversely, E-genotypes attach to chitin, the major constituent of marine snow, more efficiently than C-genotypes (20). However, once attached, C-genotypes are better protected against physiological stressors and they detach from chitin significantly more when exposed to conditions lacking oxygen (20, 21). These and other studies have laid the foundation for a hypothesis describing an effective "environmental" profile in the E-genotypes whereas C-genotypes have more of a "virulence" profile (22).

Certain stressors, such as hypoxia/anoxia, are experienced in both the environment and the host, which can provide important evidence for genotype specific responses. Phenotypically, we have previously shown that *V. vulnificus* responds to decreased oxygen in a variety of ways including decreased biofilm formation and increased detachment from chitin (21, 23). These differences were due to underlying

genotypic responses and thus we investigated here gene expression during environmental hypoxia. The present study allowed us to unveil the response of this bacterium to *in situ* hypoxia, showing the differential expression of genes involved in a multitude of functions, including stress responses, motility and attachment, putative virulence, and quorum sensing (24). These studies led us to further question the specific role that oxygen stress alone might play on global gene expression responses in both C- and E-genotypes of *V. vulnificus*. Thus, we utilized a comparative transcriptomic approach to parse the differences between each genotype in both aerobic and anaerobic conditions. The results of this study have shown, for the first time on a whole genome scale, unique E-genotype responses to aerobic and anaerobic conditions. Moreover, we found many differentially expressed genes (DEGs) in the E-genotype relative to the C-genotype that are common in both aerobic and anaerobic conditions. Finally, genes that were significantly increased in both conditions by the E-genotype further substantiates the hypothesis that this genotype is inherently better equipped in the natural environment and thus is more abundant. Here we discuss some of the important unique and shared DEGs in aerobic and anaerobic conditions and focus on their potential implications for environmental maintenance.

Materials and Methods

Bacterial strains and culture conditions

V. vulnificus strains utilized in this study are listed and described in Table 1. Strains were stored at -80°C in Bacto Luria-Bertani broth (LB) (BD Difco, NJ) with 20% glycerol and grown overnight (ON) in Bacto heart infusion (HI) broth (BD Difco, NJ). All strains were

grown ON at 30°C with aeration in a rotary incubator. The strains CMCP6 and JY1701 were chosen for RNA sequencing as they are highly characterized and representative strains of both C and E-genotypes, respectively.

RNA harvesting and purification

CMCP6 and JY1701 were grown as described above and residual HI was washed off by centrifugation at 10000xg for five minutes, followed by pouring off the supernatant and resuspending the pellet in filter-sterilized 15‰ artificial seawater (ASW: Instant Ocean; Aquarium Systems, Mentor, OH, USA). This washing step was performed three times until HI was sufficiently removed. Washed bacterial suspensions were put in modified membrane diffusion chambers as previously described by Phippen et al. (2017), and were placed into aerobic and anaerobic tanks. Covered tanks were filled with sterile 15‰ ASW, and air or 100% nitrogen (Robert Oxygen, Charlotte, NC, USA) were bubbled into each tank for two hours to achieve aerobic and anaerobic environments, respectively.

RNA harvesting and Sequencing

Chambers containing the bacterial suspensions were placed in both aerobic and anaerobic tanks in triplicate and samples were removed after two hours of incubation in each condition. Samples were removed from chambers as we have previously described (24), by removing 1 ml bacterial suspension into sterile syringes that were previously filled with 2 ml RNAprotect (Qiagen, Valencia, CA), and processed using the manufacturer's instructions. RNA extraction was performed as we and others have previously optimized using the RNeasy Mini Kit (Qiagen, Valencia, CA) (20, 23, 24). RNA quality and quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and further analyzed using the Agilent 2100 Bioanalyzer

and 6000 Nano kit (Agilent Technologies). Samples having a 23S/16S rRNA ratio >1.8, an RNA integrity number (RIN) close to 10, and lack of DNA contamination were sent to Duke University Genome Sequencing Center (DUGSC: Durham, NC) for downstream processing. mRNA enrichment, cDNA library preparations, and 1 lane of HiSeq 50bp single-read sequencing was performed on all 12 samples at DUGSC utilizing Illumina's Genome Analyzer.

Data preprocessing

On average, there were 21 million single reads generated for each sample condition containing 3 biological replicates for both C- and E-genotypes. For all downstream analyses the C-genotype was utilized as the "control" and the E-genotype was the "experimental" genotype, analyzing both aerobic and anaerobic conditions. Following methods described previously (22), quality of reads was visualized using FastQC (25) followed by trimming of adapters, low quality reads, and reads shorter than 30 bases long using Trimmomatic (26). Additionally, we removed low quality reads using a trimming window of 4 and a minimum quality score of 20 as well as removing leading and trailing bases that fell below the quality score of 3. The filtered reads were then aligned to the reference genome using Bowtie2 (27), and the resulting alignment was greater than 96% when default settings were applied. SAMTools (28) was used to convert aligned read data to the correct format for downstream analysis.

Expression Analysis, KEGG mapping, and Visualization

The CMCP6 genome was used as a reference for read summarization using FeatureCounts (29), followed by differential gene expression analysis using the EdgeR package (30). Biological variation between replicates was addressed and normalized

using the TMM method as previously described (22, 31). For all analyses the C-genotype strain was used as the baseline condition in both aerobic and anaerobic conditions. All differentially expressed genes (DEGs) that had a $p < 0.05$ and a Log_2 fold change > 1.5 or < -1.5 were categorized as significant and used for further interpretation and analysis.

Functional annotations of statistically significant DEGs were categorized using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (32, 33) PATHWAY, and BRITE modules. This annotates KEGG Orthology (KO) identifiers and this KO system links genes within the genome to biological systems in both BRITE and PATHWAY modules (33). Hypothetical proteins will not map and are not included in the KEGG annotations. We also utilized the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (34) to view predicted protein-protein interactions in the DEGs. These interactions are assigned and combined scores of their interactions are generated using a naïve Bayesian model (34). These scores describe the confidence of the interaction and are visualized in the thickness of the connecting line, the thicker the line signifying the more confidence detected in that interaction.

Competition Experiments

A previously described competition experiment was performed utilizing strains listed in Table 1, with slight modifications (35). All strains were grown as described above, washed and resuspended in 15‰ ASW before each co-culture experiment. CMCP6 was used as the C-genotype for all competitions and a variety of E-genotypes were competed against this strain. Both strains were combined in a 1:1 ratio in fresh media before spotting 20µl onto 13mm Millipore Express® PLUS Polyethersulfone membrane filters that were placed shiny-side down on ASW agar plates (ASW with the

addition of 1.5% agar). Plates were incubated at 30˚C and at two hours the plates were removed. Filters were aseptically removed and placed in tubes containing phosphate buffered solution and 0.2mg 2.9mm ZR BashingBeads™. All samples were vortexed at maximum speed for one minute to remove bacteria from filters, diluted, and plated on mannitol agar (MA: 10g D-mannitol, 15g agar, and 16g Phenol Red Broth Base; BD Difco, NJ). MA was utilized to differentiate between CMCP6 and E-genotype strains as CMCP6 can ferment mannitol and grows yellow colonies whereas the E-genotype strains utilized in this study do not, and grow pink. Competitive indices were calculated using a previously described equation (36) where the difference in CFUs/ml of CMCP6 in mono-culture compared to co-culture is compared to the difference in CFUs/ml of competing E-genotype strain in mono-culture compared to co-culture. All experiments were completed in triplicate with six technical replicates for each competition and all indices were log transformed.

Results and Discussion

RNA sequencing results

Comparative transcriptomics of the E-genotype JY701 and the C-genotype CMCP6 were performed after two-hour exposures to both aerobic and anaerobic conditions. All results are based on the “within condition” comparisons of the E-genotype relative to the C-genotype. Figure 1 depicts a linear compressed view of differentially expressed genes (DEGs) in aerobic (A) and anaerobic (B) conditions and is separated by chromosome. Comprehensive tables of all DEGs that were uniquely up-regulated in

aerobic and anaerobic conditions as well as those increased in both conditions can be found in Tables 2-4b, and contain the genes further discussed below.

At first glance, Figure 1 suggests a general trend of many significant ($p < 0.05$) DEGs between genotypes in both conditions, and that these responses are similar. Additionally, there are far more DEGs on chromosome II and of those genes it shows that there are more down-regulated genes in both conditions. To focus on those DEGs that are more likely to confer a biologically relevant phenotype, we added more strenuous filters by limiting what we classified as significant by having both $p < 0.05$ and a Log_2 fold change > 1.5 or < -1.5. A summary of filtered DEGs is shown in Figure 2, confirming that there are more DEGs in both conditions, compared to unique DEGs in each condition. In agreement with the linear compressed view we show that in all cases there was a higher percentage of DEGs on chromosome II, and regardless of the chromosome most genes were significantly down-regulated, as indicated in Figure 2.

There has always been some question of the importance of a two-chromosome system in *Vibrio* species and these results support the hypothesis that chromosome II may be more of an "experimental" chromosome. Although both chromosomes contain required housekeeping genes, chromosome II has been shown to have more plasticity, varying in size between strains/species and containing a higher proportion of uncharacterized genes (37). Our finding that a higher percentage of DEGs are found on chromosome II supports other comparative transcriptome studies, however they do not specifically investigate both genotypes of *V. vulnificus* (22). Furthermore, the fact that 554 genes were differentially expressed in both aerobic and anaerobic conditions between

genotypes supports the idea that the baseline transcriptome between these two genotypes is intrinsically different.

KEGG functional analysis

In order to functionally characterize the DEGs we utilized KEGG ontology (KO) annotations and mapped those with KEGG PATHWAY and BRITE modules (32, 33). Although this provides an overview of DEGs in functional categories, it does not represent genes that are uncharacterized or hypothetical, which is why all DEGs are not shown in this analysis. Although in both conditions there are many DEGs represented in the metabolism hierarchy, Figure 3 shows that the number of genes and categories are noticeably different. Aerobically, metabolic genes are present in all categories except glycan biosynthesis, suggesting broad metabolic potential. However, anaerobically the functional groups were more limited, showing DEGs in glycan biosynthesis, cofactor/vitamin, and lipid metabolisms, which may be indicative of a precise metabolic response to anoxia (Figure 3). There are also no genes in either condition specifically tied to biofilm formation, and translation is not represented during anaerobic conditions. We also identified the DEGS that were differentially expressed in both conditions by the E-genotype (Figure 4), which shows some functional differences. There was no difference between the genotypes in the genetic information processing hierarchy, indicating the importance for these functions in both genotypes and both conditions. Finally, this analysis suggests that E-genotypes are intrinsically more efficient at both cellular processes and environmental information processing, which again may explain why these strains are more abundant in the natural environment.

Unique up-regulated genes

There were only 41 genes significantly up-regulated by the E-genotype in aerobic conditions representing a mere 0.85% of the total genome (Figure 2; a comprehensive list can be found in Table 2). However, when analyzing the protein-protein interactions there was a significant enrichment in genes involved in aerobic metabolism (Figure 5). Although not enriched during the interactome analysis, there were also multiple genes involved in motility and chemotaxis, translation, and peptide transport mechanisms significantly up-regulated aerobically. The relatively few unique significantly up-regulated genes during aerobic conditions suggests that both genotypes have a similar baseline response to our control experimental parameters.

When comparing these same strains in anaerobic conditions we found that the E-genotype significantly up-regulated 111 genes or 2.3% of their genome (Figure 2) compared to the C-genotype. Closer examination of these genes (Table 3a-b) reveals a much more diverse repertoire of DEGs with a higher percentage occurring on chromosome II. The STRING analysis (Fig. 6) found two significantly enriched systems, quorum sensing and nitrogen metabolism, which were increased anaerobically. Another interesting finding was the high incidence of genes that are uncharacterized or encode only hypothetical proteins during this condition, with 20 compared to only 3 during aerobic conditions.

The fact that aerobic metabolism and nitrogen metabolism were up-regulated in aerobic and anaerobic conditions, respectively, is not necessarily surprising as both systems are important for metabolic function in both conditions. What is interesting however is the fact that the E-genotype increased these systems compared to the C-

genotype, which may provide it a fitness advantage in both conditions. During anaerobiosis, *V. vulnificus* must either utilize fermentation or switch from using oxygen as its final electron acceptor to another compound such as nitrate, nitrite, sulfate, or fumarate in anaerobic respiration. We found many genes involved in anaerobic nitrite/ammonia oxidation, formate-nitrite reduction, and fermentation to be significantly up-regulated by the E-genotype, suggesting that this genotype is better suited in conditions lacking oxygen. Furthermore, as some of these systems are important in marine nitrogen cycling, the finding that E-genotypes are more effective contributors to this cycle might have important implications in driving population dynamics or unique niche utilization (38).

The second anaerobically enriched system was for the interspecific quorum sensing (QS) pathway, which facilitates coordinated gene expression by bacterial communication. This communication is driven by the production of auto-inducers that accumulate in a density-dependent manner, which triggers a signaling cascade and downstream responses by the bacterium (39). Although *V. vulnificus* has two different QS systems present in their genome (22), autoinducer 2 and 3, only the interspecific system (autoinducer 2) is significantly increased during anoxia. The QS regulator (*luxO)* was increased during anoxia and has previously been shown to be important in cytotoxicity, host colonization, motility, and biofilm formation in multiple *Vibrio* species (40-42). Specifically, mutants in *luxO* are less cytotoxic, unable to colonize murine models but can form more robust biofilms, and are significantly more motile (40, 42). As there are many regulators of QS in *V. vulnificus* it is not possible in this context to completely understand what this increase means for the E-genotype biologically, but it can be

hypothesized that the increase might coordinate specific behaviors compared to the C-genotype. These results also confirm previous studies in which we saw increased expression of other QS genes during anaerobiosis, solidifying that oxygen is important in regulating this phenomenon (24). Although we do not discuss these in detail here, there are other interesting systems up-regulated but not enriched during anaerobiosis (Table 3a-b), such as the exopolysaccharide cluster I and multiple flagellar and chemotaxis proteins. These systems in combination with the enriched systems begin to differentiate the unique responses to both genotypes in a prevailing host and environmental stressor.

Genes up-regulated in both conditions

Nearly 11.5% of the genome was differentially expressed in both aerobic and anaerobic conditions between genotypes with 3.9% or 189 genes significantly increased (Figure 2). The STRING analysis (Figure 7) showed a variety of significantly enriched protein networks responsible for the type six secretion system, bacterial chemotaxis, phosphate transport, oligopeptide permease system, and siderophore transport during both aerobic and anaerobic conditions. Although we focus here largely on these enriched systems, there are many additional DEGs described in Tables 4a and 4b which are separated by chromosome I and II, respectively. Unfortunately, a large number of genes represented hypothetical proteins, however a few intriguing genes/loci might be of future interest such as the tight adherence (*tad*) pilus locus, which has been shown to be important in the ability of *V. vulnificus* to colonize oysters, form aggregates, and biofilms (43). Our finding (44) that E-genotypes increase this system relative to C-genotypes (Table 4a) could not only explain why there are few septicemia cases, but also why we

and others have reported only a small proportion of *V. vulnificus* cells in oysters and other sources in the environment to be the C-genotype (45, 46).

The ability for bacteria to effectively bring in and utilize nutrients in the marine environment is extremely important as many are quite limited. The oligopeptide permease (Opp) system has been described in a variety of bacteria, including *V. vulnificus* (47, 48), and we found it to be significantly up-regulated and enriched in E-genotypes regardless of the condition (Figure 7 and Table 4a). This system is traditionally described as having a nutritional role by assisting in the capture of peptides from the environment as a carbon source, however, it has been shown to also be important for virulence (48-50). In *V. fluvialis,* this system is essential for both biofilm production and effective hemolytic activity and others have shown that this system is an important mediator of peptide mediated cell-to-cell communication (51-53). This system has previously been shown to be up-regulated when *E. coli* is grown anaerobically, as well as with increased starvation time in *V. alginolyticus* (44, 48), both conditions of which are being experienced by *V. vulnificus* in this study.

Inorganic phosphate and iron, both essential bacterial nutrients, , are limited in the environment and are strategically depleted by hosts to protect against bacterial infection. Two systems which allow for the sequestration of phosphate and iron were enriched in both conditions (Figure 7). The former, known as the Pst system, contains four proteins: PtsS, PtsC, PtsA, and PtsB (54). These form an ATP binding cassette transporter which is responsible for transport of phosphate into the cytosol (55), and were all significantly up-regulated by the E-genotype regardless of oxygen presence (Table 4b). Iron sequestration, including the TonB3 system and additional genes for iron acquisition, were

also enriched in our study, as shown in Figure 7 and Table 4a. The *V. vulnificus* genome contains three canonical TonB systems, with the first two having been shown to be increased in conditions where iron is limited, whereas TonB3 was not increased (56). This is an interesting finding as this system has not been shown to be involved in transport of iron in limiting conditions in *V. vulnificus*, but is induced in human serum with and without the addition of iron (56). The limitation of this study is that it was done only in a C-genotype strain (CMCP6), but not in an E-genotype strain. Its inherent increase in E-genotypes in aerobic and anaerobic conditions might provide an unidentified fitness advantage which needs to be further explored.

Bacterial chemotaxis is important in *Vibrio* species for a variety of functions including both flagellar and non-flagellar mediated motility, biofilm formation, and host virulence (22, 57, 58). Our results confirm the results reported by Williams et al. (2014) in which we found that the CheA3 operon was significantly upregulated in ASW (Table 4b). However, we expand on that here to show that E-genotypes enrich that system in both conditions (Figure 7). In addition to this system there were multiple methyl-accepting chemotaxis proteins increased which were directly linked in the protein-protein interaction network (Figure 7). Although *V. vulnificus* has two chemotaxis gene clusters, this was the only one differentially expressed in the E-genotypes, which may indicate a more functional role in this genotype, and since the other system has been linked to virulence, might further explain the increased pathogenicity of C-genotypes.

The type six secretion system (T6SS) is an extremely versatile, contact-dependent, molecular weapon with the ability to kill a variety of prey. In *V. vulnificus* it has been shown to kill both prokaryotic and eukaryotic cells and can be an important

mechanism for survival and horizontal gene transfer (59). In *V. vulnificus,* there are two T6SS that were described by Church et al. (2016), T6SS1 and T6SS2, and they found that only T6SS1 provided antibacterial properties. Furthermore, unpublished data from our lab has revealed that most *V. vulnificus* strains contain the T6SS2, whereas the majority of strains possessing T6SS1 are C-genotype. The strains utilized in the present study both contain only the T6SS2, and the entire system is differentially expressed by the E-genotypes both aerobically and anaerobically (Figure 8A) and is enriched in the protein analysis (Figure 7). As this is a potential mechanism for population dynamics in estuarine environments, we hypothesized that the E-genotype would have a competitive advantage over the C-genotype.

We utilized a previously described bacterial competition assay (35) to assess the competitive index of our C-genotype strain versus multiple E-genotypes containing only the T6SS2 system. There was one key difference in our study in that all of our competitions were done in nutrient limited media (ASW) rather than complex and nutritionally robust media, which is typical for such studies. This was done in order to understand the competitive dynamics in the same conditions as our transcriptome results and to allow us to compare these studies. When competing these strains for two hours in ASW we saw that all E-genotypes tested had a competitive index > 0, indicating a clear advantage in this environment as shown in Figure 8B. This is a very compelling argument for the importance of T6SS2 in competitive dynamics in the environment, as these conditions are more comparable to the natural environment than previous studies. However, we are currently preparing mutants in both of these genotypes in order to

understand whether this competitive advantage is due to the T6SS2 or another mechanism that may be differentially expressed in our analysis.

Conclusions

To our knowledge this is the first comparative transcriptomic study investigating the profiles of both C- and E-genotypes of *V. vulnificus*, and further investigates anoxia, which is an important environmental and physiological stressor. We have shown that the E-genotypes increase a variety of unique genes in both aerobic and anaerobic conditions, and that there is a large set of genes increased in both conditions compared to the C-genotype. The enriched categories lead us to characterize the E-genotype as an "environmental specialist" when compared to the C-genotype, as it increases a variety of systems that have been shown to aid in environmental persistence. The E-genotype not only increased systems involved in the transport of nutritionally essential compounds, but also loci important for colonization, motility, attachment, and bacterial competition via the T6SS2. These findings have given us an advantageous pool of DEGs that should be investigated further, especially in the context of *in situ* responses. We have also shown that the E-genotypes have a competitive advantage in nutrient limited environments, however more investigation will need to be done to understand the mechanism behind this result and allow us to determine if it is related to their intrinsic up-regulation of T6SS2. Finally, although not discussed here, there were 803 genes significantly down-regulated by the E-genotypes over both conditions, which may reveal differentially expressed virulence mechanisms for the C-genotype that we have not yet identified during anoxia.

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Table 1 Characterization of *Vibrio vulnificus* strains utilized in this study

Strain	Genotype	Isolation Source	Biotype
CMCP6	C	Blood	1
JY1701	E	Oyster	1
E64MW	E	Wound	1
ENV1	E	Water	1
VVL1	E	Wound	1

Table 2. Significantly differentially expressed genes in *Vibrio vulnificus* that were up-regulated by the E-genotype during aerobic conditions. Only genes where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5 are included.

Gene ID	Log2 Fold Change	Protein
Chromosome I		
VV1_0155	1.81795	succinyl-CoA ligase subunit beta
VV1_0158	1.53520	succinate dehydrogenase iron-sulfur subunit
VV1_0159	1.73658	succinate dehydrogenase flavoprotein subunit, SdhA
VV1_0162	1.51353	citrate (Si)-synthase, GltA
VV1_0218	1.50274	flagellar rod assembly protein/muramidase, FlgJ
VV1_0219	1.50928	flagellar P-ring protein, FlgI
VV1_0449	1.92923	isocitrate lyase, AceA
VV1_0514	1.59361	Sodium-dependent phosphate transporter
VV1_0841	1.77356	peptide ABC transporter permease
VV1_1020	1.67554	ATP synthase subunit gamma
VV1_1022	1.63890	ATP synthase epsilon chain
VV1_1386	1.70188	ribulose-phosphate 3-epimerase, rpe
VV1_1465	1.65343	aspartate carbamoyltransferase, PyrB
VV1_1828	1.79493	hypothetical protein
VV1_1859	1.72502	30S ribosomal protein S2, RpsB
VV1_1860	1.61896	elongation factor Ts
VV1_1896	1.56566	acyl-CoA dehydrogenase
VV1_2009	1.61398	DNA-binding response regulator
VV1_2066	1.74874	chemotaxis protein
VV1_2119	1.77736	cold shock domain protein CspD
VV1_2237	1.83694	ABC transporter substrate-binding protein
VV1_2349	2.06019	lactonizing lipase
VV1_2399	1.66466	50S ribosomal protein L35, RpmI
VV1_2703	1.81758	amino acid ABC transporter substrate-binding protein
VV1_2704	2.00475	amino acid ABC transporter permease
VV1_2729	1.73682	probable AcnD-accessory protein PrpF
VV1_2730	2.07498	Fe/S-dependent 2-methylisocitrate dehydratase AcnD
VV1_2831	1.51116	acyl-CoA dehydrogenase
VV1_2975	1.93993	peptide ABC transporter permease, OppC
VV1_3066	1.74591	anthranilate phosphoribosyltransferase
VV1_3067	1.91991	bifunctional indole-3-glycerol phosphate synthase
VV1_3080	1.62573	peptide ABC transporter permease, OppC
VV1_3082	1.54535	peptide ABC transporter substrate-binding protein, OppA
Chromosome II		
VV2_0005	1.90030	phosphoenolpyruvate synthase, PpsA
VV2_0231	1.60237	hypothetical protein
VV2_0260	1.52144	2OG-Fe(II) oxygenase
VV2_0717	1.52758	membrane protein
VV2_0764	1.67874	glyceraldehyde-3-phosphate dehydrogenase, Gap
VV2_0799	1.73067	(Fe-S)-binding protein
VV2_0828	1.69421	hypothetical protein
VV2_1117	1.96668	1-pyrroline-5-carboxylate dehydrogenase

Table 3a. Significantly differentially expressed genes in *Vibrio vulnificus* that were up-regulated by the E-genotype during anaerobic conditions on chromosome I. Only genes where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5 are included.

Gene ID	Log2 Fold Change	Protein
Chromosome I		
VV1_0029	1.63270	C4-dicarboxylate ABC transporter permease
VV1_0040	1.57601	chemotaxis protein
VV1_0205	1.64645	multidrug transporter
VV1_0244	3.44836	hypothetical protein
VV1_0726	1.73781	sulfate adenylyltransferase subunit 2
VV1_0727	1.57133	uroporphyrin-III C-methyltransferase
VV1_0890	1.81839	hypothetical protein
VV1_0986	2.42292	tRNA 2-thiouridine(34) synthase TusA
VV1_1176	1.58024	RNA-binding protein
VV1_1309	1.99515	nuclease
VV1_1750	1.73376	hypothetical protein
VV1_1754	1.54290	type II/IV secretion system ATPase TadZ/CpaE
VV1_1767	1.67842	beta-galactosidase subunit alpha: ebgA
VV1_1777	1.74323	tRNA-binding protein
VV1_1856	2.29014	hypothetical protein
VV1_1939	1.50019	flagellar protein FliJ
VV1_1947	1.50901	flagellar biosynthesis protein FliR
VV1_2062	1.96880	inosine-5-monophosphate dehydrogenase
VV1_2091	1.55519	hypothetical protein
VV1_2092	1.64046	PrkA family serine protein kinase
VV1_2093	1.60609	hypothetical protein
VV1_2218	1.58352	XRE family transcriptional regulator, MetR
VV1_2274	1.70436	dihydroorotate dehydrogenase
VV1_2367	1.59691	membrane protein
VV1_2587	1.81132	(Fe-S)-binding protein
VV1_2588	1.66659	TorD family cytoplasmic chaperone
VV1_2590	1.63628	formate dehydrogenase
VV1_2591	1.86389	formate dehydrogenase
VV1_2592	1.71321	formate dehydrogenase subunit gamma
VV1_2593	1.52698	membrane protein
VV1_2656	1.51969	GGDEF domain-containing protein
VV1_2658	2.20462	anti-anti-sigma regulatory factor: syp locus
VV1_2659	1.69091	membrane protein: eps
VV1_2660	1.58013	sugar ABC transporter substrate-binding protein
VV1_2664	1.72777	sigma-54-dependent Fis transcriptional regulator
VV1_2665	1.62745	glycosyl transferase
VV1_2672	1.52425	chain-length determining protein
VV1_2684	1.58918	glucose-6-phosphate dehydrogenase
VV1_2774	2.24227	ribosomal RNA large subunit methyltransferase F
VV1_2775	1.50641	metal-dependent hydrolase
VV1_2776	1.85866	NrdH-redoxin
VV1_2815	1.56790	ATPase
VV1_2820	1.60900	DNA transformation protein
VV1_2838	1.56038	ABC transporter ATP-binding protein
VV1_2863	1.61263	nitric oxide reductase transcription regulator: nor
VV1_2929	1.67222	hypothetical protein
VV1_2931	1.80488	zinc ABC transporter permease
VV1_2932	1.87743	cyclopropane-fatty-acyl-phospholipid synthase
VV1_2935	1.58221	short-chain dehydrogenase
VV1_2961	1.71356	hypothetical protein
VV1_2964	1.75839	peptidase, TcbB
VV1_3030	1.92357	thiol:disulfide interchange protein
VV1_3031	1.51834	cytochrome c
VV1_3034	1.66641	cytochrome c nitrite reductase pentaheme subunit
VV1_3035	2.26455	nitrite reductase c552 subunit
VV1_3037	1.69781	cytochrome C
VV1_3047	1.91726	transcriptional regulator

Table 3b. Significantly differentially expressed genes in *Vibrio vulnificus* that were up-regulated by the E-genotype during anaerobic conditions on chromosome II. Only genes where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5 are included.

Gene ID	Log2 Fold Change	Protein
Chromosome II		
VV2_0049	1.55688	deacylase
VV2_0136	1.54957	DEAD/DEAH box helicase
VV2_0139	1.62674	response regulator
VV2_0155	1.88422	hypothetical protein
VV2_0157	1.57936	aerotolerance operon protein BatA
VV2_0160	1.71200	LysR family transcriptional regulator
VV2_0163	1.78250	anaerobic sulfatase maturase
VV2_0167	1.77418	NADH:ubiquinone oxidoreductase
VV2_0205	1.68875	diguanylate phosphodiesterase
VV2_0342	1.55375	transporter
VV2_0343	2.15965	membrane protein
VV2_0344	1.98239	ArsR family transcriptional regulator
VV2_0389	1.71414	nitrite reductase small subunit
VV2_0391	2.09183	nitrate ABC transporter ATP-binding protein
VV2_0392	2.55692	nitrate ABC transporter permease
VV2_0393	2.10163	nitrate ABC transporter substrate-binding protein
VV2_0394	1.59846	ANTAR domain-containing protein
VV2_0397	2.01834	uroporphyrin-III C-methyltransferase
VV2_0398	2.03216	nitrite reductase
VV2_0411	1.54321	anaerobic C4-dicarboxylate transporter DcuC
VV2_0433	1.92703	lysozyme: T6SS ImpF
VV2_0467	1.64672	hypothetical protein
VV2_0484	1.53092	RTX toxin transporter
VV2_0562	1.62080	hypothetical protein
VV2_0567	1.67592	cytochrome c oxidase subunit I
VV2_0720	2.08893	nitrate reductase
VV2_0721	1.77258	nitrate reductase catalytic subunit
VV2_0722	2.25104	nitrate reductase
VV2_0723	1.90485	cytochrome c
VV2_0930	1.68546	hypothetical protein
VV2_0931	1.57601	hybrid sensor histidine kinase/response regulator
VV2_0942	2.61010	elongation factor G
VV2_0947	1.93174	hypothetical protein
VV2_0980	1.52510	two-component system sensor histidine kinase
VV2_1009	1.58984	hypothetical protein
VV2_1038	1.58132	peptide-methionine (S)-S-oxide reductase
VV2_1045	1.62966	glutaredoxin 3
VV2_1067	2.08326	C4-dicarboxylate ABC transporter permease
VV2_1070	1.50663	uronate isomerase
VV2_1195	1.71652	chemotaxis protein
VV2_1256	1.62632	AsnC family transcriptional regulator
VV2_1316	1.90685	hypothetical protein
VV2_1320	1.52970	acriflavin resistance protein
VV2_1322	1.50876	TetR family transcriptional regulator
VV2_1324	1.58528	galactoside ABC transporter permease MglC
VV2_1397	1.58084	hypothetical protein
VV2_1398	1.95567	hypothetical protein
VV2_1430	1.92989	hypothetical protein
VV2_1549	1.59098	TonB-dependent heme receptor HutR
VV2_1550	1.65259	hypothetical protein
VV2_1666	1.82767	hypothetical protein
VV2_1681	1.75894	D-alanyl-D-alanine carboxypeptidase
VV2_1693	1.91635	phage-related tail fiber protein
VV2_1698	1.64171	hypothetical protein

Table 4a. Significantly differentially expressed genes in *Vibrio vulnificus* that were up-regulated by the E-genotype during both aerobic and anaerobic conditions on chromosome I. Only genes where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5 are included.

Gene ID	Log2 Fold Change	Protein	Gene ID	Log2 Fold Change	Protein
Chrom I			**Chrom I**		
VV1_0032	1.66239	sugar ABC transporter ATP-binding protein	VV1_2240	2.13068	sensor histidine kinase
VV1_0034	1.90338	sugar ABC transporter permease	VV1_2241	1.69322	DNA-binding response regulator
VV1_0035	1.96345	sugar ABC transporter substrate-binding protein	VV1_2301	2.99243	alanine glycine permease
VV1_0036	1.52020	two-component system sensor histidine kinase	VV1_2318	1.52074	molecular chaperone DnaK
VV1_0037	1.67036	sugar ABC transporter permease	VV1_2342	1.51593	hypothetical protein
VV1_0057	1.62695	lipoprotein	VV1_2362	2.31123	transcriptional regulator
VV1_0160	2.19640	succinate dehydrogenase, anchor protein	VV1_2372	1.66476	nicotinate phosphoribosyltransferase
VV1_0161	2.31651	cytochrome b556 large membrane subunit	VV1_2373	1.54761	NUDIX hydrolase
VV1_0216	2.09251	flagellar hook-associated protein FlgL	VV1_2516	1.86703	transcriptional regulator
VV1_0557	1.52571	hypothetical protein	VV1_2555	2.96381	hypothetical protein
VV1_0838	1.98095	peptide ABC transporter ATPase	VV1_2570	2.52792	N-acetyl-D-glucosamine kinase
VV1_0839	2.10767	ABC transporter substrate-binding protein	VV1_2661	2.29300	chromosome partitioning protein ParA
VV1_0840	2.01369	peptide ABC transporter permease	VV1_2697	1.64103	threonine transporter RhtB
VV1_0842	2.73093	TonB-dependent, OMB receptor for ferrienterochelin and colicins	VV1_2705	2.20495	amino acid ABC transporter permease
VV1_0843	2.24100	DUF3450 domain-containing protein	VV1_2706	2.35470	arginine ABC transporter ATP-binding protein
VV1_0844	1.95105	TolR biopolymer transporter ExbB	VV1_2737	1.83647	tricarboxylic transport TctB
VV1_0845	2.38704	Ferric siderophore transport system ExbB	VV1_2739	1.85011	tripartite tricarboxylate transporter TctA
VV1_0846	2.57958	biopolymer transporter ExbD	VV1_2743	2.00326	hypothetical protein
VV1_0847	2.38094	Ferric vibriobactin sugar ABC transporter substrate-binding protein	VV1_2746	1.84793	permease
VV1_0848	2.26650	TPR domain-containing protein component of TonB system	VV1_2749	1.80500	ABC transporter substrate-binding protein
VV1_0897	2.32457	methylase	VV1_2777	1.97215	LysR family transcriptional regulator
VV1_1026	1.86585	Bcr/CflA family drug resistance efflux transporter	VV1_2808	2.23165	cysteine desulfurase-like protein
VV1_1104	1.59225	hypothetical protein	VV1_2809	2.18381	agglutination protein
VV1_1237	2.67130	acetyl-coenzyme A synthetase	VV1_2851	1.60790	hypothetical protein
VV1_1239	1.63016	cyclic nucleotide-binding protein	VV1_2862	1.62119	membrane protein
VV1_1243	2.50578	cation acetate symporter	VV1_2939	2.25907	hypothetical protein
VV1_1244	3.25847	membrane protein	VV1_2960	2.42168	hypothetical protein
VV1_1308	1.72798	hypothetical protein	VV1_2963	1.71016	lipoprotein
VV1_1464	2.09072	aspartate carbamoyltransferase regulatory chain	VV1_2970	2.83515	ATPase
VV1_1723	1.63990	nucleoside transporter NupC	VV1_2971	2.48260	membrane protein
VV1_1755	1.51612	type II/IV secretion system ATP hydrolase TadA	VV1_2972	2.75423	oligopeptide ABC transporter ATPase
VV1_1757	1.74647	pilus assembly protein TadC	VV1_2973	2.38360	oligopeptide ABC transporter ATP-binding protein
VV1_1764	1.68749	amino acid permease	VV1_2974	2.81512	ABC transporter substrate-binding protein
VV1_1765	2.09950	amino acid permease	VV1_2976	2.13868	peptide ABC transporter permease
VV1_1766	2.45834	beta-D-galactosidase subunit beta	VV1_3064	2.10940	anthranilate synthase component I
VV1_1813	3.33323	hypothetical protein	VV1_3065	2.20489	glutamine amidotransferase
VV1_1814	2.59227	RNA polymerase sigma factor	VV1_3068	2.31860	tryptophan synthase subunit beta
VV1_1815	2.01118	transcriptional regulator	VV1_3069	2.46728	tryptophan synthase subunit alpha
VV1_1841	2.33887	aminobenzoyl-glutamate transporter	VV1_3078	2.14852	oligopeptide ABC transporter ATP-binding protein OppF
VV1_1944	1.56498	flagellar biosynthesis protein FliO	VV1_3124	1.51815	Ktr system potassium transporter B
VV1_1945	1.58438	flagellar biosynthetic protein FliP	VV1_3125	1.79981	sugar isomerase
VV1_1946	1.74260	flagellar export apparatus protein FliQ	VV1_3126	2.21144	phospholipase D family protein
VV1_2007	2.29199	hypothetical protein	VV1_3127	1.87284	diguanylate cyclase response regulator
VV1_2116	1.72799	23S rRNA pseudouridine synthase E	VV1_3128	1.78475	hybrid sensor histidine kinase/response regulator
VV1_2191	2.16713	MULTISPECIES: transcriptional regulator	VV1_3165	1.88586	MFS transporter
VV1_2225	2.60482	ABC transporter permease	VV1_3166	2.15216	membrane protein
VV1_2226	2.48942	ABC transporter ATP-binding protein	VV1_3227	1.68186	hypothetical protein
VV1_2238	2.57335	C4-dicarboxylate ABC transporter	VV1_3240	1.66820	hypothetical protein
VV1_2239	2.07898	C4-dicarboxylate ABC transporter	VV1_3255	1.87323	transposase

Table 4b. Significantly differentially expressed genes in *Vibrio vulnificus* that were up-regulated by the E-genotype during both aerobic and anaerobic conditions on chromosome II. Only genes where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5 are included.

Gene ID	Log2 Fold Change	Protein	Gene ID	Log2 Fold Change	Protein
Chrom II			**Chrom II**		
VV2_0043	1.59123	aspartate aminotransferase family protein	VV2_1066	1.61313	C4-dicarboxylate ABC transporter
VV2_0088	1.68203	type II secretion protein	VV2_1068	1.68429	MULTISPECIES: membrane protein
VV2_0089	1.65089	pilus assembly protein CpaF	VV2_1116	2.45583	sodium:proline symporter
VV2_0142	2.04003	surface protein	VV2_1141	2.09595	N-acetylmuramic acid 6-phosphate etherase
VV2_0158	1.71348	hypothetical protein	VV2_1142	1.79090	PTS N-acetylmuramic acid EIIBC component
VV2_0334	1.81962	aromatic amino acid aminotransferase	VV2_1146	1.66440	microbial collagenase
VV2_0336	2.35239	anaerobic ribonucleotide reductase-activating protein	VV2_1147	2.02109	hypothetical protein
VV2_0338	1.54040	hydrolase	VV2_1157	3.07506	polymerase
VV2_0390	1.86358	nitrite reductase large subunit	VV2_1158	3.49662	two-component system response regulator
VV2_0400	2.19868	alpha-amylase	VV2_1159	3.68156	anti-anti-sigma regulatory factor
VV2_0414	1.91944	succinyl-CoA synthetase subunit alpha	VV2_1160	3.03858	Methyl-accepting chemotaxis protein
VV2_0429	1.87767	hemolysin D	VV2_1161	2.89760	chemo response regulator protein, CheB
VV2_0430	1.64690	ClpV1 family T6SS ATPase	VV2_1162	2.38213	chemoreceptor glutamine deamidase CheD
VV2_0431	1.59196	Uncharacterized protein ImpH	VV2_1163	1.99204	chemotaxis protein CheR
VV2_0432	1.75548	type VI secretion system protein ImpG	VV2_1165	1.68258	Methyl-accepting chemotaxis protein
VV2_0434	2.31018	protein of avirulence locus ImpE	VV2_1167	1.56371	chemotaxis protein CheW
VV2_0435	1.64460	type VI secretion protein, IMPD	VV2_1226	1.62297	glycogen debranching enzyme
VV2_0485	1.73105	amino acid ABC transporter substrate-binding protein	VV2_1247	2.56361	transcriptional regulator
VV2_0487	2.42477	hypothetical protein	VV2_1250	2.84052	glycogen phosphorylase
VV2_0488	2.01728	3-ketoacyl-ACP reductase	VV2_1251	2.38753	4-alpha-glucanotransferase
VV2_0519	2.97777	cold-shock protein	VV2_1261	2.79305	phosphoserine phosphatase
VV2_0529	1.78445	transporter	VV2_1262	3.12530	chemotaxis protein
VV2_0530	1.81859	MexE family multidrug efflux RND transporter	VV2_1266	2.97911	NAD-dependent succinate-semialdehyde dehydrogenase
VV2_0563	1.88096	cytochrome c oxidase subunit II	VV2_1280	2.56589	oxidoreductase
VV2_0568	2.10608	cytochrome b559 subunit alpha	VV2_1282	4.05941	transcriptional regulator
VV2_0854	1.55048	tryptophanase	VV2_1283	2.42055	sugar ABC transporter substrate-binding protein
VV2_0949	2.07738	cytochrome B6	VV2_1284	2.68307	cytochrome c biogenesis protein
VV2_0950	1.78815	membrane protein	VV2_1286	2.48061	sugar ABC transporter permease
VV2_0951	1.99087	translation initiation factor 1	VV2_1287	1.89746	beta-glucosidase
VV2_0983	2.23498	hydroxyglutarate oxidase	VV2_1323	1.54762	transcriptional regulator
VV2_0984	2.92584	C4-dicarboxylate ABC transporter permease	VV2_1333	3.12465	hypothetical protein
VV2_0985	2.80215	C4-dicarboxylate ABC transporter	VV2_1371	1.73217	hypothetical protein
VV2_0986	1.96282	DNA-binding response regulator	VV2_1396	1.88325	helix-turn-helix transcriptional regulator
VV2_0987	1.93298	PAS domain-containing sensor histidine kinase	VV2_1404	2.27053	hypothetical protein
VV2_1005	1.54564	peptide ABC transporter permease	VV2_1424	2.48843	thiol:disulfide interchange protein
VV2_1006	1.79028	ABC transporter substrate-binding protein	VV2_1481	3.26102	ATPase
VV2_1007	2.54243	membrane protein	VV2_1482	4.25584	peptidase S8
VV2_1008	2.50627	membrane protein	VV2_1515	1.60598	peptidase C39
VV2_1024	1.51559	D-tagatose-bisphosphate aldolase,	VV2_1556	2.11133	phosphate ABC transporter substrate-binding protein, ptsS
VV2_1030	2.61129	ornithine decarboxylase	VV2_1557	2.24629	phosphate ABC transporter permease subunit PstC
VV2_1031	2.99708	transcriptional regulator	VV2_1558	1.87167	phosphate ABC transporter, permease protein PstA
VV2_1032	3.57532	alkyl sulfatase	VV2_1559	1.99867	phosphate ABC transporter ATP-binding protein, pstB
VV2_1036	1.70443	hypothetical protein	VV2_1560	1.72085	GGDEF domain-containing protein
VV2_1037	2.05332	peptide methionine sulfoxide reductase MsrB	VV2_1622	1.92435	alpha-amylase
VV2_1039	2.11929	glutathione peroxidase	VV2_1623	1.76560	chemotaxis protein
VV2_1064	1.53079	mannonate dehydratase			

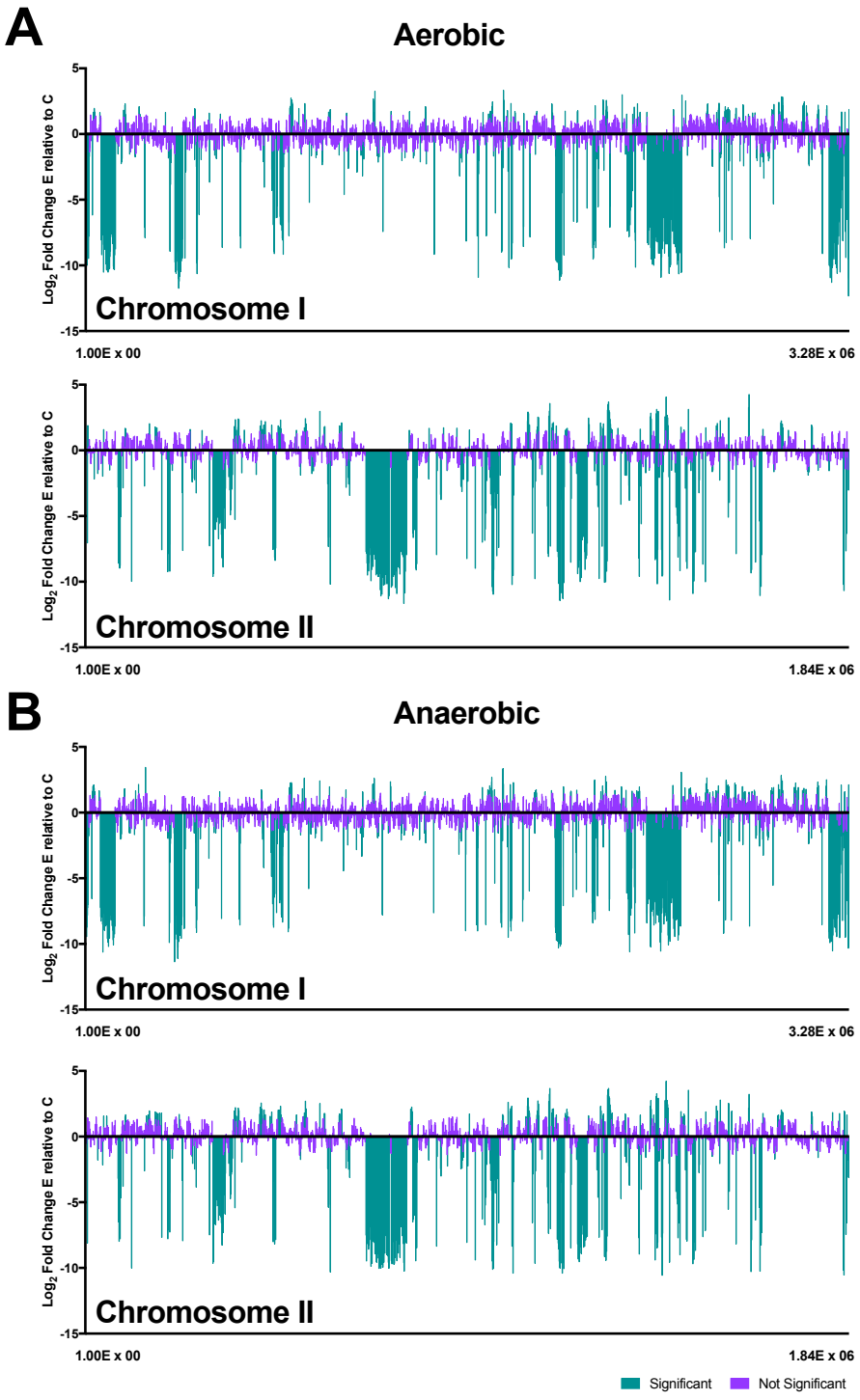

Figure 1. Linear compressed view of differentially expressed genes of E-genotype (JY1701) relative to C-genotype (CMCP6) in aerobic (A) and anaerobic (B) conditions. The y-axis shows the $\log_2$ fold change and the x-axis begins at the first position on the chromosome. Purple bars represent non-significant DE genes whereas teal bars show genes that are significantly differentially expressed ($p<0.05$) in the E-genotype.

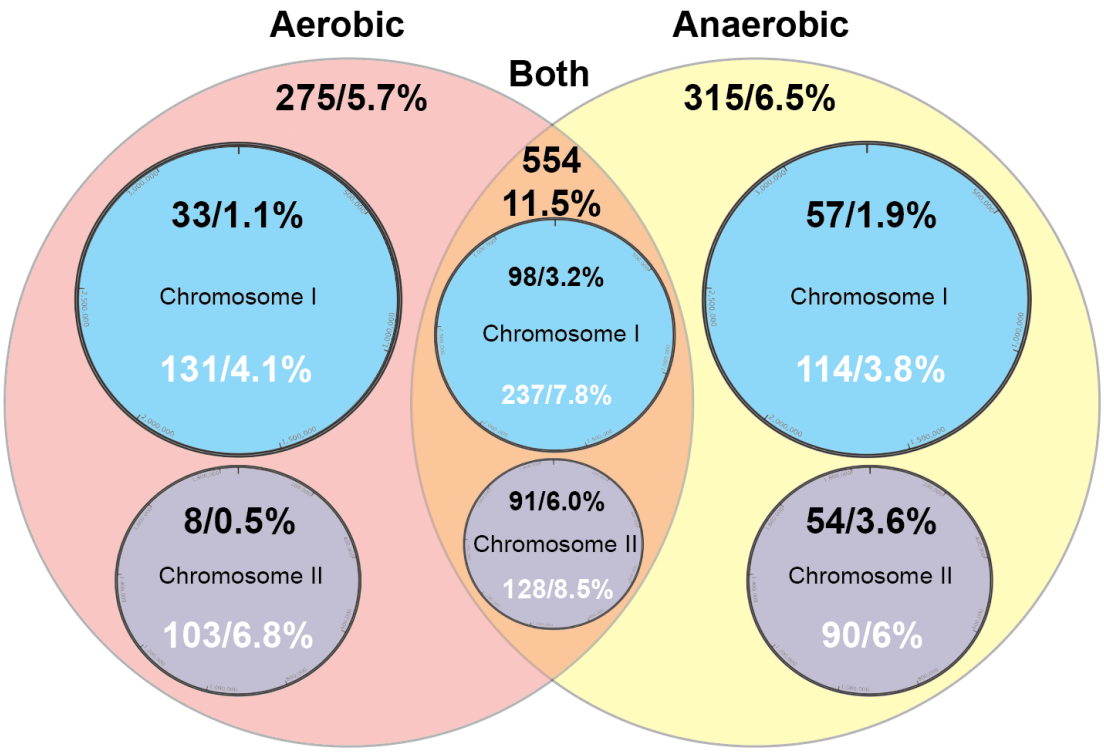

Figure 2. Venn diagram showing the comparison of significantly differentially expressed genes (DEGs) in JY1701 (E-genotype) relative to CMCP6 (C-genotype) in aerobic and anaerobic conditions ($p < 0.05$ and Log_2 fold change of greater than 1.5 or less than -1.5). Blue circles represent chromosome I and purple circles represent chromosome II of *V. vulnificus*. Inside these chromosomes, black numbers indicate the number of genes up-regulated followed by the percent of genes up regulated out of total genes and white numbers represent those that are significantly down-regulated.

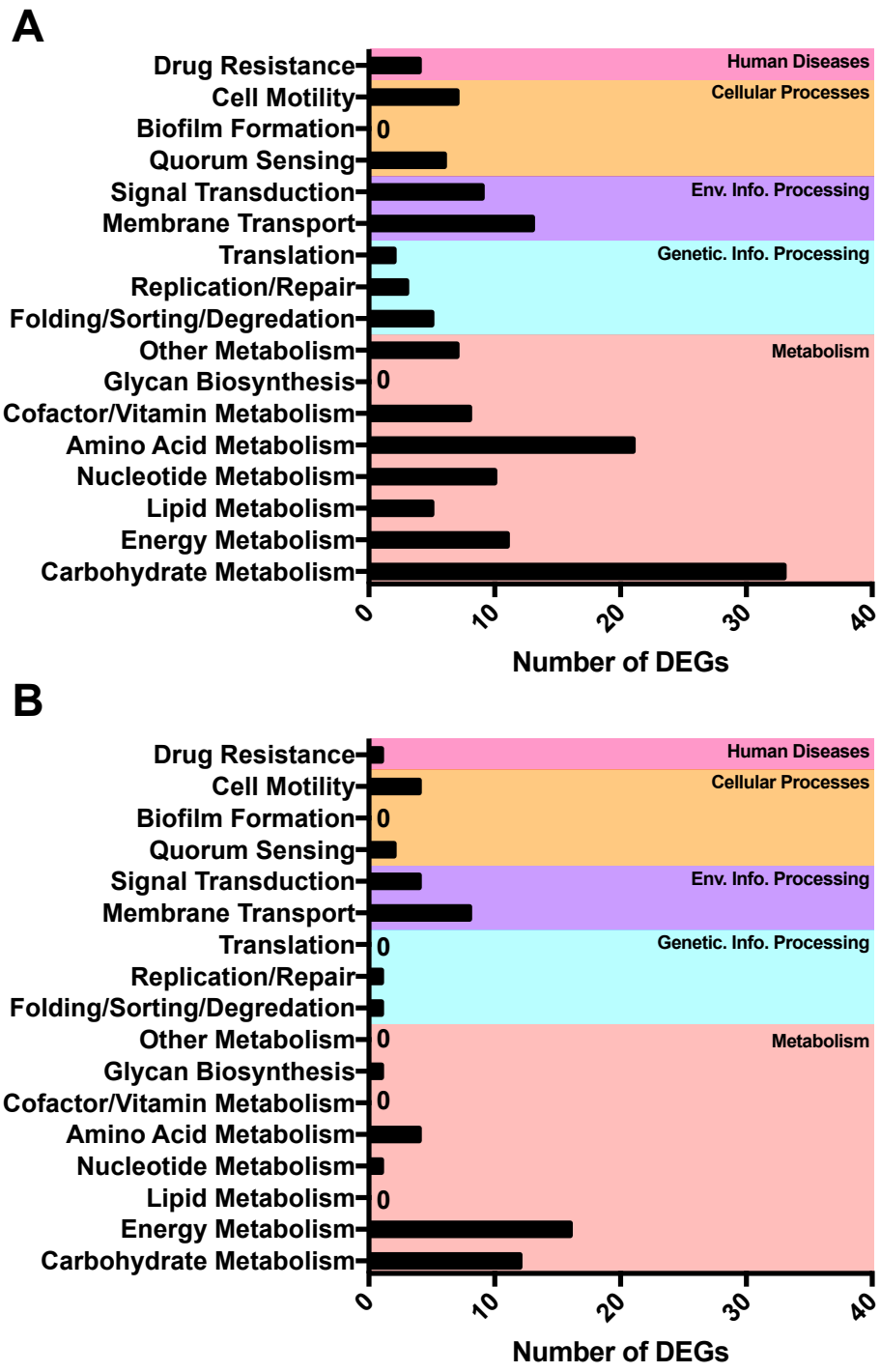

Figure 3. KEGG BRITE functional analysis showing the number of unique differentially expressed genes in the E-genotype relative to the C-genotype in aerobic (A) and anaerobic (B) conditions. All categories are made of significant DEGs where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5. Genes representing hypothetical/uncharacterized proteins are not represented.

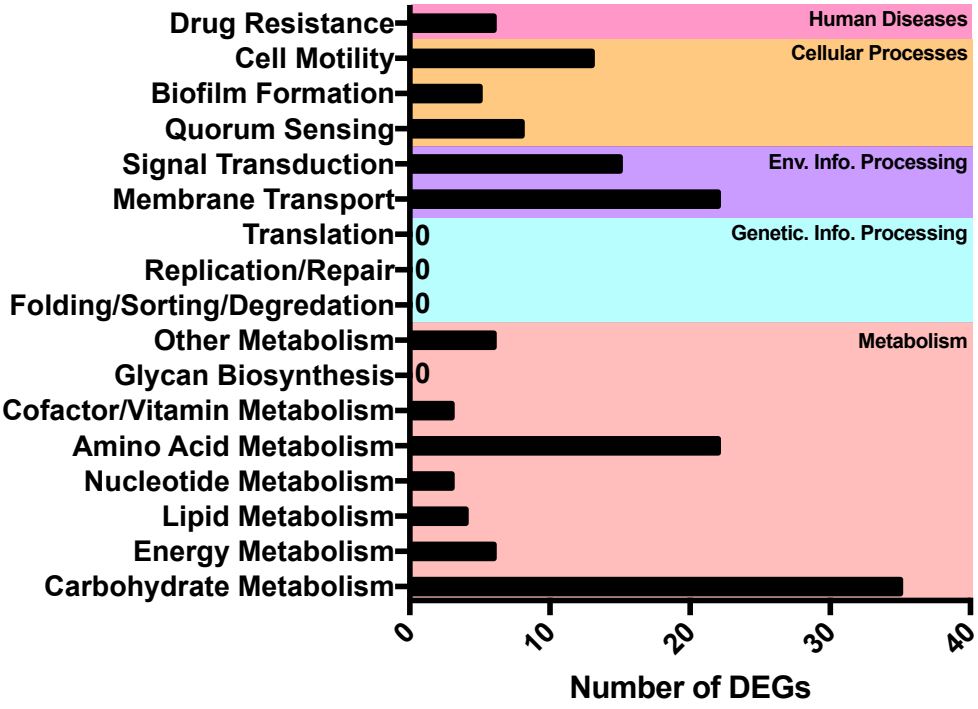

Figure 4. KEGG BRITE functional analysis showing the number of differentially expressed genes in both aerobic and anaerobic conditions in the E-genotype relative to the C-genotype in each category. All categories are made of significant DEGs where $p < 0.05$ and Log_2 fold change was greater than 1.5 or less than -1.5. Genes representing hypothetical/uncharacterized proteins are not represented.

Figure 5. Global protein network analysis using the STRING tool (version 10.5) (http://string.embl.de/) showing those proteins corresponding to significantly upregulated genes by the E-genotype during aerobic conditions. Thickness of the connecting line indicates increased confidence in the interaction. Proteins with no connecting node have been removed for ease of visualization.

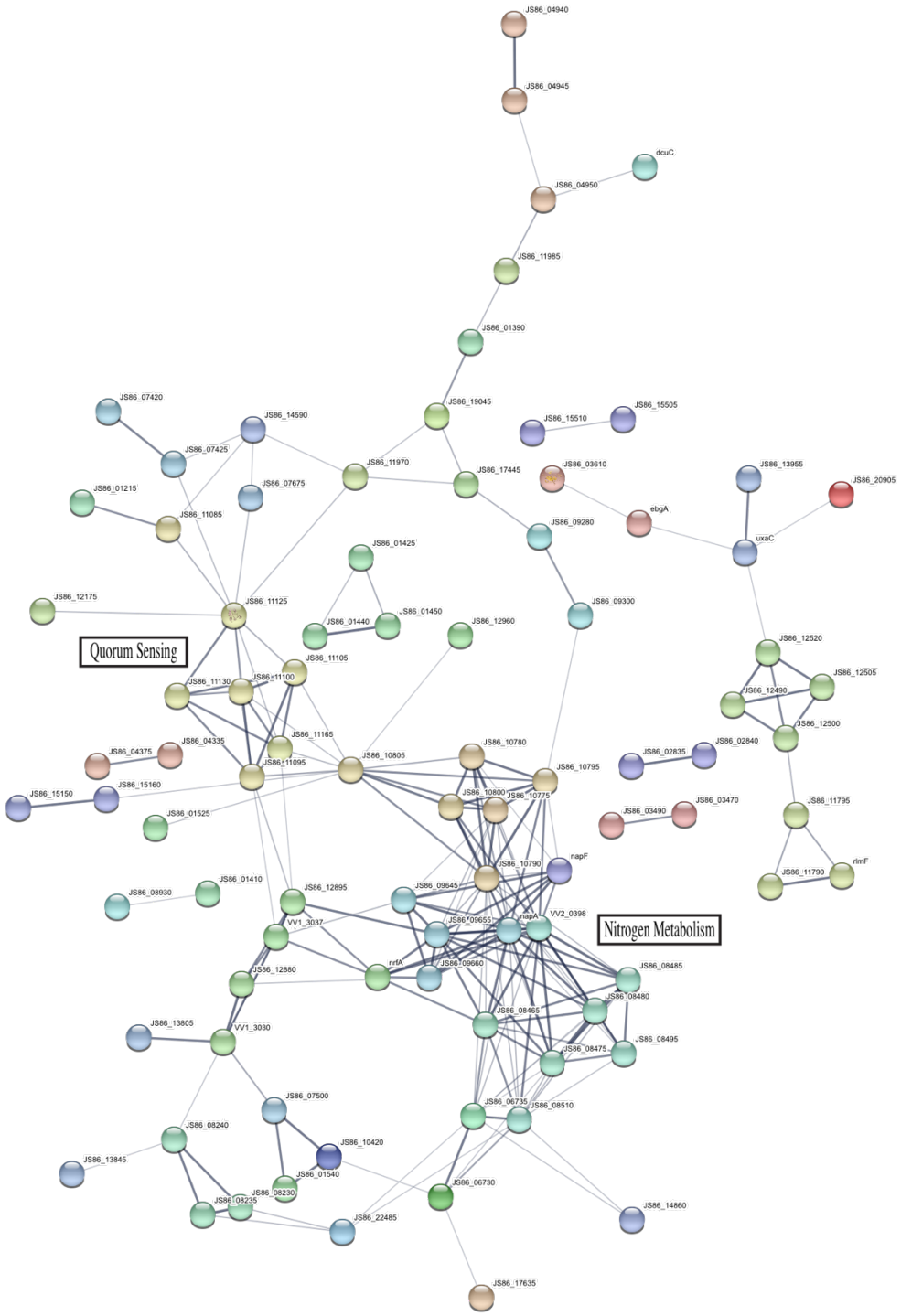

Figure 6. Global protein network analysis using the STRING tool (version 10.5)(http://string.embl.de/) showing those proteins corresponding to significantly upregulated genes by the E-genotype during anaerobic conditions. Thickness of the connecting line indicates increased confidence in the interaction. Proteins with no connecting node have been removed for ease of visualization.

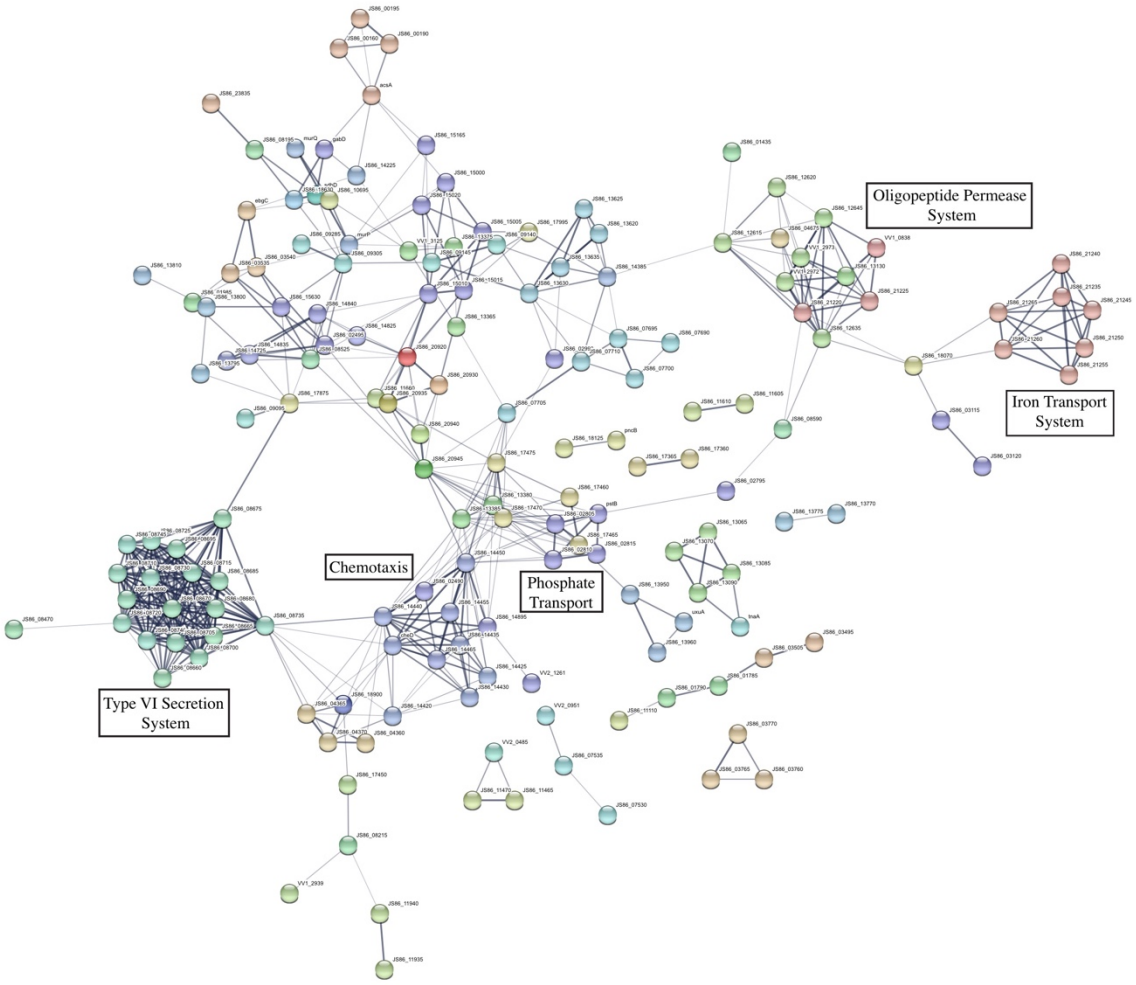

Figure 7. Global protein network analysis using the STRING tool (version 10.5)(http://string.embl.de/) showing those proteins corresponding to significantly upregulated genes by the E-genotype during both aerobic and anaerobic conditions. Thickness of the connecting line indicates increased confidence in the interaction. Proteins with no connecting node have been removed for ease of visualization.

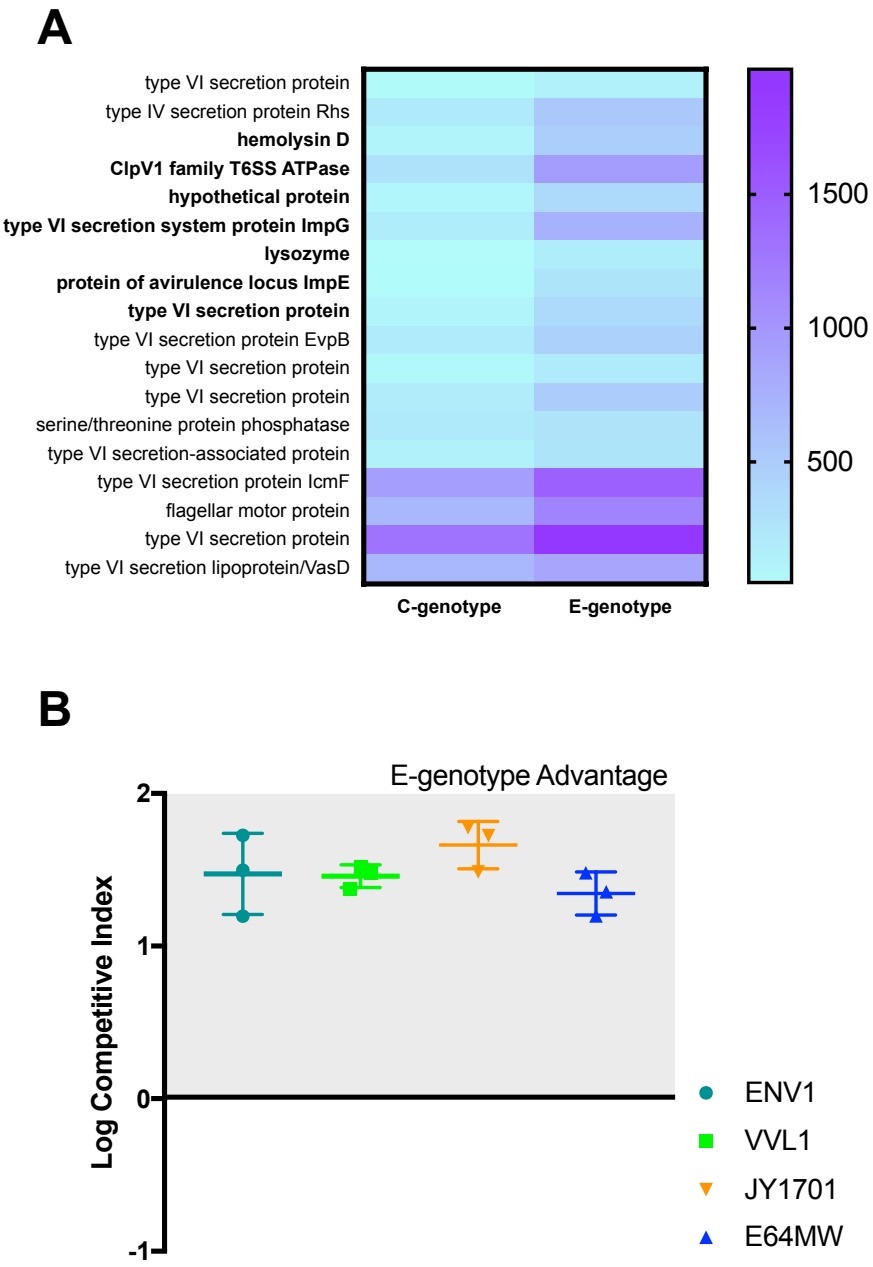

Figure 8. Averaged counts from both aerobic and anaerobic conditions corresponding to the type six secretion system (T6SS) in both genotypes (A). All genes were significantly increased in the E-genotype ($p < 0.05$) relative to the C-genotype, however, those in bold also have a Log_2 fold change greater than 1.5. (B) Log competitive indices of various E-genotypes compared to the C-genotype (CMCP6) in nutrient limited conditions. Values above 0 indicate an E-genotype advantage with each data point represents the average of 6 technical replicates.

CHAPTER 5

EFFECT OF HYPOXIA AND QUORUM SENSING ON THE VIRULENCE OF *VIBRIO CORALLIILYTICUS* AND ITS IMPACT ON THE MORTALITY AND IMMUNE FUNCTION OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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(submitted to ISME)

Abstract

Various studies have shown that oysters can survive prolonged hypoxia in estuarine systems, however, their vulnerability to bacterial pathogens during hypoxia is largely unknown. Thus, we investigated the response of the opportunistic pathogen, *V. coralliilyticus* to hypoxia, and both hypoxia and bacterial stress on the host, *Crassostrea virginica*. Oysters rely on innate immunity to survive bacterial challenges and we reveal that hypoxia impaired pathogen recognition, phagocytosis, and protein repair in oyster hemocytes. As virulence in *V. coralliilyticus* is influenced by abiotic conditions, we investigated gene expression of various factors during hypoxia. We found that hypoxia leads to significant upregulation of important virulence factors, such as the zinc-metalloprotease, multiple secretion systems, and various quorum sensing (QS) pathways, leading to rapid mortality in oysters. Moreover, as QS in many *Vibrio* species regulates virulence, we further analyzed interspecific QS (AI-2) on hypoxic host mortality and virulence potential. We showed that by stimulating or inhibiting the AI-2 system in *V. coralliilyticus,* we could influence mortality by increasing or decreasing the expression of multiple virulence factors, respectively. Together, we highlight the importance of hypoxia on host/pathogen interactions, further contributing to our understanding of virulence regulation in *V. coralliilyticus,* a pathogen with a broad host range.

Introduction

Hypoxia is a major environmental stressor in coastal ecosystems worldwide, and the number of areas which experience hypoxia and anoxia has increased exponentially in the past three decades (1). These events typically occur in summer when elevated temperatures lead to high rates of oxygen consumption by the resident biota, reduced oxygen solubility in seawater, and stratification of coastal waters. Hypoxic areas (commonly referred to as dead zones) result in fish kills, habitat constraint, and microbial community shifts (2-4). As global temperatures continue to rise, dead zones have become problematic, with frequencies, durations, and locations expanding annually (5). Additionally, the shoaling of the naturally occurring oxygen minimum zones (OMZs) decreases oxygen concentrations at ever shallower depth, impacting the resident macro/microbiota in these changing environments (6). The OMZs are expected to expand both at the upper (shallow) boundary and the lower boundary as a result of global climate change, which may impact the host/pathogen dynamics in these areas (7).

Estuarine bivalves play critical foundational roles in estuarine ecosystems by controlling water quality, stabilizing shorelines, and providing habitats for other organisms (8). *Crassostrea virginica,* an intertidal bivalve, is a champion of hypoxia and anoxia tolerance, surviving for long periods in hypoxic water (9, 10). Although oysters are well adapted to the recurring stress of anoxia and hypoxia (11-13), their close association with microbiota coupled with climate associated changes in abiotic factors may affect their ability to respond to environmental changes.

Like other invertebrates, oysters rely on their innate immune system to survive microbial challenges (14). Hemocytes (HCs) circulating in oyster hemolymph constitute

the primary immune response cells of oysters. HCs are responsible for stress sensing, phagocytosis or encapsulation of foreign particles, production of reactive oxygen species (ROS), and lysis of microbes. In addition, HCs produce a wide range of defense molecules, including defensins and lectins that work in combination with the phagocytic HCs to inactivate and degrade microbial pathogens (15). Environmental hypoxia has been previously shown to modulate immune mechanisms of marine invertebrates Specifically, hypoxia can reduce the number of circulating HCs, suppress ROS production (16), affect phagocytosis, impair lysosomal membrane integrity, and affect hemolymph antimicrobial activity (17). This repression of the immune system can be a serious threat to bivalves living in areas with hypoxic conditions and a high abundance of microorganisms (Jonson et al., 2007).

Predominant bacterial genera within the hemolymph of *Crassostrea spp.* include *Vibrio, Pseudomonas, Alteromonas* and *Aeromonas*, some species of which may become pathogenic when environmental conditions are altered. In particular, *Vibrio coralliilyticus* has the ability to infect bivalve larvae, corals, fish, crustaceans, and unicellular algae (18, 19). Its ubiquity throughout marine systems worldwide makes it a growing concern in aquaculture and marine resource management (18, 19). *V. coralliilyticus* utilizes a variety of virulence factors to cause disease in a host. This bacterium employs chemotaxis and motility to infiltrate the host, followed by increased expression of multiple secretion systems, and production of potent proteases and hemolysins (20-23). After the initial infiltration, this bacterium depends on the production of efflux pumps and other protection mechanisms against antimicrobial compounds to resist killing by the host (24, 25). Notably, the virulence of *V. coralliilyticus* is highly responsive to changing

temperatures, with virulence increasing significantly above 27°C, reiterating the importance of global climate change in its pathogenicity (21). Although recognized primarily as a coral pathogen, *V. coralliilyticus* recently caused multiple oyster hatchery crashes in Oregon and Washington states killing larvae and juveniles, though its specific impact on the morbidity and mortality on adult oysters is not yet clear. It has been suggested that recent *V. coralliilyticus* outbreaks may be due, in part, to the unusually high incidence of hypoxic/anoxic water introduced into the hatcheries from upwelling (19).

Cell-to-cell communication in *Vibrio* species is well documented through our understanding of various quorum sensing (QS) pathways [for review see (26)]. Strains of *V. coralliilyticus* have multiple QS pathways, including N-acylhomoserine lactone (AHL), autoinducer-2 (AI-2), autoinducer-3 (AI-3), and cholera autoinducer-1 (CAI-1) (23). These density-dependent systems work to coordinate genetic responses in bacteria (group behaviors), which are achieved by a signaling cascade following the production of extracellular QS molecules that interact with periplasmic binding proteins. Notably, QS can be modulated by exposure to various abiotic and biotic factors, including temperature changes and oxygen availability (2, 23, 27). As QS systems have been shown to regulate virulence, lifestyle preferences (planktonic or surface-associated) and interactions with host organisms in many *Vibrio* species (28-31), understanding their role during hypoxia might reveal novel regulatory roles.

Numerous studies have reported on the effects of abiotic stressors on oysters or bacteria alone; however, investigations of the effects of the abiotic stress on host-pathogen interactions are rare. To the best of our knowledge, the present study is the first

to examine the interactive effects of oxygen availability and *V. coralliilyticus* on morbidity and mortality of adult oysters *C. virginica*. We explored how *V. coralliilyticus* and *C. virginica* respond to hypoxia individually and as interacting host-pathogen system, and how hypoxia might influence pathogenic potential of *V. coralliilyticus* and oyster immune modulation. As QS pathways are integral in shaping host-pathogen interactions, we also sought to understand how these systems impact bacterial virulence and subsequent oyster mortality. Our studies provide insights into the modulation of host-pathogen interactions by hypoxia, which is a common stressor in coastal ecosystems, and may serve as an impetus for the future studies to investigate the link between climate change, ocean health and host-pathogen dynamics with implications for aquaculture practices and human health.

Materials and Methods

Animals

Oysters (*C. virginica*) from the North Carolina coast were purchased from a local supplier (Inland Seafood, Charlotte, NC, USA). Oysters were maintained in tanks with the artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth) at 22±1°C and salinity 30±1 practical salinity units (PSU), and fed *ad libitum* with a commercial algal blend containing *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Chlorell*a spp. (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Algal blend (2-3 ml per 20-25 animals) was added to experimental tanks every other day.

Bacterial strains and culture conditions

V. corallilyticus strain RE98 was utilized for this study and stored at -80°C in Bacto Luria-Bertani broth (LB) (BD Difco, Franklin Lakes, NJ, USA) with 20% glycerol. This strain was chosen as it has previously been sequenced and has been shown to be pathogenic to a variety of aquatic animals (19). For bacterial challenges, cells were grown overnight in Marine broth (450mM NaCl, 10mM KCl, 9mM $CaCl_2$, 30mM $MgCl_2 \cdot 6H_2O$, 16mM $MgSO_4 \cdot 7H_2O$, 5g Bacto Peptone, and 1g yeast extract) at 30°C with aeration in a rotary incubator.

Oyster challenges

To induce hypoxia, oysters were placed in covered plastic tanks (six animals in 2L ASW at 20°C and 30±1 PSU salinity), and hypoxic conditions were created by bubbling ASW with 100% nitrogen (Robert Oxygen, Charlotte, NC, USA) to achieve 0.04-0.1% O_2. Once hypoxic conditions were achieved, oysters were added to the tanks. Control oysters were maintained under the same conditions but with constant bubbling of air into the tanks to maintain normoxic environment. For bacterial challenges, overnight cultures of *V. coralliilyticus* were washed three times in sterile ASW and added at a concentration of 6 x 10^5 CFUs/mL to a large tank containing the animals under oxygenated conditions. After the initial uptake of the bacteria for 24 h, oysters were transferred to hypoxic or normoxic tanks and monitored for survival for 14 days. Hemolymph was collected from randomly chosen oysters after 24 h of exposure in all experimental treatments as well as from the control conditions.

Manipulation of AI-2 Quorum Sensing

An AI-2 precursor, 4,5-dihydroxy-2,3-pentanedione (DPD) (OMM Scientific, Dallas, TX, USA), or AI-2 inhibitor cinnamaldehyde was added to oyster exposure tanks

to stimulate or inhibit AI-2 QS pathways, respectively. The addition of DPD and cinnamaldehyde has been previously shown to stimulate and repress the production of bioluminescence in the *V. harveyi* reporter, respectively (32). To each experimental tank, a final concentration of 250 nM DPD or 150 μM cinnamaldehyde was added, which has been shown to be non-inhibitory to growth of multiple *Vibrio* species (33). We then monitored the survival of oysters for 14 days and harvested oyster hemolymph at 24 h post addition.

Hemolymph collection

Hemolymph (HL) was extracted from the adductor muscle of oysters using a sterile 10 ml syringe with a 21-gauge needle containing 1 ml ice-cold ASW to prevent aggregation of the HCs. A total of 5-6 biological replicates were obtained from individual oysters for each experimental treatment. Concentration of the HCs was determined using a Brightline hemocytometer.

Phagocytosis

Phagocytosis assays were performed as described elsewhere (34). Briefly, a suspension of Neutral Red-stained, heat-stabilized zymozan particles (Sigma-Aldrich, St. Louis, MO, USA) was added to HCs at a final concentration of 200 particles per HC. HCs were incubated for 30 min at room temperature, centrifuged at 1000 x g for 10 min and washed with ASW to remove extracellular zymozan. Cell-free suspensions of zymozan at known concentrations were used as calibration standards. HC or zymozan standards were incubated for 5 min with 1% acetic acid in 50% ethanol to extract the Neutral Red dye. The optical density of the extract was read at 550 nm on a microplate

spectrophotometer (Multiscan GO, Thermo Scientific, Waltham, MA, USA). Results are expressed as the number of ingested zymozan particles per 100 cells.

Reactive oxygen species (ROS) production

The standard method for inducing respiratory bursts was performed using non-fluorescent fluorescein analog 2'7'-dichlorofluorescin diacetate (DCFH-DA). When added to the cells, DCFH-DA diffuses across the membrane and is hydrolyzed to 2'7'-dichlorofluorescein (DCFH), becoming trapped within the cells. The intracellular DCFH is then oxidized to create a highly fluorescent, 2'7'- dichlorofluorescein (DCF) by intracellular reactive oxidative species (ROS) (35). Non-stimulated samples were prepared by combining 150 µl of HCs (0.5×10^6 cells ml^{-1}) with 150 µl of ASW followed by the addition of 10 µM DCFH-DA (ThermoFisher Scientific, Pittsburgh, PA, USA). Additional tubes were prepared containing the same suspension of HCs and ASW, with 150 µl of a zymozan suspension (200 zymozan particles per HC) was added to measure ROS induction. ROS levels were measured after a 40 minute dark incubation using a fluorescence plate reader (CytoFluorSeries 4000, Framingham, MA, USA) at the excitation and emission wavelengths of 485 nm and 530 nm, respectively. Signals measured in samples with zymozan were corrected using values from the unstimulated samples, and ROS activity was expressed as mean fluorescence in arbitrary units (AU) per 10^6 cells.

Lysosomal membrane stability

Suspensions (100 µl) of HCs were placed on microscope slides, air-dried and stained with 4 g L^{-1} Neutral Red (NR) (Sigma-Aldrich, St. Louis, MO, USA) for 5 min (36). Slides were rinsed with water, air-dried, mounted in glycerol and observed under a

Zeiss Axio Observer A1 inverted microscope equipped with AxioCam HRc digital camera (Carl Zeiss Inc, Oberkochen, Germany) using differential interference contrast illumination and a 63 x 1.4 numerical aperture plan apochromatic objective. Lysosomal alteration was scored based on the scoring system described by (37). In brief, cells that demonstrated a predominance of lysosomes retaining NR were scored as normal cells. Samples that contained cells with enlarged lysosomes and/or cells with NR that had leaked into the cytosol were scored as phagocytic cells. Cells were scored as containing autophagosomes if they presented enlarged lysosomal compartments with different strengths of coloration, and round fragmented cells were scored as cells having undergone lysis.

Quantitative real-time PCR (qRT-PCR)

For bacterial gene expression analysis, *V. coralliilyticus* was grown overnight, washed and placed in modified membrane diffusion chambers as described elsewhere (2). Chambers were placed in hypoxic or normoxic tanks for 24 h, after which the samples were collected using sterile syringes pre-filled with RNAprotect (Qiagen, Valencia, CA). RNA was extracted as described by Williams et al. (2014). For oyster gene expression analysis, HL samples were centrifuged for 10 min at 1000 x g, the HC pellet was washed once with ice-cold ASW and stored at -80°C until RNA extraction. Total RNA was extracted from HC using ZR RNA MiniPrep^{TM} kit (Zymo Research, Irvine, CA, USA) per the manufacturer's instructions. RNA was quantified and quality-controlled using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and samples with a 260/280 nm ratio of ≥1.9 and a concentration of ≥75ng/µl were stored at -80°C until further analysis. Bacterial DNA contamination was determined using end-point PCR

amplifying the housekeeping gene, bacterial DNA recombination protein (*recA*) (2). Single-stranded cDNA was obtained from 0.2 µg of the total HC RNA using 50 U μl^{-1} SMARTScribe™ reverse Transcriptase (Clontech, Mountain View, CA, USA) and 20 µmol l^{-1} of oligo$(dT)_{18}$ primers.

Primers were designed using NCBI Primer-BLAST software and primer pairs (predicted efficiencies of ≥1.5) were selected and purchased from Sigma Aldrich (Tables S1 and S2). A previously described qRT-PCR protocol was adapted for all bacterial expression studies (38). PerfeCTa SYBR green FastMix, Low ROX (Quanta Biosciences, Beverly, MA) was used to assess gene expression of *V. coralliilyticus* strain RE98 in three biological replicates with three technical replicates in each condition using a 7500 Fast Real-Time PCR System (Applied BioSystems/Life Technologies, Carlsbad, CA, USA). To correct for sampling error, normalization of target genes was performed using *recA* as the endogenous control. An algorithm proposed by Pfaffl (39) was used to calculate the change in gene expression, accounting for differences in the amplification efficiency of primer pairs.

For gene expression in the HCs the qRT-PCR reaction mixture consisted of 7.5 µl of 2 x SYBR® Green master mix, 0.3 µmol L^{-1} of each forward and reverse gene-specific primers, 1.5 µl of 10 x diluted cDNA template and water to adjust to 15 µl. The reaction mixture was subjected to the following cycling: 10 min at 95°C to denature DNA and activate Taq polymerase and 40 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C. A single cDNA sample of HCs was included as an internal cDNA standard and included in each run to test for run-to-run amplification variability. Target gene mRNA expression was standardized relative to β-actin mRNA and the internal standard (39).

Statistical analysis

Two-way ANOVA was used to test the effects of hypoxia and QS manipulation on *V. coralliilyticus*. For *C. virginica,* three-way ANOVA was used to test the effects of hypoxia, bacterial exposure and QS manipulation. Fisher's Least Significant Differences (LSD) tests were used for planned post-hoc comparisons of the pairs of means of interest. All statistical analyses were performed with JMP Pro 12 (Cary, NC, USA). Differences were considered significant if the probability of Type I error was less than 0.05. The data are presented as means ± standard error of the mean (SEM).

Results and Discussion

Hypoxia influences immune responses but not mortality of *C. virginica*

Like other marine intertidal bivalves, oysters are commonly exposed to short-term oxygen deficiency during low tide and might experience prolonged (days to weeks) seasonal hypoxia in their benthic habitats. Oysters have evolved multiple ways to tolerate and mitigate hypoxic stress including reduced metabolic activity mainly due to the suppression of protein synthesis and ion transport to conserve energy during hypoxia, high energy storage in the form of glycogen, and alternative glycolytic pathways that produce more ATP and less metabolic waste (40, 41). Therefore, it is not surprising that without *V. coralliilyticus* challenge, prolonged (14 days) hypoxia caused no mortality in oysters (Figure 1), consistent with earlier reports of high hypoxia tolerance in this species (42).

Although hypoxia did not lead to mortality in oysters, this stressor may have important sublethal effects modulating immune function and the subsequent response to

bacterial pathogens as has been shown in many marine invertebrates (43, 44). In intertidal mollusks, the mechanisms that aid in short-term survival during hypoxia and anoxia are well studied, but the effect of hypoxia on immune functions are not well understood. We found that exposure to hypoxia caused an increase of C-type lectin expression (Figure 2) and a significant inhibition of oxidative burst of HCs responding to zymozan (Figure 3A). Notably, expression of HIF-1α was slightly elevated in hypoxia consistent with the important role of this transcriptional factor in the adaptive cellular response to oxygen deficiency (45). One-day exposure to hypoxia had no effect on the ability of oysters HCs to phagocytose zymozan (Figure 3B), which supports the notion that the robust phagocytic ability of *C. virginica* HCs is maintained during hypoxia (46).

Autophagy, the controlled degradation of cellular components in lysosomes, is often upregulated during stressful conditions (such as restricted nutrients, hyperthermia or hypoxia) to recycle the nutrients and promote survival (47). We found that hypoxia caused a significant increase in the percentage of HCs containing phagolysosomes and autophagosomes, with the latter being stimulated as early as 24 h post hypoxic exposure (Figure 3C). In our study, the number of normal HCs decreased during hypoxia to a mere 33.6%, indicating that autophagy by oyster HCs during hypoxia likely contributes to its survival but might compromise immunity by decreasing the number of viable HCs.

Mortality and immune response of *C. virginica e*xposed to *V. coralliilyticus*

V. coralliilyticus is well known for its ability to cause disease in corals, yet few studies have investigated the effect of these bacteria on marine bivalves, especially in the context of the environmentally relevant hypoxia, despite their implication in oyster mortality (19). Therefore, we challenged adult oysters with *V. coralliilyticus* for two

weeks under normoxic and hypoxic conditions to assess the interactive effects of hypoxia and the pathogen on oyster survival. We found 100% survival throughout the normoxic exposure, regardless of the presence of *V. coralliilyticus* (Figure 1). However, when the oysters were simultaneously exposed to hypoxia and *V. coralliilyticus,* we saw a dramatic decrease in survival with the first deaths occurring after four days of exposure, and complete mortality by day seven (Figure 1).

To understand the normal immune response of adult oysters to *V. coralliilyticus,* we investigated key immune functions of oyster HCs during the pathogen challenge under normoxia. We found that many innate immune functions investigated in this study were rapidly activated in oyster HCs within a day of exposure to *V. coralliilyticus* (Figure 2, 3A-B). The first step of bacterial elimination is pathogen recognition either directly through membrane bound receptors (such as Toll-like receptors) or indirectly via humoral recognition factors (such as C-type lectins) present in the hemolymph (15). The Toll-like receptor pathways play a central role in initiating cellular responses to microbial infections in oysters (48, 49), and our present study showed a strong upregulation of Toll-like receptor 4 (TLR4) in HCs of oysters exposed to *V. coralliilyticus* (Figure 2). Similarly, C-type lectin was overexpressed in HCs of oysters challenged by *V. coralliilyticus* in normoxia (Figure 2). Elevated expression of the membrane-bound and humoral factors, such as TLR and lectins, that recognize and bind the pathogen-associated molecular patterns (PAMPs) might aid in pathogen recognition and killing (50), as well as to stimulate phagocytic behavior of HCs. This is due to PAMP-activated receptors in the membrane becoming clustered and activating the signaling pathways that

lead to engulfment of the bacteria (51). Indeed, a notable increase in phagocytic activity was observed in the host HCs exposed to *V. coralliilyticus* (Figure 3B).

Notably, exposure of oysters to *V. coralliilyticus* led to a significant decrease in the production of reactive oxygen species (ROS) by HCs under normoxic conditions (Figure 3A). A rapid but transient production of large amounts of ROS, termed the oxidative burst, is an important pathogen-killing mechanism and a key defense strategy of oysters against invading microorganisms (35, 52-54). A decrease in ROS production has been reported in many invertebrate species under bacterial or eukaryotic pathogen challenges and has been interpreted as an evasion strategy of the pathogens to prevent killing (55-57). It is possible that *V. coralliilyticus* employs a similar evasion mechanism to suppress the host's immune defense. Furthermore, elevated ROS production might, under some circumstances, benefit the pathogen rather than the host, for example due to the oxidative damage to the hosts' immune cells (51, 58). To prevent damage caused by these oxidative bursts, HCs can increase expression of genes involved in ROS scavenging, antioxidant defense and damage repair. Our study showed that challenge with *V. coralliilyticus* under the normoxic conditions led to upregulated expression of nucleoredoxin and metallothionein, two important ROS scavengers in *C. virginica* (Figure 2). Additionally, HSPs are important cellular chaperones induced by the damage of the cellular proteins in response to abiotic stressors and/or exposure to pathogens (59). HSP70 modulates cellular immune responses in the bay scallop, *Argopecten irradiants,* and plays a role in protection from invading pathogens (60). The lack of HSP70 upregulation in HCs of *C. virginica* challenged with *V. coralliilyticus* may indicate that

under the normoxic conditions, this infection does not induce intracellular protein damage in host HCs.

Effects of hypoxia on host-pathogen interactions

Hypoxia strongly elevates susceptibility of oysters to *V. coralliilyticus*-induced mortality (Figure 1) indicating the potential impairment of the host's immune defense and/or stimulation of *V. coralliilyticus* pathogenicity by hypoxia. In our present study, we tested both possibilities and found evidence of the weakened host immunity and strengthened pathogen virulence in hypoxic environments. Thus, hypoxic exposure negatively affected expression of the genes involved in the pathogen recognition (TLR4), as well as opsonization and killing of the pathogen (C-type lectin and Big defensin) in *V. coralliilyticus*-challenged oysters (Figure 2). Furthermore, hypoxia prevented the pathogen-induced stimulation of phagocytosis in oyster HCs (Figure 3B). Taken together, hypoxia-induced suppression of pathogen recognition, phagocytosis and microbial-killing activities may have led to inefficient elimination of *V. coralliilyticus* and contributed to the rapid mass in mortality of infected oysters in hypoxia.

Hypoxia also resulted in protein damage in HCs of oysters exposed to hypoxia alone or in combination with *V. coralliilyticus,* as indicated by the elevated expression of a molecular chaperone, HSP70 (Figure 2). This indicates that hypoxia negatively affects protein homeostasis in oyster HCs, regardless of the infection status. A massive upregulation of autophagy and cell lysis in HCs of oysters exposed to hypoxia and *V. coralliilyticus* is also consistent with the cellular damage resulting in cell death (Figure 3C). It is worth noting that autophagy may also play a protective role against intracellular pathogens by engulfing the pathogens in autophagic vesicles and targeting them to

lysosomes (61). However, pathogens can hijack this process for their own benefit. For example, *Coxiella burnettii* creates a niche within autophagosomes where it replicates and survives (62), and *V. parahaemolyticus* uses nutrients released by infected autophagic cells to support its proliferation (63). Under normoxia, *V. coralliilyticus* decreased the percentage of HCs containing phagolysosomes and autophagosomes and induced cell lysis in ~12% of HCs (Figure 3C). Bacteria can stimulate host cell lysis by several mechanisms, such as the type six secretion system (T6SS) which is present in *V. coralliilyticus.* This system plays a role in direct killing in both prokaryotic and eukaryotic cells (64) and might be employed by *V. coralliilyticus* to lyse oyster HCs. In contrast to a modest increase in the host cell lysis induced by *V. coralliilyticus* under normoxic conditions, the concomitant stress of *V. coralliilyticus* and hypoxia greatly facilitated autophagy (~50%) and cell lysis (~27%) of oyster HCs and decreased the fraction of the cells that contain phagolysosomes that could be involved in digesting the pathogen (Figure 3C). Thus, the massive (~77%) death of oyster immune cells induced by hypoxia and *V. coralliilyticus* would likely aggravate the immunosuppression caused by a decrease in bacterial recognition and elimination mechanisms of the host and contribute to the rapid morbidity and mortality of oysters under these conditions (Figure 1).

The outcome of the host-pathogen interactions between *C. virginica* and *V. coralliilyticus* under hypoxia likely depends not only on the host's immunity but also on the pathogen's virulence, which can be strongly modulated by environmental factors (20, 21, 23). To test this hypothesis, we examined gene expression of putative virulence factors that might contribute to increased pathogenicity of *V. coralliilyticus* during

hypoxia. To infiltrate their hosts, *V. coralliilyticus* utilizes a variety of mechanisms, including motility and such attachment systems as the mannose-sensitive hemagglutinin (MSHA) type IV pilus (21-23). In our present study, exposure to hypoxia upregulated expression of *mshD,* encoding a mannose-sensitive hemagglutinin pilin protein, but suppressed the expression of *fhlA* (involved in flagella biosynthesis and serving as a proxy for bacterium's motility) in *V. coralliilyticus* (Figure 4). Notably, both motility and attachment mechanisms have been implicated in *V. coralliilyticus* pathogenicity to corals (22, 23). In oysters, bacterial motility may play a less important role in determining the outcome of host-pathogen interactions, as oysters are filter feeders, and will non-specifically take up bacteria, thus allowing access to oyster tissues without the need for motility-aided transmission. Once the bacterium is in contact with the host tissues, MSHA pili become important in host colonization and switching from a planktonic to a sessile lifestyle, as was shown in many *Vibrio* species (65-68). Thus, the increased expression of MSHA may ensure attachment of *V. coralliilyticus* to oyster tissues and its persistence inside oysters, as this protein has been shown to interact with both tissues and HCs of other bivalves (69).

Once inside the host, *V. coralliilyticus* must deploy a variety of factors in order to become successfully established and cause disease, including hemolysins, proteases, and secretion systems. Our study showed that hypoxia increased the expression of *vcpA,* a zinc-metalloprotease in *V. coralliilyticus*, whereas the hemolysin *vchA* was slightly down-regulated (Figure 4). Although VcpA had previously been described as an important virulence factor, Santos et al. (2005) showed that VcpA was not required for virulence as a mutant lacking VcpA was still able to cause disease. Furthermore the

mutant showed increased expression of 18 proteins compared to the wild-type, including potent hemolysins (Santos et al. 2005). Thus, it could be hypothesized that hemolysin (VchA) and VcpA may show redundancy with regard to *V. coralliilyticus* virulence so that upregulation of one of these virulence factors eliminates the need for the other. Expression of type II, III, and VI secretion systems showed varied responses to hypoxia in *V. coralliilyticus* in our present study, with the genes involved in type II (*gpsG* and *gpsL*), and type VI (*vgrG* and *vasH*) being significantly up-regulated, and the genes encoding type III (*sepL* and *t3SS*) secretion system being significantly suppressed (Figure 4). The increased expression of the type VI secretion system (T6SS) appears particularly intriguing in the context of the hypoxia-induced virulence of *V. coralliilyticus*. In other *Vibrio* species, this system has been shown to be important in the killing of bacteria, eukaryotic microbes, and murine macrophages (70, 71), and likely provides the *V. coralliilyticus* cells with a protective or competitive advantage. To our knowledge, this is the first study that has reported an increase in T6SS in any bacterial species in response to hypoxia; however, its exact role in pathogenicity of adult oysters remains to be elucidated.

Many of the genes encoding the putative virulence factors which were the focus of our present study can be regulated by one of the four quorum sensing (QS) systems, as shown in other *Vibrio* species (72-75). *V. coralliilyticus* strain RE98 has three QS systems, AI-2, AI-3, and CAI-1. Under hypoxic conditions, we found both AI-2 (*luxS*) and AI-3 (*qseBC*)to be significantly upregulated, whereas CAI-1 (cqsA) was significantly decreased (Figure 4). This was an interesting and novel finding indicating a potential role

of QS in the virulence of *V. coralliilyticus* under different abiotic conditions, leading us to investigate the role of QS further.

Oyster mortality, bacterial virulence, and immune responses are modulated by interspecific quorum sensing

Surprisingly, although there is a clear relationship between QS and virulence (23, 74, 76), the impact of QS on host morbidity and mortality has not been extensively investigated. The number of differentially expressed putative virulence factors in *V. coralliilyticus* during hypoxia, and their potential regulation by QS, led us to evaluate mortality in oysters in response to the experimental stimulation or inhibition of interspecific QS. The AI-2 system can be easily stimulated through the addition of exogenous AI-2 (DPD), or inhibited by cinnamaldehyde. During normoxia, we saw 100% survival of oysters regardless of the addition of bacteria, DPD or the inhibitor (Figure 5). Upon the addition of DPD without *V. coralliilyticus* the oysters survived hypoxic exposure for the duration of the experiment; however, addition of DPD with *V. coralliilyticus* resulted in oyster death as early as two days exposure, and all oysters succumbed to the dual stress by day three (Figure 5). Conversely, when we exposed the oysters to hypoxia and *V. coralliilyticus* with the addition of the AI-2 inhibitor, oyster survival was prolonged to 11 days, with the first mortalities occurring at day 10. We also saw that when the inhibitor was added during hypoxia without *V. coralliilyticus*, there was some mortality beginning at day 13 and all animals died at day 14 (Figure 5).

Since the AI-2 QS system was significantly upregulated during hypoxia (Figure 4), we were interested whether this system has a regulatory effect on the other processes examined here (flagellar activity, secretion systems, hemolysins and proteases). Figure 4

shows that the stimulation of the QS system resulted in a significant increase in the expression of both type II and type III secretion systems in *V. coralliilyticus*. Many Gram-negative bacteria utilize type II secretion system for the export of proteins into the extracellular space (77, 78). For example, *V. cholerae* uses this machinery to export the cholera toxin, one of its two major virulence factors, into the host intestinal tract (79). The type III secretion system is also implicated in bacterial virulence by transporting protein effector molecules into the plasma membrane or the cytoplasm of eukaryotic cells (80). Either of these secretion systems might increase the virulence potential of *V. coralliilyticus,* and our ability to manipulate expression of these two systems through the addition exogenous AI-2 or inhibitor (Figure 4) suggests a potential role for interspecific QS in the regulation of these secretion systems. Although stimulation of the AI-2 pathway did not affect expression of *vcpA* (zinc metalloprotease) and *mshD* (type IV pili), inhibition of the QS system decreased their expression suggesting that these genes may also be, at least in part, QS regulated.

It is worth noting that our study did not investigate the effects of the AI-3 system that exhibits some crosstalk with AI-2 (81), but is not directly affected by the AI-2 stimulator and inhibitor used on our present study. AI-3 was originally described in E. *coli*, where it responds to host norepinephrine and epinephrine to trigger virulence (82). Therefore, although AI-2 manipulations play a role in modulating virulence of *V. coralliilyticus*, its virulence could also be impacted by AI-3. Further investigations are needed to fully elucidate the role of the AI-3 pathway and the AI-2/AI-3 crosstalk on virulence in *V. coralliilyticus.* Our present finding that AI-2 inhibition can prolong survival in adult oysters exposed to *V. coralliilyticus* and hypoxia by suppressing

virulence of the pathogen may have implications for aquaculture, paving the road for using AI-2 inhibitors (such as those naturally produced by some macro-algae) to mitigate oyster crashes in aquaculture (83).

Remarkably, modulation of QS not only had an effect on *V. coralliilyticus* but also impacted the immune function of oysters. Regardless of the presence of *V. coralliilyticus,* the addition of exogenous Al-2 stimulator or inhibitor to the oysters caused a significant increase in expression of thiol-based antioxidants such as nucleoredoxin and metallothionein, as well as HSP70 (Figure 6A-B) in normoxia as well as hypoxia. However, when DPD was added in the presence of *V. coralliilyticus* during normoxia, we saw a significant increase in the expression of TLR4 (Figure 6A) and a slight suppression of phagocytosis in oysters HCs (Figure 7A), which may indicate a role for this AI-2 system in preventing bacterial killing via the phagocytic pathway. Early mortality, which was observed under hypoxic conditions in the presence of DPD, can be explained by the decreased ability in oyster HCs to recognize and eliminate this pathogen, as well as dramatic increases in lysed and autophagic cells compared to those exposed to the inhibitor (Figure 7C-D). However, modulation of ROS production does not appear to be implicated in the increased mortality of oysters during hypoxia, as indicated by the lack of increase in oxidative burst (Figure 7B) and an increased expression of antioxidants increasing the ability of HCs to neutralize ROS production (Figure 6A). To our knowledge, this is the first study that has shown that by modulating the AI-2 QS pathway, we can successfully increase or decrease host mortality. Although there is some contribution of the oyster immune system, it appears that the most important mechanism involves strong upregulation of genes involved in host invasion

and killing of *V. coralliilyticus*. Further investigations are needed to fully understand this complex relationship between environmental conditions, bacterial communication, host immunity and its susceptibility to this pathogen.

Conclusions

V. coralliilyticus has been recently identified as an important larval shellfish pathogen causing mass mortalities in larvae of *Crassostrea gigas, Ostrea edulis, Pecten maximus,* and *Perna canaliculus* (19, 84, 85). However, these studies do not elucidate the molecular processes underlying the observed host-pathogen relationship. Our study demonstrates that *V. coralliilyticus* can be especially pathogenic for *C. virginica* under hypoxic conditions, and our physiological and molecular data indicate that this is due to the dual effects of hypoxia on the host and the pathogen as shown in our proposed working model (Figure 8). Specifically, during normoxia oyster HCs are able to recognize *V. coralliilyticus* by a TLR4-mediated response, which promotes ingestion of the pathogen via the phagocytic pathway. The phagocytes then fuse with the lysosomes and the bacteria can be cleared from the cells, as the virulence factors we investigated show relatively low expression levels in *V. coralliilyticus* during normoxia. During oxygen deficiency, the bacterium significantly increases the production of putative virulence factors such as VcpA, T6SS, MshD pilus, as well as interspecific and interspecies QS systems. These factors, either directly or indirectly, influence the ability of oyster HCs to recognize *V. coralliilyticus*, resulting in an increased proportion of bacterial cells that enter HCs via endocytosis and resulting in cytotoxicity to HCs. Furthermore, there is evidence for increased autophagy and cell lysis via both bacterial

mechanisms and/or the direct effects of hypoxia on HCs. Together, our results begin to unveil the complex relationship that oxygen plays on host-pathogen dynamics, demonstrates the importance of understanding abiotic factors as potential triggers of virulence in marine bacteria, and shows possible ways to manipulate those interactions by targeting global regulatory systems such as the AI-2 QS pathway.

Acknowledgements

We kindly thank Dr. Claudia C. Häse at Oregon State University for providing the *V. coralliilyticus* strains utilized in this study. We also thank Dr. Colleen R. Eade, Dr. Tiffany C. Williams, and Erik L.J.E. Broemsen for their suggestions in the preparation of this manuscript. This work was supported by the United States Department of Agriculture [2009-03571], a UNC Research Opportunities Initiative (UNC ROI) award, and by the US National Science Foundation (IOS-1557870). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Agriculture, the U.S. National Science Foundation, or the UNC ROI program.

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TABLE S1 Primers designed for *V. coralliilyticus* qRT-PCR

Gene	Primer target	5'-3' Sequence[a]	Product size (bp)
DNA recombination	*recA*	F: GAAAGCGGAAATCGAAGGCG R: TCATACGGATCTGGTTGATGA	140
Zinc-metalloprotease	*vcpA*	F: TCCATCAAAAGATGGCCGCT R: TTACGTACATCCCAACCCGC	132
Hemolysin	*vchA*	F: CAATAGCACTCGTTGGCTGC R: TCTAGTTTCCCGCCTTGCTG	112
Flagellar biosynthesis protein	*flhA*	F: ATTTACCGGCCACCACAGAG R: GAAAGCTTTTTGCGAACCCCT	175
Mannitol-sensitive hemagglutinin pilin protein	*mshD*	F: TTCGCCAGCCGTATCATCTG R: TTGAACACTGCGAAGGACGA	167
T2SS protein G	*gpsG*	F: CTTCTTGGCCATCAGCACCT R: CTACCGCGATGGTGGTTACA	125
T2SS protein L	*gpsL*	F: GTGCCATTTTTGCTCTTCCG R: AGAGCTGGATGGTCAGTGGT	178
T3SS cytoplasmic protein	*sepL*	F: ACTGCTCGCGGAAGAAGAAA R: TGATAGAGCTGGCGAACTGC	99
T3SS needle protein	*t3ss*	F: TGCTGCACTGAAAATTGGGC R: TGCACAATCAGCACAGGACA	93
T6SS protein	*vgrG*	F: CGTCCGCTTTGTATGACGTG R: CTCGCTCCGCTGATATTGGT	127
T6SS protein ImpG/VasA	*vasA*	F: CGCAAGAAGAAGATGTGCCG R: CCCCAGTGGAAAACGACAG	222
S-ribosylhomocysteinase	*luxS*	F: GACAATGACCACGCCGAAAG R: AGTGACTGCGCATGAAACCT	134
Two-component system response regulator	*qseB*	F: TTCATCCCAACCGTGCATGT R: TGACACCATTGTGGTGGGAG	178
Signal transduction histidine kinase	*qseC*	F: GCGTTGCCACCAACATAGGTA R: TTAACGCAACCGAAAACGGG	121
8-amino-7-oxononanoate synthase	*cqsA*	F: GCCCGCAACGACACCTAATA R: CGTAAGCCATCTGACAAGCG	104

TABLE S2 Primers designed for *C.virginica* qRT-PCR

Gene	Primer target	5'-3' Sequence[a]	Product size (bp)
Toll like Receptor 4	*TLR4*	F:GCCTCCGACTGATTGATTTA R:ATACCTCTGAGGATAGGACG	119
C-type Lectin	*C-type lectin*	F:ATTTGCTCAGCCTTGAATGG R:GTCCCTCCCACCCAGTAGTT	131
Big defensin	*Big defensin*	F:TGGCAGCTGCTTACGGTATC R:CCCTGTTGTTGGCACAGCTA	102
Nucleoredoxin	*Nucleoredoxin*	F:TGAAGGCAGCTTCAACGAATA R:CCTGGCCTCTACTTCCAATTATC	221
Metallothionein	*MT*	F:CACAGCCGCTTCCTCATCCTCC R:CCGGCGGATTCCATACCAAGG	150
Heat Shock Protein 70	*HSP70*	F:AATTGGGCACCTTTGAACTG R:CTTTGTCGTTGGTGATGGTG	159
Hypoxia Inducible Factor -1a	*HIF-1a*	F:CTGCTCCGATCTGATCAACA R:CATACGTGTCCCCCTGTCTT	150
β-actin	*β-actin*	F:CACAGCCGCTTCCTCATCCTCC R:CCGGCGGATTCCATACCAAGG	134

[a] F: Forward primer target, R: Reverse primer target

TABLE S3 Effect of low oxygen or QS and their interaction on the gene expression of putative virulence factors and regulatory genes in *V. coralliilyticus*

Parameters	Oxygen	QS	Oxygen x QS
luxS	**$F_{1,2}$=378.29 P<0.0001**	**$F_{2,2}$=315.33 P<0.0001**	**$F_{2,2}$=311.96 P<0.0001**
qseB	**F1,2=137.90 P<0.0001**	**$F_{2,2}$=120.67 P<0.0001**	**$F_{2,2}$=122.97 P<0.0001**
qseC	**F1,2=6.78 P=0.023**	**$F_{2,2}$=5.34 P=0.022**	**$F_{2,2}$=5.38 P=0.021**
flhA	$F_{1,2}$=0.4841 P=0.499	**$F_{2,2}$=6.9095 P=0.0101**	**$F_{2,2}$=7.2963 P=0.0084**
vcpA	**$F_{1,2}$=119.036 P<0.0001**	**$F_{2,2}$=46.77 P<0.0001**	**$F_{2,2}$=46.93 P<0.0001**
vchA	$F_{1,2}$=0.059 P=0.812	**$F_{2,2}$=5.35 P=0.022**	**$F_{2,2}$=5.48 P=0.0204**
vasH	**$F_{1,2}$=602.64 P<0.0001**	**$F_{2,2}$=302.17 P<0.0001**	**$F_{2,2}$=302.71 P<0.0001**
sepL	**$F_{1,2}$=145.011 P<0.0001**	**$F_{2,2}$=87.79 P<0.0001**	**$F_{2,2}$=86.30 P<0.0001**
cqsA	**$F_{1,2}$=4.989 P=0.0453**	**$F_{2,2}$=6.79 P=0.0107**	**$F_{2,2}$=4.2042 P=0.0413**
vgrG	**$F_{1,2}$=217.64 P<0.0001**	**$F_{2,2}$=136.09 P<0.0001**	**$F_{2,2}$=132.97 P<0.0001**
mshD	**$F_{1,2}$=19.55 P=0.0008**	**$F_{2,2}$=6.39 P=0.0129**	**$F_{2,2}$=6.326 P=0.0133**
gpsL	**$F_{1,2}$=55.67 P<0.0001**	**$F_{2,2}$=43.07 P<0.0001**	**$F_{2,2}$=43.22 P<0.0001**
t3ss	**$F_{1,2}$=633.27 P<0.0001**	**$F_{2,2}$=467.38 P<0.0001**	**$F_{2,2}$=460.95 P<0.0001**
gpsG	**$F_{1,2}$=7.70 P=0.0168**	$F_{2,2}$=1.54 P=0.2534	$F_{2,2}$=1.27 P=0.3165

TABLE S4 Effect of exposure to bacteria, low oxygen or QS modulation and their interaction on parameters HC from oyster *C. virginica*

Parameters	Bacteria	Oxygen	QS	Bacteria x Oxygen	Bacteria x QS	Oxygen x QS	Bacteria x Oxygen x QS
Total HC	P=0.5789	P=0.5008	**P<0.0001**	P=0.7396	P=0.6628	P=0.7705	P=0.5992
Phagocytosis	P=0.0825	P=0.0635	**P=0.0012**	P=0.7543	**P=0.0106**	P=0.2784	**P=0.0274**
Oxidative burst	P=0.3349	P=0.0951	P=0.3323	P=0.0854	P=0.3656	P=0.6268	P=0.6177
TLR4	P=0.9116	P=0.2606	P=0.3706	**P=0.0213**	P=0.9312	P=0.8150	P=0.4186
C-type lectin	P=0.3755	P=0.3961	**P=0.0040**	P=0.4408	P=0.3630	P=0.6806	P=0.9340
Big defensin	**P=0.0198**	**P=0.0464**	**P<0.0001**	P=0.1046	**P=0.0040**	**P=0.0041**	P=0.0970
Nucleoredoxin	P=0.2136	P=0.8193	**P=0.0051**	P=0.3056	P=0.6814	P=0.9856	P=0.6482
Metallothionein	P=0.3676	P=0.3438	**P-0.0035**	P=0.2540	P=0.1150	P=0.1314	P=0.3669
HSP70	P=0.0785	P=0.0400	**P=0.0287**	**P=0.0030**	P=0.2515	P=0.2880	P=0.5739
HIF1a	P=0.2641	P=0.1378	P=0.2925	P=0.1127	P=0.7762	P=0.8410	P=0.4102

Significant P-value are in bold, marginally significant P-value are in red

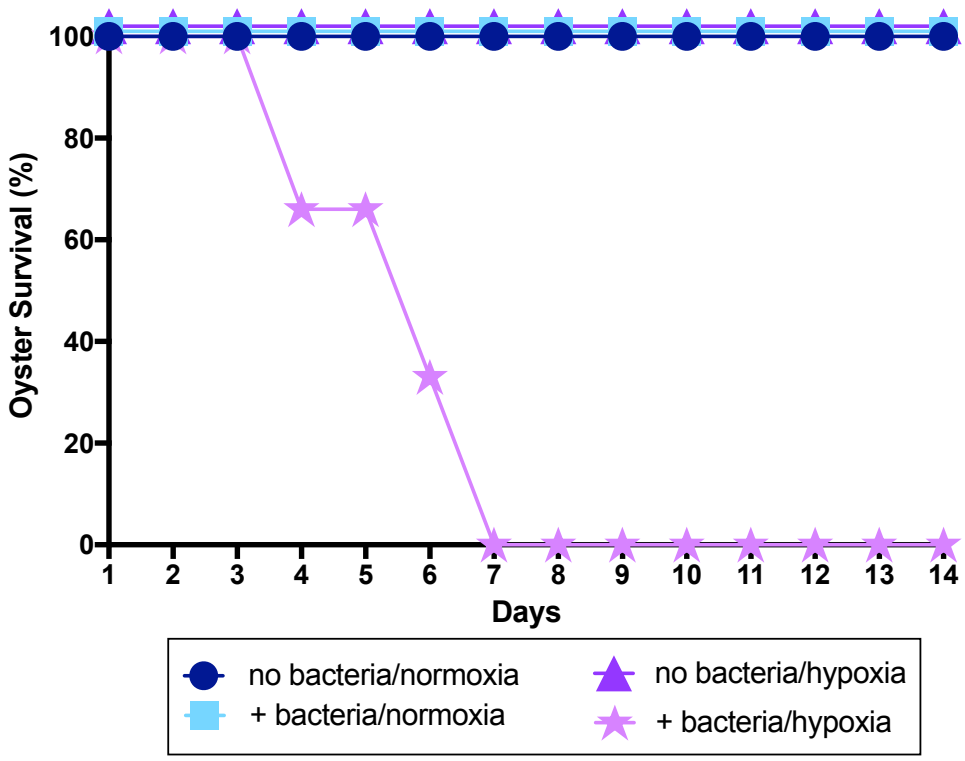

Figure 1. Percent survival of *C. virginica* exposed to the bacterium *V. coralliilyticus* under normoxia or hypoxia. Each data point represents the percent survival of at least three biological replicates with at least six oysters per experiment.

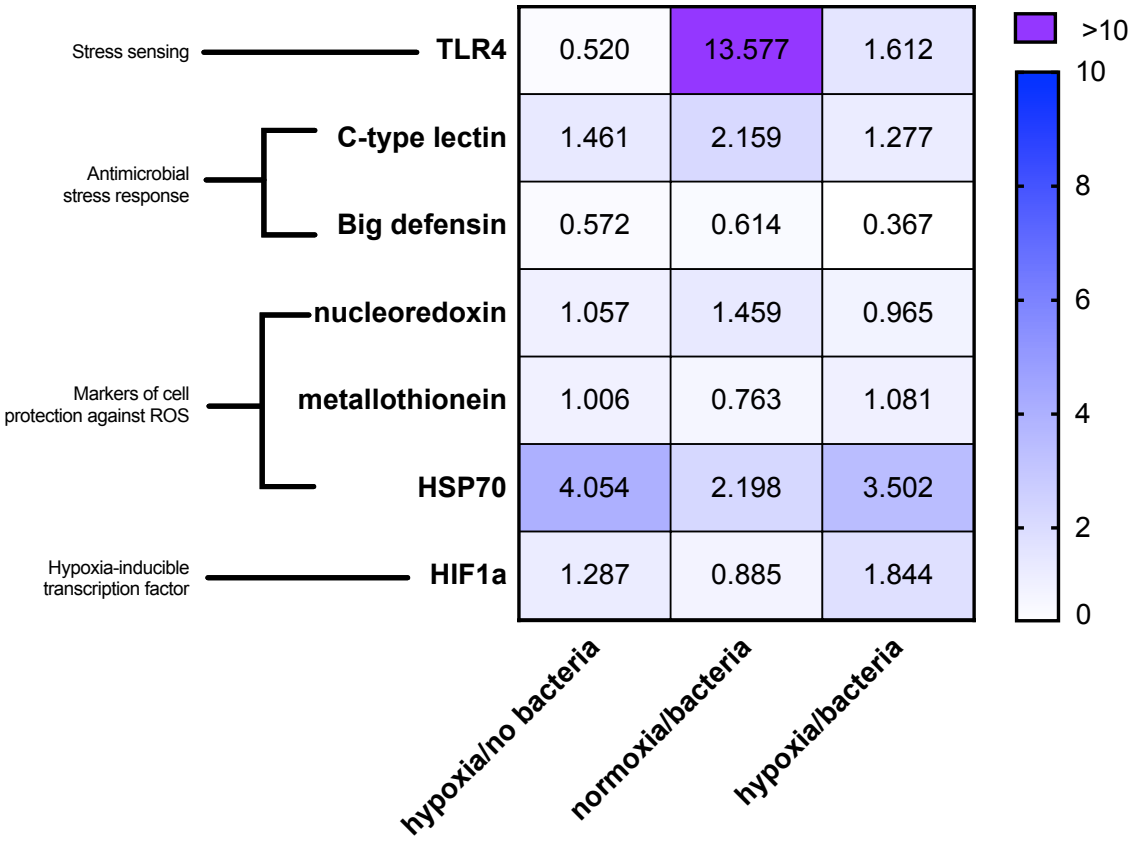

Figure 2. Relative expression of genes involved in immune-response and stress-response in hemocytes of oysters *Crassostrea virginica* exposed to hypoxia and *V. corallilyticus.* Heat map colors describe $\log_{10}$ fold change in anaerobic conditions relative to normoxia. Each box represents 4-5 biological replicates, comprising two technical replicates. Purple shaded areas indicate a $\log_{10}$ fold change > 10, which was outside of the defined range.

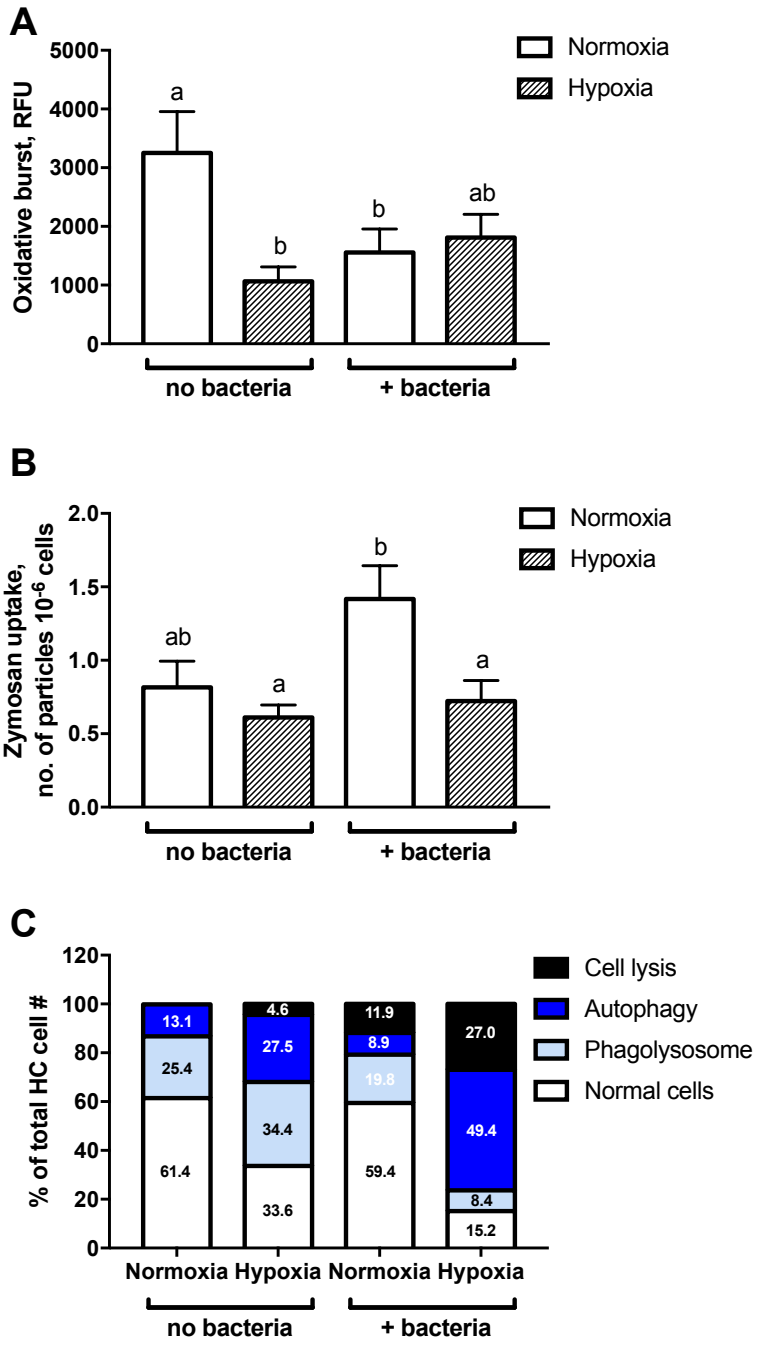

Figure 3. Effect of hypoxia and *V. coraliilyticus* exposure on immune-related traits of *C. virginica* hemocytes. (A) average rate of hemocyte phagocytosis, (B) oxidative burst, (C) cellular events activated as defense strategies visualized with neutral red staining. Columns that share letters represent values that do not significantly differ among different experimental conditions under the same QS treatment ($P<0.05$). Error bars represent the standard error of at least 4 biological replicates. For the neutral red staining for each experimental condition 100 cells were counted and scored in 3 biological replicates.

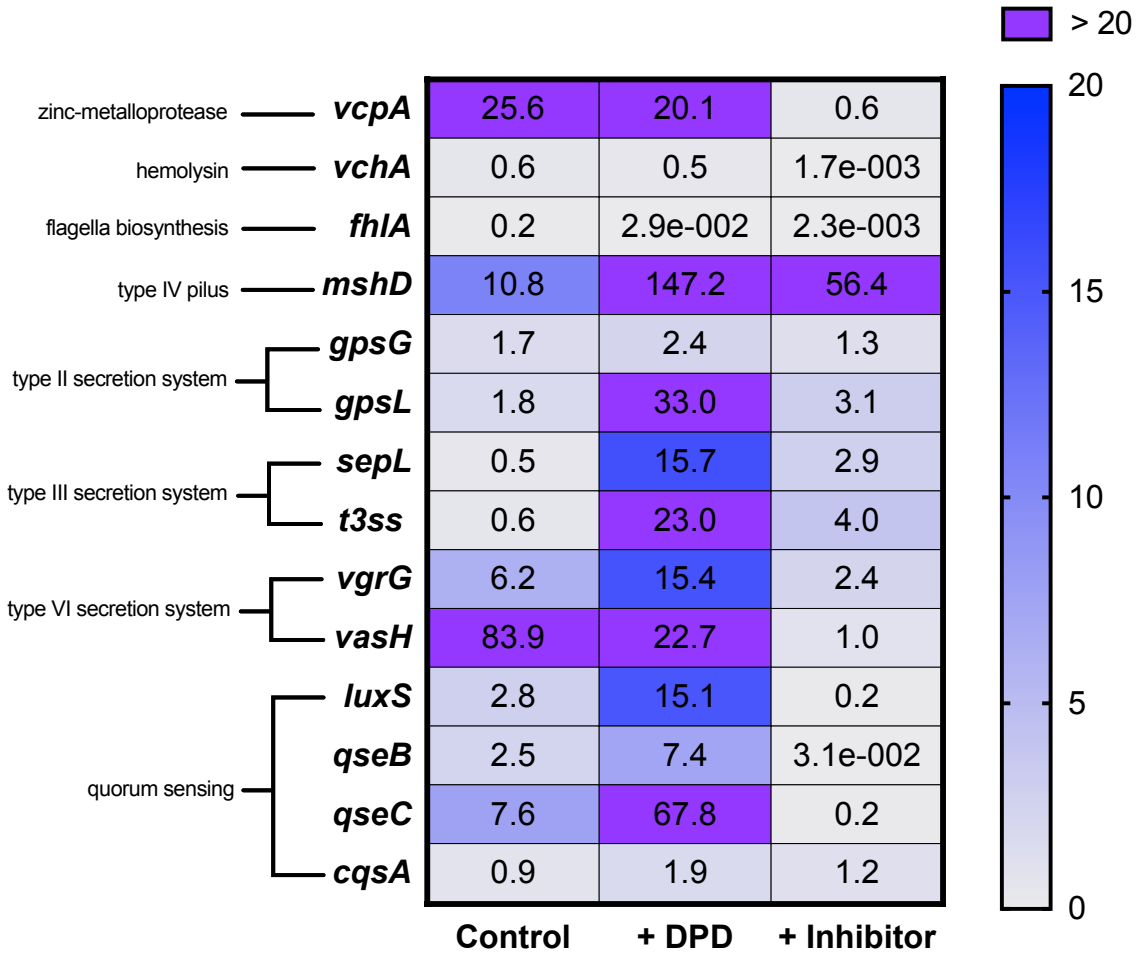

Figure 4. Relative expression of genes involved in various virulence and regulatory mechanisms in *V. corallilyticus* exposed to hypoxia, and their response to AI-2 manipulations. Heat map colors describe $\log_{10}$ fold change in anaerobic conditions relative to normoxia under each condition (hypoxia alone, hypoxia with DPD, or hypoxia with cinnamaldehyde). Each box represents at least three biological replicates (n=3), comprising three technical replicates averaged for strain RE98. Purple shaded areas indicate a $\log_{10}$ fold change > 20, which was outside of the defined range of the heat map.

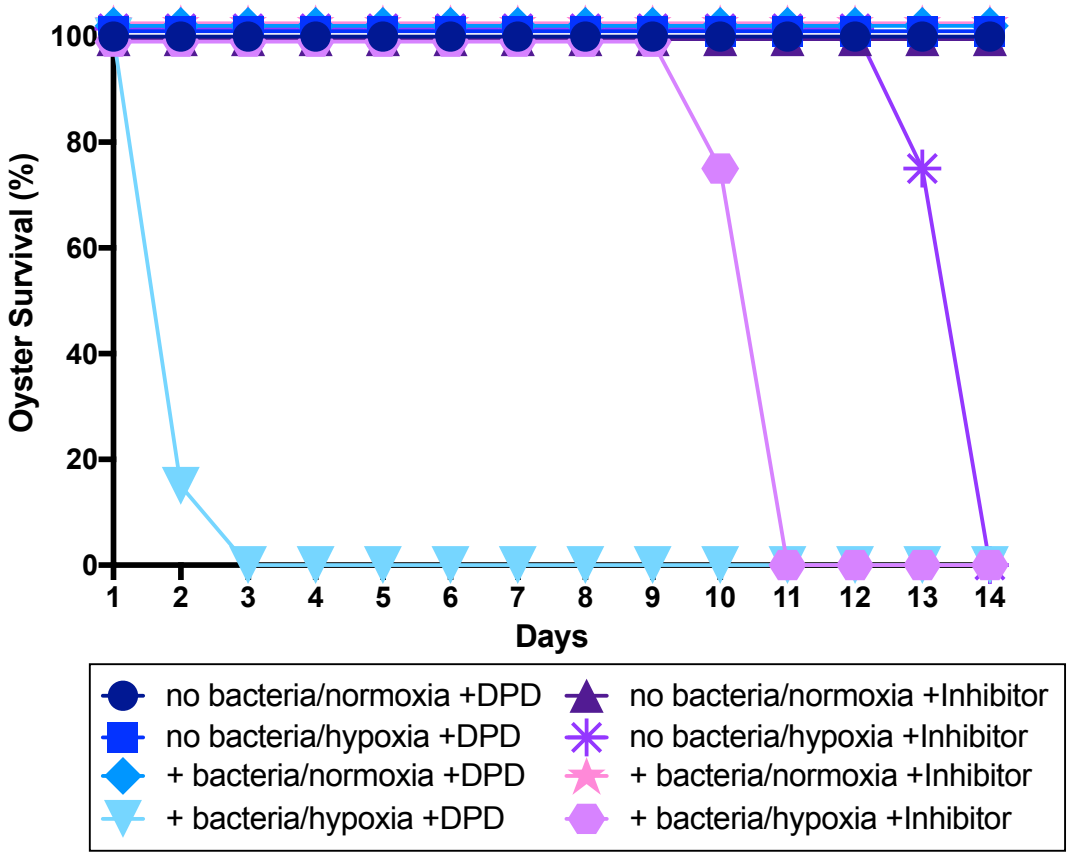

Figure 5**.** Percent survival of *C. virginica* exposed to the bacterium *V. coralliilyticus* under normoxia, and hypoxia, and their response to AI-2 manipulations. Each data point represents the percent survival of at least three biological replicates with at least six oysters per experiment.

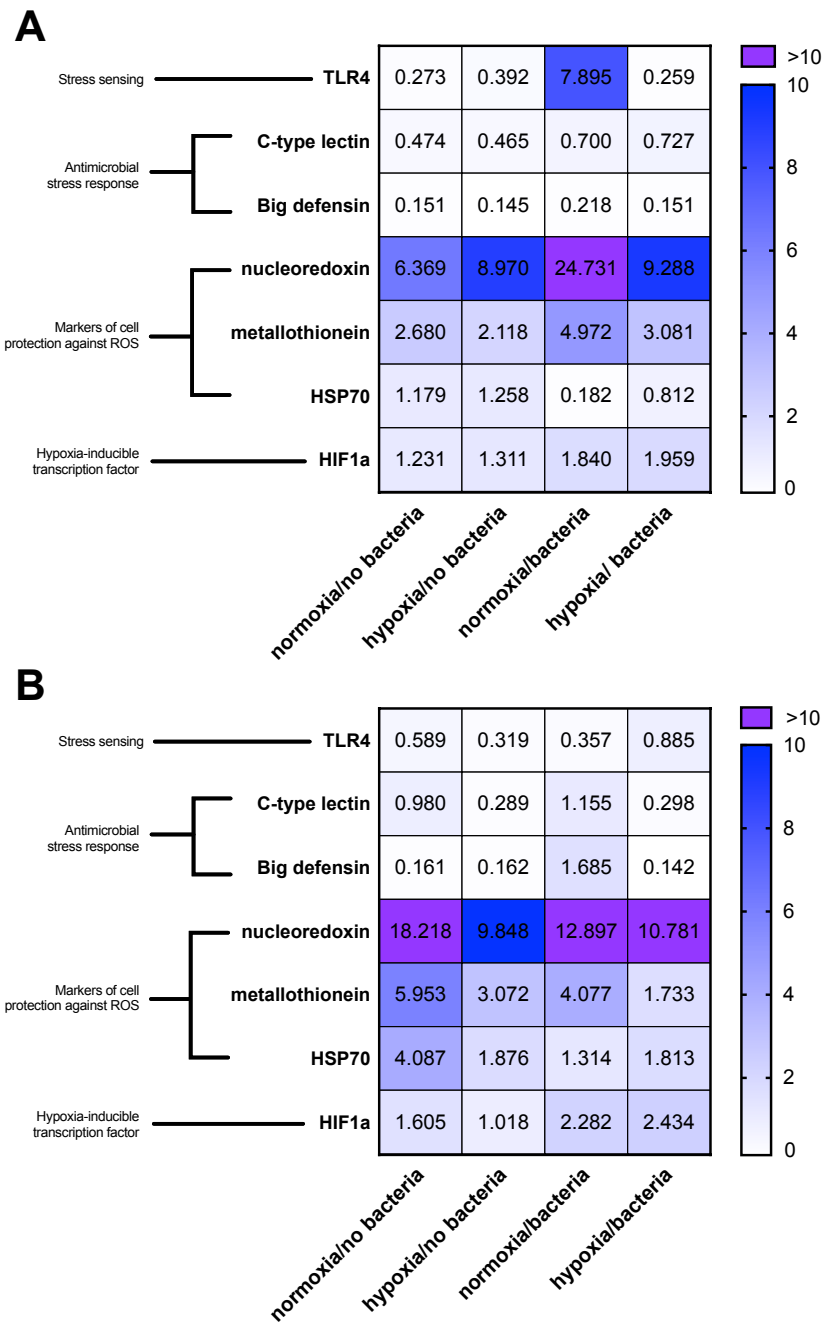

Figure 6. Relative expression of genes involved in immune-response and stress-response in hemocytes of oysters *Crassostrea virginica* exposed to hypoxia and *V. corallilyticus* in the presence of QS modulators. Heat map colors describe $\log_{10}$ fold change in anaerobic conditions relative to normoxia without QS modulation (A), upon addition of the synthetic AI-2 molecule precursor, DPD (B), or after addition of AI-2 inhibitor, cinnamaldehyde. Each box represents 4-5 biological replicates, comprising two technical replicates. Purple shaded areas indicate a $\log_{10}$ fold change > 10, which was outside of the defined range of the heat map.

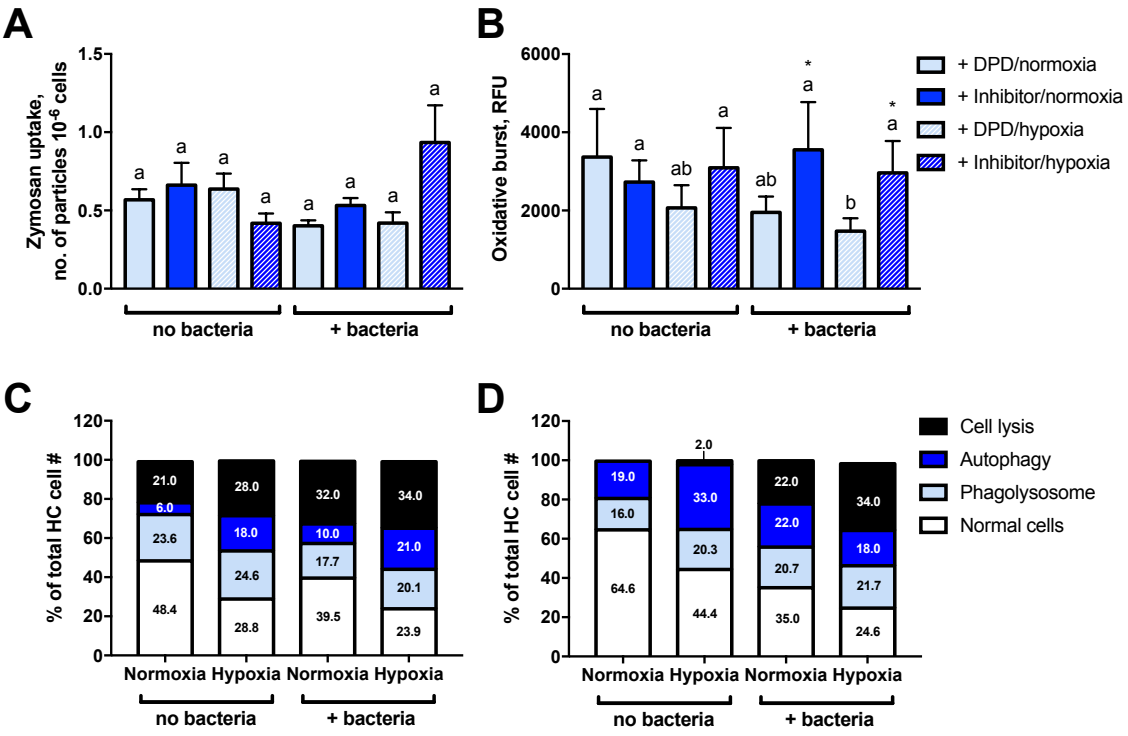

Figure 7. Effect of hypoxia and *V. coraliilyticus* in the presence of QS modulators on immune-related traits of *C. virginica* hemocytes. (A) average rate of hemocyte phagocytosis, (B) oxidative burst, (C) cellular events activated as defense strategies visualized with neutral red staining in the presence of DPD, (D) cellular events activated as defense strategies visualized with neutral red staining in the presence of AI-2 inhibitor, cinnamaldehyde. Columns that share letters represent values that do not significantly differ among different experimental conditions under the same QS treatment ($P<0.05$). Asterisks indicate values that are significantly different from the group without QS modulation at the respective oxygen and presence of *V. coralliilyticus*. Error bars represent the standard error of at least 4 biological replicates. For the neutral red staining for each experimental condition 100 cells were counted and scored in 3 biological replicates.

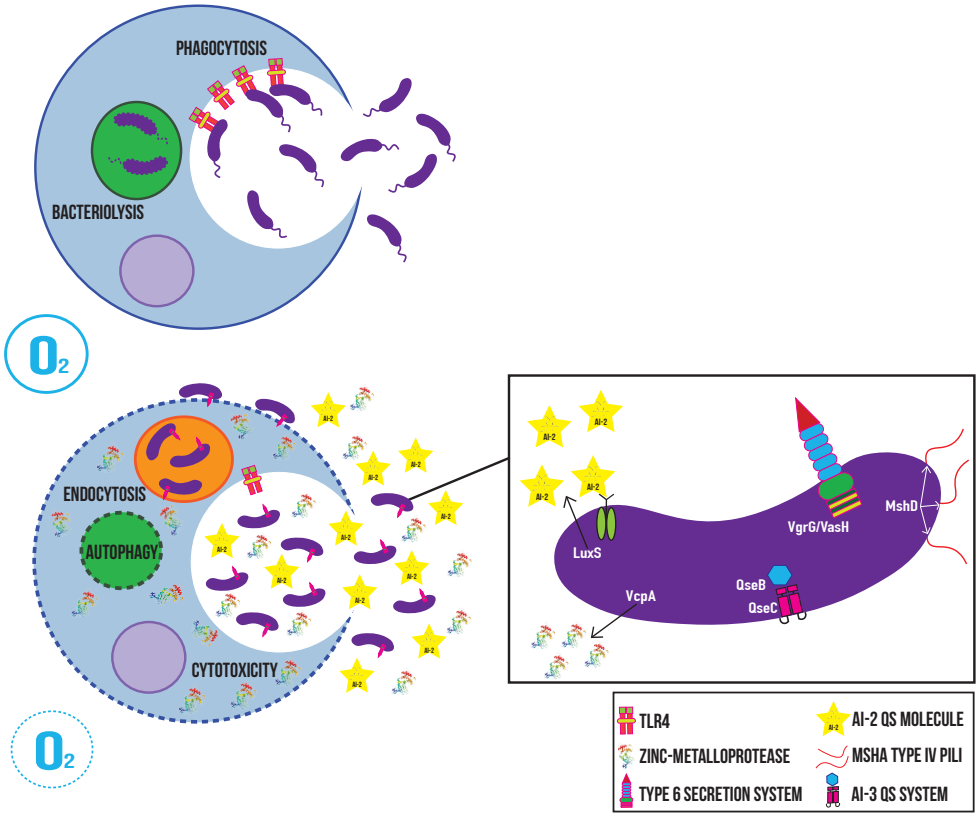

Figure 8. Schematic of molecular mechanisms involved with hypoxia induced pathogenicity of *V. coralliilyticus* as well as immune responses of *C. virginica* under concomitant stress unveiled in this study. Large blue circle represents oyster hemocytes, within which is green circle; lysosome, purple circle; nucleus, and orange circle; endosome. Dashed lines and solid lines represent compromised versus uncompromised cell membranes respectively. Image not drawn to scale.

CONCLUSIONS

This dissertation has provided an in-depth investigation on both *in vitro* and *in situ* hypoxia/anoxia in regards to responses in *Vibrio vulnificus* and *Vibrio coralliilyticus*. The specific impact of oxygen on these species had to our knowledge never been studied before, which makes this dissertation work important for both understanding the ecophysiology of these species and understanding host-pathogen dynamics. The first four chapters explore the impact of hypoxia/anoxia on *V. vulnificus* and chapter five focuses on how it impacts the relationship between *V. coralliilyticus,* and the eastern oyster *Crassostrea virginica*. As oxygen concentrations in estuaries worldwide are declining due to both natural and anthropogenic climate change, understanding these dynamics in the context of opportunistic pathogens will provide valuable information in terms of both human and ocean health.

In chapter one we proposed that when oysters harboring *V. vulnificus* are ingested, they traverse to the gut, which subsequently results in exposure to anaerobiosis. Presumably in an attached state, *V. vulnificus* would respond to the anaerobic conditions and possibly switch to a planktonic lifestyle. Our results supported this switch by the downregulation type IV pili and the upregulation of flagella, which was indicative of nonsessile living and why biofilm formation was impaired anaerobically. We also observed a reversion to an encapsulated phenotype after removal from anaerobic conditions, thus we suggested that once in the oxygenated bloodstream, genes for capsular polysaccharides were upregulated. Our lab has previously shown an innate difference in expression levels of type IV pili aerobically, and these results further validated those findings as well as expounded upon this under conditions lacking oxygen.

Chapter two was the first study to examine detachment dynamics of both clinical and environmental strains of *V. vulnificus* under aerobic and anaerobic conditions, and investigated potential molecular mechanisms that facilitated this detachment phenomenon. The finding that E-genotypes showed decreased ability to detach from chitin, with only 2% detachment in anaerobic environments, suggested a role for its maintenance in the environment, and increased propensity for sessile living. This points to E-genotypes predominating in oysters, which intuitively might lead to an increased consumption of these genotypes, but they are not typically associated with septicemia. In contrast, C-genotypes readily detached in both conditions compared to E-genotypes, which suggested an enhanced ability to switch from an attached state to a planktonic state. This ability is noteworthy, as most septicemia cases are caused by C-genotypes, and the mechanisms regulating this disparity are largely unknown. We proposed that even though the C-genotypes might not predominate in this system, they have an increased ability to detach, which will allow for their reattachment to the intestinal epithelium. We also showed that quorum sensing had a specific role in the dispersal of *V. vulnificus* from chitin, which has important implications for both clinical and environmental survival of these diverse ecotypes. Further investigation into the impact of quorum sensing inhibitors on detachment is needed, as this may effectively limit the ability for C-genotypes to attach to the intestinal epithelium and cause potentially fatal septicemia.

To our knowledge, chapter three is the only comprehensive study on the role of *in situ* hypoxia on gene expression profiles of any *Vibrio* species. We observed the largely cyclical nature of these profiles to be negatively correlated with dissolved oxygen at this site. We also examined bacterial community compositions at both high and low dissolved

oxygen sampling events, finding that they were similar, although our unweighted β-diversity suggested that rare sample occurrences might result in more diverse communities during normoxia. Importantly, this site experiences frequent nutrient inputs from a storm drain and represents a eutrophic estuarine environment, which should be explored further. These highly dynamic environments house a variety of opportunistic pathogens, including *V. vulnificus, V. parahaemolyticus,* and *V. cholerae*, all whose native ranges are expanding. This study highlighted the importance of studying not only temperature and salinity as it relates to the abundance and ecophysiology of *Vibrio* species *in situ*, but that of oxygen availability as hypoxia will only increase as global temperatures continue to rise.

The final chapter on *V. vulnificus,* chapter 4, utilized an RNA sequencing approach to tease out the response of this bacterium to *in vitro* oxygen stress alone. This was the first comparative transcriptomic study investigating the profiles of both C- and E-genotypes of *V. vulnificus*, and again further investigated anoxia, which is an important environmental and physiological stressor. We showed that the E-genotypes increased a variety of unique genes in both aerobic and anaerobic conditions, and that there were a large set of genes increased in both conditions compared to the C-genotype. The enriched categories lead us to characterize the E-genotype as an "environmental specialist" when compared to the C-genotype, as it increased a variety of systems that have been shown to aid in environmental persistence. The E-genotype not only increased systems involved in the transport of nutritionally essential compounds, but also loci important for colonization, motility, attachment, and bacterial competition via the type six secretion system. These findings have given us an advantageous pool of DEGs that should be

investigated further, especially in the context of *in situ* responses. We also showed that the E-genotypes had a competitive advantage in nutrient limited environments, however more investigation will need to be done to understand the mechanism behind this result and allow us to determine if it is related to their intrinsic up-regulation of the type six secretion system. Finally, although not discussed in detail in this chapter, there were 803 genes significantly down-regulated by the E-genotypes over both conditions, which may reveal differentially expressed virulence mechanisms for the C-genotype that we have not yet identified during anoxia.

V. coralliilyticus has been recently identified as an important larval shellfish pathogen causing mass mortalities in larvae of a variety of species. However, these studies did not elucidate the molecular processes underlying the observed host-pathogen relationship. Chapter five demonstrated that *V. coralliilyticus* can be especially pathogenic for *C. virginica* under hypoxic conditions, and our physiological and molecular data indicated that this was due to the dual effects of hypoxia on the host and the pathogen. Specifically, during normoxia oyster hemocytes could recognize *V. coralliilyticus* by a toll-like receptor four mediated response, which promoted ingestion of the pathogen via the phagocytic pathway. The phagocytes then fused with the lysosomes and the bacteria could be cleared from the cells, as the virulence factors we investigated showed relatively low expression levels in *V. coralliilyticus* during normoxia. During oxygen deficiency, the bacterium significantly increased the production of putative virulence factors such as VcpA, T6SS, MshD pilus, as well as interspecific and interspecies quorum sensing systems. These factors, either directly or indirectly, influenced the ability of oyster hemocytes to recognize *V. coralliilyticus*,

resulting in an increased proportion of bacterial cells that entered hemocytes via endocytosis and resulted in cytotoxicity. Together, our results in chapter five began to unveil the complex relationship that oxygen plays on host-pathogen dynamics, demonstrated the importance of understanding abiotic factors as potential triggers of virulence in marine bacteria, and showed possible ways to manipulate those interactions by targeting global regulatory systems such as the AI-2 quorum sensing pathway.

There are still many unanswered questions about how *V. vulnificus* and *V. coralliilyticus* is able to successfully cause disease in the human host and why some strains correlate more highly with pathogenesis. Responses to environmental parameters encountered in the host are important indicators of virulence potential, and this dissertation reports for the first time the role of hypoxia/anoxia on *V. vulnificus* and *V. coralliilyticus*.

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