INVESTIGATING THE ECOLOGICAL, PATHOGENIC, AND DORMANT LIFESTYLES OF THE ESTUARINE BACTERIUM, *VIBRIO VULNIFICUS*

by

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ABSTRACT

TIFFANY CHRISTIAN WILLIAMS. An investigation into the ecological, pathogenic, and dormant lifestyles of the opportunistic human pathogen, *Vibrio vulnificus*.
(Under the direction of DR. JAMES D. OLIVER)

Vibrio vulnificus is ubiquitous in estuarine and coastal waters worldwide and associates naturally with a variety of aquatic organisms. Notably, it is a highly invasive human pathogen capable of causing septicemia and multi-organ failure in persons consuming raw or undercooked shellfish, and can also cause flesh-eating disease upon entry into open wounds, both of which can lead to death in susceptible individuals. Of great importance is the persistent number of *V. vulnificus* cases documented in the US, and the significantly increasing rate of wound infections worldwide. Interestingly some strains of this species are more often implicated in human disease (clinical [C-] genotypes) and have identifiable genetic distinctions from strains that are typically less virulent and more often isolated from oysters and estuarine waters (environmental [E-] genotypes). The goal of this dissertation collection was to address the following three primary aspects of the biology of *V. vulnificus*: the ecology, pathogenicity, and dormancy dynamics of this organism. While *V. vulnificus* thrives in warmer waters, it can also endure cold seasons by overwintering in a dormant state referred to as the viable but non-culturable (VBNC) state which facilitates persistence of the organism in the environment. Here, we elucidate the molecular mechanisms responsible for awakening from the VBNC state, and identify quorum sensing (a wide-spread bacterial communication system) to be the phenomenon that underpins this dormancy state. To better understand the ecology of this organism, we have investigated the molecular and physical factors that facilitate

chitin attachment, a substrate known to serve as a reservoir for the persistence of *Vibrio* species in the environment. These studies revealed significant differences in the behavioral properties of C- and E-genotypes, offering insight into their documented environmental distribution. Lastly, to improve our understanding of the differential pathogenic capabilities of C- and E- genotypes, we set out to identify the molecular mechanisms that underlie these differences by use of next-generation sequencing techniques, including genome and transcriptome sequencing. This allowed for the identification of several putative virulence factors that could potentially aid this bacterium in disease progression. Furthermore, this allowed us to characterize the different gene expression profiles of C-genotypes as they transition from environmental to host-like conditions. Collectively, these studies advance our understanding of *V. vulnificus* as a natural inhabitant of the estuary, and as a human pathogen.

DEDICATION

This dissertation is dedicated to my family.

My parents: Ronnie C. Williams and Sylvia S. Williams.
My beautiful sisters: Sealy Nash and Ronii Rizzo
My nephew: Isaac Nash

As a unified couple, my parents have supported my pursuits, encouraged me to strive for my ambitions, and taught me the virtues of being dependable, responsible, and dedicated to your profession, all while maintaining a positive attitude, regardless of the circumstances. My sisters are exemplary role models as successful women in their professional fields, and have demonstrated infinite strength in the face of life's challenges. Without the loving presence and unyielding support of my family, I would truly be lost in this world. Most of all, this work is dedicated to my nephew who is the toughest little man I've ever met. He is my daily motivation to appreciate every single moment of this precious life.

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INTRODUCTION

1.1 *Vibrio vulnificus* Environmental Occurrence and Virulence

Vibrio vulnificus is a gram-negative, halophilic bacterium, which freely inhabits estuarine and coastal waters worldwide. This species can be routinely isolated from the water column or sediments, and has been found to associate with a variety of aquatic organisms such as oysters, mussels, clams, zooplankton, crabs, and fish [1]. These natural associations with other aquatic inhabitants are thought to facilitate the environmental persistence of this bacterium, serving as environmental reservoirs and/or vehicles for *V. vulnificus* [2].

V. vulnificus is a medically important opportunistic pathogen as it is a known, highly invasive pathogen of both fish and humans [2,3]. In humans, this single species is responsible for 95% of all seafood-related deaths in the US, typically from the ingestion of raw or undercooked molluscan shellfish, such as oysters. This pathogen can cause rapidly fulminating septicemia and organ failure within 24 hours after exposure and has the highest case fatality rate (>50%) of any foodborne pathogen [4,5]. Furthermore, the number of documented *Vibrio* infections in the US has increased by 85% since 1996 and is increasing in relation to other major foodborne pathogens [4,6].

In addition to host entry through ingestion, this bacterium can also enter the human body through pre-existing cuts or open wounds. Individuals exposed to water sources harboring high levels of *V. vulnificus* can succumb to severely necrotic wound infections, which often result in limb amputation, and can lead to septicemia and even death (ca. 25% mortality rate) in susceptible individuals [3]. Individuals partaking in

recreational water activities and seafood handlers are often at a higher risk of developing wound infections from *V*. *vulnificus* due to their interaction with the marine environment.

The pathogenicity of *V*. *vulnificus* is believed to arise from several putative virulence factors, including the production of iron-binding siderophores, exoenzymes, and capsular polysaccharides, as well as the ability to survive exposure to human serum [3,7-9]. While most of the proposed virulence factors remain unconfirmed, the production of capsular polysaccharide (CPS) is highly correlated with pathogenicity [3,10-12]. Previous research has shown that the LD_{50} of the encapsulated, "opaque" strains is as low as a single cell whereas non-encapsulated, "translucent" strains of *V*. *vulnificus* display little or no virulence [10,11].

Host susceptibility is a key factor in disease progression in which individuals with preexisting chronic disorders such as immunological suppression, hepatic dysfunction, or blood disease are thought to be more susceptible to developing septicemia by ingestion or wound infections [2,13,14]. Additionally, males over 40 years of age are more susceptible, with estrogen thought to provide a protective effect against disease in women [15]. Healthy individuals are considered to be at low risk to disease by septicemia but are still at risk of developing wound infections and/or gastroenteritis [16].

1.2 Genetic Diversity and Genotypic Characterization of *V*. *vulnificus* Strains

1.2.1 Biotypes

The immense disparity between the number of at risk individuals (12 – 30 million) and the number of documented cases per year (<100) has led to the suspicion that not all strains of *V*. *vulnificus* are equally pathogenic [17]. *V*. *vulnificus* is a

phenotypically and genetically diverse species, hence strains are currently organized into 3 biotypes based on biochemical characteristics [18,19]. Identification of a subset of *V*. *vulnificus* strains pathogenic to eels led to the proposal that *V*. *vulnificus* strains be subdivided into two biotypes, in which biotype 1 represents strains most often associated with disease in humans, whereas biotype 2 represents strains almost exclusively associated with disease in eels [18]. In 1996, Israel experienced an unprecedented outbreak of wound infections and bacteremia from handling fresh fish. Biochemical and genetic tests revealed these strains belonged to the species *V*. *vulnificus*, however distinct phenotypic and molecular patterns led to the proposal of a new subgroup designated biotype 3 [19].

1.2.2 Genotypes Correlate with Pathogenic Potential

Previous genetic analyses identified genetic indicators of virulence within biotype 1 strains through the identification of sequence polymorphisms (SNPs) within a specific gene (designated the virulence correlated gene – *vcg*) that strongly correlated with clinical isolates [20-22]. Subsequently, a PCR-based assay was developed for the purpose of categorizing *V*. *vulnificus* into two groups that correlate with isolation source [23]. Thus, we now further sub-type biotype 1 strains into two groups where strains with the *vcgC* allele are referred to as clinical (C-genotypes), and strains with the *vcgE* allele are designated environmental (E-genotypes). Other genotyping systems based on differences in loci such as 16S rRNA or hemolysin (*vvhA*) genes have also been devised to separate *V*. *vulnificus* populations into two genotypes which similarly correlate to environmental or clinical isolation [24-27].

While these genotyping schemes have been shown to correlate with virulence potential, it is important to note that these systems cannot strictly predict virulence [28]. For example, some strains with the *vcgE* allele have been isolated from clinical cases, often from wound infections. In paper 2, we analyze a subset of E-genotype wound isolates and demonstrate their ability to resist the bactericidal effects of human serum in a manner similar to that of C-genotype blood isolates. Thus, it appears that a subset of E-genotypes have the ability to cause human disease. This study highlights the need for more substantial indicators of virulence potential in *V. vulnificus* strains.

1.2.3 C- vs E-genotype Genome and Transcriptome Comparisons

These studies support the original hypothesis that not all strains of *V. vulnificus* are equally capable of causing disease. Interestingly, a previous analysis of several highly conserved housekeeping loci and putative virulence loci revealed that these genes also possess genetic polymorphisms and subsequent phylogenetic analyses support the aforementioned genotypic distributions [29]. Until recently, only C-genotype genomes had been fully sequenced, limiting our understanding of the scope of genetic diversity between C- and E-genotypes. In an attempt to identify key differences between these two genotypes, we sequenced 3 E-genotypes and performed a comparative analysis with three previously sequenced C-genotypes. This study, which is described in paper 3, confirmed the phylogenetic divergence of *V. vulnificus* C- and E-genotype strains, revealing a distinct branching between C- and E-genotypes, and supporting the claim that C/E differences are not restricted to a few loci but are genome-wide.

Despite the presence of numerous polymorphisms across the genome (many of which correlate with virulence), no single virulence factor has been found to be exclusively associated with clinical strains [6]. In the hunt for potential virulence genes, paper 3 reveals a more in-depth comparison of the two genotypes. This comparative analysis identified unique genes exclusive to each genotype, including 167 genes specifically associated with environmental genotypes and 278 genes specific to clinical genotypes [30,31]. Specifically, C-genotypes were found to harbor a number of genes with potential roles in pathogenesis including a 33kb genomic island (region XII), genes involved in sialic acid catabolism, mannitol fermentation, and components of the Type IV secretory pathway. Additionally, in all three C- genotypes, we identified an operon homologous to the RsbRST stress response module (or "stressosome") which commonly exists as part of the "general stress response" in gram-positive bacteria. In these organisms, this signaling hub responds to a variety of environmental insults and has potential implications for virulence. Elucidating the role of this system in *V*. *vulnificus* could potentially provide insight into the mechanisms of virulence and survival in this organism.

While this study provided significant insight into the genetic distinctions between C- and E-genotypes, further investigations are warranted to clearly understand the relevance of these genotype-unique genes in facilitating environmental survival and/or human virulence. Furthermore, it is important to note that the genetic determinants that allow for host colonization and disease progression in the human host may be more complex than the presence/absence of certain genes. Thus, the results of this study prompted, and provided the foundational basis for, the transcriptome sequencing projects

highlighted in papers 4 and 5. In paper 4, we utilized RNA sequencing to analyze the transcriptome response of C-genotypes exposed to human serum (HS) relative to artificial seawater (ASW). This experiment revealed two divergent genetic programs as cells transition from seawater into the human host environment. Specifically, when *V. vulnificus* cells encounter a host-like environment (HS), they exhibit a "virulence profile" in which biofilm formation is inhibited and virulence factor production predominates. Conversely, in an environment that simulates estuarine conditions (ASW), cells take on a low virulence, enhanced biofilm phenotype which we refer to as the "environmental profile". This dichotomous genetic switch appears to be largely governed by cyclic-di-GMP signaling, and remarkably resembles the dual life-style of *V. cholerae* as it transitions from host to environment.

This study confirmed that C-genotype specific genes were differentially expressed between the two conditions, including genes within the genomic XII region and the RsbRST stressosome module. Interestingly, the stressosome genes were upregulated in ASW, and follow-up gene expression studies revealed that C-genotypes upregulate genes for the stressosome module when incubated in natural estuarine waters. These findings suggest that this system is utilized by clinical genotypes, and prompts further interest in the role it may play in pathogenicity and/or niche colonization in strains which harbor these genes. Considering that this system acts as a signaling hub in other organisms, we speculate that this system is used to integrate various signals into a single output, thereby allowing for fine-tuning of stress responses in particular environments.

In paper 5, we further extend our transcriptomic analysis to E-genotypes and compare the transcriptome response of both genotypes as they transition from ASW to

HS. Here we found that the transition of E-genotypes between these two conditions is remarkably similar to that of C-genotypes, suggesting that these genotypes possess a similar adaptation response to human serum. In contrast to the transcriptome response *across* conditions, we discovered that *within* ASW, C- and E-genotypes have different transcriptomic profiles, and these fundamental differences may explain why these genotypes differ so greatly in their ecology. Of particular interest was the upregulation of type IV pili and EPS-associated genes in E-genotypes which suggests that these strains are more efficient in attaching to substrata and building biofilms [32,33]. Indeed, evidence for this has been documented in papers 7 and 8 (mentioned later), and in paper 5 we show that E-genotypes build more robust biofilms in the natural environment. Conversely, C-genotypes expressed genes involved in CPS production and Flp pili. Both of these cell surface structures have been implicated in virulence [12,34-36], and may serve to facilitate successful survival and colonization within the human host, should the bacterium encounter this environment. Collectively, papers 4 and 5 reveal a phenotypic and genetic dichotomy between C- and E-genotypes which, if further investigated, could offer additional insight into the pathogenic potential and environmental prevalence of these two genotypes.

1.2.4 Ecological Differences Between C- and E-genotypes

Interestingly, previous studies have shown that C- and E- genotypes display different environmental distributions, in which E-genotype strains often predominate in oysters and the water column whereas the less prevalent C-genotypes are more often implicated in disease [21]. While it is well known how salinity and seasonality influence

the diversity of *V*. *vulnificus* in the environment [37,38], the population structure of C- and E-genotypes within a given environment appears to be more complex, highlighting the need for a better understand the biotic and abiotic factors that influence the spatial and temporal distribution of C- and E-genotypes.

Deeper investigation of ecological niches, such as microenvironments, has provided considerable insight into niche differentiation of C- and E-genotypes. A study investigating the ability for *V*. *vulnificus* C- and E-genotypes to integrate into marine aggregates found that they do so with significantly different efficiencies [39]. This study found that E-genotypes incorporate into marine aggregates much more efficiently compared to their C-genotype counterparts, and this increased attachment resulted in preferential uptake and retention of E-genotypes by oysters feeding on these marine aggregates. This study offered insight into why E-genotypes have been previously documented to predominate within the oyster environment.

In paper 6, we further investigate the physiological and genetic causalities for this demonstrated genotypic heterogeneity. Marine aggregates are largely composed of chitinous substrates, thus we examined the ability of strains of each genotype to attach to chitin. This study revealed that E-genotypes attach to chitin with a significantly greater efficiency compared to C-genotypes, and that surface-associated proteins including type IV pili and mannitol sensitive hemagglutinin (MSHA) were implicated in the observed adherence to chitin. Interestingly, even in the absence of chitin, expression levels of these attachment genes were significantly higher in E-genotypes relative to C-genotypes, suggesting that E-genotypes have an inherent predisposition for adherence to substrates such as chitin. We conclude this study by proposing that C- and E-genotypes have

intrinsically divergent physiological programs which may help to explain the observed genotypic differences in oyster colonization and possibly pathogenic potential.

In paper 7, we further elaborate on this finding by identifying quorum sensing as a negative regulator of type IV pili expression, which results in decreased chitin attachment. We identify calcium as a critical component for enhanced chitin attachment by E-genotypes, and suggest that calcium in necessary for proper functioning of type IV pili. We also show that environmental stressors which induce starvation or dormancy (VBNC state) can alter the efficiency of chitin attachment which has significant implications for the environmental persistence of *V*. *vulnificus*. With the current increasing incidence of wound infections caused by *V*. *vulnificus* world-wide, we investigated a subset of E-genotypes, isolated from human wound infections, and discovered that they attached to chitin in a manner more similar to C-genotypes. Paper 2 revealed that these E-genotype wound isolates are also more resistant the bactericidal effect of human serum, suggesting that this subset of E-genotypes share virulent traits with C-genotypes, a finding which warrants further investigation. Overall, paper 7 contributes to our understanding of the molecular and physical factors that mediate chitin attachment in *V*. *vulnificus*, and provides insight into the mechanisms that facilitate environmental persistence of this pathogen in its native environment.

1.3 Understanding the mechanisms of dormancy in *V*. *vulnificus*

The environmental occurrence of *V*. *vulnificus* is favored by high temperatures (>20°C) and intermediate salinities (15 – 25 ppt), hence this bacterium can be readily isolated in environments within these physiochemical parameters [40]. However, this

organism can tolerate wide salinity ranges (1 – 35 ppt) and temperatures (7 – 31°C), which likely contributes to its natural prevalence [41,42]. Furthermore, when water temperatures drop below 4°C for extended periods of time, *V*. *vulnificus* has been documented to enter a state of dormancy referred to as the Viable but Nonculturable (VNBC) state [43,44]. This process of overwintering is thought to explain the observed seasonality of this organism in its native environment.

Numerous bacterial species have been found to enter this state of dormancy in which cells remain alive, but suppress metabolic functions, and therefore fail to grow on routinely used culture-based media [45,46]. This physiological state allows them to evade harmful environmental stresses while maintaining the ability to return to an active state once the stress is alleviated [45]. While in this state, cells are able to resist a variety of normally fatal stresses, including antibiotic treatment [47] and can resuscitate within a host, potentially leading to disease initiation [48]. Because these potentially pathogenic cells are not detectable by standard bacterial culture methods, the VBNC phenomenon is of significant public health and food safety concern. While there has been considerable growth in our knowledge of this dormancy state, very little is known regarding the molecular mechanisms responsible for the induction into and resuscitation from the VBNC state. In paper 8, we reveal that quorum sensing (QS), a mechanism for bacterial communication, is a predominate mechanism utilized by cells to awaken from this dormant state.

The VBNC state of *V*. *vulnificus* is easily studied in the laboratory by exposing the cells to low temperature incubation (4°C). These dormant cells can then be resuscitated by simple temperature upshift (room temperature), making it an ideal model

organism for the study of this physiological state. In this study we monitored QS activity of *V*. *vulnificus* cells as they resuscitate from the VBNC state and found QS signaling to be critical for the natural resuscitation process. We suggest Epstein's microbial scout hypothesis as a possible explanation for the observed phenomenon. This hypothesis proposes that a small portion of a dormant population can resuscitate stochastically acting as scouts to gauge the surrounding environment [49,50]. If environmental conditions are conducive to growth, these cells will emit a signal (in this case QS molecules) to rouse the rest of the population resulting in resuscitation.

In paper 8, we also propose a molecular mechanism for the resuscitation of VBNC cells, and show that it can be targeted to manipulate resuscitation of dormant cells. We demonstrate that addition of exogenous QS signaling molecules to VBNC populations can induce early resuscitation and conversely, a QS inhibitor can be used to delay resuscitation. We substantiate the ecological relevance of this finding by demonstrating that we can also awaken naturally dormant vibrio populations within oysters and seawater (*in vivo*). This study provides new insights into the molecular mechanisms stimulating VBNC exit from dormancy, which has significant implications on microbial ecology and public health.

The VBNC state has primarily been studied in an ecological context, however, a similar mechanism of dormancy referred to as "bacterial persistence" has been recently and extensively discussed in the literature in a clinical context [51]. While both VBNC cells and bacterial persisters have been shown to produce antibiotic-tolerant populations capable of withstanding prolonged lethal treatment [47,52], we realized that these two dormancy states are typically described as distinct processes, and are surprisingly very

rarely discussed in the same context. In paper 9, we compare the phenotypic characteristics and molecular underpinnings of these two dormancy states, and based on the evidence, argue that these two states are not distinct but are closely related phenomena.

In this paper, we make important connections detailing the similarities between the inducers, dormancy dynamics, phenotypic characteristics and molecular foundations of these dormancy states. We systematically provide evidence implicating all aspects of the proposed bacterial persistence mechanism in the VBNC state. Based on the evidence we present a novel "dormancy continuum hypothesis," which states that VBNC cells and persisters share the same mechanisms but are found in different positions in the dormancy range. We also propose future experiments to test this hypothesis. Additionally, we argue (based on published works) that, as with persisters, VBNC cells are clinically relevant with respect to recalcitrance of infections and emphasize that future efforts aimed at eradicating dormant cells should not overlook VBNC cells. The discussion within paper 9 is intended to stimulate discourse about these seemingly different but very similar dormant states, and enhance our overall understanding of the mechanisms underlying bacterial dormancy in both ecological and medically relevant contexts.

1.4 Detection, Prevalence, and Emerging Risk from Climate Change

Interestingly, foodborne outbreaks of *V*. *vulnificus* have never been documented and disease incidence appears to be sporadic, however infection rates tend to correlate with seasonality [6,21,53]. Of the estimated ~100 septicemia cases per year in the US, the

majority are associated with the ingestion of raw or undercooked oysters during warm weather months [2,6]. It has been predicted by several investigators that current trends in climate change will expand the risk season, thereby increasing the rates of infection in the future [6,54,55]. Indeed, *V*. *vulnificus* infections have been increasing in the US and world-wide, and tend to follow regional climatic trends [6,54-56].

Global climate change is anticipated to increase the frequency and intensity of extreme weather events such as heat waves, hurricanes, severe precipitation events, and droughts. Thus, there has been growing interest in how these anticipated extreme climatic and oceanographic events may impact bacterial populations and subsequently alter disease risk. Paper 10 highlights a relevant case in which an extreme weather event coincided with a remarkable change in bacterial population dynamics. In this report, we present a multi-year study which tracked *V*. *vulnificus* levels in oysters and estuarine waters during the most severe drought documented in the history of North Carolina. As a result, average water salinities increased significantly and coincided with a substantial loss in culturability of *V*. *vulnificus* from NC oysters and estuarine waters. During this drought period, we used identical isolation methods to successfully isolate high numbers of *V*. *vulnificus* from Gulf coast oysters, demonstrating that our isolation methods were sound, and that the phenomenon was indeed geographically isolated. After the drought abated and salinities returned to normal, we were again able to routinely isolate *V*. *vulnificus* from the water column, however *V*. *vulnificus* levels did not return to normal in oysters. We suggested that this was possibly a consequence of competitive displacement by a more salt-tolerant bacterium, which may have prevented recolonization by *V*. *vulnificus*.

Paper 10 presents an example of a prolonged climatic weather event that reduced the levels of pathogenic *V*. *vulnificus* in the environment. Of greater concern are events that may lead to a substantial increase in pathogenic vibrios. An example of this was documented when hurricane Katrina devastated the Gulf Coast in 2005. In the aftermath of this natural disaster, CDC COVIS (Cholera and Other Vibrio Illness Surveillance) recorded the largest number of *V*. *vulnificus* cases reported in a single month. The spike in *Vibrio* infections was attributed to the increase in number of individuals exposed to floodwaters, as the majority of the documented infections were wound-associated. Subsequent studies have also documented correlations between hurricanes, or other salinity-lowering storm events, and *V*. *vulnificus* levels, highlighting the potential for climatic weather events to increase disease risk to individuals who come into contact with or harvest food items from these waters.

As fluctuations in environmental conditions can rapidly alter *V*. *vulnificus* levels and the respective disease risk to susceptible individuals, methods for rapid and accurate detection of *V*. *vulnificus* during times of enhanced risk are needed. The most accessible and inexpensive method for detecting *V*. *vulnificus* from environmental samples involves the use of culture-based media, however due to the lack of specificity, culture-based methods can give rise to a substantial number of false positive isolates. The use of inaccurate culture-based methods necessitates molecular testing on *V*. *vulnificus* presumptive isolates to accurately identify this species. Many coastal communities and developing countries do not have access to such expensive resources. In paper 11, we describe a new, more efficient culture-based method to improve the accuracy of identifying *V*. *vulnificus* from environmental samples. By employing three selective and

differential media in tandem, we were able to substantially reduce the number of false-positive isolates which allowed for more accurate identification of *V*. *vulnificus*. We suggest this new and simple triple-plating technique will provide a more accurate, more time efficient, and more cost effective method for the initial detection of *V*. *vulnificus*. This method decreases or even eliminates the need for expensive molecular methods and therefore would be particularly useful when molecular methods are not available or too costly, and could be employed in emergency situations.

SERUM SURVIVAL OF *VIBRIO VULNIFICUS*: ROLE OF GENOTYPE, CAPSULE, COMPLEMENT, CLINICAL ORIGIN, AND *IN SITU* INCUBATION

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Article

Serum Survival of *Vibrio vulnificus*: Role of Genotype, Capsule, Complement, Clinical Origin, and *in Situ* Incubation

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Abstract: Virulence of the human pathogen, *V. vulnificus*, is associated with encapsulation, serum complement resistance, and genotype. The C-genotype of this bacterium is correlated (>90%) with virulence and with isolation source (clinical settings). E-genotype strains are highly correlated with environmental isolation (93%) but appear less virulent. In this study, we characterized the importance of genotype, encapsulation, serum complement, and *in situ* exposure to estuarine water on the survival of the two genotypes in human serum. Results confirmed the superior ability of C-genotype strains to survive exposure to human serum, as well as the significance of complement, and revealed that lack of capsule allowed serum killing of both C- and E-genotypes. Cells incubated *in situ* responded similarly to cells incubated *in vitro* with the exception of E-environmental strains. Interestingly, our studies found that those cells of the E-genotype, typically considered non-pathogenic, which were isolated from wound infections demonstrated serum survival similar to that of virulent, C-genotype, strains.

Keywords: *in situ* incubation; wound infections; capsule

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1. Introduction

Vibrio vulnificus exists in estuaries across the globe, and is associated with a variety of aquatic organisms, particularly bivalves such as the eastern oyster, *Crassostrea virginica*. Ingestion of raw or undercooked oysters carrying *V. vulnificus* can lead to rapid and severe septicemia, multi-organ failure, necrotizing fasciitis, and in some cases death [1–3]. Indeed, *V. vulnificus* accounts for ~95% of all seafood related deaths in the U.S. and has the highest case fatality rate (>50%) of any foodborne pathogen [4,5].

While most bacterial pathogens display a single mode of transmission, *V. vulnificus* has an alternate route of infection via introduction into an open wound [6]. Wound infections typically result from a wound inflicted by handling shellfish or other recreational activities in coastal environments and carry a mortality rate of *ca.* 25% [2].

Not all strains of this bacterium are equally pathogenic; of the three known biotypes of *V. vulnificus*, biotype 1 represents the majority of strains that can result in human disease upon exposure. This biotype can be further subdivided into two genotypes, aptly named for their most common sources of isolation. "C" (clinical) genotypes are typically pathogenic, and 93% of isolates from clinical settings are of this genotype [7]. Conversely, "E" (environmental) genotypes are generally non-pathogenic, and 90% percent are isolated from environmental settings, including oysters, sediment, and water samples [7]. These two genotypes can be distinguished using a simple and rapid polymerase chain reaction, in which one of two virulence correlated alleles, *vcgE* or *vcgC*, is amplified [8].

Although C- and E-genotypes significantly correlate with isolation source, it is becoming increasingly apparent that a subset of E-genotypes has acquired the ability to cause human disease, with most isolates being implicated in wound infections. This is of particular medical concern considering the documented increase in the number of *V. vulnificus* wound infections and subsequent deaths as a result of climate change [9].

The pathogenicity of this bacterium is believed to arise from several putative virulence factors, including the production of iron-binding siderophores, exoenzymes, and capsular polysaccharides, as well as the ability to survive exposure to human serum [1,10–12]. While most of the proposed virulence factors remain unconfirmed, the production of capsular polysaccharide (CPS) is highly correlated with pathogenicity [1,10,13–15]. In fact, previous research has shown that the LD_{50} of the encapsulated, "opaque" strains is as low as a single cell [13] whereas non-encapsulated, "translucent" strains of *V. vulnificus* display little or no virulence [13,14].

Our lab and others have previously reported on the ability of some *V. vulnificus* strains to survive exposure to human serum [12,16–18]. Both the C- and E- genotypes of *V. vulnificus* typically produce CPS, a trait known to aid in defense against serum, yet our lab has found the E-genotypes to show greater susceptibility when exposed to human serum than C-genotypes [16]. A variety of suggestions for the mechanisms of this phenomenon have been offered, the most significant of which may be differences in expression of the siderophore gene, *viuB* or capsule switching. Previous research from our lab indicated that C-genotype cells, all of which were *viuB* positive, showed significantly higher serum survival than E-genotype strains, most of which lacked this siderophore-encoding gene [16]. Our studies have also indicated that E-genotype cells more frequently revert to the non-encapsulated, translucent phenotype than cells of the C-genotype [19], possibly rendering them more susceptible to the bactericidal effects of serum.

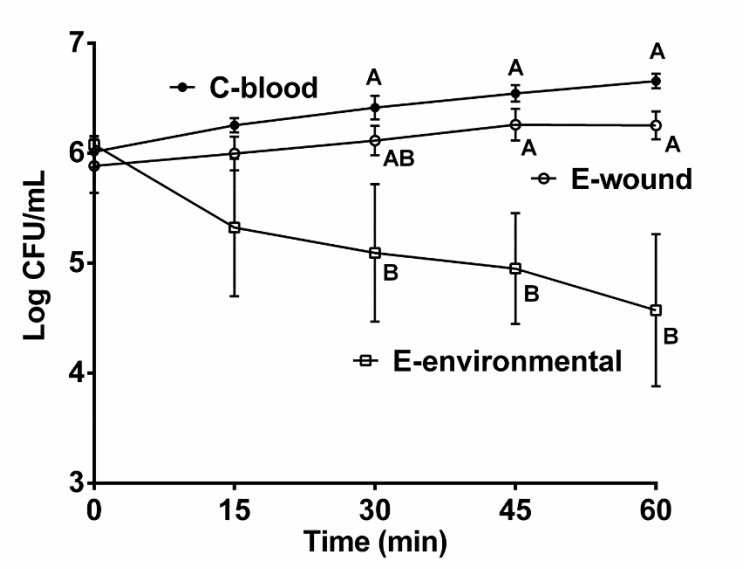
The intent of the present study was to further characterize the degree of serum resistance demonstrated by the two genotypes of *V. vulnificus,* with particular attention to clinically isolated E-genotypes. We also investigated the role of capsule in serum survival, employing strains that have undergone a permanent mutation in the *wzb* gene within the CPS operon. A unique aspect of this study involved exposing cells to environmental conditions to induce the natural physiology of *V. vulnificus* cells prior to introduction into human serum. This aspect was accomplished by subjecting the cells to 24 h of *in situ* incubation in estuarine water using membrane diffusion chambers.

2. Results and Discussion

2.1. Role of Genotype and Isolation Source in Serum Survival

Examination of four clinically isolated C-genotype and four environmentally isolated E-genotype strains revealed a significant difference in their ability to survive in human serum (Figure 1). While C-genotype strains exhibited total survival and even growth, exposure to serum was inhibitory to E-genotype growth, supporting results previously reported [16]. This difference was particularly evident after 60 min of incubation.

Figure 1. Role of genotype and isolation source on survival in human serum. Survival of clinically isolated C-genotypes (MO6-24, CMCP6, C7184, YJ016; closed circles), E-genotype wound isolates (E64MW, LSU2098, LSU1657, LSU549; open circles) and environmentally isolated E-genotypes (JY1305, JY1701, ENV1, SS108-A3A; open squares), cells exposed to human serum for 60 min. Error bars represent the standard error of the mean for four strains with three replicates each. Different letters indicate statistically significant differences (two-way ANOVA).



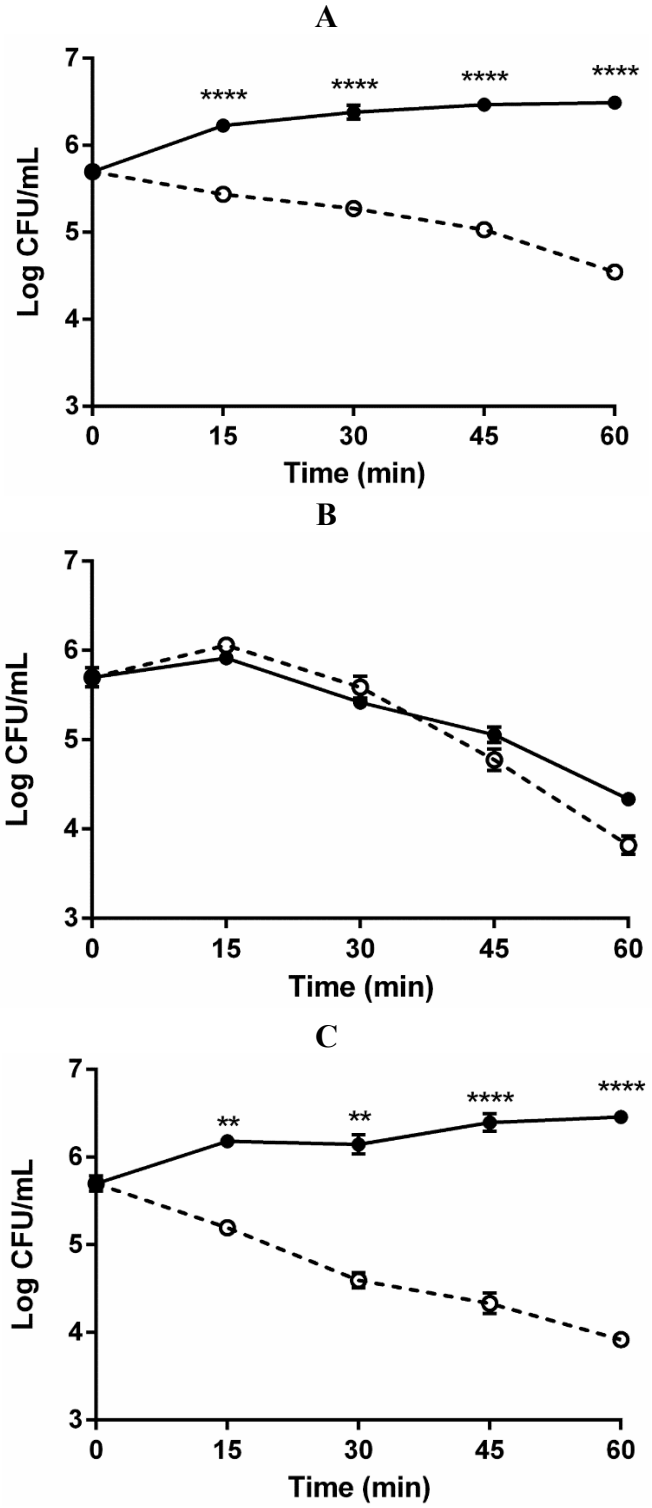
While C-genotypes predominate in septicemia cases, a considerable number of E-genotypes have been isolated from wound infections. This finding has prompted further interest into E-genotype strains, *i.e.*, do all E-genotypes have the ability to cause wound infections or is there a subset of E-genotypes that have unique virulence factors or share virulence factors with C-genotypes? Additionally, *V. vulnificus* wound infections associated with recreational water activities have become more prevalent in both the U.S. and Europe likely as a result of warming water temperatures [9,20–22]. We therefore examined if E-genotype strains isolated from wound infections differ in human serum sensitivity compared to non-clinical, environmentally isolated E-genotypes. Unlike environmental E-genotypes, wound isolates resisted the bacteriocidal effects of human serum at a rate similar to that of C-genotypes (Figure 1). Interestingly, a recent comparative genome analysis [23] revealed that the E-genotype wound isolate, E64MW, shares 43 genes with three C-genotype blood isolates (CMCP6, YJ016, and M06-25) which were absent in the two non-clinical E-genotypes (JY1305 and JY1701). From these results we suggest that a subgroup of E-genotypes have acquired mechanisms to successfully colonize and infect the human host although further investigation is required to more fully understand the relationship between C-genotypes and this subset of E-genotypes.

2.2. Role of Capsule in Serum Survival

We also analyzed the effect of capsule on serum survival by both C- and E-genotypes. All capsular polysaccharide mutants employed appeared phenotypically identical and possess genetic determinants affecting the functionality of the group 1 CPS operon which directs CPS biosynthesis and transport [14,24,25]. In contrast to the encapsulated clinical strains (C and E genotypes) which exhibited population growth, the non-encapsulated strains of both genotypes underwent more than a 1-log decrease in culturability within 60 min (Figure 2).

Culturability of the non-encapsulated environmental E-genotype strain decreased nearly 2-logs. Using the standard method for visual determination of encapsulation [13,15], none of the non-encapsulated mutants exhibited capsule when plated whereas all parent strains produced the “opaque”, encapsulated colonial phenotype. Thus, the presence of CPS appears to play a significant role in serum survival regardless of genotype or clinical/environmental source (Figure 2). This is likely due to the resistance that negatively charged CPS imparts against antimicrobial components present in human serum, or to shifts in osmolarity [26].

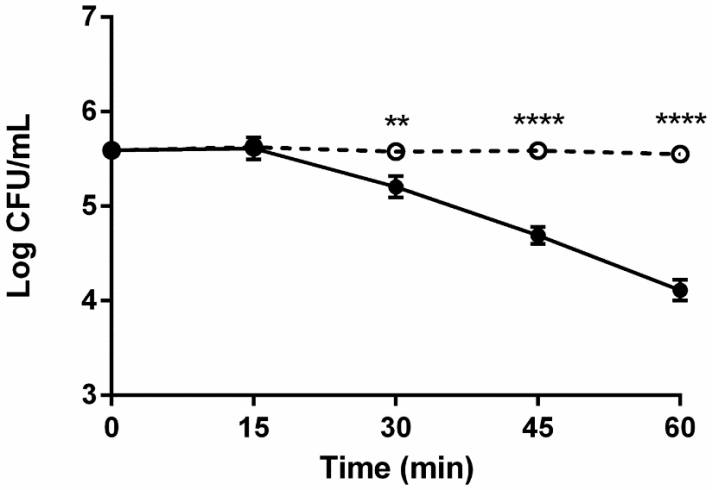
Figure 2. Role of capsular polysaccharide in survival of *V. vulnificus* exposed to human serum. (**A**) Survival of opaque and translucent clinical C-genotype (C7184/Op, closed circles; C7184/Tr, open circles); (**B**) Opaque and translucent environmental E-genotype (JY1701/Op, closed circles; JY1701/Tr, open circles); (**C**) Opaque and translucent clinical E-genotype (LSU1657, closed circles; LSU1657/Tr, open circles) exposed to human serum for up to 1 hour. Error bars represent the standard error of the mean for three replicates per strain. Two-Way ANOVA with Bonferroni *post hoc* test (** $p < 0.01$; **** $p < 0.0001$).



2.3. Role of Complement in Serum Survival

Inactivated serum allowed for more than 1-log greater survival of non-encapsulated strains compared to survival in active serum (Figure 3). These results indicate that inactivation of bacteriocidal components, including complement proteins, allows for survival and even growth of non-encapsulated strains, regardless of genotype.

Figure 3. Role of complement in bactericidal activity of serum against translucent strains of *V. vulnificus*. Effect of complement active serum on translucent strains (C7184/Tr; LSU1657/Tr; and JY1701/Tr) in complement active (closed circles) or complement inactive (open circles) serum. Error bars represent the standard error of the mean for three replicates per strain. Asterisks indicate statistically significant differences. Two-Way ANOVA with Bonferroni *post hoc* test (** $p < 0.01$; **** $p < 0.0001$).

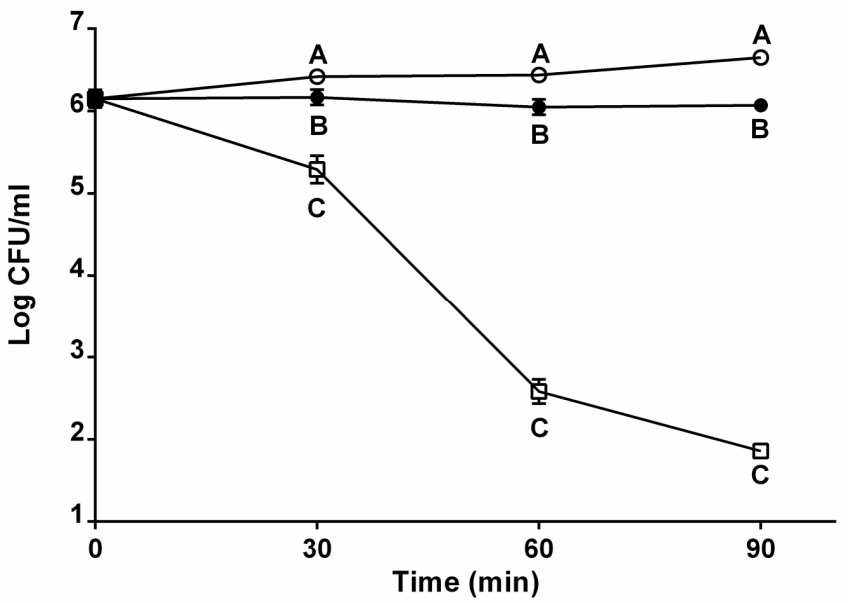


2.4. Effect of in situ Incubation on Serum Survival

To determine if cells of *V. vulnificus* present in the natural environment respond to human serum in a manner similar to what we observed for *in vitro* grown cells, we exposed cells to *in situ* conditions by placing them in an estuary for 24 h prior to their exposure to human serum. This was accomplished employing membrane diffusion chambers that house the cells while allowing them to be exposed to salinity, temperature, nutrient, viruses and other dissolved matter fluctuations naturally present in such environments. As seen with our *in vitro* studies, both clinically isolated C-genotype and wound E-genotype isolates maintained serum resistance (Figure 4) whereas environmental E-genotype strains were more susceptible to serum relative to survival *in vitro* (Figures 1 and 4).

Thus, little difference in survival was detected between cells incubated *in situ* and *in vitro* prior to exposure to serum, with the exception of E-environmental strains which displayed a weaker resistance to human serum following *in situ* incubation. This finding suggests that the environmental conditions cells experience in estuarine waters, such as fluctuating salinity, temperature, and nutrient availability, may alter the potential for serum survivability. Differences in expression of the hemolysin, *vvhA*, had previously been observed by our lab for cells of the two *V. vulnificus* genotypes incubated *in situ* [27].

Figure 4. Serum survival of *V. vulnificus* genotypes and isolate types following *in situ* incubation. Survival of clinically isolated C-genotypes (CMCP6, YJ016; open circles), clinically isolated E-genotypes (LSU2098, E64MW; closed circles), and environmentally isolated E-genotypes (JY1305, ENV1; squares) in human serum for up to 90 min after incubation *in situ* (estuarine waters). Error bars represent the standard error of the mean for two strains and three replicates. Different letters indicate statistically significant differences (two-way ANOVA).



3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

Table 1 lists the strains that were used in this study along with genotype, source of isolation, and capsule phenotype. The translucent strains used in this study, originally isolated and identified by our lab, underwent a spontaneous mutation of the *wzb* gene resulting in permanent loss of its ability to produce capsular polysaccharide. These strains are referred to as TR2 strains as previously described [24] and were genetically confirmed to lack the *wzb* gene via PCR while still possessing the flanking *wza* and wzb genes.

Bacterial cultures were taken from freezer stocks and grown overnight in heart infusion (HI) broth at 30 °C with shaking. For *in vitro* studies, overnight cultures were diluted in fresh HI broth at a 1/100 (v/v) ratio and grown to logarithmic phase (OD_{610} 0.15–0.25).

3.2. In Situ Incubations

Membrane diffusion chambers used in this study were originally designed by McFeters and Stuart [28,29]. These consist of two 76 mm, 0.2 μm hydrophilic polycarbonate filters (Midland Scientific Inc.; cat# 1220891) sandwiched between three doughnut shaped sections of Plexiglas. Using this apparatus, 25 mL of bacterial culture at a final cell concentration of *ca.* 10^{4-5} CFU/mL was aseptically injected into each autoclaved chamber which were then deployed into Calico Creek, an estuarine water body located in Beaufort, North Carolina. Water temperatures at the site of deployment were typically

20–25 °C with salinities of 15–34 ppt. After 24 h of incubation, the chambers were retrieved and the cells aseptically removed for serum studies.

Table 1. List of *V. vulnificus* strains used in this study including their respective genotypes, source of isolation, and colony opacities (capsule presence).

Strain Name	Genotype	Isolation Source	Opacity
CMCP6	C	Human Blood	Opaque
YJ016	C	Human Blood	Opaque
MO6-24	C	Human Blood	Opaque
C7184	C	Human Blood	Opaque
C7184/Tr[a]	C	Spontaneous CPS mutant	Translucent
LSU2098	E	Human wound	Opaque
E64MW	E	Human wound	Opaque
LSU549	E	Human wound	Opaque
LSU1657	E	Human wound	Opaque
LSU1657/Tr[a]	E	Spontaneous CPS mutant	Translucent
ENV1	E	Water	Opaque
SS108-A3A	E	Oyster	Opaque
JY1305	E	Oyster	Opaque
JY1701	E	Oyster	Opaque
JY1701/Tr[a]	E	Spontaneous CPS mutant	Translucent

[a] Isogenic CPS mutants lacking the *wzb* gene of the CPS operon, referred to as a TR2 genotype by Chatzidaki-Livanis *et al.* [24].

3.3. Human Serum Exposure

Pooled male human serum (MP Biomedicals, Santa Ana, CA, USA) was used for all studies. To determine the significance of bacteriocidal components, including the complement cascade, in serum sensitivity, serum was heat-inactivated by incubation of the serum at 56°C for 30 min. *In vitro* studies were adapted from Bogard and Oliver [16]. To achieve a final cell concentration of *ca.* $10^{5–6}$ cells/mL serum, log-phase cells (12 µL) were inoculated into 788 µL serum, or in the case of *in situ* incubation, 100 µL of cells were added to 1 mL of serum. In all cases cells were incubated in serum at 37°C for up to 2 h, with culturability assessed at 15 or 30 min time intervals by serially diluting into PBS followed by plating onto HI agar to determine CFU/mL after 24 h incubation at 30°C.

3.4. Statistical Analysis

Each experiment was performed with at least three replicates per strain. Log transformed data were analyzed using GraphPad Prism (v. 5.0; GraphPad Software Inc. San Diego, CA, USA). Statistical analyses were performed using one and two-way analyses of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparisons. Significance was determined using a 95% confidence interval.

4. Conclusions

The findings reported here support previous studies and demonstrate that genotype and capsular polysaccharide significantly impact *V. vulnificus* serum survivability and likely pathogenicity, and are therefore important virulence determinants for this organism. However, E-genotype strains isolated from wound infections were found to exhibit serum resistance similar to that of the clinically isolated C-genotypes, and this resistance was maintained regardless of *in vitro* or *in situ* incubation prior to exposure to serum. Thus, while genotype largely correlates with source of isolation, further genetic distinctions within E-genotypes are necessary to predict pathogenic potential.

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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.

Author Contributions

James Oliver conceived and supervised the studies. Heather Ryan initiated the research as a portion of her Honors in Biology thesis. Tiffany Williams and Mesrop Ayrapetyan developed the mutants and significantly expanded on the studies.

Conflicts of Interest

The authors declare no conflict of interest.

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PYROSEQUENCING-BASED COMPARATIVE GENOME ANALYSIS OF *VIBRIO VULNIFICUS* ENVIRONMENTAL ISOLATES

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Contributions by Tiffany C. Williams:

Comparative genome analysis, parts of manuscript preparation, editing of manuscript

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Pyrosequencing-Based Comparative Genome Analysis of *Vibrio vulnificus* Environmental Isolates

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Abstract

Between 1996 and 2006, the US Centers for Disease Control reported that the only category of food-borne infections increasing in frequency were those caused by members of the genus *Vibrio*. The Gram-negative bacterium *Vibrio vulnificus* is a ubiquitous inhabitant of estuarine waters, and is the number one cause of seafood-related deaths in the US. Many *V. vulnificus* isolates have been studied, and it has been shown that two genetically distinct subtypes, distinguished by 16S rDNA and other gene polymorphisms, are associated predominantly with either environmental or clinical isolation. While local genetic differences between the subtypes have been probed, only the genomes of clinical isolates have so far been completely sequenced. In order to better understand *V. vulnificus* as an agent of disease and to identify the molecular components of its virulence mechanisms, we have completed whole genome shotgun sequencing of three diverse environmental genotypes using a pyrosequencing approach. *V. vulnificus* strain JY1305 was sequenced to a depth of 33×, and strains E64MW and JY1701 were sequenced to lesser depth, covering approximately 99.9% of each genome. We have performed a comparative analysis of these sequences against the previously published sequences of three *V. vulnificus* clinical isolates. We find that the genome of *V. vulnificus* is dynamic, with 1.27% of genes in the C-genotype genomes not found in the E- genotype genomes. We identified key genes that differentiate between the genomes of the clinical and environmental genotypes. 167 genes were found to be specifically associated with environmental genotypes and 278 genes with clinical genotypes. Genes specific to the clinical strains include components of sialic acid catabolism, mannitol fermentation, and a component of a Type IV secretory pathway *VirB4*, as well as several other genes with potential significance for human virulence. Genes specific to environmental strains included several that may have implications for the balance between self-preservation under stress and nutritional competence.

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Introduction

Of all seafood-associated pathogens, none are as critical as those of the genus *Vibrio*, and of all food-borne pathogens, the US Centers for Disease Control reported that only infections by those of this genus increased (by 78%) between 1996 and 2006 [1]. In the United States, 95% of all deaths resulting from seafood consumption are caused by a single bacterium, *Vibrio vulnificus* [1]. *V. vulnificus* is part of the normal bacterial flora of estuarine waters and occurs in high numbers in molluscan shellfish around the world [2]. In the 10-year period between 2000 and 2009, 303 cases involving oyster ingestion occurred in the United States, of which 148 were fatal (Oliver, unpublished). A COVIS dataset suggest that there were over 1800 *V. vulnificus* cases reported in the USA from 1988–2010, with over 500 associated fatalities (Baker-Austin et al., unpublished). Infections occur rapidly, with median incubation times to onset of symptoms being as little as 7 hours [1]. Most (~85%) cases occur in males, because females are protected to some extent, from the *V. vulnificus* endotoxin by estrogen [3]. Nearly all infections (~95%) occur in individuals who are immunocompromised, have diabetes, or who have underlying diseases or syndromes that result in elevated serum iron levels, primarily liver cirrhosis secondary to alcohol abuse/alcoholism [4]. These relatively common conditions put a large number of persons at risk for serious injury or death from *V. vulnificus*, and we would expect to see a far greater number of cases than are typically reported each year. The question then arises as to why so few of these infections are reported each year in the USA.

Understanding the mechanism of *V. vulnificus* virulence and the molecular basis of its interaction with human and oyster hosts is the key to this question. Despite a high degree of phenotypic and genotypic heterogeneity among *V. vulnificus* strains all known putative virulence determinants have been found to be expressed in both clinical and environmental isolates [1]. Despite this, Starks et al. (2000) [5] found clinical isolates (n = 3) to be significantly more virulent than environmental strains (n = 3) in both an

intraperitoneal and subcutaneous mouse model, and we have found 81% of 16 C-genotype strains examined to be virulent ($LD_{50} \leq 10^3$), but only 31% of 13 E-genotype strains in an iron-overload mouse model (Oliver, unpublished).

Several approaches have been used to identify genotypic factors that distinguish between virulent and avirulent isolates of this pathogen. Aznar et al. [6] identified two groups (termed A and B) of *V. vulnificus* strains based on 16S rDNA gene polymorphism, and Nilsson et al. [7] showed that these two groups were associated with clinical (B) or environmental (A) isolation. Despite employing a variety of population genetics methods, however, Gutacker et al. [8] found no association between their grouping and environmental or clinical origin. Recently, Okura et al. [9] employed a PCR-based assay, using a primer pair derived from a group-specific sequence of a RAPD-PCR fragment encoding a hypothetical protein, to distinguish pandemic strains of *V. parahaemolyticus* from non-pandemic strains. Using the same strategy, we identified an approximately 200 bp RAPD-PCR amplicon significantly associated with clinical isolates [10]. Analysis of this *vcg* (termed the Virulence Correlated Gene) led to a PCR-based assay that can separate *V. vulnificus* into two groups which strongly correlate to the source (clinical or environmental) of their isolation [11]. In a subsequent study of the distribution of the C- and E-genotypes in oysters and the surrounding estuarine waters, we found that while an almost equal distribution of the two genotypes existed in water, the E-genotype accounted for over 84% of those present in oysters [12]. This suggests that either E-genotypes are preferentially taken up by oysters, or that they survive better than do C-genotypes following uptake. More recently, Baker-Austin et al. [13] developed a rapid, real-time PCR method for *in situ* detection of C-genotype *V. vulnificus* strains present in raw oysters. These two genotypes may in fact be different ecotypes, as the genetic dimorphisms are not limited to the *vcg* gene, but occur throughout the chromosome and appear to dictate the species' environmental preference [11,14]. Despite the growing recognition of the existence of these two genotypes and their relevance to human disease, only clinical strains of the C-genotype have been completely sequenced to date [15,16,17]. Recently, a comparative genomic analysis study using short read data was performed on four *V. vulnificus* strains, including three E-strains and ATCC 33149 [18]. However, that study employed ABI SoLID sequencing to produce very short fragment reads. Such reads cannot be assembled *ab initio*, but must be mapped to the C reference genomes. This approach left the possibility that regions of the E genome for which there is no reference in the C sequence remained undetected. In the present study, we report on the sequencing of three strains of the E-genotype of *V. vulnificus*, using Roche 454 GS Titanium sequencing. Genomes have been assembled *ab initio* into large contigs, and the genomic sequences are estimated to be over 99% complete. These newly sequenced genomes have been compared to three previously published C-genotype genomes, strains CMCP6, YJ016, and MO6-24/O. The results of our comparison indicate several significant differences in gene content between the C- and E-genotypes of this pathogen, including genomic regions unique to the E-genotypes, which provide initial insights into the functional basis of pathogenicity in *V. vulnificus*.

Results and Discussion

Genome Sequencing and Assembly

188,710,063 nucleotide bases were generated for *V. vulnificus* strain JY1305. Given the known sizes and expected variability of *V. vulnificus* genomes, we estimated that this is equivalent to ~33× coverage depth of the *V. vulnificus* JY1305 genome, of estimated size 5.7 Mb. We obtained 671,521 reads of average length 281 bp. The data were assembled into 159 large contigs and 9,184 unassembled fragments using the MIRA assembler, version 3.0 [19]. Table 1 has the complete assembly results for the three E-strain genomes. The coverage of each of these genomes is significantly above the recommended genome coverage (6–10×) for a whole prokaryote genome study established in a recent exhaustive simulation of outcomes of Roche 454 type sequencing in prokaryotes [20]. In Figure 1, we show the assembled contigs from each of the newly sequenced E genomes, aligned to the *V. vulnificus* CMCP6 genome [21]. *V. vulnificus* CMCP6 was recently re-annotated and is regarded as the most complete and accurate of the published *V. vulnificus* clinical strain genomes [22]. Assembled contigs were deposited in the NCBI whole genome shotgun archive, and are available under project IDs 49015 (JY1305), 67135 (E64MW) and 67137 (JY1701). The GenBank accession IDs are AFSW00000000 (JY1305), AFSX00000000 (E64MW), and ASFY00000000 (JY1701) in the NCBI Whole Genome Assembly database. Complete sequence data will be made available via the NCBI Short Read Archive and at http://gibas-research.uncc.edu.

General properties of the *Vibrio* E strain genomes

The genome of *V. vulnificus* JY1305 is composed of 2 circular chromosomes with an estimated total of approximately 5.7 Mb of genomic DNA. *V. vulnificus* E64MW is estimated to be nearly identical in size, with *V. vulnificus* JY1701 slightly smaller at 5.6 Mb. Some *Vibrio* strains are known to have plasmids, but the *V. vulnificus* JY1305 sequence data contained no evidence of extrachromosomal DNA. PCR validation was performed to verify this finding and no plasmid DNA was found in the genomic DNA preps. It is unknown if *V. vulnificus* E64MW and *V. vulnificus* JY1701 contain plasmid DNA, but no plasmid sequence with homology to known *V. vulnificus* YJ016 plasmid sequences was identified, either in the assembled genomic sequence, or among the unassembled reads.

Table 1 summarizes the general characteristics and predicted gene content of each sequenced draft genome. Complete gene lists for each of the newly sequenced genomes are provided in Supplement S1.

Locally collinear blocks highlight extensive synteny in the *Vibrio vulnificus* genomes

LCBs (locally collinear blocks) are defined as conserved segments that appear to be internally free from genome rearrangements relative to the other genomes in the set under study [23]. The newly sequenced *V. vulnificus* strains were co-analyzed with genome sequences of strains CMCP6, YJ016, and MO6-24/O to identify LCBs common to C and E strains. The *V. vulnificus* CMCP6 genome was used as the reference genome in this analysis. At a size threshold of 1% of the genome, or 57 kb or greater, there are a total of 24 locally collinear sequence blocks that are conserved in the six *V.vulnificus* genomes. All of these large blocks are found in each of the six *V. vulnificus* strains, and they cover approximately 68.5% of the genome. At a size threshold of 90 aa (270 bp) or greater, we find an additional 186 LCBs. At this size threshold, LCBs are not necessarily conserved across all six genomes, and may correspond to individual genes or genomic islands that differentiate among the sequenced strains. Figure 2 shows the global arrangement of LCBs identified among the *V. vulnificus* strains (CMCP6, JY1305, E64MW, and JY1701) used in this study. Table S1 contains a table that summarizes the conservation of locally collinear blocks in all 6 *V. vulnificus* genomes, and Supplement S2 contains all LCBs identified, along with their genomic coordinates.

Table 1. Summary of assembly and annotation characteristics for the *V. vulnificus* JY1305, E64MW, and JY1701 genomes.

Genomic Characteristic	*V. vulnificus* JY1305	*V. vulnificus* E64MW	*V. vulnificus* JY1701
# of reads	671,521	376,287	321,091
# of nucleotides sequenced	188,710,063 bp	96,530,017 bp	73,115,338 bp
Average read length	281 bp	257 bp	228 bp
# of contigs	159	271	329
N50	237659 bp	69696 bp	36756 bp
N90	54287 bp	14424 bp	9249 bp
Largest Contig	489256 bp	163962 bp	112761 bp
Depth Coverage	~33×	~17×	~13×
Estimate Genome Size	5.7 Mb	5.7 Mb	5.6 Mb
Genome Coverage	~99.9%	~99%	~99%
Chromosome Number	2	2	2
Plasmid	None	N/A	N/A
G+C content %	46.7%	46.7%	46.5%
Predicted Genes	4235	4301	4425
# of predicted tRNAs	115	109	96
# of predicted rRNAs	23	17	15

doi:10.1371/journal.pone.0037553.t001

Genome content comparison

After annotation of the newly sequenced E-genotype *Vibrio vulnificus* genomes as described in Materials and Methods, we performed a comparative analysis of the presence or absence of individual genes. We compared the E-genotype genomes to the group of previously sequenced C-genotype *V. vulnificus* genomes as well as to a broader group of all 16 previously completely sequenced genomes belonging to the genus *Vibrio* (See Materials and Methods). When we subsequently refer to comparisons of E, or C and E types against "all *Vibrio* spp.", we are referring to this

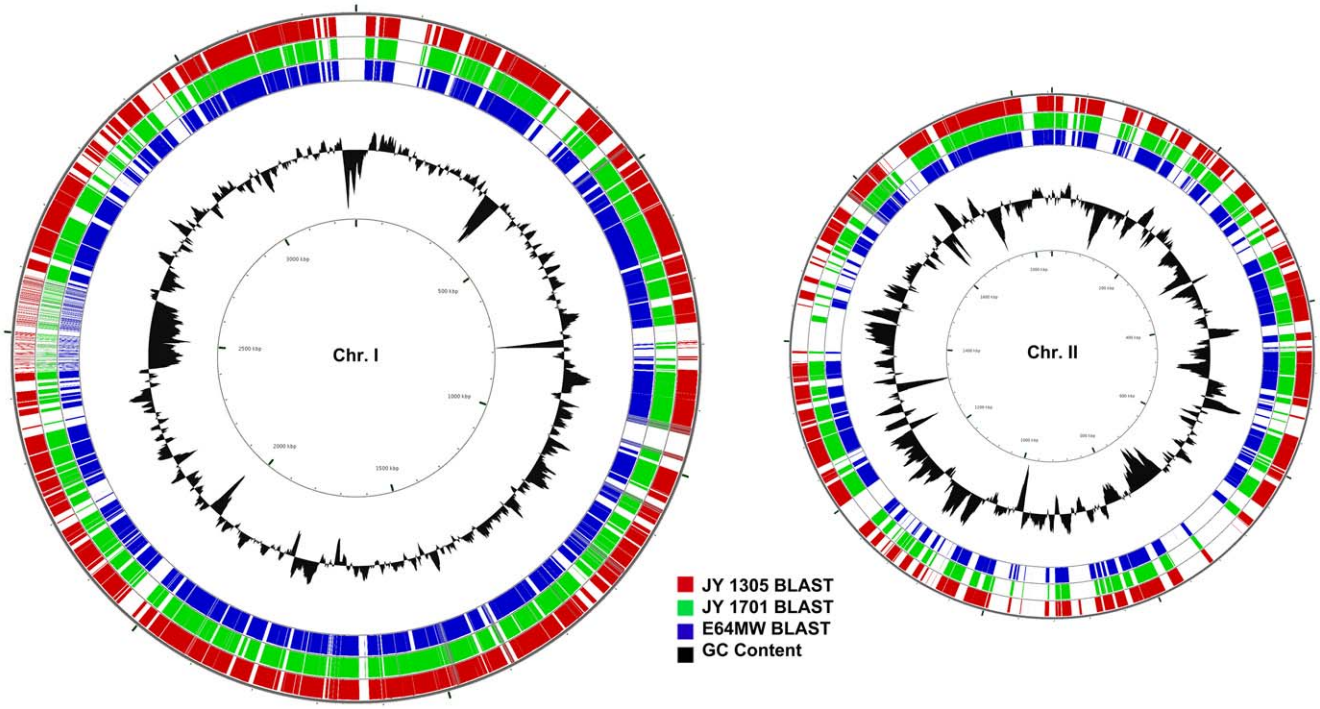

Figure 1. Circular maps of the sequence contigs of *V. vulnificus* JY1305, JY1701, and E64MW. From the outside in, the first circle (red) represents *V. vulnificus* JY1305 genomic contigs, the second circle (green) represents *V. vulnificus* JY1701 genomic contigs, and third circle (blue) represents *V. vulnificus* E64MW genomic contigs. The circles represent BLAST alignment of contigs against the *V. vulnificus* CMCP6 reference genome. Circle 4 shows GC content. Figure generated using CGView [21].
doi:10.1371/journal.pone.0037553.g001

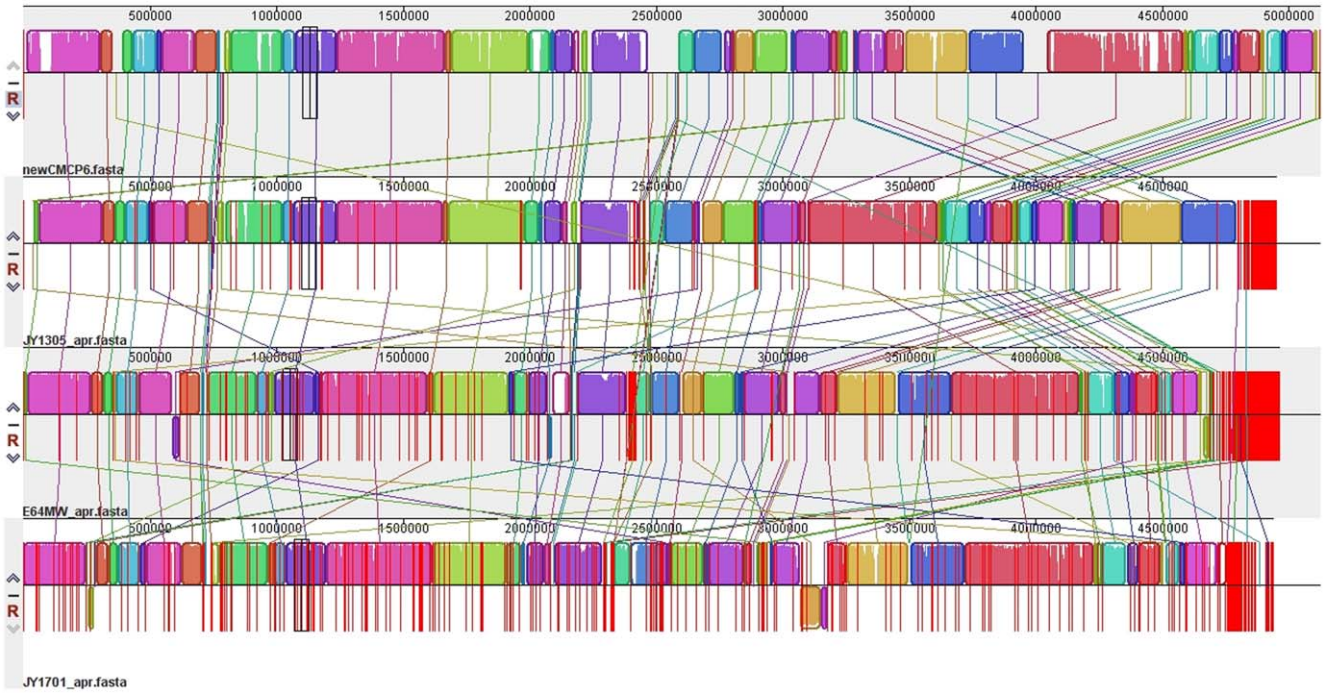

Figure 2. Genomic alignment of *Vibrio vulnificus* Biotype 1 strains CMCP6, YJ016, MO6-24/O, JY1305, E64MW, and JY1701. Locally conserved block based alignment between the reference genome CMCP6 and the newly sequenced genomes of JY1305, E64MW, and JY1701 as locally collinear blocks (LCB). Figure generated using Mauve [23].
doi:10.1371/journal.pone.0037553.g002

group. Figure 3 summarizes the gene count differentials for the six *V. vulnificus* strains included in this study. Genes were clustered together on the basis of a shared sequence similarity of 70% or greater for the purpose of defining orthology, as described in Materials and Methods. The counts represent differential presence or absence of a gene ortholog in a given genome.

The conserved core of *V. vulnificus*, and commonalities among *Vibrios*

We identified 1748 core genes that are common to the three draft E-genotype genomes and the 16 *Vibrio* genomes that have been completely characterized at the time of this writing. Each of these genes has a single-copy ortholog in each of the genomes analyzed. 192 genes were identified as core genes to all other *Vibrio* spp. but were not present in any of the *V. vulnificus* genomes, whether C-genotype or E-genotype. 940 genes were identified as core genes found in the six *V. vulnificus* genomes, but were not present in any other *Vibrio* spp. The gene VV2 0404 (*vvhA*), which is commonly used in combination with other markers to distinguish *V. vulnificus* from other *Vibrio* spp. in molecular assays, were found, as expected, in all six *V. vulnificus* strains, which gives us confidence in the sequencing and differential analysis. A related gene, VVA0964, the cytolysin secretion protein gene *vvhB* [24], is unique to the *V. vulnificus* genomes and may have potential as a diagnostic marker. The gene encoding zinc metalloprotease, VV2-0032 (*vvpE*), another commonly-used diagnostic marker, was identified by Gulig et al. 2010 as being common to both E-genotypes and C-genotypes [18], and we found this to be true in our analysis, as well.

Also found in the list of 940 core *V. vulnificus* genes are the Flp pilus genes. We believe this is a novel observation, as we have not seen it discussed elsewhere. The E- and C-genotypes of *V. vulnificus* contain a nearly identical operon for the assembly of an Flp pilus, a type IV pilus that mediates adherence, including genes for Flp pilus assembly *CpaB, CpaC*, a conserved unknown protein, and *CpaE*. The Tad assembly proteins of the Flp pilus, including *TadA*, *TadB*, *TadC*, and *TadD*, are also highly conserved and identically ordered in C7184 and YJ016. Both E- and C- strains of *V. vulnificus* contain all the components of the Tad assembly proteins except *TadD*, while other *Vibrio* spp. do not. These genes may be part of a *tad* (tight adherence) locus, found in a wide variety of bacteria, that is characteristic of horizontal gene transfer. *tad* loci are generally present as part of a mobile genetic element, specifically the "widespread colonization island" [25]. Loci such as these have been shown to be related to diseases, both human and animal, playing a role in colonization and/or pathogenicity. In non-pathogens, *tad* loci are proposed to facilitate environmental niche colonization [26].

Table S2 summarizes key differences between the *V. vulnificus* (Table S2A) and the other *Vibrio* spp (Table S2B). Supplement S3 contains a complete list of all the genes that are differentially present or absent in the *V. vulnificus* C and E strains, relative to all other fully-sequenced *Vibrio* spp.

Phylogeny of *V. vulnificus* and other *Vibrios*

Figure 4 is a phylogeny of the genus *Vibrio* based on common single copy orthologs. The consensus of the three trees is consistent with the evolutionary relationships previously observed within the genus *Vibrio* [*Vibrio* Phylogeny, PATRIC]. The *V. vulnificus* isolates cluster together, and segregate from the other *Vibrio* spp. Within the *V. vulnificus* clade there is a deep branching between the E-genotypes and C-genotypes and the branchings within the E- and C-genotype groupings are very shallow. This branching suggests a fundamental divergence between the genotypes, which correlates with the divergent lifestyle preferences of E- and C- isolates of *V. vulnificus* [12]. Rosche et al. introduced the concept of distinctive

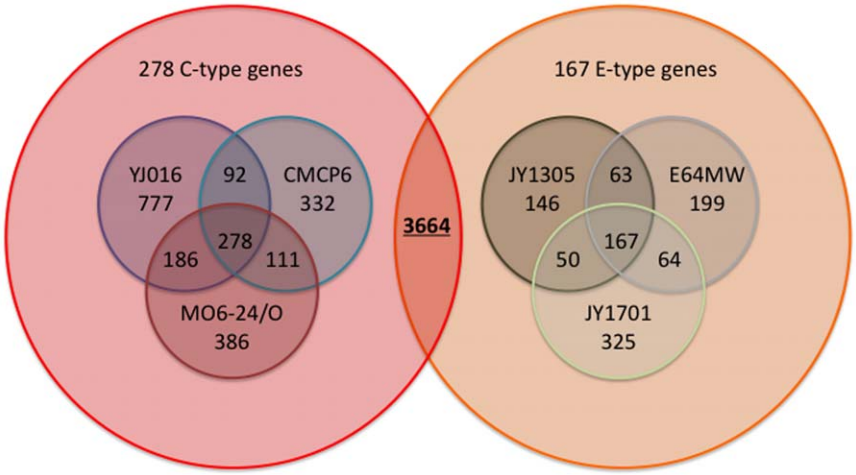

Figure 3. *Vibrio vulnificus* genomic content differential Venn diagram. A 6-way Venn diagram representing the differential and shared gene counts between the *V. vulnificus* YJ016, CMCP6, MO6-24/O, JY1305, E64MW, and JY1701. The main Venn diagram represents the overlap between C- and E- genotype groups, while the nested Venn diagrams represent the content relationships among the individual C-genotype or E-genotype strains. Gene counts are based on presence or absence of orthologs, where orthology is defined by OrthoMCL [74], using as a clustering criterion shared sequence similarity of 70% or greater.
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ecotypes in *V. vulnificus* based on eight housekeeping and virulence loci [27] and this distinction is supported by analysis on the genome-wide scale.

V. vulnificus Gene Differentials

We identified an average of 3664 orthologs common to all of the *V. vulnificus* strains analyzed in this study. An in-depth comparison between the two genotypes of *V. vulnificus* revealed 278 genes found only in the C-genotype strains, and 167 genes found only in the E-genotype strains. We also identified 43 genes common to the three C-genotype blood isolates, CMCP6, YJ016, MO6-24/O, and the E-genotype wound isolate, E64MW. Supplement S4 has a complete list of all the genes for these differential categories.

In Table 2 and Table 3, we summarize key differences between C and E genomes, summarizing genes that are shared between the strains of a specific genotype, but excluded from the other genotype. Supplement S4 has a complete list of differentials between the C-genotypes and E-genotypes. A few of those differentiating genes, of significance to human virulence or to survival in the estuarine/oyster environment, are noted here.

Functional Classification of Differentiating Genes

For functional comparison purposes, it is helpful to identify genes and other features using a controlled vocabulary. Therefore, functional classifications between the C-genotypes and E-genotypes were categorized based on the gene ontology annotation schema (GO) [28]. The Gene Ontology (GO) provides standardized terms for the description of gene products in terms of biological processes, cellular location, and molecular function [28,29]. GO categories and individual genes having functionally significant enrichment or depletion between genomes at the species or genus level were identified using the Gene Ontologizer [30]. A detailed description of how GO terms are identified as significant is given in (Cain et al., in review) [31].

Figure 5 summarizes differences in GO function content between the C-genotypes and E-genotypes of *V. vulnificus*. The differential functional analysis shows that GO terms mannitol-1-phosphate 5-dehydrogenase and N-acetylneuraminidase are significantly enriched in the C-types with an adjusted p-values of 2.42^{E-04} and 1.13^{E-05}, respectively. Specifically, 35% of the genes associated with mannitol-1-phosphate 5-dehydrogenase activity and nearly100% of the genes with associated N-acetylneuraminidase function are found to be unique to C-types. Additionally, GO terms "chondroitin AC lyase activity" and "arylsulfatase activity" are significantly enriched with adjusted p-values of 0.0068 and 0.048, respectively. In both categories, close to 100% of the genes are only found in the C-genotype differentials. In contrast, the E-genotypes appear to be strongly enriched in genes associated with the GO functions "urea metabolic process" and "nickel ion binding". Nearly all of the genes that fall under these GO categories are only found in the E-genotypes. Both show up as statistically significant differentials with adjusted p-values of 1.52^{E-09} and 4.37^{E-07}, respectively. Additionally, E-genotypes appears to have several unique genes that fall into GO categories associated with carbohydrate transport and transmembrane transporter activity for a variety of sugars and sugar derivatives.

Understanding the overall significance of these genotypic GO functional differences will require further investigation, however we propose that these differentiating functional categories may be relevant to the SPANC hypothesis which describes the balance between self-preservation and nutritional competence in bacterial genomes [32,33].

Chromosomal location of differential genes

It has been previously suggested that the second chromosome in the *Vibrionaceae* family may play a role in adaptation to environmental changes [34]. Our genome comparison revealed that the majority of the C-genotype differential genes (Table 2) are located on the second chromosome of each strain, with the exception of a small number of genes in *V. vulnificus* MO6-24/O (VVMO6_02633, VVMO6_02634, and VVMO6_02635). Based on the location of the E-genotype differentiating genes in the LCB alignments (Figure 2), we were able to approximate the likely chromosomal location of the E-genotype differentials in Table 3. If a gene was located inside a conserved block that appeared in Chr. 1 in the CMCP6 reference genome, we assigned it to Chr. 1 or likewise to Chr. 2 if it was found in a conserved block matching Chr. 2. This analysis suggests that E-genotype differentials such as

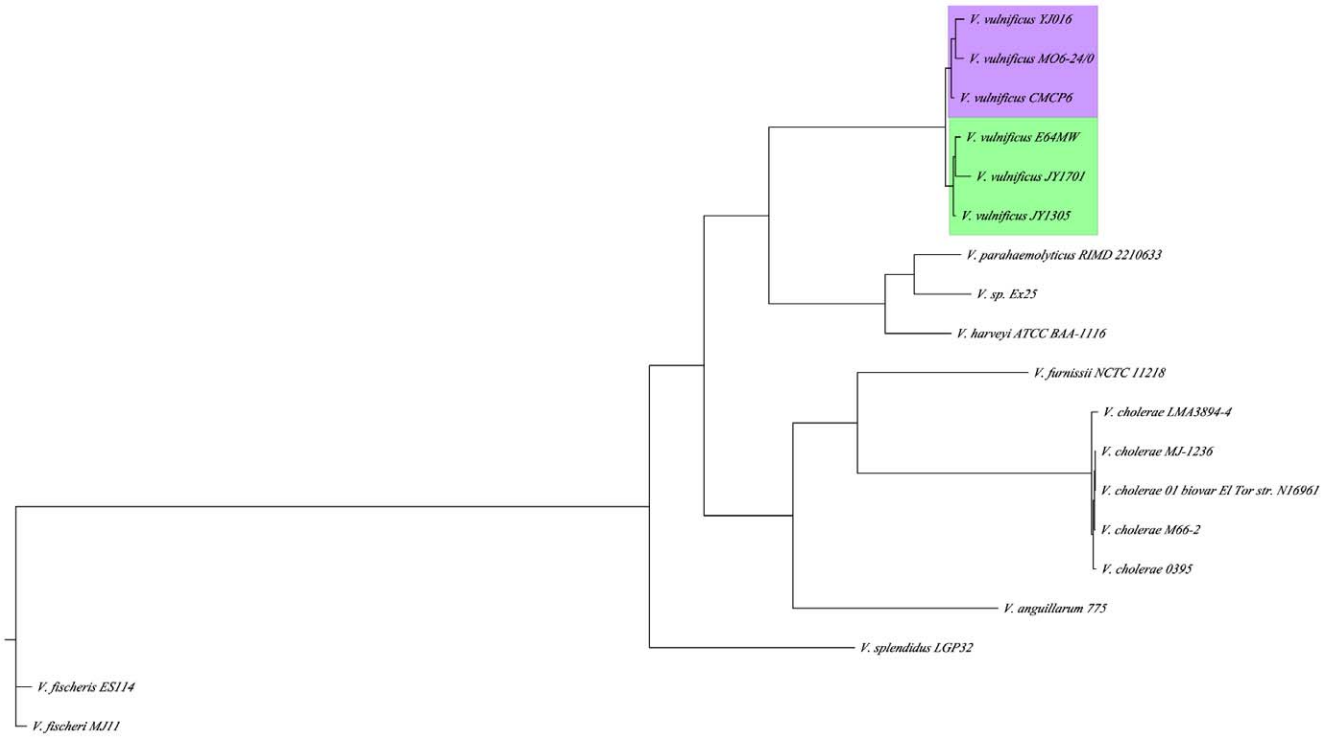

Figure 4. Phylogenetic relationships among sequenced *Vibrio* genomes. Phylogenetic relationships computed using maximum likelihood estimation, from a random sampling of 175 single copy gene ortholog sequences common among the newly sequenced E-genotype genomes and other sequenced *Vibrio* species. Three randomly sampled replicates produce trees with highly similar topologies. Purple box indicates strains classified as C-genotypes and green box indicates strains classified as E-genotypes for *V. vulnificus*.
doi:10.1371/journal.pone.0037553.g004

the PKD domain-containing protein and the PTS system are found on Chr. 2, while a permease locus is most likely found on Chromosome 1. The location of differentiating genes with a urea metabolic process GO term identification can't be definitively determined based on this assembly. Our observations are consistent with increased plasticity of the second chromosome, which could potentially offer further insight into the genetic diversity found within the species, and the proposed divergence of this species into two distinct ecotypes.

Characteristic features of the E-genotype genomes

As previously mentioned, *V. vulnificus* C- and E-genotypes have been shown to exhibit differences in pathogenicity and environmental distribution. In addition, previous examination of several housekeeping and putative virulence-associated genes has revealed a number of genetic polymorphisms suggesting that these two genotypes are in the process of diverging into distinct ecotypes [11,27]. One hypothesis of particular interest, referred to as the SPANC (self-preservation and nutritional competence) balance, could potentially offer insight into the niche adaptation and differentiation seen in *V. vulnificus* C- and E-genotypes. The SPANC hypothesis has been well characterized in *E. coli* and demonstrates that clonal populations can experience genetic mutations and phenotypic changes as a result of physiological stress under conditions such as nutrient starvation. These changes often lead to variations in the activity of the global gene regulator, sigma factor σ^S (*rpoS*), which governs the general stress response. Decreased RpoS activity can lead to the development of specialized populations which are less resistant to stress but have broader nutritional capabilities and a higher affinity for low nutrient concentrations, whereas the original population is more stress tolerant but less nutritionally competent [32,33]. In aquatic environments, in which nutrients are often limiting and competition for resources is intense, such modifications could confer a selective advantage for these bacterial strains.

It seems plausible that this trade-off between self-preservation (stress resistance) and nutritional competence could be a factor driving the diversification of *V. vulnificus* species. By completely sequencing three E-genotypes of *V. vulnificus*, we were able to examine what genes are unique to E-genotypes. As noted above, the GO functional gene content differences between C- and E-genotypes showed that the sequenced E genomes have significant enrichment for genes associated with metabolic functions such as urea and nitrogen cycle metabolism (Figure 5), suggesting that the E-genotypes may possess versatile metabolic capabilities. Previous laboratory studies support this finding demonstrating that when *V. vulnificus* C- and E-genotypes are grown in co-culture, E-genotypes are favored under nutrient rich conditions (Rosche and Oliver, unpublished). In addition, both C- and E-genotypes are enriched for GO functions associated with transmembrane transport of various organic compounds (Figure 5). Indeed, these genes appear to possess the same functionality for C- and E-genotypes, however multiple sequence alignment of the protein sequences reveals very little homology. This finding suggests that the genes associated with these GO terms have either diverged considerably between the two genotypes or are completely different genes that serve the same function. Future investigations should be performed to examine what effect these genetic differences could have on the metabolic capabilities of each genotype.

In the E-genotypes, we also identified genes associated with specific attachment proteins which would likely facilitate environmental survival. Polycystic kidney disease I domain (PKD) is a

Table 2. Key differential genes between found in *V. vulnificus* C-genotypes that are NOT present in the E-genotypes.

Strain	Chr.	Locus tag	Product Description	GO id	GO Term
CMCP6	2	VV2_0726	Sialic acid-induced transmembrane protein YjhT	GO:0005975	carbohydrate metabolic process*
	2	VV2_0729	Salic acid utilization regulator RpiR family	GO:0005975	carbohydrate metabolic process*
	2	VV2_0730	N-acetylneuraminate lyase	GO:0008747	N-acetylneuraminate lyase activity*
	2	VV2_0731	TRAP-type transport system large permease component	GO:0016021	Integral to membrane+
	2	VV2_0732	TRAP-type transport system small permease component	N/A	N/A
	2	VV2_0733	TRAP-type system periplasmic component	GO:006810	transport+*
	2	VV2_1509	Putative two –component response regulator & GGDEF family protein YeaJ	GO:0009190	cyclic nucleotide biosynthetic process+*
	2	VV2_1510	Response regulator	GO:0000156	two-component response regulator activity+*
	2	VV2_1106	Arysulfastase A	GO:0008484	sulfuric ester hydrolase activity*
	2	VV2_1107	Arylsulfatase regulator	GO:0008152	metabolic process+*
	2	VV2_1108	Arylsulfatase A	GO:0008449	N-acetylglucosamine-6-sulfatase activity*
	2	VV2_1109	Arysulfatase	GO:0008484	Sulfuric ester hydrolase activity*
	2	VV2_0074	RsbS, negative regulator of sigma-B	N/A	N/A
	2	VV2_0075	anti-sigma B factor RsbT	GO:0005524	ATP binding+
	2	VV2_0076	Serine phosphatase RsbU, regulator of sigma subunit	GO:0008152	metabolic process+*
	2	VV2_0077	Two-component system sensor protein	GO:0004673	protein histidine kinase activity+*
	2	VV2_0735	N-acylmannosamine kinase	GO:0009384	N-acylmannosamine kinase activity*
MO6-24/0	2	VVMO6_03282	Putative two-component response regulator & GGDEF family protein YeaJ	GO:0009190	cyclic nucleotide biosynthetic process+*
	2	VVMO6_03283	Putative two-component response regulator	GO:0003677	DNA binding*
	2	VVMO6_04101	Sialic acid-induced transmembrane protein YjhT	GO:0005975	Carbohydrate metabolic process*
	2	VVMO6_04102	Salic acid utilization regulator RpiR family	GO:0005975	Carbohydrate metabolic process*
	2	VVMO6_04103	N-acetylneuraminate lyase	GO:0008747	N-acetylneuraminate lyase activity*
	2	VVMO6_04104	TRAP-type transport system large permease component	GO:0016021	integral to membrane+
	2	VVMO6_04105	TRAP-type transport system small permease component	N/A	N/A
	2	VVMO6_04106	TRAP-type system periplasmic component	GO:0006810	transport+*
	2	VVMO6_04498	Arysulfastase A	GO:0008484	sulfuric ester hydrolase activity*
	2	VVMO6_04499	GALNS arysulfatase regulator (Fe-S oxidoreductase)	GO:0008152	metabolic process+*
	2	VVMO6_04500	Choline-sulfatase	GO:0008449	N-acetylglucosamine-6-sulfatase activity*
	2	VVMO6_04501	Arysulfastase	GO:0008484	Sulfuric ester hydrolase activity*
	2	VVMO6_03523	rsbS, negative regulator of sigma-B	N/A	N/A
	2	VVMO6_03524	anti-sigma B factor RsbT	GO:0005524	ATP binding+
	2	VVMO6_03525	serine phosphatase RsbU, regulator of sigma subunit	GO:0003824	Catalytic activity+*
	2	VVMO6_03526	two-component system sensor protein	GO:0004673	protein histidine kinase activity+*
	1	VVMO6_02633	PTS system, mannitol-specific IIC component	GO:0016301	kinase activity+*
	1	VVMO6_02634	Mannitol-1-phosphate 5-dehydrogenase	GO:0008926	mannitol-1-phosphate 5-dehydrogenase activity*
	1	VVMO6_02635	Mannitol operon repressor	N/A	N/A
YJ016	2	VVA0202	Transcriptional regulator	GO:0003677	DNA binding*
	2	VVA0325	Putative fimbrial protein Z, transcriptional regulator	GO:0003677	DNA binding*
	2	VVA0326	GGDEF family protein	GO:0009190	cyclic nucleotide biosynthetic process+*
	2	VVA0327	Putative fimbrial protein Z, transcriptional regulator	GO:0003677	DNA binding*
	2	VVA1199	Putative N-acetylneuraminate lyase	GO:0008747	N-acetylneuraminate lyase activity*
	2	VVA1200	TRAP-type C4-dicarboxylate transport system, large permease component	GO:0016021	integral to membrane+*

Table 2. Cont.

Strain	Chr.	Locus tag	Product Description	GO id	GO Term
	2	VVA1201	TRAP-type C4-dicarboxylate transport system, small permease component	N/A	N/A
	2	VVA1202	TRAP-type C4- dicarboxylate transport system, periplasmic component	GO:0006810	Transport+*
	2	VVA1632	Arysulfastase A	GO:0008484	sulfuric ester hydrolase activity*
	2	VVA1633	Arylsulfatase regulator	GO:0055114	oxidation-reduction process+*
	2	VVA1634	Arylsulfatase A	GO:0008449	N-acetylglucosamine-6-sulfatase activity*
	2	VVA1635	Arysulfatase A	GO:0008484	sulfuric ester hydrolase activity*
	2	VVA0581	anti-anti-sigma regulatory factor	N/A	N/A
	2	VVA0582	anti-sigma regulatory factor	GO:000552	ATP binding+
	2	VVA0583	indirect negative regulator of sigma-B activity	GO:0003824	Catalytic activity+*
	2	VVA0584	conserved hypothetical protein	GO:0016310	phosphorylation+*

*indicates there are more than 1 GO term at the lowest level for this gene. +indicates that no significant GO term was associated with gene. Significance adjusted-p value <.005. Box highlights genes that are found on Chromosome 1 of *V. vulnificus* CMCP6. All other differential genes are found on Chromosome 2.
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unique domain that can be found within chitinases, which has been proposed to enhance the hydrolysis of insoluble chitin [35]. Manual annotation of individual gene differentials revealed that E-genotypes have a unique PKD domain-containing protein, which is most closely related to its homolog in *Alteromonadales* TW-7, with a significant blast hit (e-value of 7.00^{e-62}). Using site directed mutagenesis of conserved aromatic residues within the PKD domain, Orikoshi *et al.* [36] were able to demonstrate that the PKD domain of chitinase A in *Alteromonas* sp. strain *O-7* was required for effective binding and hydrolysis of chitin. As noted by Grimes *et al.* [37], several chitinases and putative chitinases have been identified in two previously sequenced clinical strains of *V. vulnificus* (CMCP6 and YJ016). However, the PKD gene found in the newly sequenced E strains may be a chitinase that is unique to E-genotypes. The ability to attach to chitin is important for *Vibrio* spp. as it facilitates DNA transformation, believed to be critical for horizontal gene transfer in this genus [38].

The ability to cope with the rapid and potentially stressful transition from the oyster environment to the human host likely requires a variety of stress resistance genes that provide the bacterium with protection and the ability to survive in this seemingly hostile environment. Previous studies have demonstrated the need for stress regulators to aid in survival under a variety of stressful conditions, such as starvation, osmotic stress, low pH, non-optimal temperatures, and oxidative damage [39]. Studies investigating the ability of *V. vulnificus* to survive stressful conditions

Table 3. Key differential genes found in *V. vulnificus* E-genotypes but not in C-genotypes.

Strain	Chr. Alignment	Locus tag	Product Description	GO id	GO Term
JY1305	No LCB alignment	VvJY1305_2152	Hypothetical protein	GO:0019627	urea metabolic process*
	LCB in Vv. CMCP6 chr 1	VvJY1305_1632	Permease	GO:0016020	membrane+*
	LCB in Vv. CMCP6 chr 2	VvJY1305_2975	PTS system, glucose-specific IIBBC component	GO:0006810	transport+*
	LCB in Vv. CMCP6 chr 2	VvJY1305_3160	PKD domain containing protein	N/A	N/A
E64MW	No LCB alignment	VvE64MW_4158	Hypothetical protein	GO:0016151	nickel ion binding*
	LCB in Vv. CMCP6 chr 1	VvE64MW_1434	Permease	GO:0015128	gluconate transmembrane transporter
	LCB in Vv. CMCP6 chr 2	VvE64MW_3479	PTS system, glucose-specific IIBBC component	GO:0005351	hydrogen symporter activity+*
	No LCB alignment	VvE64MW_3886	PKD domain containing protein	N/A	N/A
JY1701	No LCB alignment	VvJY1701_4279	Hypothetical protein	GO:0019627	urea metabolic process*
	LCB in Vv. CMCP6 chr 1	VvJY1701_1508	Permease	GO:0016020	membrane+*
	LCB in Vv. CMCP6 chr 2	VvJY1701_3646	PTS system, glucose-specific IIBBC component	GO:0006810	transport+*
	LCB in Vv. CMCP6 chr 2	VvJY1701_4020	PKD domain containing protein	N/A	N/A

*indicates there are more than 1 GO term at the lowest level for this gene. +indicates that no significant GO term was associated with gene. Significance adjusted-p value <.005. Box highlights differential genes which aligned to locally conserved blocks in Chromosome 1 of *V. vulnificus* CMCP6, suggesting a possible location on Chromosome 1 in the E-genotype genomes.
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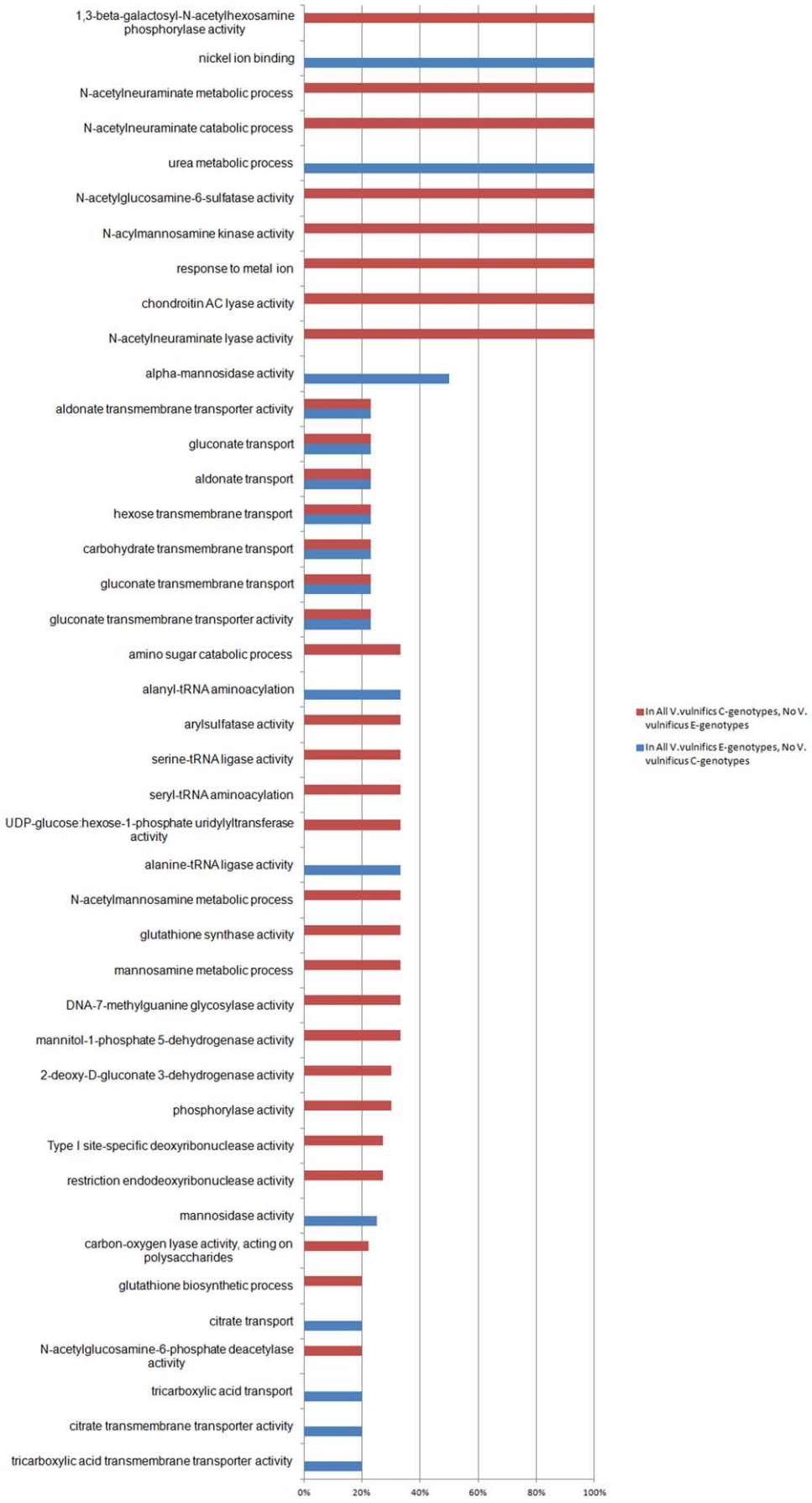
1,3-beta-galactosyl-N-acetylhexosamine phosphorylase activity
nickel ion binding
N-acetylneuraminate metabolic process
N-acetylneuraminate catabolic process
urea metabolic process
N-acetylglucosamine-6-sulfatase activity
N-acylmannosamine kinase activity
response to metal ion
chondroitin AC lyase activity
N-acetylneuraminate lyase activity
alpha-mannosidase activity
aldonate transmembrane transporter activity
gluconate transport
aldonate transport
hexose transmembrane transport
carbohydrate transmembrane transport
gluconate transmembrane transport
gluconate transmembrane transporter activity
amino sugar catabolic process
alanyl-tRNA aminoacylation
arylsulfatase activity
serine-tRNA ligase activity
seryl-tRNA aminoacylation
UDP-glucose:hexose-1-phosphate uridylyltransferase activity
alanine-tRNA ligase activity
N-acetylmannosamine metabolic process
glutathione synthase activity
mannosamine metabolic process
DNA-7-methylguanine glycosylase activity
mannitol-1-phosphate 5-dehydrogenase activity
2-deoxy-D-gluconate 3-dehydrogenase activity
phosphorylase activity
Type I site-specific deoxyribonuclease activity
restriction endodeoxyribonuclease activity
mannosidase activity
carbon-oxygen lyase activity, acting on polysaccharides
glutathione biosynthetic process
citrate transport
N-acetylglucosamine-6-phosphate deacetylase activity
tricarboxylic acid transport
citrate transmembrane transporter activity
tricarboxylic acid transmembrane transporter activity
0%
20%
40%
60%
80%
100%
In All V.vulnifics C-genotypes, No V. vulnificus E-genotypes
In All V.vulnifics E-genotypes, No V. vulnificus C-genotypes

Figure 5. Gene Ontology (GO) functional differences between C- and E- genotypes. Figure shows GO functional categories which are enriched in C-genotypes of *V. vulnificus* relative to E-genotypes (blue) or E-genotypes relative to C-genotypes (red). Percentages represent percent of genes under each category that are differential between the genotypes. Percentages of less than 20% are not depicted.
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have shown that C-genotypes are significantly better able to survive in complement-activated human serum than E-genotypes [40]. Rosche et al. demonstrated that C-genotypes exhibit better cross-protection when exposed to multiple stresses, such as osmotic shock followed by H_20_2 exposure or elevated temperature [27]. C-genotypes appear to be physiologically more stress tolerant, and this suggest that the SPANC hypothesis may apply in *Vibrio vulnificus*, in that C-genotypes are more capable at self-preservation, while E-genotypes carry additional genes that suggest they may be more capable of nutritional competence. Sequence alignments of the *rpoS* gene for all six sequence strains did not indicate any major genetic polymorphisms, and only resulted in a few amino acid substitutions. The nucleic acid sequence is ~99% identical and the coded protein 98.5% identical. Other genes that may affect the SPANC balance [41] are similarly well conserved. Future studies will need to be performed to investigate the roles of E- genotype specific genes under relevant conditions such as nutrient limitation in order to validate this hypothesis.

Characteristic features of the C-genotype genomes

Genotype classification with Mannitol transport and fermentation genes. Mannitol transport and fermentation genes were found to be present in the C-genotype strains but not in the newly sequenced E-genotype strains. Mannitol has been correlated with virulence-associated genotypes (*vcgC* and 16S rDNA type B) [25]. This lack of a mannitol operon (consisting of a dehydrogenase, a phosphotransferase system component, and an operon repressor) in the sequenced E-type strains was identified in a previous study, and confirmed by our sequencing [42,43]. This differentiating feature was also identified in a recent analysis of short-read sequence fragments from four other E-type strains [18]. It is important to note that while many E-genotype strains lack the mannitol operon, phenotypic and molecular testing by our laboratory has shown that 40% of 73 total tested E-type strains contain the mannitol operon and are able to ferment this sugar [42,43]. The strains sequenced in this study and in the study by Gulig et al. [18] were among those previously known, before sequencing, to be unable to ferment mannitol, and future sequencing should include E-genotype strains that are able to ferment mannitol, to provide a more extensive comparison between these two phenotypes.

Genomic XII region. Cohen et al. (2007) used MLST data to identify a 33-kb genomic island (region XII) on the second chromosome of V. vulnificus [14]. This region contained an arylsulfatase gene cluster, a sulfate reduction system, two chondroitinase genes, and an oligopeptide ABC transport system, none of which were found in their "lineage II" (our E-genotype) isolates. They suggested that this region may play a role in the pathogenic process, as both arylsulfatases (see discussion below) and the chondroitin sulfate proteoglycan degrading chondrotinase have been speculated to be involved in the penetration of epithelial cells [44,45]. The authors thus speculated that region XII, along with others, could give cells of the C-genotypes a selective advantage in their relationships with aquatic environments or human hosts, or both. Gulig et al. (2010), in their V. vulnificus sequencing study, suggested that the ability to scavenge sulfate groups could facilitate survival in the human host, where free sulfur is limited [18]. Cohen et al. (2007) identified region XII in 32 of the 37 lineage I genotypes (including reference C-genotypes, V. vulnificus CMCP6, V. vulnificus MO6-24/O and V. vulnificus YJ016) they examined, but in only 3 of the 6 lineage II strains [14]. Consistent with their findings, we identified 83.3% of this region as being present only in the C-genotypes, and not in the three E-genotypes we sequenced here.

Arylsulfatases. Arylsulfatases occur in virtually all organisms, and are found in high levels in the digestive glands of oysters and other mollusks [46]. These enzymes hydrolyze arylsulfate ester bonds, releasing free sulfate, which is critical for microbial growth [14]. Interestingly, arylsulfatase synthesis in enteric bacteria is regulated by norepinephrine, among other monoamine compounds, which is believed to be involved in quorum sensing in the human gut [47]. A role for arylsulfatases is suggested by the finding that, in *E. coli*, they facilitate invasion of the blood-brain barrier [32]. In a major study of the genomics of *V. vulnificus* [14], the authors found that the clinical (C-genotype) strains possess a 33 kb genomic island ("region XII") which contains an arylsulfatase gene cluster [14]. We did not observe this gene cluster in the E-genotype strains, suggesting it may be important in the pathogenesis of the C-genotype strains, possibly by allowing survival in the human gut where free sulfur is limited [14]. In the present study, VVA1632, VVA1633, VVA1634, and VVA1635, which make up the arylsulfatase gene cluster, are among the genes differentiating C-type from E-type strains (Table 3). This difference may be one component of the reduced pathogenicity of E-genotype strains relative to C-type strains.

Sialic acid catabolism. Sialic acids are a family of nine-carbon sugar acids that are typically located at the terminal carbohydrate ends of mucin proteins. The most abundant sialic acid is N-acetylneuraminic acid (Neu5Ac), with all of the other sialic acids being derivatives of this compound [48]. Sialic acids are found in both eukaryotes and prokaryotes and occur on many types of cells, including epithelial cells in humans [4]. Sialic acids are commonly used as a carbon and nitrogen source for enteropathogenic bacteria, and can serve as a vital substrate for invasion and survival within the human host [49,50]. Mucin is abundant in the mucus layer overlaying intestinal epithelial cells where *V. vulnificus* adheres and begins its infective route, and previous studies have demonstrated the importance of this gene for growth, adhesion, and survival within the jejunum and colon tissues of the mouse intestine [50]. The Nan cluster (*nanA, nanE, and nanK*) is responsible for sialic acid and catabolism has been identified in several major intestinal pathogens including *V. vulnificus* [51,52]. The E-genotypes sequenced in this study lack the major components of the sialic acid catabolism gene cluster including *nanA*. However, recent work in our lab (Taylor and Oliver, unpublished) and others investigating the presence of *nanA* in a larger number of clinical and environmental genotypes has revealed the presence of this gene in some E-genotypes [53]. However, the *nanA* gene is less prevalent in E-genotypes and there also appears to be some correlation between the presence of this gene and C-genotypes, highlighting the need for further investigation into the function of sialic acid catabolism as a virulence factor for *V. vulnificus*.

The RsbRST Operon. A hallmark method for responding and adapting to environmental fluctuations involves the use of alternate sigma factors which compete for RNA polymerase and subsequently initiate the transcription of a specific subset of genes [54]. In gram negative bacteria such as *V. vulnificus*, stressful

conditions such as carbon starvation, non-permissive pH values, and hyperosmolarity will induce a stress response in which sigma S (σ^S) competes with the housekeeping sigma factor (σ^D) thus redirecting gene expression to respond to the stress. Gram positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, use a comparable alternative sigma factor (σ^B) which governs the "general stress response" and plays an important role in virulence in these organisms. This is accomplished by activating the transcription of over 125 genes in response to a variety of stressful conditions, including temperature shifts, ethanol, salt and acid stress, and starvation [54,55,56]. In *B. subtilis*, σ^B activation is partly regulated by a large signaling complex called the RsbRST stress module (or stressosome), and a PP2C-type phosphatase, RsbU [55].

In all three C- genotypes, we identified an operon homologous to the RsbRST stress response module, the PP2C-type phosphatase, and a downstream two-component regulatory system. With the exception of a single gene encoding *rsbR*, which we found in E64MW and JY1701, this stressosome was absent in the E-genotypes we sequenced. To our knowledge, *V. vulnificus* does not possess the σ^B subunit of RNA polymerase, thus the role of this signaling system in *V. vulnificus* is not clear. It has been proposed by some investigators that the function of these modules may vary considerably amongst bacteria as a result of niche expansion in which incoming signals are relayed to a diverse array of regulatory systems, such as alternative sigma factors and two-component signal transducing systems [55,56]. Further investigation should be performed to determine if the presence of this system strongly correlates with *V. vulnificus* genotype. Additionally, elucidating the role of this system in *V. vulnificus* would be of great interest and could potentially provide insight into the mechanisms of virulence and survival in this organism.

Cyclic-di-GMP. Cyclic-di-GMP is an intracellular signaling molecule that acts as a second messenger for integrating environmental signals and has been demonstrated to regulate several distinct cellular processes such as motility, biofilm formation, virulence, and rugose colony morphology [57]. Cyclic di-GMP levels in the cell are controlled by the activity of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), resulting in the synthesis and degradation, respectively, of cyclic-di-GMP. DGCs are characterized by a conserved GGDEF domain, whereas PDEs contain a conserved EAL or HD-GYP domain. *Vibrio* spp. have been shown to possess a large number of these regulators indicating the importance of cyclic-di-GMP signaling in this genus in their adaptation to new environments [58]. Sequence comparisons revealed that C-genotypes possess unique GGDEF family proteins that were not present in the currently sequenced E-genotypes. Interestingly, we identified one of these GGDEF family proteins (GGDEF family protein YeaJ) located in an operon with a putative two-component response regulator and a fimbrial protein Z transcriptional regulator. In *E. coli*, *yeaJ* is one of the many GGDEF domain encoding genes that differentially mediates switching between motility and curli-fimbrial mediated adhesion [59].

In other pathogens, such as *Salmonella enterica serovar Typhimurium*, *FimZ* acts as a positive transcriptional activator of type I fimbrial expression, therefore modulating ability to attach or swim in a given environment [60]. Clegg and Hughes [60] found that an increase in *FimZ* results in a lack of motility due to the down-regulation of the *flhDC* master flagellar operon. Clegg's group has also shown that *FimZ* plays a crucial role in regulating the expression of phenotypes associated with adherence to, and invasion of, eukaryotic epithelial cells [60].

Type IV secretory system. Type IV secretory system gene *VirB4* (VV2_0638) was found to be present in C-genotype strains (*V. vulnificus* YJ016 and *V. vulnificus* CMCP6) but absent in the newly sequenced E-genotype isolates and *V. vulnificus* MO6-24/O. Type IV bacterial secretion systems (T4SS) are responsible for the translocation of molecules such as DNA, proteins, and toxins out of the cell and into the immediate environment or the host cell [61,62]. This system is composed of the T-pilus and membrane-associated complex which are constructed from 12 VirB proteins, several other Vir proteins, and a coupling protein (*VirD4*) [63,64]. Of these proteins, *VirB4* serve as energizing components as these genes are associated with ATPase functionality [63,65]. Because this system is associated with the transfer of DNA (conjugation) and also toxins, it is also often implicated with pathogenicity. Our *V. vulnificus* E-genotype strain sequencing suggests that these T4SS components are active in infections caused by C-genotypes (*V. vulnificus* YJ016 and *V. vulnificus* CMCP6). 70% of the predicted *virB* operon sequence of the T4SS has been observed to be present in the C-genotypes (*V. vulnificus* YJ016 and *V. vulnificus* CMCP6) and not in M06-24 or the E-genotypes [66]. Sequencing of more C- and E-genotypes should be performed to investigate whether the presence of this operon displays a trend towards virulent strains in *Vibrio vulnificus*.

Summary

In conclusion, three E-genotype strains of *Vibrio vulnificus* have been sequenced to over 99% completion. The genomes have been assembled using *ab initio* methods and contig sequences have been deposited in the NCBI Whole Genome Shotgun archive. Additional Illumina sequencing is underway with the aim of complete closure of the strain JY1305 genome. We expect that effort to provide insights into structural rearrangements among the C-genotype and E-genotype strains, but we do not expect the additional sequencing to significantly alter the findings of strain-differentiating genes reported herein. Current work in progress also includes the genomic sequencing of a larger collection of *V. vulnificus* strains, encompassing the entire genomic spectrum of pathogenicity. That data will provide additional insights into the distinct genomic differences between pathogenic and non-pathogenic strains (Baker-Austin, unpublished). The comparative analysis of C- and E- genotypes confirms previous observations of putative virulence determinants that differentiate *V. vulnificus* isolated from wounds in clinical settings from environmental strains. However, the analysis also points the way to dozens of differentiating genes specific to the E-genotypes. Some of the genes potentially fit into existing functional hypotheses such as the SPANC theory, while others are as yet functionally uncharacterized.

Although the presence or absence of a particular gene in a specific genotype provides initial targets for functional differentiation, this current sequencing effort provides the *V. vulnificus* community with a valuable reference for functional study of determinants of virulence, and facilitates the future use of high-throughput approaches to assess functional differences via study of the *V. vulnificus* transcriptome. Future studies will aim to analyze gene locations and gene neighborhoods to determine if there are genotypic differences here that could account for differences in physiology; e.g. our mannitol study revealed differences in the gene arrangement of a putative hemolysin, mannitol transporter, and mannitol fermentation operon that has been shown to correlate with clinical C-genotypes [42]. We also recognize that the presence of gene homologs (e.g. virulence-related genes) in both genotypes does not necessarily indicate equivalent function - even single base pair changes can alter protein function of a

particular gene, and a detailed investigation of the impact of cross-genotype differences on protein sequences is planned as a follow-up to this study.

Materials and Methods

Strains, Growth Conditions, and DNA Isolation

V. vulnificus strain JY1305 was grown overnight in Bacto™ Heart Infusion (HI) broth (BD, New Jersey) at 30°C with vigorous shaking. Cells were pelleted by centrifugation and supernatants discarded. The cells were washed three times with phosphate buffered saline (PBS) before being resuspended to a final approximate concentration of 5×10^8 cell/ml. The MagMax™ Total Nucleic Acid Isolation Kit (Ambion) and All Prep DNA/RNA/Protein Mini Kit (Qiagen) were used for DNA extraction. The quality and quantity of DNA was evaluated spectrophotometrically with the NanoDrop ND1000 (Thermo Scientific, Wilmington, DE). A concentration of 50 ng/µL was used for next gen sequencing.

V. vulnificus strains JY1701 and E64MW were grown overnight with shaking in 10 ml of ASPW. Cells were pelleted by centrifugation and resuspended in 100 µl of ice-cold PBS. DNA was extracted using DNAzol (Invitrogen) according to manufacturer instructions, followed by incubation with RNase A. Subsequently, samples were purified using a phenol/chloroform/isoamyl alcohol extraction protocol. Briefly, 40 µl of 3 M sodium acetate was added to each DNA sample, followed by 440 µl of phenol/chloroform/isoamyl alcohol. Samples were centrifuged (5 min, 13,000 rpm) and ~400 µl of supernatant was removed and mixed with an equal volume of phenol/chloroform/isoamyl alcohol. This solution was centrifuged (5 min, 13,000 rpm) and the supernatant (~300 µl) removed and mixed with an equal volume of 24:1 chloroform/isoamyl alcohol. Samples were subsequently centrifuged for 5 min (13,000 rpm) and the supernatant (~200 µl) was subjected to ethanol precipitation. The DNA pellet was re-dissolved in 50 µl 1×TE buffer and stored at −80°C. The quality and quantity of DNA was subsequently ascertained spectrophotometrically using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE).

Genome sequencing and assembly

V. vulnificus JY1305 was sequenced at the Virginia Commonwealth University using Roche/454 Titanium technology [67]. One complete sequencing plate was used for this genome. *V. vulnificus* E64MW and JY1701 were sequenced at the BBSRC Genome Analysis Centre (Norwich, UK) also using the Roche/454 Titanium technology [67]. Quarter plates were used for both. A total of 671521, 376290, and 321096 single end reads were generated for JY1305, E64MW, and JY1701 respectively. De novo assembly with Newbler version 2.3 initially constructed 179, 269, and 269 contigs for JY1305, E64MW, and JY1701. An additional assembly was performed using the MIRA 3.2.1 de novo assembler. The default parameters for MIRA were used, except that the assembly quality parameter was changed from "normal" to "accurate", and trace information was excluded from the assembly. MIRA constructed 159, 271, 329 contigs for JY1305, E64MW, and JY1701 respectively. Assembled contigs were compared by constructing sequence alignments using Mummer 3.0 [68]. Supplement S5 provides details of the assembly approach. A comparison of homologous contigs generated by MIRA [19] and Newbler [67] is provided in Supplement S5.

Genome sequence comparison

Contigs from each assembly were aligned to reference genomes using the Mauve software [23]. The three E-genotype genomes, *V. vulnificus* JY1305, E64MW and JY1701, were aligned to three *V. vulnificus* C strain reference sequences [AE016795.3, CP002469.1, and BA000037.2] and longest common blocks (LCBs) were identified in each genome. *V. vulnificus* YJ016 and MO6-24/O and each set of assembled contigs for JY1305, E64MW, and JY1701 were aligned against the reference sequence *(V.vulnificus* CMCP6) to produce separate, optimal pair-wise alignments for each query sequence. The pair-wise alignments were then used to produce a multiple alignment of the newly sequenced strains, and *V. vulnificus* YJ016 and MO6-24/O, using *V. vulnificus* CMCP6, which has recently been re-annotated [22].

The LCB alignment results suggested that a plasmid sequence, present in YJ016 and thought to be present in other *V. vulnificus* strains, was absent in the newly sequenced E-strain genomes [23]. To confirm this, a PCR assay was performed on the extracted JY1305 DNA during prep, and validated the extraction of two chromosomes, and the absence of plasmid DNA. The primers used to verify chromosomal identity were *csrA* F2, *csrAR2*, *rpod* UP, *rpod* DOWN, *vvhA* F, *vvhA* R, *pepRF* F2 and *pepR3*. These were designed based on known features of the C-type genomes. The primers used to test for the presence of a YJ016-type plasmid were *vvSSF1*, *vvSSR1*, *vvF2* forward primer, and *vvR2* reverse primer. Using Primer3 [69], two sets of primers were generated for conserved regions of plasmid YJ016 and PC4602-1, with expected product length of 244 and 209 bps. The conserved sequences used for primer generation were compared to the genomic sequence of *V. vulnificus* CMCP6 and YJ016 strains using BLAST to ensure that they exclusively matched the two plasmid sequences. Primer sequences are provided in Table S3.

Genome and gene characterization

Draft annotation of the sequences was performed using a pipeline of published microbial annotation tools. Feature determination for each strain was performed on the contig set from each sequence assembly. The feature identification methods that were used were Glimmer3.02 and GeneMark.hmm [70,71]. Both packages are widely used feature determination applications recognized and accepted by NCBI, and both are publicly available. Glimmer3.02 was used with default parameters. An exception was that the circular chromosomes were treated as linear in the analysis. This setting was used to prevent each contig from being treated as an individual circular chromosome. GeneMark.hmm was used with default parameters. The models used for training were the two *V. vulnificus* reference organisms (CMCP6 and YJ016). Spacer sequence was added to the ends of each contig to mimic start and stop signals. The spacer sequence was 32 nucleotides in length. We used the sequence NNNNNCACACACTTAATTAATTAAGTGTGTGNNNNN, which is used at JCVI to merge contigs [http://www.jcvi.org/cms/research/projects/annotation-service/submission-guide/].

For gene identification in each of the newly sequenced strains, one of the following criteria had to be met: (1) A gene will be included in the gene list if it can be predicted by either Glimmer or GeneMark, as long as it amino acid sequence length is equal to or greater than 150. (2) A gene must be predicted by both Glimmer and GeneMark to be included in the gene list, if its amino acid sequence length less than 150. (3) A gene prediction will only be included in the gene list if it occurs in a cluster of known or hypothetical genes found in other *Vibrio* spp. The first two criteria were derived from Chen et al., 2003 [15] and were used to ensure as much consistency as possible between gene prediction methods

used among all the genomes being compared. Supplement S1 describes the annotation procedure in greater detail. Supplement S1 contains the gene counts based on each criterion. When there was a conflict between a predicted gene's start position from different feature identification methods, BLASTP was used to compare the predicted gene to the sequence of its products, if available, and a start site was chosen on that basis. The target database consisted of all completely characterized bacterial genomes. ptt files. Preliminary locus tags were generated for all genes in each E-genotype genome.

tRNAScanSE was used to predicted the tRNAs in the MIRA contigs for each strain [72]. RNAHMMER was used to predict the rRNAs from the MIRA contigs for each strain [73]. In both cases, default parameter settings were used.

The reference genomes used for the comparative genomic content analysis include all the available and completely characterized *Vibrio* genomes GenBank identifiers [*Vibrio anguillarum* 775; CP002284.1, *Vibrio cholerae* LMA 3984-4; CP002555, *Vibrio cholerae* M66-2; CP001233.1, *Vibrio cholerae* MJ-1236; CP001485.1, *Vibrio cholerae O1 biovar El Tor str.* N16961; AE003852.1, *Vibrio cholerae* 0395; CP000626.1, *Vibrio fischeri* ES114; CP000020.2, *Vibrio fischeri* MJ11; CP001133.1, *Vibrio furnissii* NCTC 11218; CP002377, *Vibrio harveyi* ATCC BAA-116; CP000789.1, *Vibrio parahaemolyticus* RIMD 2210633; BA000031.2, *Vibrio sp.* Ex 25; CP001805.1, *Vibrio splendidus* LGP32; FM954973.2, *Vibrio vulnificus* CMCP6; AE016795.3, *Vibrio vulnificus* MO6-24/O; CP002469.1, and *Vibrio vulnificus* YJ016; BA000037.2].

Gene clustering

OrthoMCL version 2.0 was used to cluster newly predicted genes with genes from other *Vibrio* spp. [74]. OrthoMCL has been shown to outperform other stand-alone methods for ortholog clustering. OrthoMCL uses an all-against-all blastp comparison of sequences as an input step followed by application of a Markov clustering procedure. The e-value cutoff for the BLASTP was 1e-5. Default parameters were used for OrthoMCL except that clusters were formed based on a shared sequence similarity of 70%, instead of the OrthoMCL default parameter value of 50%. The increase in stringency to 70% shared sequence similarity resulted in more constrained gene clusters, and reduced the chance of inappropriate clustering of partial homologs into ortholog clusters. The newly sequenced genomes were clustered first with the previously sequenced *V. vulnificus* C-type strains, and then with the 16 fully sequenced *Vibrio* species, to determine the impact of different reference sets on the orthology analysis outcome.

Gene content comparison

The OrthoMCL clustering generated during the annotation step was used as the basis for identification of differentiating genes. Identified gene features and OrthoMCL results were stored in a locally developed OLAP data warehouse (GenoSets) that supports queries across aggregate data generated by a variety of genomic annotation and comparison methods. This system is fully described in (Cain et al. 2011, in review) [31]. Annotations for the published C-strain genomes were downloaded and parsed from the EMBL-Bank public repositories. Annotations for the novel E-strain genomes reported herein were generated as described above. Once feature boundaries were determined from the annotation and stored, gene presence-absence queries were formulated within the GenoSets system at different levels of the taxonomy hierarchy, in order to identify gene features that differentiate the three E-strains from each other, from the C-strains, and from other *Vibrio* spp.

In order to provide a standard means of comparison for feature attributes we establish relationships between features using two methods. First, we estimate orthologous relationships between genes using OrthoMCL, which uses a Markov Cluster algorithm to group putative homologs based on sequence similarity, as the primary ortholog clustering method in GenoSets. OrthoMCL has been shown to outperform other stand-alone methods for ortholog clustering [74]. For functional analysis, gene features identified in the newly sequenced *V. vulnificus* strains were associated with GO terms using homology determined through OrthoMCL clustering of BLASTP results. For functional comparison purposes, it is helpful to identify genes and other features using a controlled vocabulary. The Gene Ontology (GO) provides standardized terms for the description of gene products in terms of biological processes, cellular location, and molecular function [28,29]. If a GO term was associated with any gene within an ortholog cluster, all genes within that cluster were also associated with that GO term. In Figure 5, we show the GO classifications, with the quantity of differentiating genes shown as a percentage of all E- and C-genotype genes.

Phylogenetic Analysis

We identified 1748 single-copy ortholog clusters within 19 *Vibrio* spp. We performed a phylogenetic analysis following the methods used in Suzuki et al. and Hasan et al. [75,76]. We randomly selected protein sequences of 10% of the single-copy ortholog clusters identified (175 genes) and used the sample as a basis for construction of a maximum likelihood tree, following the approach used in Hasan et al. [76]. ClustalW was used to align sequence members of each ortholog cluster independently, to minimize gene rearrangement within the multiple sequence alignment [77]. Once each individual protein alignment was built, the independent alignments were concatenated. phyML 3.0, a maximum likelihood method, was used to generate a phylogenetic species tree with 100 replicates for bootstrapping [78]. The tree was visualized with Figtree [79]. Three independent samplings were tested and all three produced trees with highly similar topologies.

Supporting Information

Table S1 Conservation of locally collinear blocks (LCBs) in *V. vulnificus* genomes.
(DOCX)

Table S2 Summary of key gene differences between *V. vulnificus* and other *Vibrio* spp.
(DOCX)

Table S3 Primer sequences used to validate presence of genomic DNA and plasmid DNA in extracted samples.
(DOCX)

Supplement S1 Complete list of predicted genes for the draft assemblies of *V. vulnificus* JY1305, E64MW, and JY1701 and alignment position in the *V. vulnificus* CMCP6 reference genome.
(XLSX)

Supplement S2 Complete listing of LCBs (locally collinear blocks) identified by Mauve among the newly sequenced E-genotypes and the C-genotype reference strains.
(XLSX)

Supplement S3 Complete list of genes that differentiate *V. vulnificus* from other *Vibrio spp.*
(XLSX)

Supplement S4 Complete list of genes differentiating E-genotype strains from C-genotype strains.
(XLSX)

Supplement S5 Details that support the rationale for aspects of the bioinformatic analysis.
(PDF)

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Author Contributions

Conceived and designed the experiments: CJG CB-A JDO. Performed the experiments: SSM RH DV-J. Analyzed the data: SSM AC BF TW CT JDO CB-A CJG. Contributed reagents/materials/analysis tools: AC BF JDO CB-A CJG. Wrote the paper: SSM AC BF TW CT JDO CB-A CJG.

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TRANSCRIPTOME SEQUENCING REVEALS THE VIRULENCE AND ENVIRONMENTAL GENETIC PROGRAMS OF *VIBRIO VULNIFICUS* EXPOSED TO HOST AND ESTUARINE CONDITIONS

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PLOS ONE

RESEARCH ARTICLE

Transcriptome Sequencing Reveals the Virulence and Environmental Genetic Programs of *Vibrio vulnificus* Exposed to Host and Estuarine Conditions

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Abstract

Vibrio vulnificus is a natural inhabitant of estuarine waters worldwide and is of medical relevance due to its ability to cause grievous wound infections and/or fatal septicemia. Genetic polymorphisms within the virulence-correlated gene (*vcg*) serve as a primary feature to distinguish clinical (C-) genotypes from environmental (E-) genotypes. C-genotypes demonstrate superior survival in human serum relative to E-genotypes, and genome comparisons have allowed for the identification of several putative virulence factors that could potentially aid C-genotypes in disease progression. We used RNA sequencing to analyze the transcriptome of C-genotypes exposed to human serum relative to seawater, which revealed two divergent genetic programs under these two conditions. In human serum, cells displayed a distinct ''virulence profile'' in which a number of putative virulence factors were upregulated, including genes involved in intracellular signaling, substrate binding and transport, toxin and exoenzyme production, and the heat shock response. Conversely, the ''environmental profile'' exhibited by cells in seawater revealed upregulation of transcription factors such as *rpoS, rpoN*, and *iscR*, as well as genes involved in intracellular signaling, chemotaxis, adherence, and biofilm formation. This dichotomous genetic switch appears to be largely governed by cyclic-di-GMP signaling, and remarkably resembles the dual life-style of *V. cholerae* as it transitions from host to environment. Furthermore, we found a ''general stress response'' module, known as the stressosome, to be upregulated in seawater. This signaling system has been well characterized in Gram-positive bacteria, however its role in *V. vulnificus* is not clear. We examined temporal gene expression patterns of the stressosome and found it to be upregulated in natural

estuarine waters indicating that this system plays a role in sensing and responding to the environment. This study advances our understanding of gene regulation in *V. vulnificus*, and brings to the forefront a number of previously overlooked genetic networks.

Introduction

Vibrio vulnificus is a free-living inhabitant of estuarine and coastal waters worldwide, and associates with a variety of aquatic organisms [1]. This bacterium is also a highly invasive pathogen of both fish and humans, and is the primary cause of sea-food related deaths in the US, typically from ingestion of raw or undercooked molluscan shellfish [2]. This medically and economically relevant organism can cause rapidly fulminating septicemia when ingested. If able to gain entry through an open cut or wound, this pathogen can also cause necrotizing fasciitis, often resulting in limb amputation, and can lead to fatal septicemia in susceptible individuals [3].

Not all strains of *V. vulnificus* are equally pathogenic, thus strains are grouped into biotypes and genotypes. Strains within biotype 1 represents those most often associated with disease in humans, whereas biotype 2 represents strains are almost exclusively associated with disease in eels [4]. The most recently discovered biotype 3 consists of strains which are genetically distinct from biotypes 1 and 2, a pathogen which to date is geographically limited to Israel [5, 6]. A PCR-based assay can be used to separate *V. vulnificus* biotype 1 strains into two groups that strongly correlate with source of isolation, such that ''environmental isolates'' possess the *vcgE* allele, whereas ''clinical isolates'' have the *vcgC* allele [7, 8]. Thus, we further subtype biotype 1 strains into two groups: clinical (C-genotypes) and environmental (E-genotypes). Multilocus sequence typing and phylogenetic analyses of conserved housekeeping loci and putative virulence loci have further substantiated the observed dimorphism between *V. vulnificus* biotype 1 strains [9, 10]. Additionally, a phylogenetic analysis of 175 genes present in all currently sequenced *Vibrio* species revealed the same trend, with all six *V. vulnificus* strains examined grouping into one clade, although a distinct branching between C- and E-genotypes was observed [11]. These results indicate that the C/E differences observed are not restricted to a few loci, but are genome-wide and led to the proposition that these two genotypes represent distinct ecotypes [9].

Undoubtedly, *V. vulnificus* C- and E-genotypes display significant differences in their ecology, physiology, genome content, and genetic responses. C-genotypes can resist the bactericidal effects of human serum and even grow in this environment, whereas E-genotypes largely succumb to these bactericidal effects and rapidly decline in number shortly after exposure [12]. Indeed, human serum has become a popular model for predicting virulence amongst environmentally isolated strains [13, 14]. This correlation between genotype and virulence has been

further substantiated in the mammalian model of disease, where C-genotypes are more likely to cause systemic infection and death relative to E-genotypes [15]. Furthermore, a recent comparative genomic analysis of three C- and three E-genotype strains revealed that while these share approximately 3664 genes, they also possess genes unique to each genotype. Of the 278 genes unique to C-genotypes, many were found to represent putative virulence factors, whereas 167 E-specific genes were associated with metabolic functions and may have implications for nutritional competence [11]. Nevertheless, the elusive question of which specific genetic features contribute to the observed differences in environmental distribution and pathogenic potential still stands.

The goal of the current study was to analyze the transcriptome profile of two clinically isolated *V. vulnificus* C-genotypes exposed to human serum (HS) or artificial seawater (ASW). Using RNA sequencing, we screened the transcriptome for clinically relevant genes (or sets of genes), to provide a snapshot of the gene content within clinical strains under these two conditions. We identified several genetic features that likely contribute to survival in the natural aquatic environment as well as the host environment, many of which are relatable to one another. Additionally we found several differentially expressed genes to be unique to C-genotypes and lacking in E-genotypes. From a holistic perspective, our results indicate that in ASW cells take on a low virulence, enhanced biofilm phenotype which we refer to as the ''environmental profile'', whereas in HS, cells exhibit a ''virulence profile'' in which biofilm formation is inhibited and virulence factor production predominates. Notably, this dichotomy in genetic programming between HS and ASW remarkably resembles the genetic and phenotypic switch documented in *V. cholerae* cells as they transition from host to environment. Here, we highlight some of these differentially expressed genes, and discuss the potential relevance of each gene set, thereby setting the stage from which future studies can be directed.

Results and Discussion

RNA sequencing results

Two blood isolates of *V. vulnificus* (CMCP6 and YJ016) were exposed to human serum (HS) or 10ppt artificial seawater (ASW), and cDNA prepared from mRNA isolated from each strain was subjected to Illumina sequencing. Comparative transcriptome analysis of *V. vulnificus* cells exposed to human serum relative to artificial seawater resulted in a total of 469 and 653 differentially expressed (DE) genes (p-value <0.0001) in CMCP6 and YJ016, respectively (**Table 1**). **Fig. 1** depicts a smear plot of DE genes in CMCP6 with a distinction between those transcripts deemed to be significantly DE (**S1 Figure** depicts the smear plot for YJ016). **Fig. 2** shows a compressed linear view of DE genes in CMCP6 by chromosome. **S2 Figure** depicts the same information for YJ016. A full list of differentially expressed genes in CMCP6 and YJ016 when grown in HS relative to ASW can be found in **S1 File**. Each file lists the protein number (NP) associated

Table 1. Summary of differentially expressed genes ($p<0.0001$).

	CMCP6	YJ016
No. of differentially expressed genes		
Genome-wide	469	653
Chromosome I	220	302
Positively expressed	59	79
Negatively expressed	161	147
Chromosome II	249	347
Positively expressed	120	223
Negatively expressed	129	200
Plasmid	N/A[a]	4
Percent of differentially expressed genes		
Genome-wide	10.3%	12.6%
Chromosome I[b]	7.3%	8.9%
Chromosome II	16%	20.3%
Plasmid	N/A	5.8%

[a]N/A – not applicable.
[b]Chromosomal percentages were calculated as number of DE genes divided by total number of genes on each chromosome.

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with each gene, respective product descriptions, expression levels (expressed as log fold change), direction of regulation (+ or −), and p-values to indicate level of significance. S2 **and** S3 Files contain organized files of selected DE genes

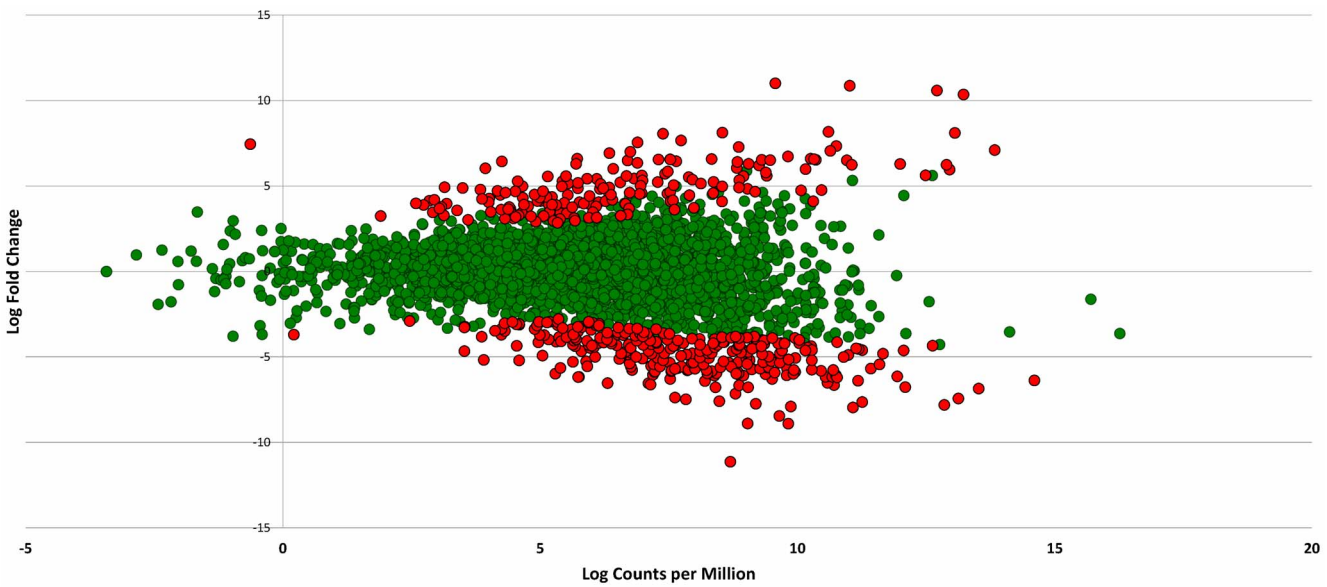

Fig. 1. Smear plot of differentially expressed genes in *V. vulnificus* CMCP6 exposed to human serum (relative to artificial seawater). The smear plot shows the relationship between the log fold change and log counts per million. Green points represent non-significant DE genes whereas red points show genes that are significantly differentially expressed ($p<0.0001$) in relation to artificial seawater.

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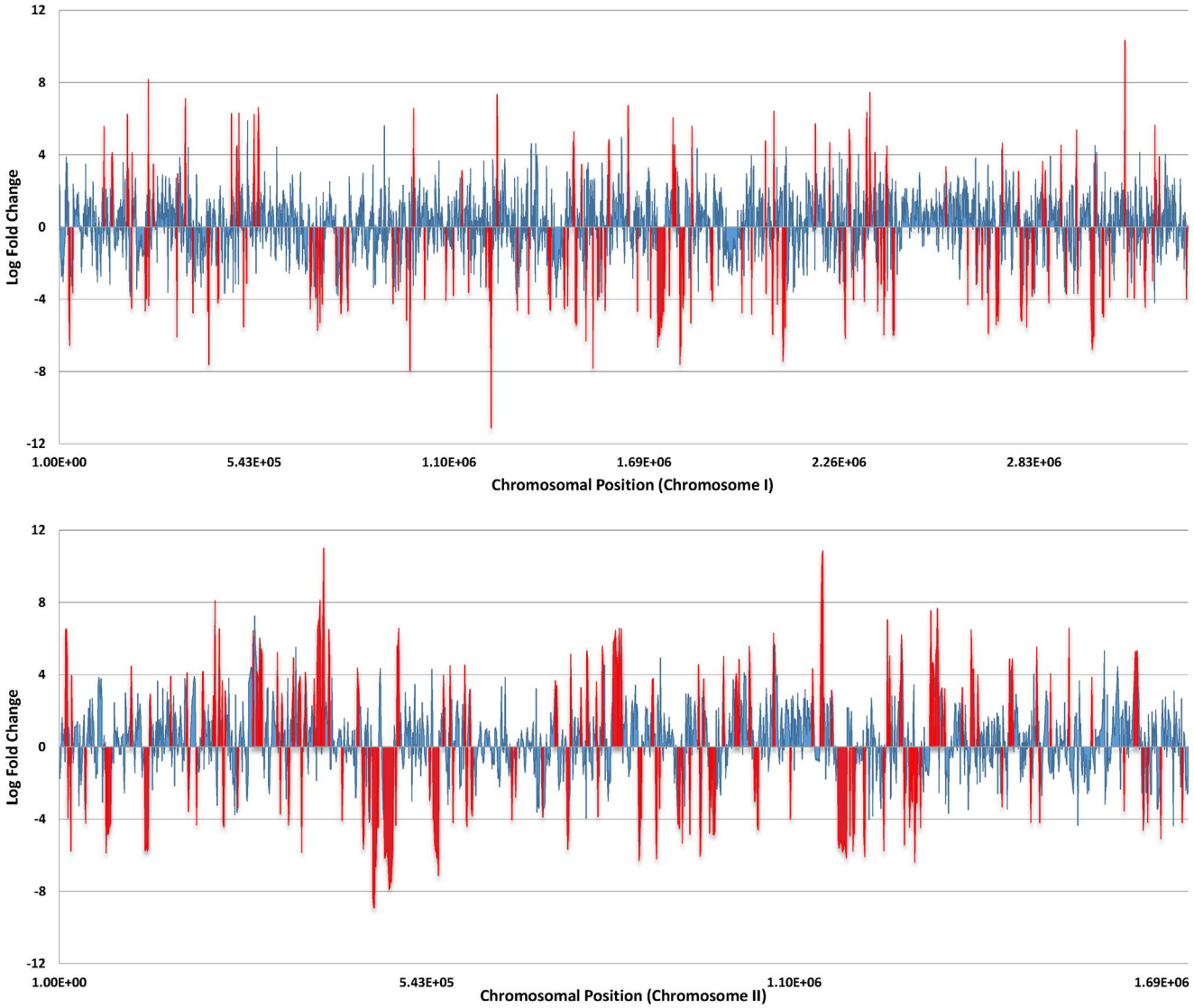

Fig. 2. Linear compressed view of differentially expressed genes in *V. vulnificus* exposed to human serum (relative to artificial seawater). These charts show a compressed view of the differentially expressed genes in *V. vulnificus* CMCP6 by chromosome (top; chromosome I, bottom; chromosome II) and allows quick identification of clusters of differentially expressed genes, both positive and negative. The y-axis shows the log fold change and the x-axis is the nucleotide position of the chromosome. Blue bars represent non-significant DE genes whereas red bars show genes that are significantly differentially expressed ($p<0.0001$) in relation to artificial seawater.

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discussed in detail in this study. S2 File lists genes upregulated in HS and S3 File lists genes upregulated in ASW.

An interesting aspect of our study was the observed imbalance of differentially expressed genes between the two chromosomes of *V. vulnificus*. A higher percentage of genes were upregulated on chromosome II relative to chromosome I. This phenomenon can be easily visualized using a compressed linear view of the differentially expressed genes based on chromosomal position (Fig. 2 and S2 Figure). These charts (assembled using edgeR output, featureCounts output, and

Excel) allow for quick identification of differentially expressed genes (depicted in red), both positive and negative.

In *Vibrio* spp., the larger chromosome (I) is relatively constant in size and contains most of the housekeeping genes essential for growth and viability, whereas the smaller chromosome (II) has a tendency to vary in size and contains many unknown or hypothetical genes [16]. Interspecies comparisons of chromosome II reveals a large degree of genomic variation and this diversity has been suggested to play a role in adaptation to environmental changes and may be important for species-specific functions [16]. Our study highlights the utility of chromosome II in facilitating survival in an unnatural environment for *V. vulnificus*.

GO term enrichment analysis

Gene Ontology (GO) term enrichment analysis of the differentially expressed genes in the two strains revealed a consistent pattern of functional enrichment. 15 enriched terms were identified for CMCP6 and 16 for YJ016, with the primary differences being the ranking of terms. In the cellular component term hierarchy, which describes the localization of the gene, the only significantly enriched term for both CMCP6 and YJ016 was GO:0016020 (membrane). In the molecular function hierarchy, which describes molecular activities, the only significantly enriched term, again appearing in both species, was GO:0060089 (molecular transducer activity). All remaining significantly enriched terms identified were within the biological process hierarchy, and included such biological roles as cellular response to stimulus, signal transduction, cellular communication, formamide and other amide metabolism, and (in the case of YJ016), taxis (**Table 2**).

Genes induced in human serum

Substrate binding and transport

In the human host, *V. vulnificus* must overcome a number of physiological challenges to survive and multiply. *In vivo* growth requires the acquisition of scarce but essential nutrients, such as iron and sulfur, which are critical to cell function. Indeed we identified genes involved in iron acquisition to be upregulated in human serum. Iron is an essential micronutrient for *V. vulnificus*, and is critical for growth and virulence [17], thus this bacterium employs siderophore-based systems to chelate iron from high-affinity binding proteins such as ferritin, transferrin, and lactoferrin. *V. vulnificus* also makes use of an OM receptor, HupA, to acquire iron from heme-containing compounds such as hemoglobin and cytochromes [18]. In our transcriptome analysis, we found the receptor and transport genes for the hydroxamate siderophore, the biosynthetic, transport, and receptor genes for the catechol siderophore (vulnibactin), and hupA to be induced upon exposure to human serum. This finding corroborates a previous study in which *V. vulnificus* expression of *vuuA* and *fhuA* (encoding the

Table 2. Enriched Gene Ontology Categories of Differentially Expressed Genes in human serum and artificial seawater.

	CMCP6				YJ106			
	Human serum		Seawater		Human serum		Seawater	
Description	No. DE genes	Fold exp. range	No. DE genes	Fold exp. range	No. DE genes	Fold exp. range	No. DE genes	Fold exp. range
Membrane	41	2.8 to 8.1	58	7.6 to 2.8	48	2.7 to 9.8	68	8.1 to 2.6
Response to stimulus	13	2.9 to 7.1	51	7.6 to 3.1	18	3.2 to 7.9	56	6.3 to 2.6
Cellular response	6	2.9 to 6.3	45	7.6 to 3.2	9	3.5 to 5.6	49	6.3 to 2.6
Cellular communication	6	2.9 to 6.3	43	7.6 to 3.2	9	3.5 to 5.6	46	3.6 to 2.6
Molecular transducer activity	6	2.9 to 5.2	37	7.6 to 3.2	9	3.5 to 3.9	39	6.3 to 2.6
Signal Transduction	5	2.9 to 4.8	42	7.6 to 3.2	9	3.5 to 5.6	45	6.3 to 2.6
Signaling	5	2.9 to 4.8	42	7.6 to 3.2	9	3.5 to 5.6	45	6.3 to 2.6
Single organism signaling	5	2.9 to 4.8	42	7.6 to 3.2	9	3.5 to 5.6	45	6.3 to 2.6
Regulation of RNA metabolism	15	2.9 to 6.7	12	7.4 to 3.9	14	2.7 to 7.0	20	8.3 to 2.5
Regulation of macromolecule biosynthesis	15	2.9 to 6.7	12	7.4 to 3.9	14	2.7 to 7.0	20	8.3 to 2.5
Regulation of cell macromolecules	15	2.9 to 6.7	12	7.4 to 3.9	14	2.7 to 7.0	20	8.3 to 2.5
Histidine catabolism	0	NA[a]	4	6.0 to 5.4	0	NA	4	6.0 to 5.4
Imidazole containing compounds	0	NA	4	6.0 to 5.4	0	NA	4	6.0 to 5.4
Formamide metabolism	0	NA	4	6.0 to 5.4	0	NA	4	6.0 to 5.4
Formate metabolism	0	NA	4	6.0 to 5.4	0	NA	4	6.0 to 5.4
Taxis	NA	NA	NA	NA	1	3.5	13	−6.3 to −2.9

[a]– not applicable.

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vulnibactin and hydroxamate siderophore receptors, respectively), was significantly enhanced in human serum compared to natural seawater [19].

V. vulnificus utilizes three TonB systems (TonB1, TonB2, and TonB3) to transport both the hydroxamate-type siderophore and vulnibactin, as well as heme and hemoglobin [20]. The TonB systems consist of three integral inner membrane proteins (TonB, ExbB, and ExbD), which generate the energy needed to activate the OM TonB receptor proteins. The operons coding for TonB1 and TonB2 were found to be significantly upregulated in HS, however TonB3 was not differentially expressed. This finding strongly supports a previous study from the Crosa lab which found *V. vulnificus* TonB1 and TonB2 systems to be induced under iron-limiting conditions whereas TonB3 transcription did not change in response to iron concentrations [21].

Our study found the operon (*potABCD*) encoding spermidine transport to be up-regulated in human serum. Polyamines such as spermidine, are a class of small organic compounds with a hydrocarbon backbone and multiple amino groups, are known to play a crucial role in maintaining optimal conformation of nucleic acids, and are therefore essential for normal cellular growth and multiplication [22]. However, research has unveiled some novel functions of polyamines in microorganisms linking these compounds with biofilm formation, escape from

phagolysosomes, bacteriocin production, toxin activity, and protection from oxidative and acid stress [22]. Upregulation of spermidine transport genes in HS indicates that polyamines could serve an important function for *V. vulnificus* within the human host and this proposition deserves further attention.

Clinical isolates of *V. vulnificus* harbor a 33-kb genomic island (region XII) located on chromosome II, which contains an arylsulfatase gene cluster, a sulfate reduction system, two chondroitinases, and an oligopeptide ABC transport system [10]. It has been proposed that acquisition of this genomic island may serve to enhance fitness in the aquatic environment and/or human host, however studies to validate this proposition for *V. vulnificus* have yet to be reported. We found a subset of genes within the genomic region XII to be upregulated in human serum, particularly those within the arysulfatase gene cluster. Arysulfatases hydrolyze arylsulfate ester bonds to release free sulfate, which is essential for bacterial growth and survival [10, 23]. These are typically expressed under conditions of sulfur starvation, but have also been shown to be regulated by monoamine compounds such as norepinephrine, which has been implicated in quorum sensing signaling within the human host [24].

Intracellular signaling

Cyclic-di-GMP is an intracellular second messenger that integrates environmental signals and has been demonstrated to regulate several distinct cellular processes in vibrios such as motility, biofilm formation, virulence, and rugose colony morphology [25–27]. This signaling molecule has been proposed to be a critical component for the transition of *V. cholerae* from the aquatic environment to the host [28]. The intracellular concentrations of this second messenger are regulated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) which synthesize and degrade c-di-GMP, respectively. Studies have shown that phosphodiesterase activity of the response regulator, VieA, reduces intracellular c-di-GMP levels which allows for optimal gene expression of virulence factors, specifically *toxT*, the transcriptional activator of toxin-coregulated pili (*tcp*) (the major colonization factor of *V. cholerae*) as well as cholera toxin (*ctx*) [28]. Furthermore, through the action of c-di-GMP, VieA negatively regulates expression of *vps* genes which contribute to exopolysaccharide production and biofilm formation in *V. cholerae* [29]. A similar regulatory response system (RocR/SadR) has been identified in *Pseudomonas aeruginosa* which was also shown to control biofilm and virulence phenotypes in a reciprocal manner in response to environmental signals [30]. Interestingly, our study found the sensor histidine kinase *vieS* and the cognate response regulator *vieA* to be significantly upregulated in *V. vulnificus* when exposed to human serum. Considering that *V. vulnificus* appears to lack *toxT*, *tcp* and *ctx* homologs, the downstream effect of this two-component system deserves further investigation as it may play a critical role in the transition from environment to host, as is the case for *V. cholerae*.

In *V. cholerae*, the ToxR regulon encodes over 20 genes that aid in intestinal colonization, toxin production and survival within the host [31]. Homologs of the *toxRS* operon have been identified in *V. vulnificus* and the ToxRS signal

transduction system has been shown to stimulate hemolysin production and alter the outer membrane protein expression profile [32]. In addition to upregulation of *toxRS* expression in HS, we found increased expression of three hemolysins, along with differential expression of several outer membrane proteins.

Intracellular signaling also occurs through quorum sensing (QS) in which signaling molecules (autoinducers) accumulate extracellularly, bind a membrane-bound sensor to effect a collective change in gene expression patterns within the population. This results in the modulation of processes such as bioluminescence, biofilm formation, virulence, and fitness factor production (see [33] for a review on QS). Three systems of bacterial communication exist in Gram-negative bacteria and each system utilizes chemically different autoinducers. Only one functional quorum sensing pathway (autoinducer-2, involving interspecies communication) has been described in *V. vulnificus*, but it has been proposed that this bacterium possesses other, not yet identified, compensatory channels for quorum sensing [34]. The AI-3 system involves inter-kingdom cross-signaling in which bacteria can sense and respond to host-derived signals [35]. The sensor kinase, QseC, of this two-component signaling system acts as an ''adrenergic receptor'' that senses and responds to epinephrine, norepinephrine, and AI-3 in enterohemorrhagic *E. coli*, subsequently activating virulence genes [35, 36]. Interestingly, we identified the *qseBC* homolog in *V. vulnificus* to be upregulated in cells exposed to HS. Whether the QseBC system functions as a genuine inter-kingdom signaling network for *V. vulnificus* is an area of research that deserves prompt investigation as this system may be used to detect and respond to eukaryotic adrenergic signals present in the GI tract and/or human serum, thereby assisting the bacterium's navigation throughout the human host.

It is unclear whether *V. vulnificus* employs AI-1 (intraspecies) signaling as a LuxI/LuxR pair has yet to be genetically identified in this species [37, 38]. However, we identified a gene encoding a LuxR-family transcriptional regulator to be upregulated in human serum. In AI-1 signaling, LuxR serves as the response regulator which binds AHL signals and subsequently interacts with DNA to effect changes in gene expression in response to the stimulus. Interestingly, many bacteria (both with and without AI-1 signaling capabilities) have been found to possess LuxR-family proteins for which there is no obvious cognate LuxI synthase [39]. Recent research suggests these ''LuxR-family orphans'' may help in fine tuning of existing QS regulatory networks, control independent regulons, or allow bacteria to detect and respond to neighboring interspecies or inter-kingdom signals [39, 40]. Indeed, these propositions suggest the need to investigate the relevance of these LuxR-family orphans in pathogens such as *V. vulnificus*.

Response to heat/protein stabilization

Exposure to temperature stress can have deleterious effects on cellular components, particularly due to protein denaturation. In response, bacterial cells will activate the heat shock response (HSR) in order to protect the cell from physiological stress. This signaling pathway is governed by the transcription factor, σ^{32}, which rapidly induces transcription of several heat-shock proteins

(HSPs) including molecular chaperones and ATP-dependent proteases that aid in repair or disposal of damaged proteins. Exposing *V. vulnificus* to HS at 37℃ (compared to ASW at room temperature) resulted in a remarkable upshift in σ^{32}, hsp chaperones, and proteases. Interestingly, this effect was still prominent at two hours of incubation in serum. HSR typically occurs within minutes of temperature upshift and reaches a new steady-state level within ca. 30 m [41]. Considering the cross-protective qualities of HSPs [42], it is plausible that these genes also promote cellular stability in the presence of antimicrobial constituents within human serum.

Toxins and exoenzymes

V. vulnificus produces cytotoxic effects to human cells by secreting hemolysins and extracellular proteases [3]. Hemolysins, such as the *vvhA* cytolysin, cause cellular damage by forming pores in the cell's membrane which can lead to apoptosis, vascular permeability and hypotension [43]. Extracellular proteases, such as the metalloprotease *vvpE* and elastase, cause tissue necrosis and cutaneous lesions as well as vascular permeability and edema. While the pathogenic effects of these putative virulence factors have been thoroughly investigated, some studies indicate that they may not play a significant role in virulence [44–46]. This conclusion is supported in our current study, which found no change in expression of *vvhA* between ASW and HS, and down-regulation of *vvpE* and elastase in HS. However, we found three other putative hemolysins and several proteases (including two zinc-dependent proteases and two collagenases) to be upregulated in human serum. Further evaluation of these putative toxins is necessary to assess if and how they contribute to virulence.

Genes induced in artificial seawater

Transcription factors

In *V. vulnificus*, the alternative sigma factor, RpoS, aids in adaptation to environmental stress (such as osmotic shock, nutrient stress, and oxidative stress) and has been shown to be required for full motility [47]. We found *rpoS* expression to be enhanced in ASW, highlighting the necessity of the general stress response in the environment. Additionally, we found the Crl transcriptional regulator to be upregulated in ASW. In *E. coli*, Crl is an auxiliary factor that binds to σ^{S} and enhances its affinity for certain promoters, including the genes necessary for curli fimbriae formation [48]. These extracellular matrix components facilitate surface colonization and biofilm formation, and expression is positively regulated by curli subunit gene D (CsgD) [48, 49]. Previous studies have identified CsgD homologs in *V. vulnificus, V. parahaemolyticus*, and *V. cholerae* [50]. In *V. cholerae*, the CsgD homolog, VpsT, has been shown to enhance biofilm formation and activate genes involved in VPS exopolysaccharide production [51]. In our study, expression of a VpsT homolog was upregulated in *V. vulnificus* cells (CMCP6 only) in ASW.

Expression of the gene encoding the alternative sigma factor RpoN (σ^{54}) was also upregulated in ASW. This transcription factor regulates over 30 operons in *E. coli*, nearly half of which are involved in nitrogen assimilation and metabolism [52]. The remaining σ^{54}-dependent genes have been proposed to neutralize adverse conditions and/or prevent depletion of metabolites and energy resources in limiting environments [52]. In some *Vibrio* spp., σ^{54} has been documented to upregulate genes involved flagellar synthesis and motility [53–55]. Interestingly, deletion of *rpoN* in *V. cholerae* reduces the competitive fitness of cells (relative to the wild-type) when colonizing the infant mouse model, however this deletion enhances fitness in intestinal colonization by *V. parahaemolyticus* in the adult mouse model [53, 55]. Furthermore, this sigma factor has been implicated in regulating EPS in *V. vulnificus* and *V. fischeri* [56, 57]. Thus, in ASW, RpoN may be regulating a number of processes including nitrogen metabolism, motility, colonization, and biofilm formation and further investigation into this regulatory network in *V. vulnificus* is prompted.

IscR is a transcription factor that regulates the *isc* operon in *E. coli* and controls the expression of over 40 genes including those involved in Fe-S cluster biosynthesis, anaerobic respiration, and biofilm formation [58]. Recently, an IscR homolog was identified in *V. vulnificus* that regulates a number of genes possibly involved in pathogenesis, including genes involved in motility and adhesion, hemolytic activity, and oxidative stress response. Interestingly, our study revealed upregulation of five out of eight genes within the *isc* operon, including *iscR*, when cells were exposed to ASW. Additionally, IscR regulated genes involved in chemotaxis, methyl-accepting chemotaxis proteins, and a glutaredoxin were also upregulated in our study, reaffirming the relationship identified by Lim et al. (2014). While IscR appears to play a critical role in protecting the cell from reactive oxygen species generated by host cells during infection [59], it may be that IscR also plays an important role in environmental survival.

Adherence and Biofilm formation

The *tad* (tight adherence) genes encode the machinery needed to assemble Flp pili (fibrils) that mediate adherence to surfaces. The *tad* genes have been found to be essential for adherence, biofilm formation, colonization, and pathogenesis in a number of genera, and are considered to be instrumental in the colonization of diverse environmental niches [60–63]. These genes are present on a genomic island referred to as the ''widespread colonization island'' which is present in a wide variety of bacteria, including several human pathogens [61]. Some species harbor more than one distinct tad locus, including *V. vulnificus*, which contains two loci on chromosome I and a third locus on chromosome II. One of these three *tad* loci was differentially expressed in our study with expression being enhanced in ASW relative to HS. The function(s) of the *tad* loci in *V. vulnificus* have yet to be investigated but it is reasonable to conjecture that this widespread colonization island provides important functions for this organism specifically regarding adherence and colonization. Indeed, a previous RNAseq study found that (when compared to growth in a nutrient-rich medium), a *V. vulnificus*

biotype 3 strain isolated from a wound infection expressed the Flp-coding region [64].

Additionally, we found several genes involved in biogenesis of the type IV mannose-sensitive haemagglutinin (MSHA) pilus to be upregulated in ASW. In V. cholerae, MSHA facilitates attachment to chitinous substrates, such as zooplankton exoskeletons, allowing for subsequent biofilm formation [65, 66]. In contrast to the toxin-coregulated pilus (a predominant virulence factor in *V. cholerae*), MSHA biogenesis has been shown to be repressed *in vivo*, acting as an "anticolonization factor" in the human host [67]. Our results suggest that MSHA may play a similar role in *V. vulnificus* in which this pilus mediates attachment to substrates in the aquatic environment.

V. vulnificus produces extracellular surface polysaccharides (EPS) and capsular polysaccharides (CPS) both of which are involved in biofilm formation. CPS is recognized as an essential virulence factor for *V. vulnificus* and negatively impacts biofilm formation [68, 69], whereas EPS production is essential for bacterial attachment and biofilm formation [57]. *V. vulnificus* has at least three kinds of EPS and we found one of these polysaccharide biosynthetic loci to be significantly upregulated in ASW. This set of EPS genes (homologous to the "*syp* genes" originally identified in *V. fischeri*) appears to be transcriptionally regulated by σ^{54} [56], and produces wrinkled colonies, pellicle formation, and matrix production when induced [70]. Upregulation of *syp*-like genes in ASW suggests this EPS locus may play an important role in environmental persistence.

Intracellular signaling and chemotaxis

As previously mentioned, the signaling pathway regulator c-di-GMP controls phenotypes associated with biofilm formation and planktonic growth in a reciprocal manner. Intracellular levels of c-di-GMP increase through the action of diguanylate cyclases (DGCs) which synthesize this second messenger. Subsequently, c-di-GMP levels can be reduced through the action of phosphodiesterases (PDEs) which degrade this molecule. We identified 17 DGCs and six PDEs to be upregulated in ASW, as well as two PDEs to be downregulated in ASW. This result suggests that cells in this environment have higher intracellular levels of c-di-GMP. In *V. vulnificus*, c-di-GMP positively regulates biofilm formation by regulating production of EPS, thus cells in ASW would presumably exhibit enhanced biofilm formation and possibly decreased virulence potential [25, 71]. This speculation is further supported through the observed upregulation of *rpoS* expression in ASW, as RpoS has been shown to modulate c-di-GMP levels by enhancing expression of DGCs.

Chemotaxis is initiated by membrane-bound chemoreceptors called methyl-accepting chemotaxis proteins (MCPs) which bind a ligand resulting in a signal transduction cascade to direct the flagellum's activity. The role of chemotaxis in the process of infection has been examined in several bacteria/enteric pathogens and for many of these, motility is an important virulence factor. Interestingly, chemotaxis in *V. cholerae* inhibits its ability to colonize the small intestine of infant mice and is inversely regulated with expression of virulence traits [72].

V. cholerae has three chemotaxis gene clusters, denoted *cheA*-1, *cheA*-2, and *cheA*-3, although only *cheA*-2 has been found to be essential for chemotaxis in growth media [73]. In the current study, we identified two of these homologs (*cheA*-2 and *cheA*-3) in *V. vulnificus* and found the *cheA*-3 homolog (located on chromosome II) to be upregulated in ASW. Additionally, many of the MCPs located throughout the genome were significantly expressed in this condition. In *V. cholerae cheA*-3 is not required for chemotactic control of flagellar motility, thus it has been suggested that this operon could regulate flagellum-independent motility (e.g. twitching motility) [73], a proposition that could be extended to *V. vulnificus*. The intimate relationship between chemotaxis and virulence expression that has been documented in *V. cholerae* prompts further investigation into these mechanisms in *V. vulnificus*. For both of these pathogens, chemotaxis is assuredly a vital fitness factor in both environmental and pathogenic settings.

V. vulnificus C-genotypes possess an operon homologous to the RsbRST stress module (or stressosome) which is classically found in Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* [11] and governs the "general stress response", sometimes playing a role in virulence. This supramolecular signaling complex perceives signals related to environmental stress or nutrient limitation, triggering a signal transduction phospho-relay that ultimately activates the sigma factor (σ^B), which will then bind to the core RNA polymerase to direct transcription of over 150 genes involved in stress adaptation [74]. For organisms such as *V. vulnificus* that possess stressosome orthologs, but lack σ^B, the stressosome has been proposed to be involved in regulating aerotaxis, two-component signaling systems, and the biosynthesis of secondary messenger signaling molecules [74]. We assessed 20 C-genotypes and 29 E-genotypes for the presence of this operon and found this module to be specific to C-genotypes, with 75% of C-genotypes and none of E-genotypes possessing the entire operon (**S1 Table**). Furthermore, we found these genes to be upregulated in ASW relative to HS, suggesting that the stressosome serves an important function for the organism.

We examined gene expression of the *rsbRST* operon using relative qRT-PCR and found these genes to be repressed more than 100-fold in HS relative to ASW (**Fig. 3**), validating our RNAseq transcriptome findings. To further characterize expression of the stressosome we examined how this operon is regulated temporally using relative qRT-PCR. Interestingly we found stressosome genes to be upregulated over time in ASW (**Fig. 4A**), as well as in HS (**Fig. 4B**), albeit to a lesser extent. The enhanced expression of stressosome-related genes in ASW relative to HS suggests an ecological function for this system, thus we used membrane diffusion chambers to examine temporal gene expression of *rsbR* and *rsbT*, *in situ*. When placed in their native estuarine environment, cells upregulated *rsbR* and *rsbT* over 10-fold within 6 hours and continued to express these genes for up to 48 hrs (**Fig. 5**).

Further studies are warranted to assess if and how the stressosome module contributes to pathogenicity and/or niche colonization in strains of *V. vulnificus* harboring these genes. It may be that this system has been optimized to allow for

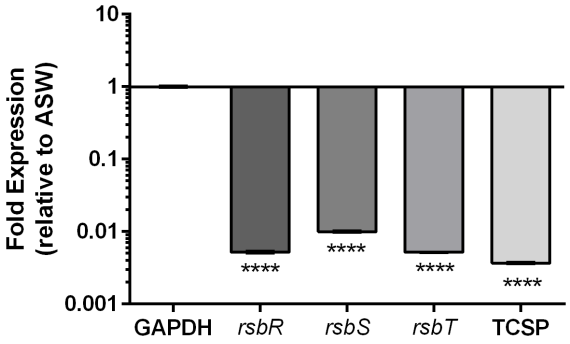

Fig. 3. Gene expression of the stressosome in human serum relative to artificial seawater. Relative qRT-PCR was performed on CMCP6 to examine expression of select stressosome genes (*rsbRST* and a downstream two-component sensory protein, here denoted ''TCSP'') confirming RNAseq results. GAPDH was used to normalize expression data. Asterisks represent significant differences in expression $p<0.0001$ (One-way ANOVA).

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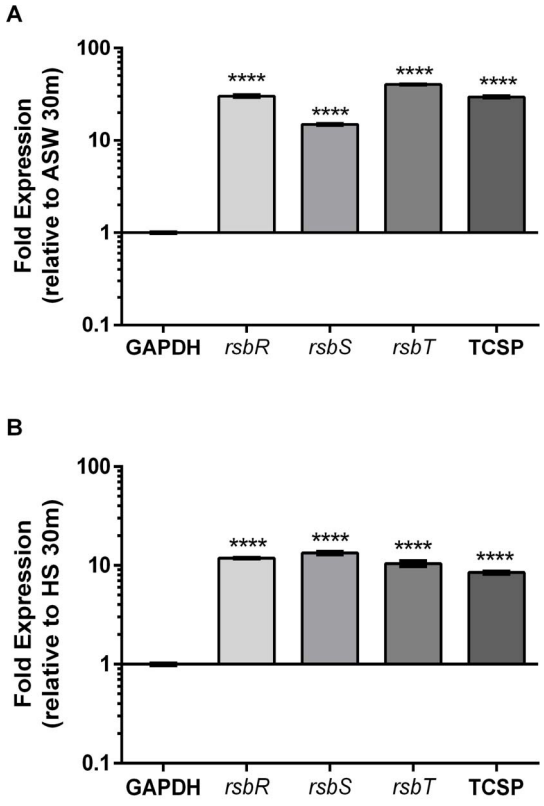

Fig. 4. Temporal gene expression of the stressosome during exposure to A) artificial seawater or B) human serum. Relative qRT-PCR was performed on CMCP6 to examine expression of select stressosome genes (*rsbRST* and a downstream two-component sensory protein, here denoted ''TCSP'') after incubation in each condition for 2 hrs relative to 30 min. GAPDH was used to normalize expression data. Asterisks represent significant differences in expression $p<0.0001$ (One-way ANOVA).

doi:10.1371/journal.pone.0114376.g004

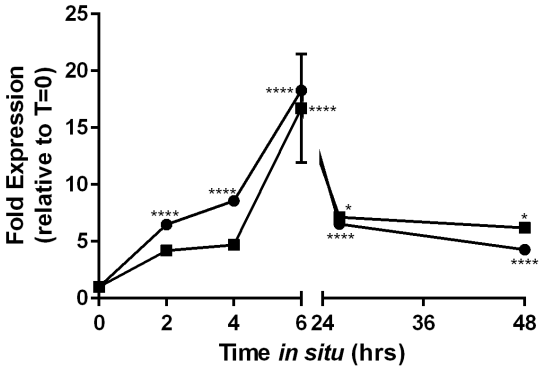

Fig. 5. Temporal gene expression of the stressosome in natural estuarine waters. Cells of CMCP6 were incubated in natural estuarine waters for 2 days using membrane diffusion chambers. Relative qRT-PCR was performed on samples collected at several time points and expression of select stressosome genes (*rsbR*, circles; *rsbT*, squares) was analyzed relative to 0 hrs of incubation (prior to *in situ* exposure). GAPDH was used to normalize expression data. Asterisks represent significant differences in expression (One-way ANOVA).

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integration of various signals into a single output, thereby allowing for fine-tuning of stress responses in a particular environment. Expression of this operon *in situ* also suggests a role for this system in the environment and it may have been acquired to aid in niche expansion.

Conclusions

The goal of the current study was to investigate the transcriptome profile of two clinically isolated C-genotypes of the human pathogen, *V. vulnificus*. Overall, this study unveiled a picture of the ''virulence profile'' cells exhibit in human serum and the ''environmental profile'' displayed in cells exposed to estuarine-like conditions. From this picture emerges a phenotypic dichotomy that closely resonates with previous findings regarding the dual life-style of *V. cholerae*. **Figs. 6 and** 7 feature diagrams of differentially expressed genes that were upregulated in human serum and artificial seawater and attempt to depict the potential phenotypic outcomes for existence in these two diverse environments. As with all high-throughput methods, a detailed investigation of the current results is necessary to establish their functional relevance for bacterial regulation, physiology and pathogenicity.

Our study reveals that when *V. vulnificus* cells encounter human serum, they exhibit a ''virulence profile'' similar to what has been seen in the initial stage of *V. cholerae* infection [28, 31], in which a number of genes encoding sensory proteins (*vieSA*, *toxRS*) and virulence-associated genes (such as *toxRS*, *vieSA*, hemolysins and proteases) and were upregulated in this environment (Fig. 6). Additionally, expression of the *qseBC* operon (a system which has been previously shown to be involved in inter-kingdom communication in other organisms) was also

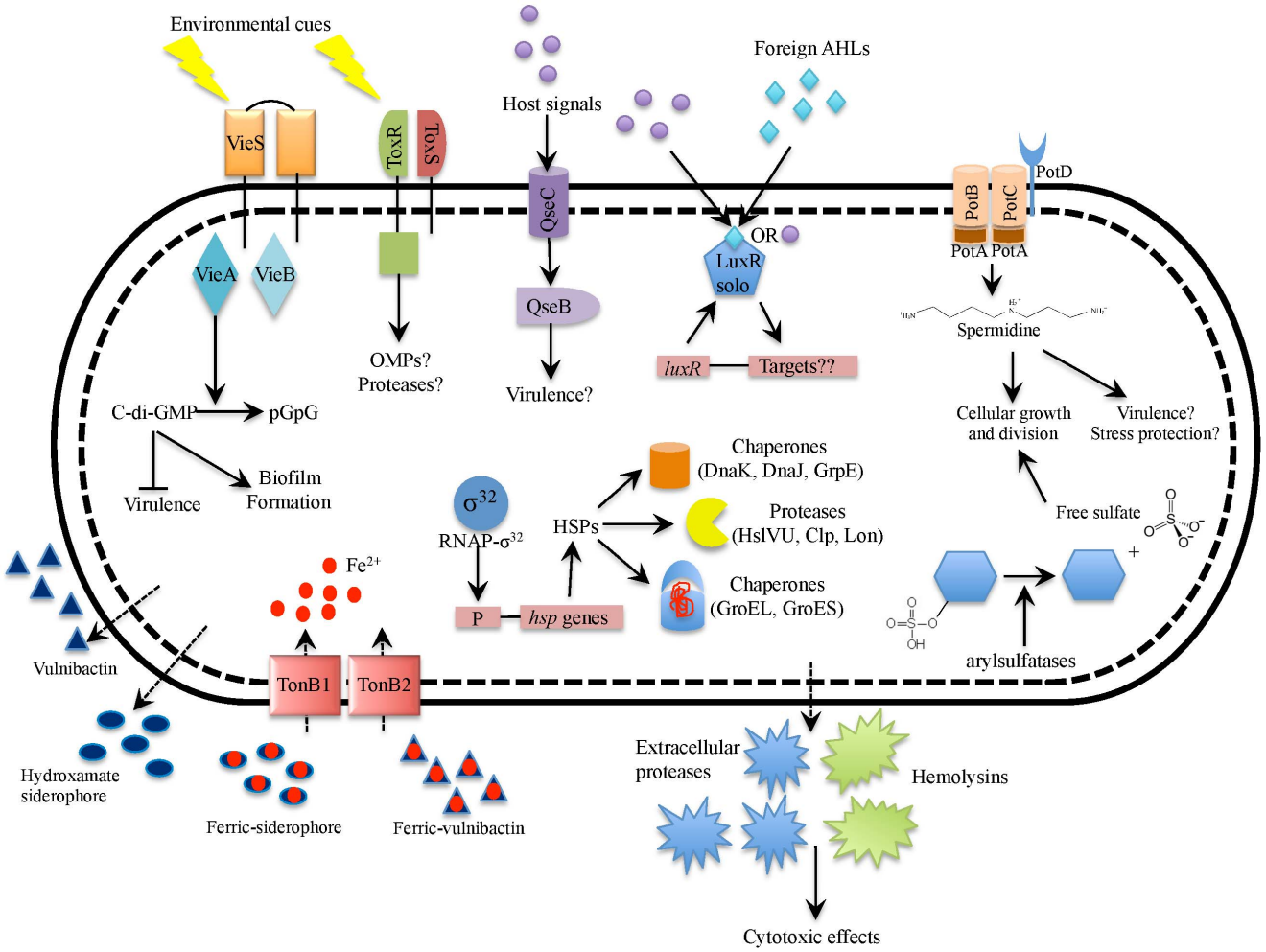

Fig. 6. Diagrammatic summary of genes upregulated in human serum relative to artificial seawater. Depicted in this figure are genes (or sets of genes) upregulated in HS along with the inferred phenotypic outcomes. In this condition, the cell scavenges for nutrients by utilizing processes involved in substrate binding and transport including iron chelators and transport systems (hydroxamate-siderophore, vulnibactin, TonB1, and TonB2), spermidine transport (PotABCD), and sulfate scavenging arylsulfatases. In response to unknown environmental cues, the cell also engages in intracellular signaling using systems such as a two component signal transduction system (VieSA) involved in c-di-GMP signaling, the ToxRS signal transduction system, and a LuxR-family orphan possibly involved in quorum sensing signaling, and the cell possibly senses and responds to host hormones using a two-component signaling system (QseBC) involved in inter-kingdom cross-talk. The cell responds to human body temperature (37˚C) by upregulating genes involved in the heat shock response (sigma factor RpoH, chaperones and proteases). The cell also secretes several putative virulence factors such as cytotoxic hemolysins and extracellular proteases. Note, this diagram is developed based solely on transcriptomic data and further investigation is necessary to establish the proposed model.

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upregulated in HS. Regulation of this system is a finding which we believe to be novel for *V. vulnificus*.

In artificial seawater, genes involved in stress response, chemotaxis, adhesion, and biofilm formation were upregulated (Fig. 7). In *V. cholerae*, expression of many chemotaxis genes and motility genes are dependent on RpoS, which has been shown to orchestrate the "mucosal escape response" in the later stages of infection, and coincides with down-regulation of virulence-gene expression [75]. This switch in genetic programming is thought to prepare the organism for the

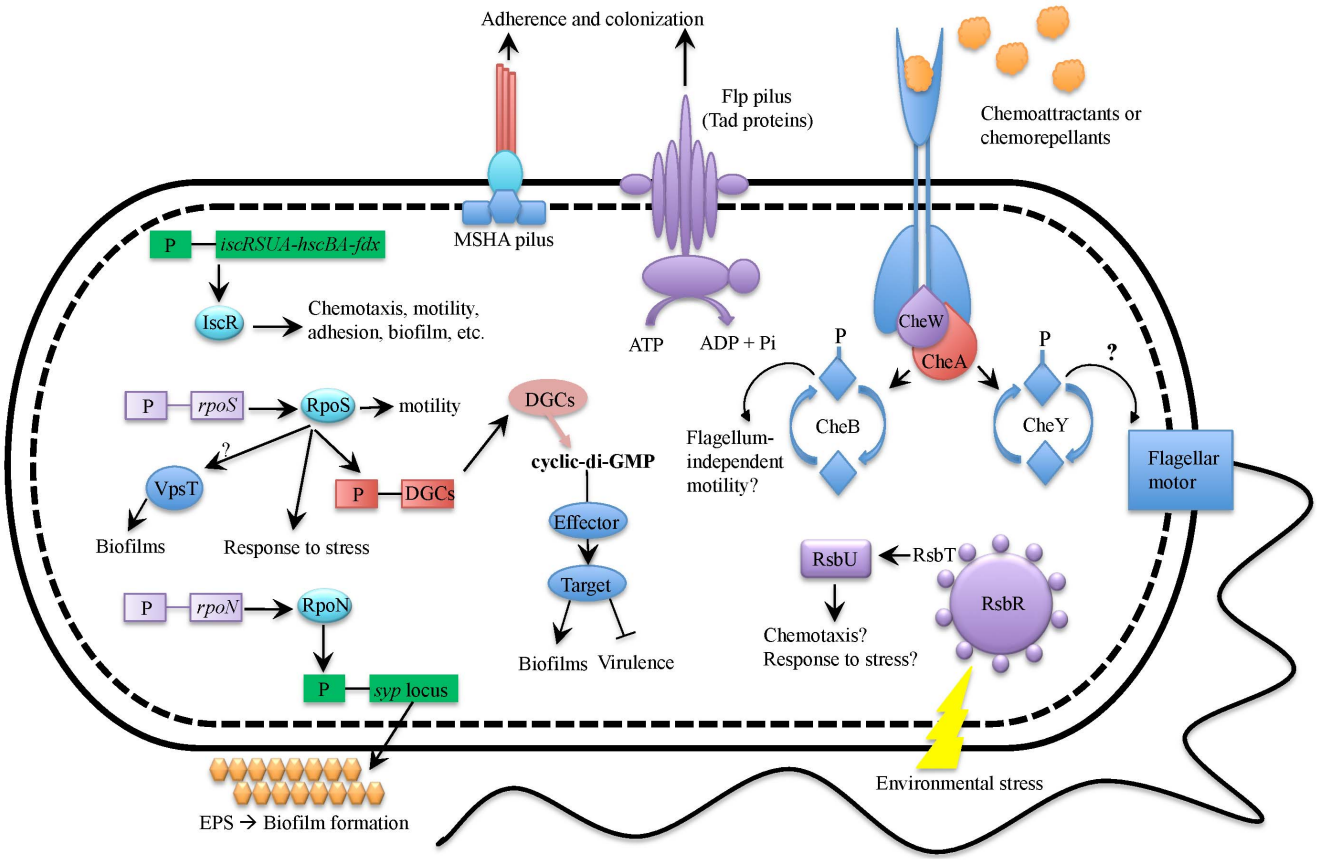

Fig. 7. Diagrammatic summary of genes upregulated in artificial seawater relative to human serum. Depicted in this figure are genes (or sets of genes) upregulated in ASW along with the inferred phenotypic outcomes. The cell expresses several transcription factors involved in stress response, motility, and biofilm formation (RpoS, RpoN and IscR). The cell also expresses genes involved in attachment and biofilm formation including Flp pilus (tad locus), MSHA pilus, and EPS (syp locus). Through the action of diguanylate cyclases (DGCs), the cell has high intracellular concentrations of c-di-GMP which enhances biofilm formation and possibly reduces virulence factor production. In response to chemoattractants and chemorepellents, the cell also engages in chemotactic sensing and signaling which may result in modulation of flagellar rotation and/or flagellum-independent motility. As a result of some unknown environmental cue, the cell also expresses genes involved in the stressosome module (RsbRSTU) which is likely involved in chemotaxis and/or stress response. Note, this diagram is developed based solely on transcriptomic data and further investigation is necessary to establish the proposed model.

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next stage in its life cycle, dissemination back into the environment. Here, we discovered a similar genetic profile in which transcription factors including *rpoS*, and genes involved biofilm formation (e.g. EPS and pili-associated genes) were enhanced, and this phenotype appeared to be governed (at least in part) through c-di-GMP signaling. We also observed upregulation of a distinct signaling network, referred to as the stressosome in distantly related bacteria, which is unique to C-genotypes of *V. vulnificus*. This is the first report to investigate expression of this enigmatic network in *V. vulnificus*, and we believe future investigations will be fruitful in enhancing our understanding of this human pathogen.

To our knowledge, this is the first transcriptome-based analysis of *V. vulnificus* biotype 1 strains, and we identified a number of genes which appeared to "trend" together. These findings provide a more holistic vision of how *V. vulnificus*

behaves in a given environment, and provides a foundation on which future studies on this human pathogen can be built. Currently, we are in the process of investigating the transcriptome response of E-genotype cells of *V. vulnificus* in the same conditions in order to compare 1) how this genotype responds to the transition from environment to host, and 2) how C- and E-genotypes differentially express genes within the same environment.

Materials and Methods

Experimental design and sample preparation

Experimental design

Two clinically isolated strains of *V. vulnificus* (CMCP6 and YJ016) were selected due to availability of their genome sequences. Strains were grown in heart infusion (HI) broth (BD, New Jersey) at 30℃ overnight at 200 rpm and then diluted 1:100 v:v into fresh HI and grown at 30℃ for ca. 90 m at 200 rpm until cells reached logarithmic phase (OD_{610} 0.15–0.25). Cells were pelleted at 5000 × g at room temperature for 10 m, resuspended in 10 parts per thousand (ppt) artificial seawater (ASW), and inoculated into either 10 ppt ASW or normal human serum (MP Biomedicals) to a final concentration of ca. 1e8 CFU/ml. Cells were incubated in 10 ppt ASW at room temperature (22℃), or human serum at 37℃, on a rotisserie for 2 hours. ASW at 22℃ was chosen to represent a controlled version of the natural estuarine waters and temperature this bacterium encounters in its native environment. Human serum at 37℃ was chosen to reflect the host environment and human body temperature. Pooled human serum (HS) used in this study was collected from male donors and contained nutrients in the form of glucose (96 mg/dl) with total protein levels of 5.5–7.5 g/dl [76].

RNA extraction and purification

Two biological replicates were performed for each condition and after 2 hours of incubation cells were treated with 2 volumes of RNAprotect (Qiagen) following manufacturer's instructions. Preserved pellets were resuspended in TE buffer, pH 8.0 (Ambion) with 1 mg/ml lysozyme (Sigma Aldrich) and vortexed at medium speed for 30 m. RNA was extracted using RNeasy Midi Kit (Qiagen) following protocol with on-column DNase I treatment. RNA was eluted from the column using nuclease-free water and a second post-extraction DNase treatment using TURBO DNA-free (Life Technologies) following the rigorous DNase treatment protocol. RNA quality and quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific) and RNA samples having a 260/280 ratio $<$1.7 were not used in downstream applications.

To confirm the complete removal of DNA, endpoint PCR was performed on RNA samples using Promega's Go-Taq DNA polymerase, 5X Green GoTaq Reaction Buffer, 10 mM dNTP mix, and primers targeting the species-specific *vvhA* gene [7]. Cycling parameters were according to the manufacturer's recommendations, with an annealing temperature of 53.1℃ and 40 cycles of

amplification. Any amplification of the *vvhA* gene was indicative of DNA contamination in which case the RNA was not used for downstream processes.

RNA integrity was assessed using an Agilent 2100 Bioanalyzer and 6000 Nano kit (Agilent Technologies). The 23S/16S rRNA ratio and RNA integrity number (RIN) were confirmed to be >1.8 and close to 10, respectively. Due to the enhanced sensitivity of this technique, the electropherogram for each sample was also screened for residual DNA contamination. Samples yielding bands >4000 nt were not used in downstream applications. Total RNA samples were quantified using a NanoDrop Spectrophotometer (Thermo Scientific).

mRNA Enrichment and cDNA Library Preparation

To enhance the sequencing coverage of mRNA transcripts, we depleted highly expressed rRNA transcripts using Epicentre's Ribo-Zero kit (Epicentre Biotechnologies). Remaining mRNA was assessed for quality and levels of rRNA contamination using the Agilent Bioanalyzer and RNA 6000 Pico Chip (Agilent Technologies). Samples having >1% rRNA contamination were not used for downstream applications. mRNA was quantified using Qubit's 2.0 Fluorometer and RNA High Sensitivity Assay Kit (Life Technologies).

DNA libraries complementary to mRNA sequences were prepared for each sample using ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies). This kit, compatible with Illumina Technologies, enabled directional, paired-end sequencing. Libraries were prepared following manufacturer's instructions. The size and quality of each cDNA library was assessed using Agilent Bioanalyzer's High Sensitivity DNA kit (Agilent Technologies). Insert sizes were approximately 400 bp+/−35 bp. cDNA library quantification was performed using Qubit's 2.0 Fluorometer and Quant-iT dsDNA High Sensitivity Assay Kit (Life Technologies). Samples were stored at −20℃ and transported to the David H. Murdock Research Institute (Kannapolis, N.C.) for sequencing using Illumina's Genome Analyzer.

RNA sequencing data analysis and statistics

Data preprocessing

Approximately 61 to 79 million paired-end raw reads were generated for each set of biological replicates (S2 Table). RNA sequencing data is publicly available in NCBI's BioProject database (BioProject ID PRJNA252365). Raw Illumina sequence reads were filtered to remove reads which fell below the sequencer quality threshold. A second round of filtering using fastq-mcf [77] removed low-quality regions of sequence using a quality trimming window of size 4 and a minimum quality score of 15. The resulting reads were of high average quality and read lengths were tightly clustered around 100 nucleotides. The remaining filtered reads were aligned to reference genomes using Bowtie2 [78]. The alignment rate using Bowtie2 with default settings for paired-end reads was greater than 95% for all samples in the study indicating high quality and reproducibility of the data set

(S2 Table). The view and sort functions of SAMTools [79] were used to convert the aligned read data into the correct format for subsequent analysis.

Expression analysis

Read counts were summarized against the CMCP6 and YJ016 reference genomes using FeatureCounts [80]. Differential gene expression analysis was performed using the EdgeR package [81] following the approach outlined by Jenkins [82]. EdgeR identifies significant differentially expressed genes out of genome-scale count data using exact tests based on the negative binomial distribution. Replicate data for each condition was used for normalization using the TMM method [83]. The artificial sea water (ASW) condition was treated as the baseline condition for analysis. Genes identified as differentially expressed in human serum (HS) relative to ASW, with a p-value less than 0.0001 were selected for further analysis.

Gene Ontology (GO) enrichment analysis

Gene category enrichment analysis was performed using the Ontologizer software [84]. The study sets of differentially expressed genes generated by EdgeR were compared to the population set of GO-annotated genes in the published CMCP6 and YJ016, respectively. The Ontologizer makes several different enrichment analysis methods available. The Parent-Child Union method, which avoids detection artifacts due to the hierarchical structure of the GO, was used to identify enriched GO term categories, and the Benjamini-Hochberg [85] method for multiple-testing correction was applied in order to minimize the false discovery rate. Reference Gene Ontology OBO file [86] and genome-specific GO annotations for *V. vulnificus* CMCP6 and YJ016 were downloaded from UniProt-GOA [87] on April 1, 2014. 3066 genes in CMCP6 and 3107 genes in YJ016 had available GO term mappings, resulting in 331 of 469 DE genes in CMCP6 and 406 of 661 DE genes in YJ016 being included in the enrichment analysis. Differential expression is summarized by GO terms identified as enriched in the study set relative to the population set, with a p-value <0.05.

PCR and qRT-PCR analysis of stressosome module

PCR analysis of RsbRST stressosome module

Using PCR, 20 *V. vulnificus* C-genotypes and 29 E-genotypes were assessed for the presence of stressosome-associated genes (*rsbR*, *rsbS*, *rsbT*, *rsbU*, and a downstream two-component sensor protein). Primers (Sigma Aldrich) were designed for each gene using all three sequenced C-genotype strains of *V. vulnificus* (CMCP6, YJ016, and M06-24) reported in NCBI's database. Primer sequences are listed in S3 Table. Optimal primer quality and fidelity were assessed using IDT OligoAnalyzer 3.1 software, and primer specificity was initially analyzed using *in silico* PCR [88]. DNA extracted from each strain was subjected to 30 cycles of PCR with Promega's 5X Green Go*Taq* Reaction Buffer, 10 mM PCR Nucleotide Mix, 1.25 U Go*Taq* DNA Polymerase, and 0.5 μM of each primer. Thermal cycling parameters were followed according to manufacturer's

recommendations (Promega) and PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Gene expression of RsbRST stressosome module

Gene expression of selected genes associated with the stressosome module was examined in human serum relative to 10 ppt ASW using relative quantitative reverse transcription-PCR (qRT-PCR) as previously described [89]. Primers were designed to be suitable for qRT-PCR and PCR amplification efficiency was assessed using parameters previously described [89]. Cells of CMCP6 were treated and RNA was extracted as described in the ''experimental design and sample preparation'' section above. Total RNA (1 μg) was reverse transcribed using qScript cDNASuperMix (Quanta Biosciences) and 50 ng of cDNA template was carried over for quantitative PCR (qPCR). qRT-PCR was performed on three technical replicates for each sample using PerfeCTa SYBR green FastMix, LowROX (Quanta Biosciences). Negative controls and ''no-RT'' controls were employed to rule out the influence of DNA contaminants or residual genomic DNA, respectively. Expression levels of each gene were normalized by using an endogenous control gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) to correct for sampling errors. Fold changes in expression levels were measured using the Pfaffl equation [90], taking into account the differences in PCR efficiencies between primer sets. Significant differences in gene expression were assessed using a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. Significance was determined by using a 95% confidence interval. Gene expression data were analyzed by using GraphPad Prism (version 5.0; GraphPad Software Inc.).

To further investigate the expression pattern of the stressosome module, gene expression was analyzed over time in human serum and 10 ppt ASW (2 hrs relative to 30 min) using the protocol described above. Additionally, *in situ* gene expression by cells incubated in natural estuarine waters was also performed as previously described [91]. Briefly, laboratory grown cells were diluted into 15 ppt ASW, injected into sterile (0.22 μm) membrane diffusion chambers, and deployed in estuarine waters along Core Creek in Beaufort, North Carolina. Chambers were deployed in September of 2012 and samples were collected periodically for up to 2 days. For the duration of the experiment, estuarine water temperatures ranged from 13.8℃ to 17.2℃ and salinity ranged from 17 ppt to 22 ppt. Samples were treated with RNAprotect (Qiagen) within 10 minutes of collection. RNA extraction followed by qRT-PCR was performed as previously mentioned.

Supporting Information

S1 Figure. Smear plot of differentially expressed genes in *V. vulnificus* YJ016 exposed to human serum (relative to artificial seawater). The smear plot shows the relationship between the log fold change and log counts per million. Green points

represent non-significant DE genes whereas red points show genes that are significantly differentially expressed ($p<0.0001$) in relation to artificial seawater.
doi:10.1371/journal.pone.0114376.s001 (TIFF)

S2 Figure. Linear compressed view of differentially expressed genes in *V. vulnificus* exposed to human serum (relative to artificial seawater). These charts show a compressed view of the differentially expressed genes in *V. vulnificus* YJ016 by chromosome (top; chromosome I, bottom; chromosome II) and allows quick identification of clusters of differentially expressed genes, both positive and negative. The y-axis shows the log fold change and the x-axis is the nucleotide position of the chromosome. Blue bars represent non-significant DE genes whereas red bars show genes that are significantly differentially expressed ($p<0.0001$) in relation to artificial seawater.
doi:10.1371/journal.pone.0114376.s002 (TIFF)

S1 Table. Presence or absence of stressosome genes in *V. vulnificus* strains by PCR analysis.
doi:10.1371/journal.pone.0114376.s003 (DOCX)

S2 Table. Summary of RNAseq coverage data using the Illumina genome analyzer.
doi:10.1371/journal.pone.0114376.s004 (DOCX)

S3 Table. Primers designed for this study.
doi:10.1371/journal.pone.0114376.s005 (DOCX)

S1 File. Significantly differentially expressed gene lists for CMCP6 and YJ016 ($p<0.0001$).
doi:10.1371/journal.pone.0114376.s006 (XLSX)

S2 File. Select differentially expressed genes upregulated in human serum.
doi:10.1371/journal.pone.0114376.s007 (XLSX)

S3 File. Select differentially expressed genes upregulated in artificial seawater.
doi:10.1371/journal.pone.0114376.s008 (XLSX)

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Author Contributions

Conceived and designed the experiments: TCW SSM CJG JDO. Performed the experiments: TCW. Analyzed the data: TCW SSM ERB CJG. Contributed reagents/materials/analysis tools: CJG JDO. Wrote the paper: TCW CJG. Critical review and editing of paper: JDO.

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SERUM SURVIVAL OF *VIBRIO VULNIFICUS*: ROLE OF GENOTYPE, CAPSULE, COMPLEMENT, CLINICAL ORIGIN, AND *IN SITU* INCUBATION

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TRANSCRIPTOME-BASED COMPARATIVE ANALYSIS OF CLINICAL AND ENVIRONMENTAL STRAINS OF THE OPPORTUNISTIC HUMAN PATHOGEN *VIBRIO VULNIFICUS*

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ABSTRACT

Vibrio vulnificus is a natural inhabitant of estuaries and coastal waters worldwide and is of medical relevance due to its ability to cause grievous wound infections and/or fatal septicemia in susceptible individuals. Clinical (C-) and environmental (E-) genotypes of this species display significant differences in their ecology, physiology, genome content, and molecular genetic responses. However, the reasons why C-genotypes are more capable of causing human disease, whereas the E-genotypes are more prevalent in the environment, remains elusive. The recent sequencing of both C- and E-genotype genomes has set the stage for which to build transciptomic studies. Previously, we used RNA sequencing technologies to reveal the "environmental transcriptomic profile" of C-genotypes incubating in seawater, which was strikingly different from the "virulence profile" exhibited by these cells when exposed to human serum (HS). In the current study, we further elaborate on this project by sequencing the transcriptome of E-genotypes exposed to the same conditions (seawater and human serum). We find that the transition of E-genotypes between these two conditions is remarkably similar to that of C-genotypes, suggesting that these genotypes possess similar adaptation responses to human serum. One interesting difference was the upregulation of carbohydrate metabolism genes by C-genotypes and we suspect that utilization of these pathways could contribute to this genotype's pathogenic capabilities. Furthermore, we discovered that C- and E-genotypes have different transcriptomic profiles when incubating in seawater, and these fundamental differences may explain why these genotypes differ so greatly in their ecology. Of particular interest was the upregulation of type IV pili and EPS-associated genes in E-genotypes which suggests that these strains are more efficient in attaching to substrata and building biofilms. Indeed, evidence for this has been documented in previous studies and here we show that E-genotypes build better biofilms in the natural environment. Conversely, C-genotypes expressed genes involved in CPS production and Flp pili. Both of these cell surface structures have been implicated in virulence and may serve to facilitate successful survival and colonization within the human host, should the bacterium encounter this environment.

INTRODUCTION

V. *vulnificus* is natural inhabitant of estaurine environments throughout the world, and naturally associates with a variety of acquatic inhabitants including shellfish. This bacterium is coincidentally capable of causing potentially fatal disease in humans, and this is largely due to the common practice of consuming raw or undercooked seafood such as oysters. Upon entry into the human host via ingestion, if this organism is able to survive the hostile transition into the host, evade host immune responses, successfully colonize the host, and enter into the blood-stream, it can cause rapid septicemia and organ failure, which may ultimatly lead to death of the patient. Furthermore, individuals with pre-exisiting cuts or lesions exposed to waters harboring this organism are at risk to developing severe wound infections and necrotizing fasciitis.

Understanding the molecular mechanisms for how this opportunistic human pathogen is able to 1) colonize oysters and 2) cause disease in humans has been a long-standing inquiry. Previous studies have discovered enhanced pathogenicity in some strains of this human pathogen and genetic polymorphisms within the virulence correlated gene (*vcg*) serve as a genetic marker to distinguish clinical (C-) genotypes from environmental (E-) genotypes [1-3]. Previous studies have provided further distinctions between these genotypes; specifically, we have found that C-genotypes demonstrate superior survival in human serum relative to E-genotypes [4,5], and genome comparisons have allowed for the identification of several putative virulence factors that could potentially aid C-genotypes in disease progression [6,7].

In a recent study, we sequenced the transcriptome of two C-genotypes of *V*. *vulnificus* exposed to seawater or human serum which revealed two divergent genetic programs under these two conditions [8]. In that study, we found that C-genotypes cells incubated in seawater exhibit an “environmental profile” in which genes involved in intracellular signaling, chemotaxis, adherence, and biofilm formation predominated. Once cells transitioned from the natural environment to host-like conditions they switched their transcriptomic response to reflect a “virulence profile” in which a number of putative virulence factors were upregulated. This transition appeared to be largely regulated by cyclic-di-GMP signaling, striking a close resemblance to the transitory genetic switch documented in *V*. *cholerae* cells as they travel from host to environment [9,10].

In an attempt to identify key genetic distinctions that underlie the different physiological responses between C- and E- genotypes, we made use of RNA sequencing technologies to analyze the transcriptome response of E-genotypes exposed to human serum relative to artificial seawater. The genetic response of E-genotypes was compared to that of C-genotypes, and we also compared the genetic profile of each genotype within seawater to identify potential underlying differences in gene regulation. Lastly, to obtain

a better understanding of why E-genotypes often predominate in estuarine environments, we examined *in situ* survival and biofilm formation of C- and E-genotypes in natural estuarine waters, which allowed us to understand these transcriptomic findings in an ecological context. Overall, this study provides new distinctions between C- and E-genotypes, and highlights candidate genes for further investigation.

METHODS

Experimental conditions:

The conditions utilized in this study were chosen to simulate the natural estuarine environment in which *V*. *vulnificus* resides (artificial seawater, ASW), or the clinical environment that *V*. *vulnificus* cells would experience in the human host (human serum, HS). Previously, we have shown that *V*. *vulnificus* C-genotypes can effectively resist the bactericidal effects of human serum, whereas E-genotypes largely succumb to these effects, with culturability dropping significantly minutes after exposure [4]. Thus, it is evident that C-genotypes possess some unique trait(s) that provides these strains with a selective advantage in this hostile environment, and this motivated us to investigate gene expression in this condition.

For the purpose of performing transcriptome comparisons, we used ASW as the "control condition" as this nutrient-depleted medium mimics the chemical composition of seawater (e.g. salinity, osmolarity, and pH) without the natural, uncontrollable variables that contribute to the dynamic composition of natural sea water (e.g. biotic constituents, organic compounds, etc). Further, this medium does not provide a selective advantage for either genotype, and provides a controlled environment similar to the natural habitat of *V*. *vulnificus*.

Experimental design:

Two clinically isolated C-genotypes of *V*. *vulnificus* (CMCP6 and YJ016) and two E-genotypes isolated from oysters (JY1305 and JY1701) were selected for this study. Genetic characteristics of each strain are listed in Table 1. Cells were treated as previously described [8]. Briefly, strains were grown overnight in heart infusion (HI) broth (BD, New Jersey) at 30°C, and then grown to logarithmic phase (OD_{610} 0.15-0.25) in fresh HI. Cells were pelleted and resuspended in 10 parts per thousand (ppt) artificial seawater (ASW) to remove HI nutrients. Cells were then inoculated into either 10ppt ASW or normal human serum (MP Biomedicals) to a final concentration of ca. 1e8 CFU/ml and incubated on a rotisserie for 2 hours at 22°C or 37°C, for ASW or HS, respectively. ASW at 22°C was chosen to represent a controlled version of the natural estuarine waters and temperature this bacterium encounters in its native environment. Human serum at 37°C was chosen to reflect the host environment and human body temperature. Pooled human serum (HS) used in this study was collected from male

donors and contained nutrients in the form of glucose (96mg/dl) with total protein levels of 5.5 – 7.5g/dl [11].

RNA extraction and purification:

Two biological replicates were performed for each strain and after 2 hours of incubation cells were treated with 2 volumes of RNAprotect (Qiagen) following manufacturer's instructions and subsequently subjected to RNA extraction following the protocol developed by [8]. Briefly, cells were treated with 1mg/ml lysozyme (Sigma Aldrich) and vortexed at medium speed for 30m to facilitate cell lysis. RNA was extracted using RNeasy Midi Kit (Qiagen) following protocol with on-column DNase I treatment. A second DNA digestion was performed using TURBO DNA-free (Life Technologies) following the rigorous DNase treatment protocol. RNA quality and quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific) and RNA samples were confirmed to have a 260/280 ratio <1.7 before proceeding.

RNA was confirmed to be free of DNA contamination by performeing PCR on RNA samples using Promega's Go-Taq DNA polymerase, 5X Green GoTaq Reaction Buffer, 10mM dNTP mix, and primers targeting the species-specific *vvhA* gene [1]. Cycling parameters were according to the manufacturer's recommendations, with an annealing temperature of 53.1°C and 40 cycles of amplification. Amplification of the *vvhA* gene was indicative of DNA contamination in which case the RNA was not used for downstream processes. RNA integrity was assessed using an Agilent 2100 Bioanalyzer and 6000 Nano kit (Agilent Technologies). The 23S/16S rRNA ratio and RNA integrity number (RIN) were confirmed to be >1.8 and close to 10, respectively.

mRNA Enrichment and cDNA Library Preparation:

Prior to cDNA library preparation, mRNA transcripts were enriched for using Epicentre's Ribo-Zero kit (Epicentre Biotechnologies) following manufacturer's instructions. mRNA was assessed for quality and levels of rRNA contamination using the Agilent Bioanalyzer and RNA 6000 Pico Chip (Agilent Technologies). Samples having >1% rRNA contamination were not used for downstream applications. mRNA was quantified using Qubit's 2.0 Fluorometer and RNA High Sensitivity Assay Kit (Life Technologies).

DNA libraries complementary to mRNA sequences were prepared for each sample using ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies) following manufacturer's instructions. The size and quality of each cDNA library was assessed using Agilent Bioanalyzer's High Sensitivity DNA kit (Agilent Technologies). Insert sizes were approximately 400bp +/- 35bp. cDNA library quantification was performed using Qubit's 2.0 Fluorometer and Quant-iT dsDNA High Sensitivity Assay Kit (Life Technologies). Samples were stored at -20°C and transported to the David H.

Murdock Research Institute (Kannapolis, N.C.) for sequencing using Illumina's Genome Analyzer.

RNA sequencing data analysis and statistics:

Data preprocessing:

Approximately 61 to 79 million paired-end raw reads were generated for each set of biological replicates (Table 2). RNA sequencing data is publicly available in NCBI's BioProject database (BioProject ID PRJNA252365). Raw Illumina sequence reads were filtered to remove reads which fell below the sequencer quality threshold. A second round of filtering using fastq-mcf [12] removed low-quality regions of sequence using a quality trimming window of size 4 and a minimum quality score of 15. The resulting reads were of high average quality and read lengths were tightly clustered around 100 nucleotides. The remaining filtered reads were aligned to reference genomes using Bowtie2 [13]. The alignment rate using Bowtie2 with default settings for paired-end reads was greater than 95% for all samples in the study indicating high quality and reproducibility of the data set (Table 2). The view and sort functions of SAMTools [14] were used to convert the aligned read data into the correct format for subsequent analysis.

Expression analysis:

Read counts were summarized against the CMCP6 reference genome using FeatureCounts [15]. It is important to note that using a closely related but non-identical reference genome may lead to mapping errors. The available methodological choices in this experiment were to 1) use a related but non-identical closed genome as the reference for mapping, 2) use draft-assembled genomes that remain unclosed as the reference for the E-genotypes while having closed references for the C-genotypes, or 3) use de novo assembled reference transcriptomes as the reference for mapping for all genotypes. Each of these approaches has the potential to produce errors of different types: erroneous mapping in the first case, missing data due to absence of regions in the reference in the second, and modeling of spurious transcripts in the third. Mapping the four closely related strains to the same closed reference genome is a current standard of practice in genomic bioinformatics, and we felt that this approach was likely to produce the least ambiguous results. We are currently investigating and contrasting the results of the three possible analysis approaches and will present that work in a future manuscript.

Differential gene expression analysis was performed using the EdgeR package [16] following the approach outlined by Jenkins [17]. EdgeR identifies significant differentially expressed genes out of genome-scale count data using exact tests based on the negative binomial distribution. Replicate data for each condition was used for normalization using the TMM method [18]. Transcriptome comparisons were performed for each genotype across conditions using artificial sea water (ASW) as the baseline

condition for analysis. For within conditions comparisons, CMCP6 was used as the reference genome and expression profiles of E-genotypes were analyzed relative to the CMCP6 baseline. Genes identified as differentially expressed between or within conditions, with a p-value less than 0.0001 were selected for further analysis.

Figure 1 depicts a multiscale-dimensional scaling (MDS) plot which allows for the visualization of differences or similarities between transcript pools. This plot shows that biological replicates for each strain/condition are located in close proximity to one another indicating the reproducibility of experimental replicates. Within each condition, E-genotypes group closely together, indicating the two strains have very similar transcript profiles in each condition. E-genotypes separate nicely from C-genotypes, however a curious finding is the distance between the two C-genotypes. It appears that YJ016 and CMCP6 are just as different from each other as they are from E-genotypes. This finding raises concern in grouping these two strains into one category, so for the purposes of this study we eliminated YJ016 from the within conditions comparison until we are able to further substantiate the cause(s) for this difference.

In situ survival and biofilm formation:

To examine survival of C- and E-genotypes in the natural estuarine environment, we used membrane diffusion chambers, originally designed by McFeters and Stuart [19]. These chambers consist of two 0.2μm polycarbonate filters sandwiched between three doughnut shaped sections of Plexiglas. Using this apparatus, 25ml of bacterial culture can be aseptically injected and placed in the desired environment. Subsequently, samples can be removed aseptically from the chamber and analyzed. Previous studies have demonstrated that these chambers provide an effective (i.e. good diffusivity), and statistically reproducible means of examining bacteria in their natural environment [20]. To set up chambers for deployment, overnight laboratory grown cultures of *V. vulnificus* were washed to remove nutrients, diluted into sterile 20ppt ASW at a final concentration of ca. 1e4 CFU/ml, and asceptically injected into chambers. Chambers were employed over several days during which samples were periodically extracted to quantify cell numbers. Culturability was measured by serial dilutions into ASW followed by the standard plate count method using HI agar. Plates were incubated at 30°C for 24h to calculate CFU/ml.

Biofilm formation by C- and E-genotypes incubated in natural estuarine waters was also assessed using membrane diffusion chambers. Briefly, laboratory grown cells of all four strains utilized in this study (Table 1) were diluted into 20ppt ASW and injected into individual sterile (0.22μm) membrane diffusion chambers at a final concentration of ca. 2e8 CFU/ml. Each chamber contained two sterile glass coverslips which served as the substrata for attachment and biofilm formation. A control chamber containing cover slips but no bacterial inocula were included to account for non-specific crystal violet staining (see below). Chambers were immediately deployed in estuarine waters along Core Creek

in Beaufort, North Carolina, and incubated for 24 hours. Chambers were deployed in August of 2013 and for the duration of the experiment, estuarine water temperatures were ca. 33°C and salinity ranged from 23- 27ppt.

After 24 hours, chambers were collected and brought back to the lab for immediate testing. Cover slips were removed from each chamber, stained in crystal violet solution for 15 minutes, washed several times with 20ppt ASW, and allowed to dry. Biofilm bound crystal violet was then eluted with ethanol and the optical density (OD_{610}) of each eluate was analyzed using a Spectrophotometer.

RESULTS AND DISCUSSION

1. Comparing C- and E-genotypes transcriptional profiles across conditions (ASW vs HS)

Recently, we sequenced the transcriptome of two C-genotypes of *V. vulnificus* exposed to ASW or HS. That study revealed two divergent genetic programs under these two conditions [8]. In human serum, cells displayed a distinct "virulence profile" in which a number of putative virulence factors were upregulated, including genes involved in intracellular signaling, substrate binding and transport, toxin and exoenzyme production, and the heat shock response. Conversely, the "environmental profile" exhibited by these C-genotype cells in seawater revealed upregulation of several transcription factors including *rpoS, rpoN,* and *iscR*, as well as genes involved in intracellular signaling, chemotaxis, adherence, and biofilm formation. Notably, this dichotomy in genetic programming between HS and ASW appeared to be largely governed by cyclic-di-GMP signaling and remarkably resembled the genetic and phenotypic switch documented in *V. cholerae* cells as they transition from host to environment [9,10].

In the current study, we sequenced the transcriptome of two E-genotypes exposed to the same conditions (ASW and HS) in order to compare the transcriptonal response of this genotype to the previously analzed C-genotype as the cells transition from the natural environment to host-like conditions. Surprisingly, the transcriptional response of E-genotype cells as they transtion from ASW to HS was strikingly similar to what we had observed for C-genotype cells. This finding was quite surprising given the different HS resistance patterns demonstrated by these two genotypes.

Athough the transcriptional patterns were unexpectedly similar, they were not identical. Previously, we identified genes previously found to be specific to C-genotypes [6] that were differentially expressed across ASW and HS, including arylsulfatases and the stressosome module [8]. As E-genotypes do not possess these genes, these C-genotype unique genes may provide some competitive advantage to C-genotypes, and these C-specific genes should be considered for further investigation. Additionally, we identified a large set of genes involved in maltose and maltodextrin utilization to be significantly upregulated by C-genotypes exposed to HS (Table 3). The importance of

these genes in human serum survival is not immediately apparent, although previous studies have demonstrated the importance of maltose and maltodextrin utilization for pathogens such as Group A *Streptococcus* and *E. coli* [21,22].

Although carbohydrate transport and metabolism pathways are not often thought of in the context of microbial pathogenesis, it has been repeatedly shown that utilization of these pathways can contribute to a bacterium's pathogenic capabilities [23-25]. Indeed, *V. cholerae* has been shown to accumulate and store glycogen inside granules within the host, which is thought to prepare the organism for dissemination back into the aquatic environment [26]. From that study, glycogen metabolism was concluded to be critical for the transtion of *V. cholerae* between host and aquatic environments. Furthermore, a recent study used Tn-Seq to screen for genes involved in dissemination of *V. cholerae* into the environment and identified a number of metabolic and catabolic genes to be important during host infection [27]. To our knowledge, the ability for *V. vulnificus* to form glycogen granules has never been investigated although our findings appear to warrant such studies. Table 3 documents that numerous of genes involved in carbohydrate metabolism were upregulated by C-genotypes in HS, highlighting the need for more thorough investigation into the contribution of these pathways to virulence, if any.

2. Comparing C- and E-genotype transcriptome profiles within ASW

To identify any inherent differences between C- and E-genotypes, we examined their transcriptome profiles in ASW using the C-genotype CMCP6 as the "baseline" and measured log-fold expression of E-genotypes relative to that baseline. Thus, genes which had positive log-fold change represent those which are upregulated by E-genotypes (relative to C-genotypes), and genes having a negative log-fold change represent those which are upregulated by C-genotypes (relative to E-genotypes) in ASW.

A previous study investigating the attachment capabilites of C- and E-genotypes found that E-genotypes attach to chitin more efficiently compared to C-genotypes [28]. That study identified surface-associated proteins including type IV pili and mannitol sensitive hemagglutinin (MSHA) to be involved in adherence to chitin. Interestingly, even in the absence of chitin, expression levels of these attachment genes were significantly higher in E-genotypes relative to C-genotypes, suggesting that E-genotypes have an inherent predisposition for adherence to substrates such as chitin [28]. That study concluded with the proposition that C- and E-genotypes have intrinsically divergent physiological programs which may contribute to the observed genotypic differences in oyster colonization and pathogenic potential.

By comparing the transcriptome profiles of C- and E-genotypes in ASW we see that this proposition is supported; genes involved in type IV pili structure and function were upregulated in E-genotypes. Furthermore, transcriptional comparisons revealed the upregulation of exopolysaccharide (EPS) associated genes in E-genotypes, which

indicates that these strains are indeed more efficient in attaching to surfaces and building biofilms. Three EPS loci have been idenified in *V*. *vulnificus* and we found two of these polysaccharide biosynthetic loci to be significantly upregulated by E-genotypes relative to C-genotypes. These EPS loci, referred to as the "vps-like" (VV2_1575 – VV2_1582) and "syp-like" loci (VV1_2658 - 2673), have been found to produce wrinkled colonies, pellicle formation, and matrix production when induced [29].

Conversely, C-genotypes upregulated *tad* (tight adherence) genes which encode the Flp pilus machinery. The *tad* genes have been found to be essential for adherence, biofilm formation, colonization, and pathogenesis in a number of genera, and are considered to be instrumental in the colonization of diverse environmental niches [30-33]. *V*. *vulnificus* contains three tad loci (VV1_1742 – VV1_1757, VV1_2330 – VV1_2337, and VV2_0084 – VV2_0096). Interestingly, we previously found the VV1_1742 locus to be significantly upregulated by C-genotypes in ASW relative to HS [8]. In the present study we find that the VV1_2330 locus is upregulated by C-genotypes in ASW relative to E-genotypes. Together, these findings underscore the potential importance of these Flp pili for C-genotypes. The VV1_2330 *tad* locus has previously been implicated in virulence as it was signficantly expressed in *V*. *vulnificus* cells isolated directly from a wound infection [34]. A separate study found this locus to be under the control of iron in a Fur-dependent manner, suggesting that iron mediates attachment and colonization of biotic and abiotic surfaces in the evironment and/or within the host [35].

C-genotypes also upregulated genes involved in capsular polysaccharide (CPS) biosynthesis, as well as *tad* (tight adherence) genes which encode the Flp pilus machinery. CPS (VV1_0773 – VV1_0786) is recognized as an essential virulence factor for *V*. *vulnificus* and negatively impacts biofilm formation [36-38]. Although CPS genes were not upregulated during the transition from ASW to HS, enhanced expression of CPS in ASW could indicate that C-genotypes have a thicker, more complete, CPS layer which conceptually could provide better protection from the host immune response, should the organism enter the human host.

3. *In situ* survival and *in situ* biofilm formation of C- and E-genotypes

Given the documented predominance of E-genotypes in oysters and the surrounding estuarine waters, we made use of membrane diffusion chambers to examine survival of C- and E-genotypes incubated in natural estuarine waters for seven days (Figure 2). Results revealed no overall drop in culturability over this time course, and no significant difference between survival of C- and E-genotypes. Thus, we do not observe the same genotypic ratio as seen in the wild, at least over this seven day period. Given the design of these chambers (cells housed within 0.2μm filter chamber) it could be that these size selective filters are preventing exposure to the top-down or bottom-up controls on growth that regulate *V*. *vulnificus* survival and growth in the estuarine environment. For example, it has been shown that protozoan grazing can significantly affect mortality in

Vibrio species such as *V*. *cholerae*, and survival depends on the bacterium's ability to resist these grazing pressures [39]. Abiotic factors such as salinity and temperature are known to influcence *V*. *vulnificus* abundance, however the factor(s) that promote success of one genotype over the other have yet to be indentified and are of significant interest.

Examing *in situ* survival did not offer any insight into the observed distribution anomoly that is typically observed in C- and E-genotypes. What provides E-genotypes with a competitive advantage in colonizing and persisting in oysters? Considering the robust expression of EPS and type IV pili genes observed in E-genotypes, we inserted microscope cover slips into each chamber and incubated C- and E-genotypes in situ for 24 hours to assess biofilm formation. Figure 3 reveals that E-genotypes form significantly better biofilms *in situ* relative to C-genotypes, confirming our previous findings. Indeed, it appears that E-genotypes form more robust biofilms and this is likely due to enhanced expression of type IV pili and EPS.

CONCLUSIONS

The main objective of this study was to disect the transcriptomic profiles of C- and E-genotypes within ASW, and to compare the profiles of each genotype as they transition from this natural environment into a host-like environment (human serum). We found that C- and E-genotypes possess strikingly similar adaptation responses when transitioning from seawater to human serum. This finding was quite perplexing until we compared the gene expression profiles of E-genotypes relative to C-genotypes within the seawater condition. This comparison revealed that C- and E-genotypes have naturally different transcriptomic profiles within seawater and suggests differences in the ecology and pathogenic potential of these two genotypes is a result of fundamental differences in genetic programming rather than differences in the genetic response. Of particular interest was the differential regulation of attachment and exopolysaccharide associated genes when cells are incubated in ASW. E-genotypes express type IV pili and EPS loci, whereas C-genotypes express more Flp pili and CPS biosynthesis genes. These transcriptomic results were complemented with the finding that E-genotypes form more robust biofilms *in situ* compared to C-genotypes. This phenotypic and genetic dichotomy between C- and E-genotypes could have significant implications for the pathogenic potential and environmental prevalence of these two genotypes.

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FIGURES

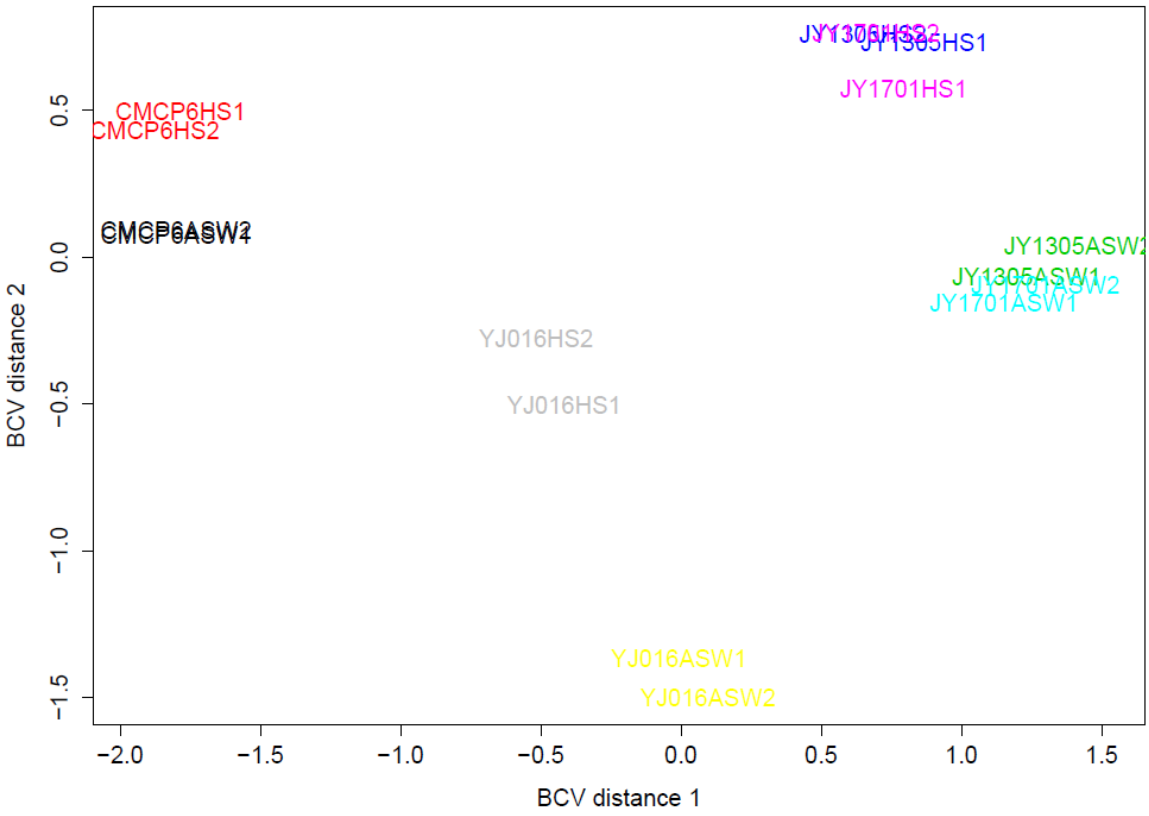

FIGURE 1: Multiscale-dimensional scaling (MDS) plot of *V*. *vulnificus* transcript pools by strain, condition, and biological replicate. This plot depicts differences or similarities between transcript pools.

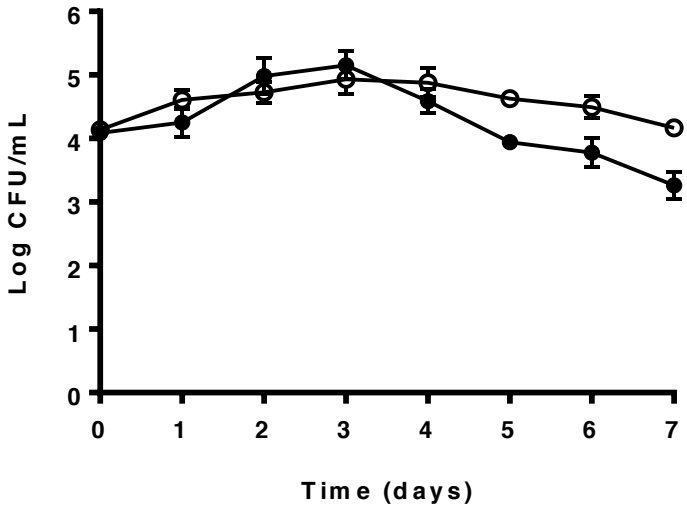

FIGURE 2: Survival of *V. vulnificus* C-genotypes and E-genotypes. C-genotypes (open circles) and E-genotypes (closed circles) were incubated in membrane diffusion chambers placed in natural estuarine waters and culturability was assessed over a period of 7 days. Error bars represent the standard errors of three biological replicates for each genotype.

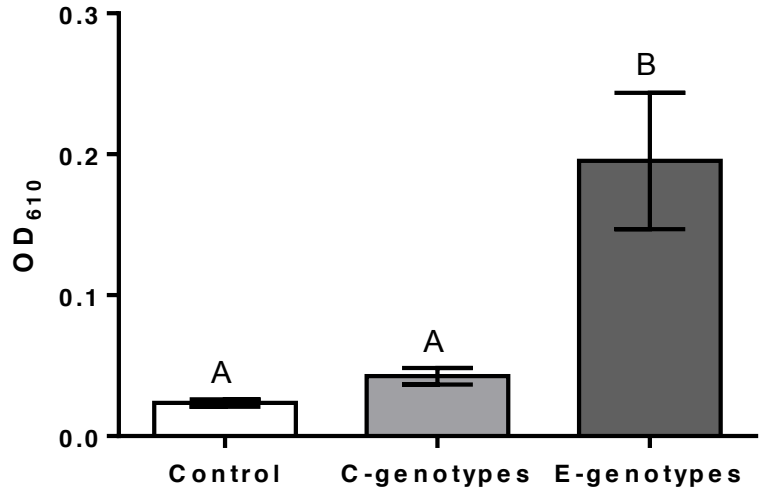

FIGURE 3: *In situ* biofilm formation by C- and E-genotypes of *V. vulnificus*. Cells were incubated in membrane diffusion chambers placed in natural estuarine waters and the ability of each strain to form biofilms on glass cover slips was measured after 24 hours. Error bars represent the standard errors of the means of two technical and two biological

replicates for each genotype. Control represents chambers with no bacteria added. Different letters indicate statistically significant differences (One-Way ANOVA; $p<0.05$).

TABLE 1: Strains used in this study

Strain name	Source	Genotypes[a]	Lineage[b]	Genomic region XII[c]
JY1701	Environmental	A, E	II	-
JY1305	Environmental	A, E	II	-
CMCP6	Human blood	B, C	I	+
YJ016	Human blood	B, C	I	+

[a] – Genotype groupings according to polymorphisms within the 16s rRNA [40], and virulence correlated gene [1], respectively.

[b] – Lineage groupings based on MLST analysis of six housekeeping genes (Cohen *et al*. 2007)

[c] – +/- indicates the respective presence or absence of the 33-kb genomic island (region XII) [41].

TABLE 2: Summary of RNAseq coverage data using the Illumina genome analyzer

Sample[a]	Total number of reads	Filtered reads	Reads mapped to genome	Overall Alignment Rate (%)
CMCP6, HS (1)	27,099,015	825,725	26,273,290	97.37
CMCP6, HS (2)	34,668,387	1,075,167	33,593,220	96.66
YJ016, HS (1)	45,576,619	1,403,108	44,173,511	98.16
YJ016, HS (2)	34,291,544	1,049,630	33,241,914	97.18
CMCP6, ASW (1)	36,508,024	1,080,730	35,427,294	97.57
CMCP6, ASW (2)	31,957,944	825,277	31,132,667	96.74
YJ016, ASW (1)	45,245,390	1,374,713	43,870,677	95.41
YJ016, ASW (2)	17,022,254	435,673	16,586,581	96.14

TABLE 3: Maltose and maltodextrin utilization genes upregulated in C-genotypes exposed to HS

Gene #	Annotation	Fold Exp	P-value
VV2_1582	Undecaprenyl-phosphate glucose phosphotransferase	2.860433	0.000542
VV2_1584	Maltose/maltodextrin transport ATP-binding protein MalK	3.190686	0.00614
VV2_1585	Maltose/maltodextrin ABC transporter substrate binding periplasmic protein MalE	4.445094	0.001849
VV2_1586	Maltose/maltodextrin ABC transporter permease protein MalF	3.60778	0.002651
VV2_1227	Maltoporin (maltose/maltodextrin high-affinity receptor phage lambda receptor protein)	7.056403	9.15E-05
VV2_1228	Maltose operon periplasmic protein MalM	2.409057	0.007779
VV2_1230	Maltose/maltodextrin ABC transporter substrate binding periplasmic protein MalE	5.057104	6.80E-05
VV2_1247	Transcriptional activator of maltose regulon MalT	6.203433	3.97E-08
VV2_1250	Glycogen phosphorylase	5.216028	0.000337
VV2_1251	4-alpha-glucanotransferase	3.663698	0.004735
VV2_1290	hypothetical protein	3.490478	5.15E-05
VV2_1291	ABC-type dipeptide transport system periplasmic component	7.551806	4.03E-07
VV2_1292	ABC-type dipeptide/oligopeptide/nickel transport system permease component	4.008378	0.000705
VV2_1293	ABC-type dipeptide/oligopeptide/nickel transport system permease component	4.618453	8.86E-05

VV2_1294	ABC-type dipeptide/oligopeptide/nickel transport system ATPase component	4.68908	5.03E-05
VV2_1295	ABC-type oligopeptide transport system ATPase component	4.243894	7.49E-05
VV2_1296	hypothetical protein	3.686629	0.000411
VV2_1297	Transcriptional regulator	2.717039	0.000257
VV2_1298	hypothetical protein	5.182407	6.44E-05
VV2_1300	Beta-glucanase precursor	5.562536	3.90E-06
VV2_1301	Maltoporin (maltose/maltodextrin high-affinity receptor%2C phage lambda receptor protein)	7.700691	2.78E-06
VV2_1302	AraC-type DNA-binding domain-containing protein	4.791357	2.88E-08
VV2_1304	Beta-glucosidase-related glycosidase	2.764799	0.000152
VV2_1306	Na+ driven multidrug efflux pump	2.805016	0.000241
VV2_1307	hypothetical protein	3.12323	0.000247
VV2_1308	Multidrug resistance efflux pump	3.268445	7.91E-05
VV2_1309	Permease of the drug/metabolite transporter (DMT) superfamily	2.415911	0.002945
VV2_1328	hypothetical protein	2.40331	0.003058
VV2_1329	Maltoporin (maltose/maltodextrin high-affinity receptor phage lambda receptor protein)	2.329287	0.010562
VV1_1788	Glycerol uptake facilitator protein	3.384994	0.009292

IMPLICATIONS OF CHITIN ATTACHMENT FOR THE ENVIRONMENTAL PERSISTENCE AND CLINICAL NATURE OF THE HUMAN PATHOGEN *VIBRIO VULNIFICUS*

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Implications of Chitin Attachment for the Environmental Persistence and Clinical Nature of the Human Pathogen *Vibrio vulnificus*

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Vibrio vulnificus **naturally inhabits a variety of aquatic organisms, including oysters, and is the leading cause of seafood-related death in the United States. Strains of this bacterium are genetically classified into environmental (E) and clinical (C) genotypes, which correlate with source of isolation. E-genotype strains integrate into marine aggregates more efficiently than do C-genotype strains, leading to a greater uptake of strains of this genotype by oysters feeding on these aggregates. The causes of this increased integration of E-type strains into marine "snow" have not been demonstrated. Here, we further investigate the physiological and genetic causalities for this genotypic heterogeneity by examining the ability of strains of each genotype to attach to chitin, a major constituent of marine snow. We found that E-genotype strains attach to chitin with significantly greater efficiency than do C-genotype strains when incubated at 20°C. Type IV pili were implicated in chitin adherence, and even in the absence of chitin, the expression level of type IV pilin genes (*pilA*, *pilD*, and *mshA*) was found to be inherently higher by E genotypes than by C genotypes. In contrast, the level of expression of *N*-acetylglucosamine binding protein A (*gbpA*) was significantly higher in C-genotype strains. Interestingly, incubation at a clinically relevant temperature (37°C) resulted in a significant increase in C-genotype attachment to chitin, which subsequently provided a protective effect against exposure to acid or bile, thus offering a clue into their increased incidence in human infections. This study suggests that C- and E-genotype strains have intrinsically divergent physiological programs, which may help explain the observed differences in the ecology and pathogenic potential between these two genotypes.**

Vibrio vulnificus is a highly invasive opportunistic human pathogen indigenous to estuarine and coastal waters worldwide (1). Importantly, this bacterium is the causative agent of frequently fatal septicemia in susceptible persons consuming raw or undercooked oysters and is the leading cause of seafood-related death in the United States (2). *V. vulnificus* is a free-living bacterium but also exists as part of the normal microflora within bivalves (3). As filter feeders, these molluscs tend to concentrate *V. vulnificus* within their tissues and therefore represent an important ecological niche for this bacterium (4). The mechanisms as to how this opportunistic human pathogen survives and proliferates in the environment and is capable of disease production in humans have been long-standing questions.

V. vulnificus strains are genetically and phenotypically diverse and are grouped into biotypes and genotypes based on biochemical and genetic traits, respectively. Biotype 1 strains are responsible for the majority of human infections and are the focus of this study (5, 6). Genetic polymorphisms within the virulence-correlated gene (*vcg*) serve as a primary feature to distinguish strains of clinical (C) genotypes from those of environmental (E) genotypes, the former being more often associated with disease (7). Similarly, polymorphisms within the 16S rRNA gene can be used to distinguish between clinically and environmentally associated genotypes, referred to as types B and A, respectively (8). The use of multilocus sequence typing and phylogenetic analyses of sequenced genomes has further delineated C and E genotypes into two distinct evolutionary lineages (9, 10). Indeed, previous studies have shown that C- and E-genotype strains display different ecologies, in which E-genotype strains seem to have a distinctive advantage in inhabiting oysters, whereas C-genotype strains are more successful in infecting the human host (7, 11–13). Furthermore, genome comparisons have allowed the identification of several putative virulence factors (such as the genomic XII region) that could potentially aid C-genotype strains in disease progression (10, 14, 15).

Froelich et al. recently demonstrated that E-genotype cells integrate into marine aggregates more efficiently than do C-genotype cells, thereby resulting in a greater uptake of E-genotype strains by oysters feeding on these aggregates (16). Additionally, experiments with C- and E-genotype cocultures resulted in a significantly greater uptake of E-genotype cells than C-genotype cells into oysters. This finding offered a possible explanation for the predominance of E-genotype strains in oysters; however, the mechanisms for this increased integration of E-genotype strains remain unknown. Marine aggregates are naturally forming conglomerates of organic and inorganic detritus, of which chitin is a primary constituent (17). Chitinous substrates are considered to play a vital role in the ecology of vibrios, serving as a critical reservoir for the survival and persistence of pathogens such as *Vibrio cholerae* (18). Importantly, these associations with chitin are thought to influence the overall metabolism and physiology of *Vibrio* spp. (18, 19). The primary goal of the present study was to comparatively assess the efficiencies of chitin attachment by *V. vulnificus* C- and E-genotype strains in order to further investigate the specific mechanisms responsible for the genotypic disparity observed within marine aggregates and oysters.

Considering the ecological relevance of vibrio associations with chitinous substrates, surface-associated proteins aiding in

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adherence to chitin have been studied extensively in a variety of *Vibrio* spp. (19–26). *N*-Acetylglucosamine (GlcNAc) binding protein A (GbpA) binds to GlcNAc-containing carbohydrates such as chitin. In *V. cholerae*, GbpA has been demonstrated to bind chitin and the mucin of intestinal epithelial cells, thus linking a single colonization factor critical for both environmental and host survival (19, 27). GbpA also contributes to the persistence of *V. cholerae* within bivalve tissues, particularly through colonization of mussel hepatopancreas cells (28). Two type IV pili have been identified in *V. vulnificus*, the mannose-sensitive hemagglutinin pilus (MSHA) and the PilA pilus (also referred to as the chitin-regulated pilus ChiRP in some *Vibrio* species), both of which are processed by a unique prepilin peptidase (PilD) to form a mature pilus structure that extends from the surface of the cell and interacts with the environment (29). In *V. vulnificus*, the type IV pilin subunit PilA has been shown to play a role in biofilm formation, adherence to human epithelial cells, and oyster colonization (30, 31). Additionally, *pilA* and *mshA* mutants of both *Vibrio parahaemolyticus* and *V. cholerae* show a lower fitness for adherence to chitinous substrates (20, 22, 23, 26). In *V. cholerae*, MSHA is considered to be important for environmental persistence, aiding in attachment to zooplankton exoskeletons, but unlike GbpA, it has been shown to interact with bivalve hemocytes and act as an anticolonization factor in human disease (4, 20, 22, 32). In the current study, the role of these genes in facilitating chitin attachment was assessed for both C- and E-genotype strains.

Stauder et al. demonstrated that increasing temperatures positively affect expression of both *mshA* and *gbpA* and consequently enhance *V. cholerae* attachment to chitin (33). Additionally, due to its ability to resist digestion by acid, chitin has been shown to provide *V. cholerae* with protection from lethal acid stress, offering an effective means for gastric transit inside the human host (34, 35). *V. vulnificus* C-genotype strains have been shown to resist the bactericidal effects of human serum compared to their E-genotype counterparts and also to exhibit a more robust cross-protective response in the presence of multiple stressors (12, 13). To our knowledge, the effect of *V. vulnificus*-chitin interactions on stress resistance has not yet been investigated. Thus, we examined the differences in attachment efficiency between C- and E-genotype cells at the human physiological temperature (37°C) compared to an environmental temperature of 20°C and also examined the ability of chitin-bound cells to resist exposure to acid and bile stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. vulnificus* strains used in this study are listed in Table 1. Bacterial cultures were stored at −80°C in Bacto Luria broth (LB) (BD Difco, NJ) containing 20% glycerol. Clinical isolates selected for this study were characterized as having the *vcgC* and 16S rRNA type B alleles and have been classified as lineage II strains, possessing genomic region XII. Conversely, environmental isolates were characterized as having *vcgE* and 16S rRNA type A alleles and have been classified as lineage I strains, lacking genomic region XII (14). All strains were grown in Bacto heart infusion (HI) broth (BD Difco, NJ) for 24 h at 30°C with shaking. PilA and PilD type IV pilus mutants were grown with 25 μg/ml and 50 μg/ml of streptomycin and spectinomycin, respectively.

For coculture competition experiments, in which *V. vulnificus* C- and E-genotype cells were allowed to attach to chitin simultaneously, 1% mannitol agar containing 1.6% BBL phenol red broth base (BD Difco, NJ) and 1% D-mannitol (Sigma-Aldrich), autoclaved for 5 min at 121°C, was used to differentiate between the two genotypes. C- and E-genotype strains used in this study form yellow and pink colonies on mannitol agar, respectively (36).

TABLE 1 Strains used in this study

Strain	Source	Genotypes[a]	Lineage[b]	Presence of genomic region XII[c]
JY1701	Environmental	A, E	II	−
JY1305	Environmental	A, E	II	−
SS108-A3A	Environmental	A, E	II	−
Env1	Environmental	A, E	II	−
CMCP6	Clinical	B, C	I	+
YJ016	Clinical	B, C	I	+
M06-24	Clinical	B, C	I	+
C7184[d]	Clinical	B, C	I	+
C7184ΩA[e]	*pilA* mutant	B, C	I	+
C7184ΩD[e]	*pilD* mutant	B, C	I	+

[a] Genotype groupings according to polymorphisms within the 16S rRNA (8) and virulence-correlated (7) genes, respectively.
[b] Lineage groupings based on multilocus sequence typing analysis of six housekeeping genes (9).
[c] + or − indicates the respective presence or absence of the 33-kb genomic island (region XII) (9).
[d] Parental wild-type strain of type IV pilus mutants.
[e] Type IV pilin mutants were kindly provided by Rohinee Paranjpye of the NOAA Northwest Fisheries Science Center (Seattle, WA).

Chitin attachment assay. A chitin attachment assay was performed as previously described, with slight modifications (37). Chitin magnetic beads (New England BioLabs) were washed twice by vortexing in phosphate-buffered saline (PBS). Bacterial cultures were washed twice in PBS by centrifugation and diluted 10-fold into fresh PBS (or 15 ppt artificial seawater [ASW] for temperature experiments). In a 1.5-ml microcentrifuge tube, 900 μl of washed culture was added to 100 μl of washed chitin magnetic beads at a concentration of 5×10^7 cells/ml. The mixture was allowed to incubate at 20°C or 37°C for 1 h on a rotisserie at 8 rpm. The supernatant, containing the unattached cells, was removed by placing the tube onto a 1.5-ml microcentrifuge magnetic stand (Life Technologies) and gently washing the beads three times with PBS. Chitin beads, along with the remaining attached bacteria, were suspended in 1 ml of PBS and prepared as described below for cell quantification using either culture-based methods or fluorescence microscopy.

Culture-based quantification. A total of 0.2 g of 0.5-mm ZR Bashing-Bead was added to the washed chitin beads suspended in 1 ml of PBS, and the tube was vortexed vigorously for 60 s to detach the bound bacteria. Chitin beads were separated from the supernatant by using a magnetic stand, and the cell suspension containing the detached cells was serially diluted and plated onto HI agar for individual strain experiments or spread plated onto 1% mannitol agar for competition experiments.

Fluorescence microscopy-based quantification. A total of 10 μl of beads harboring attached *V. vulnificus* cells was dropped onto a clean microscope slide and allowed to dry. The beads were then fixed with 95% ethanol, stained by using acridine orange, and visualized under a fluorescence microscope. To compare chitin attachment efficiencies, average numbers of cells per bead were quantified for each strain.

Acid/bile stress assay. For acid stress exposure, washed chitin beads with attached *V. vulnificus* cells were exposed for 5 min at 20°C to 1 ml of PBS adjusted to pH 3 by using hydrochloric acid. For bile exposure, washed chitin beads with attached cells were exposed to 1 ml of 1% bile (Ox gall powder; Sigma-Aldrich) in PBS for 30 min at 20°C. After incubation, the stress was immediately removed by washing the beads with PBS through gentle inversion of the tube five times. Culture-based quantification was performed as outlined above. The amount of chitin-attached cells that survived exposure to stress was compared to the number of chitin-attached cells that were not exposed to stress and expressed as percent survival. For comparison,

TABLE 2 Primers designed for this study

Gene	Strain(s) amplified	Primer target[a]	Sequence (5′–3′)	Expected product size (bp)
Glyceraldehyde phosphate dehydrogenase	C7184 and JY1305	*gapdh* F	TGAAGGCGGTAACCTAATCG	97
		gapdh R	TACGTCAACACCGATTGCAT	
Type IV pilin protein subunit	JY1305 only	*pilA* F	TCATTGGTGTGTTAGCCGCA	73
		pilA R	GCTGAGGCAGCTTCTGACTT	
Type IV pilin protein subunit	C7184 only	*pilA* F	GCACAGCTCCAACCAGTAGT	57
		pilA R	TTGGCGGCACTTCAACAATG	
Type IV pilin protein prepilin peptidase	JY1305 only	*pilD* F	GGCTTACTGGTAGGCAGCTT	126
		pilD R	GGTTTCTGTCGGCGGTGATA	
Type IV pilin protein prepilin peptidase	C7184 only	*pilD* F	TTGGCTTACTGGTAGGCAGC	128
		pilD R	GGTTTCTGTCGGTGGTGTGA	
N-Acetylglucosamine binding protein	C7184 and JY1305	*gpbA* F	TTGAGTGGACCTTTACCGCC	91
		gpbA R	CGGGCAAGTGGTTGATTTGG	
Mannitol-sensitive hemagglutinin	C7184 and JY1305	*mshA* F	CAAGGCGGTTTCACCCTGAT	90
		mshA R	CAGATTTAGAAAACGCGGAGCC	

[a] F, forward; R, reverse.

percent survival of planktonic cells incubated without chitin but exposed to the same stress conditions was also measured.

Gene expression. (i) RNA harvesting. *V. vulnificus* strains C7184 (C genotype) and JY1305 (E genotype), grown in HI broth for 24 h, were washed twice and then resuspended into 15 ppt ASW to a final concentration of ca. 1×10^8 CFU/ml. Each strain was incubated on a rotisserie for 1 h and then treated with RNAprotect (Qiagen) according to the manufacturer's instructions. Cell pellets were resuspended in Tris-EDTA (TE) buffer at pH 8.0 with 10 mg/ml lysozyme and then vortexed at medium speed for 30 min. RNA from lysed cells was extracted by using the RNeasy Minikit (Qiagen) according to the manufacturer's protocol, with optional on-column DNase I treatment. In the final step, RNA was eluted from the column by using nuclease-free water, and a second postextraction DNase treatment was performed by using Turbo DNA-free (Ambion) according to the "rigorous DNase treatment" protocol. RNA quality and quantity were assessed by using a NanoDrop spectrophotometer (Thermo), and all RNA samples were found to have a 260/280-nm absorbance ratio of ≥1.7. RNA samples were stored at −80°C.

(ii) PCR to confirm removal of DNA contamination. Endpoint PCR was performed on RNA samples, targeting the species-specific *vvhA* gene (7), to confirm the complete removal of DNA. This was done by using Promega Go-*Taq* DNA polymerase, 5× Green GoTaq reaction buffer, 10 mM deoxynucleoside triphosphate (dNTP) mix, and primers for *vvhA*. Cycling parameters were performed according to the manufacturer's recommendations, with an annealing temperature of 53.1°C and 40 cycles of amplification. Any amplification of the *vvhA* gene was indicative of DNA contamination, in which case the RNA was not used for downstream processes.

(iii) Primer design for qRT-PCR. Primers were designed for each gene of interest by using all three sequenced C-genotype strains of *V. vulnificus* (CMCP6, YJ016, and M06-24) reported in the NCBI database. Primers were designed to also target the three sequenced E-genotype strains of *V. vulnificus* (JY1701, JY1305, and E64MW) by using whole-genome shotgun contigs deposited in the NCBI database. ClustalW gene alignments were performed to identify conserved nucleotide regions, and primers were designed by using NCBI Primer-BLAST software. Optimal primer quality and fidelity were assessed by using IDT OligoAnalyzer 3.1 software. Primer specificity was analyzed by using *in silico* PCR (38), and the PCR efficiency of each primer pair was evaluated by using an *in silico* PCR efficiency estimation tool (39). Primers were purchased from Sigma-Aldrich and validated by using endpoint PCR to ensure specific amplification of the DNA targets of interest for each strain under investigation. Primers used in this study are listed in Table 2.

(iv) Relative qRT-PCR. The PCR amplification efficiency of each primer set was analyzed by generating a standard curve and evaluating the slope. Standard curves were performed for all genes and all strains under investigation by using five 1:5 serial dilutions of cDNA. To measure relative expression, 1 μg of total RNA was reverse transcribed by using qScript cDNA SuperMix (Quanta Biosciences). cDNA was then diluted, and 50 ng of cDNA template was carried over for quantitative PCR (qPCR). Relative quantitative reverse transcription-PCR (qRT-PCR) was performed on three technical and three biological replicates for each sample by using PerfeCTa SYBR green FastMix, Low ROX (Quanta Biosciences), thus generating nine threshold cycle (C_T) values per target. Negative controls consisting of water (in place of cDNA) were employed to rule out the influence of DNA contaminants or other artifacts (e.g., primer-dimers). "No-RT" controls consisting of an equivalent quantity of RNA were added to control wells to rule out the presence of residual genomic DNA. Expression levels of each gene were normalized by using an endogenous control gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) to correct for sampling errors. Fold changes in expression levels were measured for E-genotype strains relative to C-genotype strains by using the Pfaffl equation (40), taking into account the differences in PCR efficiencies between primer sets.

Statistical analysis. For culture-based quantification, attachment was expressed as the percentage of the total number of cells attached to the chitin beads (output) divided by the total number of cells added to the system (input), multiplied by 100. Attachment was also measured by using fluorescence microscopy, with the number of cells attached to 10 beads being averaged. Data were analyzed by using unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons. Significance was determined by using a 95% confidence interval. All data were analyzed by using GraphPad Prism (version 5.0; GraphPad Software Inc.). Relative fitness (w) of chitin attachment between genotypes (20, 23) was calculated as $w(C,E) = \mathrm{sqrt}(C_I/C_O)/\mathrm{sqrt}(E_I/E_O)$, where C_I and E_I represent the initial CFU input and C_O and E_O represent the output CFU at the end of the experiment. The relative fitness for each type IV pilus mutant was also calculated relative to the fitness of the parental wild-type strain. Gene expression levels of E-geno-

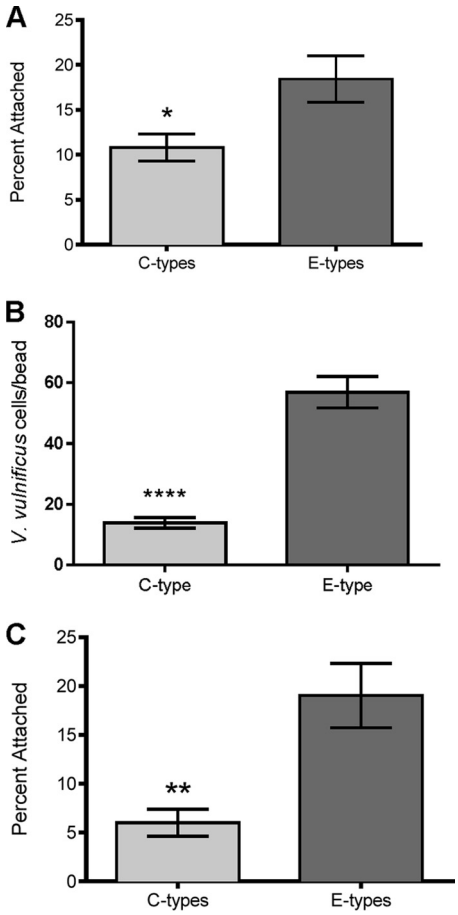

FIG 1 (A) Attachment of *V. vulnificus* C-genotype strains (C7184, CMCP6, M06-24, and YJ016) and E-genotype strains (JY1305, JY1701, ENV1, and SS108-A3A) to chitin magnetic beads. Error bars represent the standard errors of the means for three replicates of the four strains. E-genotype attachment to chitin was significantly greater than C-genotype attachment ($P = 0.0182$ by an unpaired Student *t* test). (B) Microscopic quantification of chitin attachment by *V. vulnificus* C-genotype (C7184) and E-genotype (JY1305) strains. Error bars represent the standard errors of the means for 10 replicate beads. JY1305 attachment to chitin was significantly greater than that of C7184 ($P < 0.0001$ by an unpaired Student *t* test with Welch's correction). (C) Competitive chitin attachment of *V. vulnificus* C- and E-genotype strains incubated in coculture. Two competitions were performed (M06-24 versus Env1 and C7184 versus JY1305). Error bars represent the standard errors of the means for the two competition studies with triplicate replicates. In coculture, E-genotype cell attachment to chitin was significantly greater than C-genotype cell attachment ($P = 0.0045$ by a Student *t* test).

type strains relative to C-genotype strains were analyzed by using the Mann-Whitney (nonparametric) rank-sum test.

RESULTS AND DISCUSSION

Attachment of *V. vulnificus* strains of clinical and environmental genotypes to chitin. The strains selected for this study have several distinct genetic features which have been used to demarcate the evolutionary lineages of clinical and environmental strains (7, 8, 13, 14, 41). We investigated the abilities of four C- and four E-genotype strains to attach to chitin magnetic beads, and while all isolates tested were able to adhere to chitin within 1 h of incubation, E-genotype strains were significantly more capable of chitin binding than were C-genotype strains (Fig. 1A). This

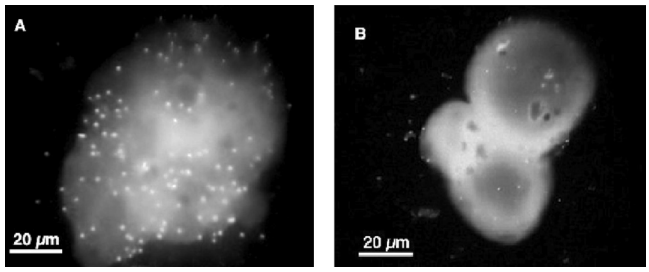

FIG 2 Representative fluorescence microscopy images of *V. vulnificus* E-genotype (A) and C-genotype (B) cells attached to a single chitin magnetic bead. Image color, contrast, and brightness were applied to each image by using the Macintosh Preview application.

finding was further substantiated by visually quantifying the number of cells attached to individual chitin particles using fluorescence microscopy (Fig. 1B and 2). To more accurately reflect the natural environment, C- and E-genotype cells were incubated with chitin in cocultures to examine competitive attachment. Again, E-genotype cells were significantly more efficient in adhering to chitin than were their C-genotype counterparts (Fig. 1C). Differences in attachment efficiency between C- and E-genotype strains were assessed by calculating relative fitness values, in which values of <1 indicated a defect in the competitiveness of the designated strain (see Materials and Methods). In monoculture, the overall fitness of C-genotype strains relative to E-genotype strains was 0.68. This relative fitness was reduced to 0.56 when C- and E-genotype strains were incubated in cocultures with chitin. This indicates that E-genotype strains have a competitive advantage in chitin colonization, which offers an explanation for the enhanced integration of E-genotype strains into marine aggregates along with the subsequent persistence of E-genotype strains within oyster tissues that we have previously reported (16).

Role of surface proteins in chitin attachment. We suspected that type IV pili are important for attachment to chitin; thus, we investigated the attachment efficiencies of two *V. vulnificus* mutants deficient in type IV pilin expression. Both *pilA* and *pilD* mutants displayed significantly decreased attachment compared

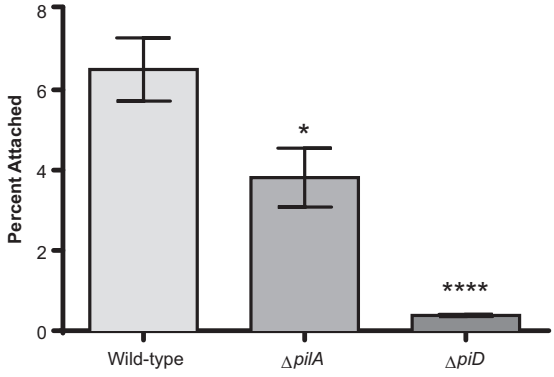

FIG 3 Chitin attachment of *V. vulnificus* type IV pilin mutants. Depicted are the differences in chitin attachment between parent strain C7184 and strains with mutations in type IV pilin protein A (*pilA*) (C7184ΩA) and prepilin peptidase (*pilD*) (C7184ΩD). Error bars represent the standard errors of the means for seven replicates. Asterisks indicate significant differences relative to the parental strain. One-way ANOVA indicated that the loss of type IV pilin genes significantly reduces the ability of C7184 to attach to chitin.

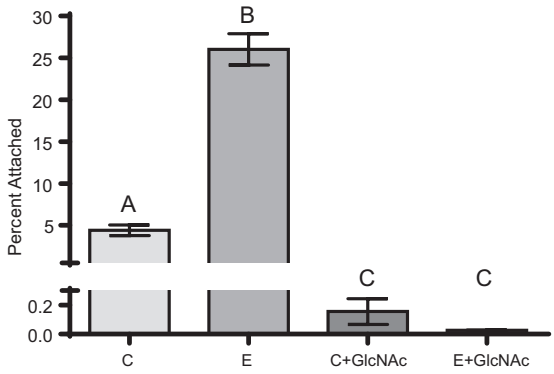

FIG 4 Effect of *N*-acetylglucosamine (GlcNAc) on *V. vulnificus* attachment to chitin. Depicted is the attachment efficiencies of two C-genotype (CMCP6 and M06-24) and two E-genotype (ENV1 and JY1305) strains incubated with chitin in the presence or absence of 125 mg/ml GlcNAc. Error bars represent the standard errors of the means for two replicates of two strains. Incubation with GlcNAc resulted in a significant decrease in attachment for strains of both genotypes. Different letters indicate statistically significant differences determined by using one-way ANOVA.

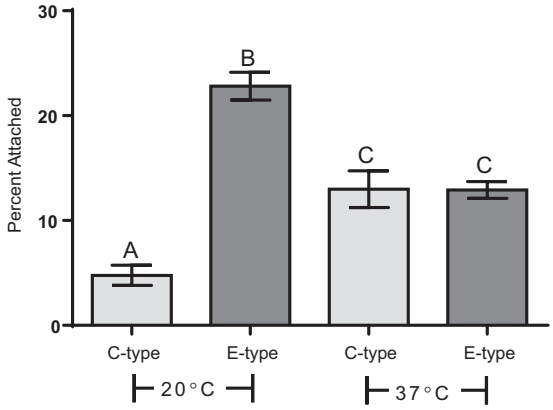

FIG 6 Effect of incubation temperature on attachment of C- and E-genotype strains to chitin. Depicted is attachment of two C-genotype strains (CMCP6 and M06-24) and two E-genotype strains (ENV1 and JY1305) incubated with chitin at room temperature (20°C) or the human physiological temperature (37°C). Error bars represent the standard errors of the means for two replicates of two strains. Different letters indicate statistically significant differences determined by using one-way ANOVA. Increasing the incubation temperature from 20°C to 37°C resulted in a significant increase in attachment for C-genotype strains and a significant decrease in attachment for E-genotype strains.

to the parent strain (Fig. 3), with relative fitness values of 0.64 and 0.13, respectively. The much lower fitness value of *pilD* suggests a crucial role in chitin attachment. It has been demonstrated that a *V. vulnificus pilA* mutant still expresses pili, whereas the *pilD* mutant results in a complete loss of surface pili, suggesting that PilD processes both MSHA and PilA type IV pili of *V. vulnificus* (31, 42). Because chitin is composed of *N*-acetylglucosamine (GlcNAc) monomers, cells were treated with this sugar to ascertain the specificity of surface proteins in chitin attachment. Incubation with GlcNAc in the presence of chitin resulted in a significant reduction in attachment efficiency for both C- and E-genotype strains (Fig. 4).

Our study confirmed the relevance of type IV pili in chitin attachment; however, it is important to note that the parent for these mutants was a C-genotype strain. Thus, to further characterize the role of these genes in chitin attachment, we examined relative gene expression levels of C- and E-genotype strains in the presence and absence of chitin. Interestingly, neither of these genes was induced (in strains of either genotype) when cells were incubated with chitin for 1 h (data not shown). This led us to the hypothesis that there may be basal differences in gene expression levels between these two genotypes. Hence, we examined relative gene expression levels between the genotypes when incubated in ASW without chitin addition. We discovered that E-genotype strains inherently express more *pilA* and *pilD*, by 23-fold and 9-fold, respectively, than do C-genotype strains (Fig. 5). Additionally, *mshA*, encoding the principal subunit of the type IV MSHA pilus, was expressed at 2-fold-higher levels in E-genotype strains (Fig. 5).

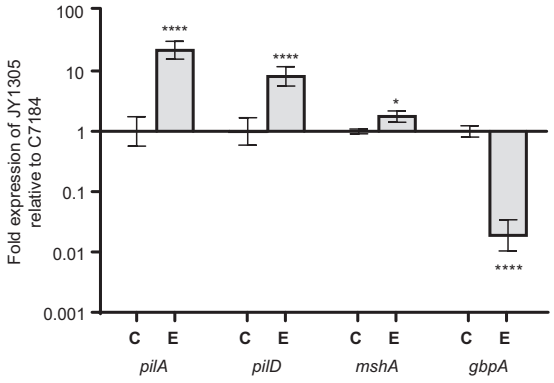

FIG 5 Expression of attachment genes by E-genotype (JY1305) relative to C-genotype (C7184) strains incubated in ASW for 1 h. Error bars represent the standard deviations of three biological and three technical replicates. Asterisks represent statistically significant differences between C- and E-genotype strains for each gene of interest by using one-way ANOVA. JY1305 intrinsically expresses significantly higher levels of *pilA*, *pilD*, and *mshA* than does C7184, whereas C7184 expresses higher levels of *gbpA* than does JY1305.

The role of *N*-acetylglucosamine binding protein (*gbpA*) was also investigated, and we discovered that C-genotype strains expressed significantly (46-fold) more *gbpA* than did E-genotype strains (Fig. 5). In *V. cholerae*, GbpA plays an essential role in chitin binding as well as in colonization of the human intestine; thus, the differential expression of *gbpA* by clinical strains of *V. vulnificus* likely has very important implications regarding the enhanced pathogenicity of these strains. It is plausible that increased expression levels of *gbpA* may provide enhanced attachment to epithelial cell surfaces in the human gastrointestinal tract; however, further studies are needed to validate this hypothesis.

Effect of temperature on chitin attachment. To investigate the effect of temperature on chitin attachment, C- and E-genotype strains were incubated with chitin beads at 20°C and 37°C, in parallel. As has been documented for *V. cholerae* (33, 43, 44), increasing the incubation temperature resulted in a significant increase in attachment for C-genotype strains (Fig. 6). As a result, the fitness of C-genotype strains relative to E-genotype strains in attachment to chitin increased from 0.450 at 20°C to 0.997 at 37°C. In contrast, this increase in temperature caused a significant decrease in attachment for E-genotype strains (Fig. 6). This inverse effect of temperature on chitin attachment likely has important clinical and ecological relevance and warrants further investigation.

Protective effect of chitin. We examined whether adherence to chitin allowed *V. vulnificus* C- and E-genotype strains to withstand stressors that would be encountered by this bacterium upon entry into the human host. To this end, cells attached to chitin beads were incubated in acidified PBS. Chitin particles were then washed to remove the acid stress, and the percentage of culturable cells remaining on chitin particles was quantified. Despite the rapid lethality (>99%) of pH 3 to planktonic (control) cultures of *V. vulnificus*, C-genotype cells significantly survived acid exposure when attached to chitin particles (Fig. 7A). Conversely, E-genotype cells were not significantly protected by chitin in the face of acid stress, emphasizing the difference in genetic programming between these two genotypes. Along with a temperature upshift, the acidic environment of the human stomach is likely to be one of the first physiological stresses encountered by the bacterium when ingested. *V. vulnificus* is known to possess several mechanisms for acid resistance and adaptation (45, 46), such as the *cadBA* operon, which is induced under conditions of low pH and which neutralizes the external pH through simple acid/base chemistry (47). Nonetheless, the genetic mechanisms responsible for combating acid stress in C-genotype strains may be superior to those in E-genotype strains, and it is tempting to speculate that attachment to chitin may grant C-genotype strains with temporary protection upon entry into the human host, thus providing them with a competitive advantage over E-genotype strains. Notably, although there is no clear role for chitin in oyster-associated infections, it is well known that bacterial cells in an attached state exhibit observable physiological changes relative to their planktonic counterparts (48); thus, attachment to some other more relevant oyster-based substratum may offer similar protection during the transition into the human host.

A bacterial pathogen attempting to successfully traverse the human gastrointestinal tract will inevitably encounter the antimicrobial properties of bile, an emulsifying fluid produced by the liver and excreted into the small intestine. A variety of enteric bacteria have been shown to respond to and subsequently develop resistance to bile exposure (49). Furthermore, pathogenic *Vibrio* spp., such as *V. cholerae* and *V. parahaemolyticus*, have been shown to possess mechanisms for bile resistance resulting in modulated expression of virulence factors (50–52). We exposed planktonic and chitin-attached cells to bile stress for 30 min and observed that while planktonic E-genotype cells survived bile stress significantly better than planktonic C-genotype cells, chitin-attached C-genotype cells survived bile stress significantly better than chitin-attached E-genotype cells (Fig. 7B). This opposing trend highlights the divergent physiological responses of strains of these two genotypes when exposed to clinically relevant stress. Further investigation into the acid and bile stress resistance of chitin-attached C-genotype cells would likely provide important insights into the virulent nature of strains of this genotype. Our recent comparative analysis of C- and E-genotype genomes revealed a number of genes unique to C-genotype strains, many of which could bestow the cell with enhanced stress resistance (10). We are currently investigating genes unique to C-genotype strains that may equip them with this heightened stress response.

Conclusion. *V. vulnificus* is widely recognized as a human pathogen, although genetic analyses have revealed that strains of this species are not equally pathogenic. Genotyping has revealed differences not only in pathogenic potential but also in the ecological niches in which these strains reside (11, 12, 53). Genetic

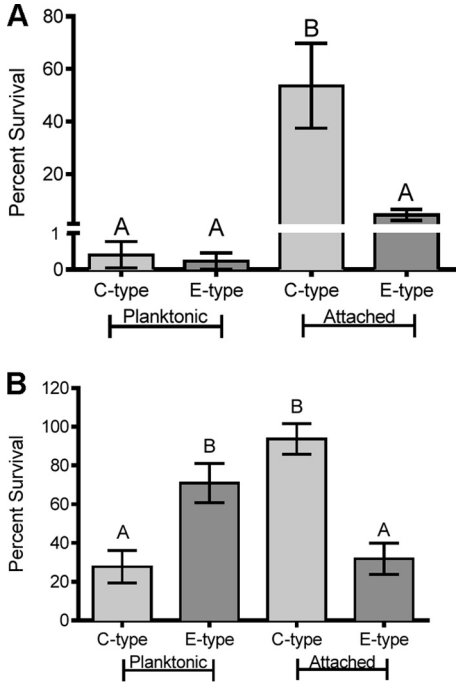

FIG 7 (A) Protective effect of chitin attachment upon exposure to acid stress. Depicted is the survival of two C-genotype strains (CMCP6 and M06-24) and two E-genotype strains (ENV1 and JY1305) when exposed to acid stress (pH 3) for 5 min in the planktonic state or when attached to chitin. Error bars represent the standard errors of the means for four replicates of two strains. Different letters indicate significant differences determined by using one-way ANOVA. Attachment to chitin provided a significant protective effect against acid stress for C-genotype strains only. (B) Effect of chitin attachment on exposure to bile stress. Depicted is the survival of two C-genotype strains (CMCP6 and M06-24) and two E-genotype strains (ENV1 and JY1305) when exposed to 1% bile for 30 min in the planktonic state or when attached to chitin. Error bars represent the standard errors of the means for two (planktonic) or four (attached) replicates of two strains. Different letters indicate significant differences determined by using one-way ANOVA. E-genotype strains survived bile stress significantly better than did C-genotype strains when in the planktonic state, while C-genotype strains survived bile stress significantly better than did E-genotype strains when attached to chitin.

dimorphisms among *V. vulnificus* strains have been documented on a genome-wide scale; thus, it has been proposed that C- and E-genotype strains may reflect distinct ecotypes (10, 13). Chitin is considered to play a pivotal role in the ecology of vibrios, and adherence to this substrate has been demonstrated for a number of species, including *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* (18, 25, 34, 54). The ability to associate with chitin in the aquatic environment has a profound influence on the life-style of *Vibrio* spp., providing them with a number of advantages, including food availability, adaptation to environmental nutrient gradients, tolerance to stress, and protection from predators (33, 55).

The ecologically distinct C- and E-genotype strains examined here displayed different degrees of chitin attachment, providing deeper insight into the natural ecology of this organism. Investigation of a larger set of C- and E-genotype strains should be performed to further substantiate this phenomenon. In this study, type IV pili were found to be important for chitin adherence and appear to be a key player in attachment by E-genotype strains. Conversely, *gbpA* expression was enhanced in C-genotype strains

compared to E-genotype strains, which we suspect has significant implications in human intestinal colonization and survival. Attachment to substrates such as chitin is likely to shift the physiological regimen of the bacterium, resulting in different adaptive capabilities in the face of stress. As demonstrated here, chitin-associated cells exhibited substantially different stress responses compared to those of free-living planktonic cells, particularly for C-genotype strains.

Sequence analyses of type IV pili among several *Vibrio* spp. have revealed interesting clues into the immense genetic diversity observed within this genus. For many bacterial species, including numerous pathogens, type IV pili have been implicated in environmental survival as well as host colonization. Aagesen and Hase proposed that the heterogeneities among *pilA* and *mshA* sequences are likely a result of positive selective pressures, which would be expected to have ecological implications for the bacterium (29). Sequence alignment of *V. vulnificus* type IV pilus genes (*pilA*, *pilD*, and *mshA*) as well as *gbpA* revealed a considerable number of genetic polymorphisms, many of which correlate with genotype. Indeed, our study reveals that C- and E-genotype strains differentially express these four genes within the same environment, even in the absence of chitin. Thus, the observed differences in chitin attachment do not appear to be a result of induction of gene expression but rather an inherent predisposition for adherence to chitin. We propose that this variance can help explain the observed genotypic differences in oyster colonization and possibly pathogenic potential. The environmental and molecular mechanisms facilitating chitin attachment deserve further examination and may provide a better understanding of the ecological and clinical divergence of these two genotypes.

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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 or the Duke University Marine Laboratory.

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IDENTIFYING THE MOLECULAR AND PHYSICAL FACTORS THAT INFLUENCE *VIBRIO VULNIFICUS* CHITIN ATTACHMENT

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IDENTIFYING THE MOLECULAR AND PHYSICAL FACTORS THAT INFLUENCE *VIBRIO VULNIFICUS* CHITIN ATTACHMENT

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ABSTRACT

The human pathogen, *Vibrio vulnificus*, is the leading cause of seafood-related deaths in the United States. Strains are genotyped based on alleles that correlates with isolation source, with clinical (C)-genotypes being more often implicated in disease, and environmental (E)-genotypes being more frequently isolated from oysters and estuarine waters. Previously, we have shown that the ecologically distinct C- and E-genotype strains of *V*. *vulnificus* display different degrees of chitin attachment, with C-genotypes exhibiting reduced attachment relative to their E-genotype counterparts. We identified type IV pili to be part of the molecular basis for this observed genotypic variance as E-genotypes exhibit higher expression of these genes relative to C-genotypes. Here, we demonstrate that quorum sensing is a negative regulator of type IV pili expression, which results in decreased chitin attachment. Furthermore, we identify calcium as an important component for chitin attachment by E-genotypes, which suggests the need for calcium for proper functioning of the type IV pili. We also found that starvation or dormancy can alter the efficiency of chitin attachment, which has significant implications for the environmental persistence of *V*. *vulnificus*. With the increasing incidence of wound infections caused by *V*. *vulnificus*, we investigated a subset of E-genotypes, isolated from human wound infections, and discovered that they attached to chitin in a manner more similar to C-genotypes. This study enhances our understanding of the molecular and physical factors that mediate chitin attachment in *V*. *vulnificus*, providing insight into the mechanisms that facilitate persistence of this pathogen in its native environment.

INTRODUCTION

The opportunistic human pathogen, *Vibrio vulnificus*, is part of the normal microflora of the estuarine environment and is found concentrated within bivalves and other organisms that inhabit these waters. *V. vulnificus* classically expresses its virulence after the ingestion of raw or undercooked shellfish, with the potential to cause fulminating septicemia and organ failure, particularly in individuals with liver disease or immunodeficiency. With a case-fatality rate of up to 50%, the majority (95%) of deaths associated with seafood in the U.S. are caused by this pathogen [1-3].

V. vulnificus is an exceptional pathogen in that it has more than one portal of entry. In addition to septicemia caused by ingestion of raw or undercooked seafood, this bacterium causes grievous wound infections and necrotizing fasciitis via entry into pre-existing cuts or wounds. In dthe latter case, infection can result from exposure to seawater or shellfish harboring sufficient quantities of the pathogen, which can occur during fishing, swimming, or other coastal activities. The incubation period post exposure is relatively short, averaging only 16 hours and often requires tissue debridement, skin grafts, and even amputation of the affected limbs [4]. Approximately 25% of wound infections result in death of the patient. Unlike patients who suffer septicemia from ingestion, victims suffering from wound infections often do not have underlying diseases [4,5]. Interestingly, the incidence of wound infections in the US has been increasing over the last few decades and is now the predominant form of infection caused by this pathogen [6].

Previous studies have discovered a predisposition for enhanced pathogenicity in some strains of this human pathogen. Genetic polymorphisms within specific genes have been found to highly correlate with the source of isolation. Currently, we use an allele referred to as the virulence correlated gene (*vcg*) to distinguish between strains more often implicated in human disease (clinical, or C-genotypes), from those which are more frequently isolated from the natural environment (environmental, or E-genotypes) [7-9]. Previous studies have provided further distinctions between these genotypes. Specifically, we have found that C-genotypes demonstrate superior survival in human serum relative to E-genotypes [10-12], and genome comparisons have allowed for the identification of several putative virulence factors that could potentially aid this bacterium in disease progression [13,14].

Despite these studies, no single, causal virulence factor has been has been found to be exclusively associated with clinical strains [15]. While the C/E genotyping scheme strongly correlates with virulence potential, this method does not strictly predict virulence [15]. For example, some strains with the *vcgE* allele have been isolated from clinical cases, often from wound infections. Previously, we analyzed a subset of E-genotype wound isolates and demonstrated their ability to resist the bactericidal effects of human

serum in a manner similar to that of C-genotype blood isolates [11]. Thus, it appears that a subset of E-genotypes have the ability to cause human disease, highlighting the need for better predictors of virulence potential within E-genotypes.

The environmental occurrence of *V. vulnificus* is favored by high temperatures (>20°C) and intermediate salinities (15 – 25 ppt), hence this bacterium can be readily isolated in environments within these physiochemical parameters [16]. However, this organism can tolerate wide salinity ranges and temperatures, which likely contributes to its natural prevalence [17,18]. Furthermore, when water temperatures drop below 4°C for extended periods of time, *V. vulnificus* has been documented to enter a state of dormancy referred to as the Viable but Nonculturable (VNBC) state [4,19]. This process of overwintering is thought to explain the observed seasonality of this organism in its native environment.

While it is well known how salinity and seasonality influences the diversity of *V. vulnificus* in the environment [20,21], the population structure of C- and E-genotypes within a given environment appears to be more complex. In their natural environment, C- and E-genotypes are routinely found to have different environmental distributions, with E-genotype strains often predominating in oysters and the water column (ca. 85%) relative to C-genotypes (ca. 15%) [22]. This distribution anomaly highlights the need for a better understanding of the biotic and abiotic factors that influence the spatial and temporal distribution of C- and E-genotypes.

Deeper investigation of ecological niches, such as microenvironments, has provided considerable insight into niche differentiation of C- and E-genotypes. A study investigating the ability for *V. vulnificus* C- and E-genotypes to integrate into marine aggregates found that they do so with significantly different efficiencies [23]. That study found that E-genotypes incorporate into marine aggregates much more efficiently compared to their C-genotype counterparts, and this increased attachment resulted in preferential uptake and retention of E-genotypes by oysters feeding on these marine aggregates. That study offered insight into why E-genotypes have been previously documented to predominate within the oyster environment, however the physiological and genetic causalities for this demonstrated genotypic heterogeneity remain unexplained.

Chitin is a major constituent of marine aggregates, and chitinous substrates are considered to play a pivotal role in the survival and persistence of vibrios, serving as a critical reservoir for pathogens such as *Vibrio cholerae* [24]. Importantly, a number of *Vibrio* spp. have been shown to adhere to chitinous substrates and these associations with chitin are thought to influence the organism's overall metabolism and physiology [24-27]. This substrate provides the organism with a number of advantages including food availability, tolerance to stress, and protection from predators [24,28,29]. Given the abundance of chitin in marine aggregates, and the significance of chitin in the ecology of vibrios, we subsequently investigated the efficiency of C- and E-genotypes to attach to

chitin particles [30]. Our study revealed that E-genotypes attach to chitin with a significantly greater efficiency compared to C-genotypes, and surface-associated proteins including the type IV pili and mannitol sensitive hemagglutinin (MSHA) were implicated in the observed adherence to chitin. Interestingly, even in the absence of chitin, expression levels of these attachment genes were significantly higher in E-genotypes relative to C-genotypes, suggesting that E-genotypes have an inherent predisposition for adherence to chitin. From these findings, we proposed that C- and E-genotypes have intrinsically divergent physiological programs which may help to explain the observed genotypic differences in oyster colonization and possibly pathogenic potential.

Collectively, these studies provided significant insight into the genotypic differences documented in environmental abundance and oyster colonization. Here, we further elaborate on the molecular mechanisms that facilitate differential chitin attachment of C- and E-genotypes by revealing a role for quorum sensing in this process. Additionally, we explore how fluctuations in environmental conditions can influence chitin attachment and show that starvation and cold temperatures can alter attachment efficiency by *V. vulnificus*. Lastly, given the increasing prevalence of E-genotype wound isolates, we compare the attachment efficiency of representative isolates and reveal that they behave more like C-genotypes. Overall, these studies contribute to our understanding of the behavior of C- and E-genotypes in the environment, and reveal how environmental influences can alter the efficiency of attachment.

METHODS

Bacterial strains and growth conditions:

Strains of *V. vulnificus* used in this study are summarized in Table 1. Cultures were stored at -80˚C in Bacto Luria broth (LB) (BD Difco, NJ) containing 20% glycerol. All strains were grown in Bacto heart infusion (HI) broth for 24 h at 30˚C with aeration. Strain JDO1 is a quorum sensing, *luxS* mutant derived from parent strain C7184 and is unable to make 4,5-dihydroxy-2,3-pentanedione, an essential precursor to the autoinducer AI-2. JDO1 was grown with 2µg/ml chloramphenicol.

Chitin attachment assay:

Chitin attachment was performed as previously described [30]. Briefly, chitin magnetic beads (New England BioLabs) were washed twice by vortexing in ½ strength artificial seawater (15 ppt ASW). Bacterial cultures were washed twice in ½ ASW by centrifugation and added to washed chitin beads at a final concentration of 5 X 10^7 cells/ml (1ml final volume). The mixture was allowed to incubate at 20˚C for 1 h on a rotisserie at 8rpm. The supernatant, containing the unattached cells, was removed by placing the tube onto a 1.5-ml microcentrifuge magnetic stand (Life Technologies) and gently washing the beads three times with ½ ASW. Chitin beads, which harbor the attached cells, were suspended in 1ml ½ ASW and prepared for quantification. To

quantify cells, 0.2g of 0.5-mm ZR Bashing Bead was added to the washed chitin beads suspended in 1ml ½ ASW, and the tube was vortexed vigorously for 60 s to detach the bound bacteria. Chitin beads were separated from the supernatant by using the magnetic stand, and the cell suspension containing the detached cells was serially diluted and plated onto HI agar.

Effect quorum sensing on chitin attachment:

To test the role of quorum sensing on chitin attachment, the ability of the *luxS* mutant to attach to chitin was evaluated as described above. To test whether the addition of AI-2 to the *luxS* mutant would allow the mutant to attach to chitin similar to the parent strain, the mutant was grown overnight in HI broth supplemented with 250nM AI-2 prior to performing the chitin attachment assay. AI-2 was prepared as previously described [31]. Chitin attachment was performed in ½ ASW supplemented with 250nM AI-2. To test whether the addition of a quorum sensing inhibitor (cinnamaldehyde) would allow the parent strain to attach to chitin at similar levels as the *luxS* mutant, the parent strain was grown overnight in HI broth supplemented with 150 μM cinnamaldehyde prior to performing the chitin attachment assay. Chitin attachment was performed in ½ ASW supplemented with 150 μM cinnamaldehyde.

Expression of Type IV pilin genes by the *luxS* mutant:

To analyze gene expression of the quorum sensing *luxS* mutant relative to the wild-type strain we performed relative quantitative reverse transcription PCR (qRT-PCR) using methods previously described [30]. Briefly, *V*. *vulnificus* strains C7184 and JDO1 were exposed to 15ppt ASW for 1 hour after which cells were subjected to RNA extraction using Qiagen's RNAprotect Bacterial Reagent with the RNeasy Minikit and on-column DNase I treatment (Qiagen). Post RNA extraction cells were subjected to a second DNase treatment using Turbo DNA-free (Ambion). Endpoint PCR was performed on RNA samples to confirm complete removal of DNA [30].

Primers were designed, analyzed, and validated as previously described [30] and the PCR amplification efficiency of each primer set was validated by generating standard curves and evaluating the slope. To measure gene expression, 1ug of total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences) and 50ng of cDNA template was used for quantitative PCR (qPCR). qRT-PCR was performed on three technical and two biological replicates for each sample using PerfeCTa SYBR green FastMix, Low ROX (Quanta Biosciences). Negative water controls and "No-RT" controls were employed to rule out the influence of contaminants or residual genomic DNA, respectively. Expression levels of each gene were normalized using an endogenous control gene (DNA gyrase subunit B [*gyrB*]) to correct for sampling errors. Fold changes in expression levels were measured for the *luxS* mutant relative to the wild-type strain

using the Pfaffl equation [32], taking into account differences in PCR efficiencies between primer sets.

Effect of calcium on attachment to chitin:

To test the effect of calcium on the attachment efficiencies of C- and E-genotype *V. vulnificus,* strains were grown overnight in HI broth, and chitin attachment was performed as described above. To test whether calcium has an effect on attachment, we incubated cells with chitin in either normal ½ ASW (containing 0.018% Ca^{2+}) or ½ ASW lacking Ca^{2+}. Both ASW types were maintained at 15 ppt salinity. To test whether calcium has an effect on chitin attachment of the *luxS* mutant, chitin attachment was performed for the mutant using ASW with and without calcium chloride.

Effect of starvation and low temperature on chitin attachment:

Cultures of C7184 were grown overnight in HI broth, washed, resuspended in ½ ASW and allowed to attach to chitin as described above (control). Separate aliquots of this culture were left in ASW at 20˚C and 4˚C for 18 days to induce starvation and the VBNC state, respectively. The ability to attach to chitin for each of these cultures was assessed after 2 and 18 days of incubation in these conditions. Cells were determined to be in the VBNC state using methods previously described [33]. Briefly, cell cultures were quantified every other day until *V. vulnificus* was no longer culturable on HI agar (<10CFU/ml detectable). Cells were confirmed to be VBNC by successfully resuscitation after cultures were returned to 20°C for 24 hours. For cultures that entered the VBNC state due to 4˚C incubation (18 days), the chitin attachment assay was modified to allow for the detection of VBNC cells, since they are not detectable on HI agar. Chitin attachment was performed as described above, however after bead-bashing to detach the cells that had adhered to the chitin beads, cultures were incubated at 20˚C for 24 h to allow VBNC cells to resuscitate (to allow for their detection on HI agar). Cells were then quantified by serially diluting and plating on HI agar.

Statistical Analysis:

For culture-based quantification, attachment was expressed as the percentage of the total number of cells attached to the chitin beads (output) divided by the total number of cells added to the system (input), multiplied by 100. Data were analyzed by using unpaired Student's t test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Significance was determined by using a 95% confidence interval. All data were analyzed by using GraphPad Prism (version 5.0; GraphPad Software Inc.).

RESULTS AND DISCUSSION

Quorum sensing reduces chitin attachment by negatively regulating expression of type IV pili:

Previously, we have shown that the ecologically distinct C- and E-genotype strains of *V. vulnificus* display different degrees of chitin attachment, with C-genotypes exhibiting reduced attachment relative to their E-genotype counterparts [30]. We identified the type IV pili to be part of the molecular basis for this observed genotypic variance as E-genotypes express these genes more heavily relative to C-genotypes. This difference in gene expression was documented even in the absence of chitin suggesting that the observed differences in chitin attachment are not a result of induction of gene expression but rather an inherent predisposition for adherence to chitin [30].

The phenomenon known as quorum sensing (QS) is the production of and response to signals (autoinducers) generated by bacteria of the same or other species in an effort to gauge the population density in a certain environment or to evaluate the cells' environmental location [34]. This leads to global changes in gene expression and has been shown to orchestrate multicellular behaviors in participating communities. Biofilm formation is a behavior that depends on community activities hence bacteria rely on quorum sensing regulators to control stages of biofilm formation [35]. In *V. cholerae*, cells initiate biofilm formation in a low cell density state and allow quorum sensing regulators to mediate natural maturation during biofilm development [35,36]. A similar phenomenon has been documented in *V. vulnificus* in which biofilm of a quorum sensing mutant was initially robust, but ultimately lead to collapse of the biofilm architecture [37]. That study suggested that the QS system regulates proper biofilm development and maintenance in *V. vulnificus*.

Pili, such as the type IV pili (MSHA and PilA), have been repeatedly implicated in facilitating cell-surface and cell-cell interactions in several *Vibrio* species, including *V. vulnificus*, and these cell surface structures mediate critical steps of attachment and biofilm formation [38-43]. Previously, an inverse relationship between pili and quorum sensing has been suggested in *V. vulnificus* since a QS mutant was shown to phenotypically exhibit significantly more pili and fimbriae relative to the wild-type strain [37].

Considering the suggested role of QS in pili production and biofilm formation, we examined the ability of a *V. vulnificus* QS mutant to attach to chitin particles and found a significant increase in attachment relative to the wild-type strain (Figure 1). Addition of synthetic AI-2 to the QS mutant restored attachment to the wild-type level. Further, addition of an AI-2 inhibitor (cinnamaldehyde) to the wild-type strain significantly enhanced attachment, to levels similar to that of the QS mutant. Collectively, these results strongly suggest that reduced QS enhances chitin attachment.

Previously, we have shown that mutations within the type IV pili result in significantly reduced chitin attachment [30]. To determine if the involvement of QS in chitin attachment is an effect on type IV pili we examined gene expression of the type IV pili genes, *pilA* (pilin subunit A) and *pilD* (prepilin peptidase), in the QS mutant relative to the wild-type strain. Figure 2 illustrates a significant up-regulation of both *pilA* and *pilD* in the QS mutant relative to the wild-type, indicating that type IV pili are negatively regulated by QS. Overall, these studies imply that the regulatory QS network functions quite differently between C- and E-genotypes and that this difference likely contributes significantly to the ecological success and pathogenic capabilities of the two genotypes.

Calcium deficiency impairs chitin attachment in E-genotypes:

One of the environmental conditions that can vary in natural the aquatic habitat of *V*. *vulnificus* is calcium [44]. Further, calcium levels have been shown to affect biofilm formation in *V*. *cholerae* [45]. To observe the effect of calcium on chitin attachment, we incubated C- and E-genotypes in the presence of chitin while incubating in artificial seawater with or without calcium. While the absence of calcium appeared to have no impact on chitin attachment by C-genotypes, a dramatic drop in attachment efficiency was observed in E-genotypes (Figure 3). Previous studies have shown that some pili possess a calcium-binding motif and, as a result, calcium can regulate type IV pili-mediated adhesion [46,47]. To further assess this potential in *V*. *vulnificus* we examined the effect of calcium on attachment by the QS mutant, which was previously shown to have enhanced chitin attachment and upregulation of type IV pili genes. While the wild-type strain (C-genotype) showed no change in chitin attachment in the presence or absence of calcium, we noticed a significant drop in chitin attachment by the QS mutant in the absence of calcium (Figure 4). Thus, lack of calcium affected the QS mutant in a manner similar to E-genotypes suggesting that this reduction in chitin attachment is likely due to a defect in type IV pili function when calcium is not available. Interestingly, attachment efficiency of the E-genotype was higher than that of the QS mutant, suggesting that E-genotypes possess additional characteristics beyond type IV pili that enhance attachment efficiency.

Environmental fluctuations can alter chitin attachment efficiency:

Environmental conditions fluctuate considerably in estuarine environments, thus bacterial inhabitants are perpetually faced with nutrient starvation, iron limitation, or changes in salinity and temperature. To cope with the “feast-to-famine lifestyle”, bacteria possess robust stress resistance mechanisms that help the organism adapt to these unpredictable instabilities [48]. One such adaptation is the ability to attach to abiotic or biotic surfaces, and subsequently build protective biofilms. In addition to the inherent stress resistance provided by biofilms, attachment to biotic substrates such as chitin can also provide direct access to nutrients [27,49]. Indeed, surface adhesion is often thought

of as a survival strategy, allowing bacteria to persist in nutrient-limited natural environments [49].

Previously, we demonstrated that environmental changes (such as temperature upshift) can alter chitin attachment efficiency in C- and E-genotypes of *V*. *vulnificus*. By increasing the attachment incubation temperature from 20°C to 37°C we demonstrated a significant increase in chitin attachment by C-genotypes [30]. This increased fitness in chitin attachment prompted us to further investigate into how common environmental fluctuations, such as nutrient starvation and cold temperatures, can influence the attachment efficiency of *V*. *vulnificus*.

To investigate the role of nutrient starvation on *V*. *vulnificus* attachment to chitin we incubated cells in artificial seawater (ASW) at room temperature (ca. 22°C) to induce starvation. At 2 and 18 days of starvation, cells were tested for their ability to attach to chitin particles. Figure 5 shows that cells are significantly more capable of attaching to chitin after 2 and 18 days of starvation, although attachment efficiency dropped at 18 days relative to 2 days.

Environmental stress such as nutrient deprivation, salinity fluctuations, and water temperature can also induce a state of dormancy referred to as the viable but nonculturable (VNBC) state [50,51]. Cells that enter the VBNC state are still viable but lose culturability due to a reduction in metabolic activity. In this state of dormancy, cells phenotypically exhibit a significant reduction in cell size, dwarfing of cell structure into a coccoid morphology, and changes in cell wall composition [50,51]. Our results show that, during exposure in ASW for 2 and 18 days at 4°C, cells retain the ability to attach to chitin, though significantly less efficiently when cells are in the VBNC state (Figure 5). Reduced attachment by VBNC cells may be attributed to cell rounding, cell surface modifications, and reduced metabolism, which have been documented to take place in VBNC cells.

The VBNC state is recognized as an important bacterial strategy for survival in adverse conditions and here we show that despite a significant reduction, these cells still maintain the capability to attach to chitin. Both starvation-induced attachment and the VBNC state are ecologically relevant phenomena that likely promote the persistence of *V*. *vulnificus* in the estuarine environment. Furthermore, some chitin-binding ligands in *V*. *cholerae* have been shown to act as “dual role colonization factors”, facilitating human intestinal colonization in addition to chitin attachment [52]. Thus, the environmental cues that modulate chitin attachment can potentially have significant implications for the pathogenic potential of *V*. *vulnificus*.

Clinical E-genotype strains attach to chitin in a manner similar to C-genotype strains:

Recently, it has become increasingly apparent that a subset of E-genotypes may have enhanced pathogenic potential relative to other environmentally isolated E-genotypes. These E-genotype wound isolates are of particular interest given the recent

rise in wound isolates within the US as well as in other countries. The genetic factors that distinguish this subset of E-genotypes remains unknown, however we recently demonstrated that these strains are capable of surviving human serum in a manner nearly identical to C-genotypes [11]. In the current study, we assessed the ability of these wound isolates to attach to chitin and found that again, these isolates behave more like C-genotypes (Figure 6). Furthermore, studies examining biofilm formation found that E-genotype wound isolates behave similarly to C-genotypes, forming very poor biofilms whereas E-genotype environmental isolates forming much more robust biofilms in the same condition (data not shown). Considering the various lines of study, evidence suggests that that E-genotype wound isolates phenotypically behave much like C-genotypes, indicating that these strains may share some genetic commonalities that can be attributed to their success in colonizing the human host. This suggestion warrants further investigation to identify the factor(s) responsible for this observation. Currently, genome sequencing of multiple E-genotype wound isolates is underway and will hopefully allow for further insight into this currently ambiguous phenomenon.

CONCLUSION

The primary goal of this study was to elaborate on previous studies investigating the interactions of C- and E-genotypes with chitinous substrates, with the intent of obtaining a more thorough understanding of the molecular foundations that facilitate superior attachment by E-genotypes. Previously, we revealed the involvement of type IV pili in facilitating chitin attachment in *V*. *vulnificus*, particularly in E-genotypes. In this study, we identify quorum sensing to be a negative regulator of type IV pili expression, as a QS mutant displayed enhanced chitin attachment in a manner similar to E-genotypes. This finding suggests that C- and E-genotypes have inherently different regulatory mechanisms underlying their ability to adhere to chitin. This suggestion could have significant implications in our understanding of the ecological and clinical characteristics of these two genotypes. We also found calcium deficiency to impair chitin attachment in E-genotypes and suggest that this reduction in chitin attachment is likely due to a defect in the function of type IV pili when calcium is not available.

Environmental parameters such as increasing temperatures has been previously shown to alter chitin attachment efficiency in *V*. *vulnificus* and *V*. *cholerae* [30,53], thus we further investigated how additional environmental fluctuations such as nutrient starvation and cold temperatures can influence chitin attachment. Short term (2 day) starvation and cold temperature incubation both significantly enhanced chitin attachment indicating that this biotic substrate may serve as a critical reservoir for *V*. *vulnificus* in suboptimal conditions. In the natural environment, planktonic cells must successfully navigate through the vast and dynamic estuary towards a suitable niche. In the face of starvation or death, locating a chitinous oasis within a vast aquatic expanse can turn the

tides for the organism by providing access to nutrients, protection from predators, and setting the stage for growth and biofilm formation. Even dormant cells of *V*. *vulnificus* retained their attachment properties (albeit to a lesser extent), despite the significant reduction in cell size and changes in cell wall structure. Overall, these results reveal that physical parameters can alter the attachment efficiency of *V*. *vulnificus* and these factors likely contribute to the environmental persistence of this organism.

Geographical regions which have experienced warming patterns have been linked with the emergence of *Vibrio* outbreaks [6,54]. Indeed, *V*. *vulnificus* infections have been increasing in the US and world-wide, with wound infections becoming the more prevalent disease manifestation [6,54]. Despite genotypic schemes which correlate with pathogenicity, a subset of E-genotypes have been isolated from wound infections. Previous studies demonstrated the ability of these strains to survive in human serum [11] and here we show again that these strains behave more like C-genotypes compared to archetypal E-genotypes. These results indicate that E-genotype wound isolates share some virulence characteristics with C-genotypes and identification of these features would likely aid our understanding of the pathogenic capabilities of *V*. *vulnificus*.

FIGURES

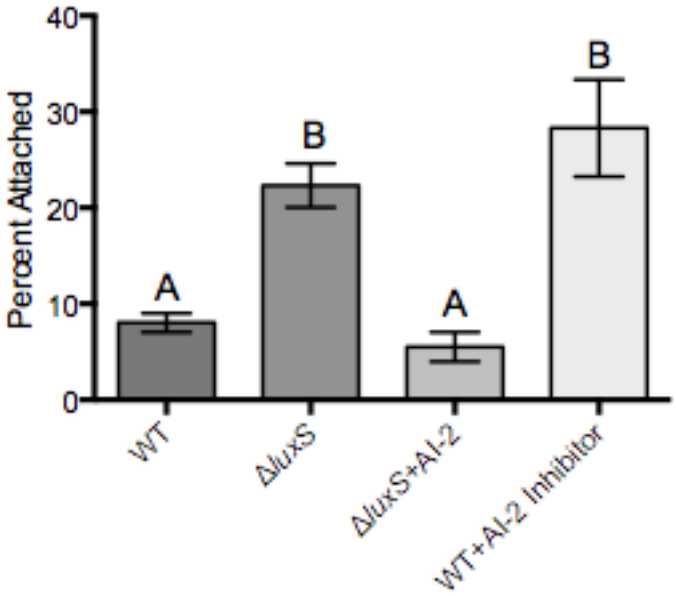

FIGURE 1: Effect of quorum sensing on chitin attachment. Attachment efficiency of a C-genotype wild-type strain (C7184; first bar), *luxS* quorum sensing mutant (JDO1; second bar), *luxS* mutant supplemented with the quorum sensing molecule AI-2 (third bar), and the parent strain supplemented with a quorum sensing inhibitor (fourth bar). Error bars represent the standard errors of the means for six replicates. Quorum sensing via autoinducer-2 inhibits chitin attachment. Different letters indicate statistically significant differences (One-way ANOVA; $p<0.0001$).

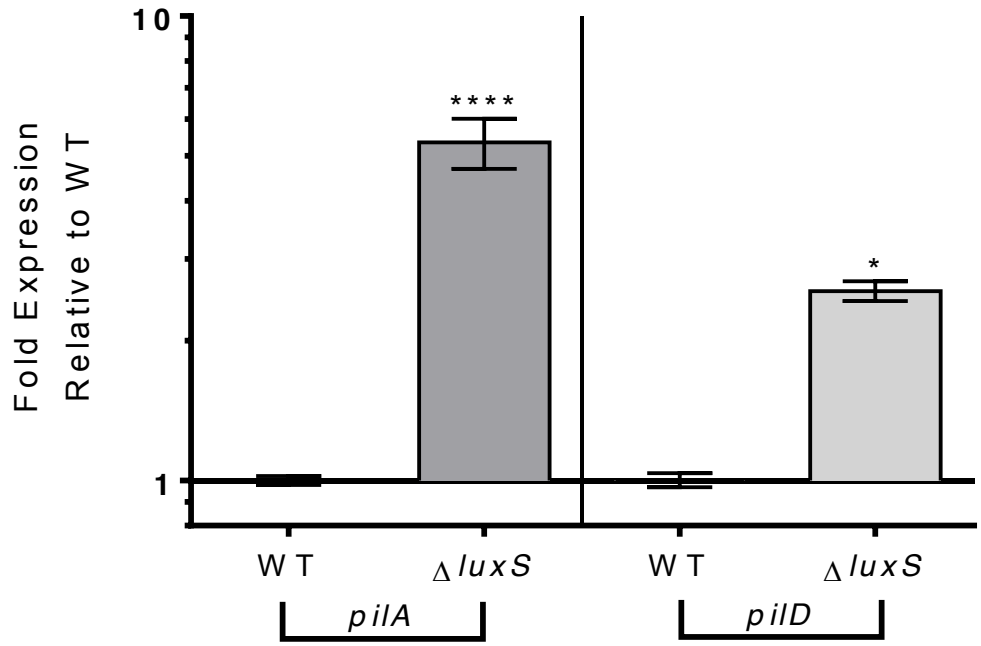

FIGURE 2: Expression of attachment genes by a quorum sensing mutant relative to the wild-type strain (C7184). Error bars represent the standard error of two biological and three technical replicates. Asterisks represent statistically significant differences between the *luxS* mutant and the wild-type strain for each gene of interest by using a one-way ANOVA. The *luxS* mutant intrinsically expresses significantly higher levels of *pilA* and *pilD* compared to the wild-type strain (****$p<0.0001$, *$p<0.05$). There was no statistically significant difference in the expression of *gbpA*.

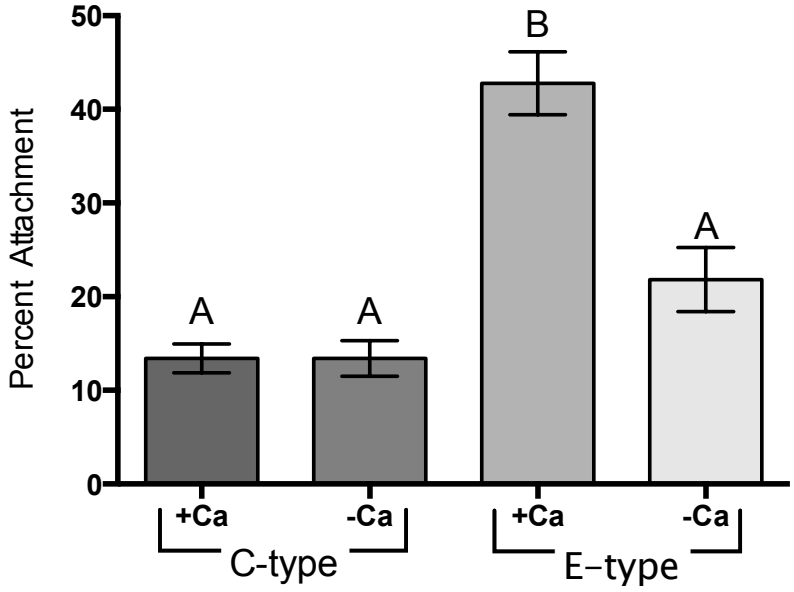

FIGURE 3: Effect of calcium on C- and E-genotype attachment to chitin. Chitin attachment efficiencies of two C-genotypes (C7184 and MO6-24) and two E-genotypes (JY1305 and JY1701) incubated in ½ ASW either with or without 0.018% Ca^{2+} (w:v). Error bars represent the standard errors of the means of three biological replicates for each strain and condition. Calcium enhances the attachment efficiency of E-genotypes to chitin but has no effect on C-genotype attachment. Different letters indicate statistically significant differences (One-way ANOVA; $p<0.0001$).

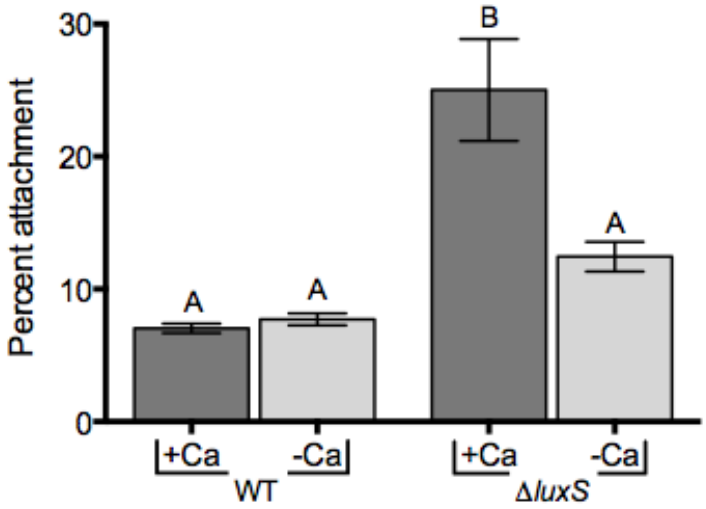

FIGURE 4: Effect of calcium on chitin attachment of the *luxS* mutant. Chitin attachment efficiencies of the wild-type strain (C7184) and the *luxS* quorum sensing mutant incubated in ½ ASW either with or without 0.018% Ca^{2+} (w:v). Error bars represent the standard errors of the means of three biological replicates for each strain and condition. Calcium enhances the attachment of the *luxS* mutant to chitin. Different letters indicate statistically significant differences (One-way ANOVA; p=0.0008).

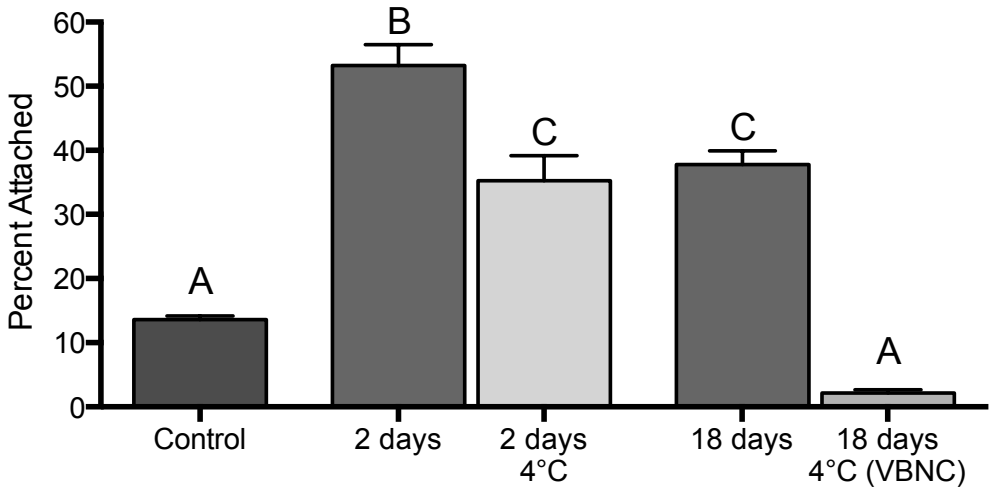

FIGURE 5: Effect of starvation and cold temperature (4˚C) on chitin attachment. Chitin attachment efficiency of a C-genotype strain (C7184) during prolonged starvation in ½ ASW at 20˚C or 4˚C. Cells were viable but non-culturable (VBNC) at day 18 at 4˚C. Error bars represent the standard errors of the means of three biological replicates. Starvation increases the ability of *V*. *vulnificus* to attach to chitin. However, longer-term starvation (18 days) leads to decreased attachment to chitin relative to short-term starvation (2 days). There was significantly less chitin attachment by cells incubated at 4˚C for 2 and 18 days compared to cells incubated at 20˚C. Different letters indicate statistically significant differences (One-way ANOVA; $p<0.0001$).

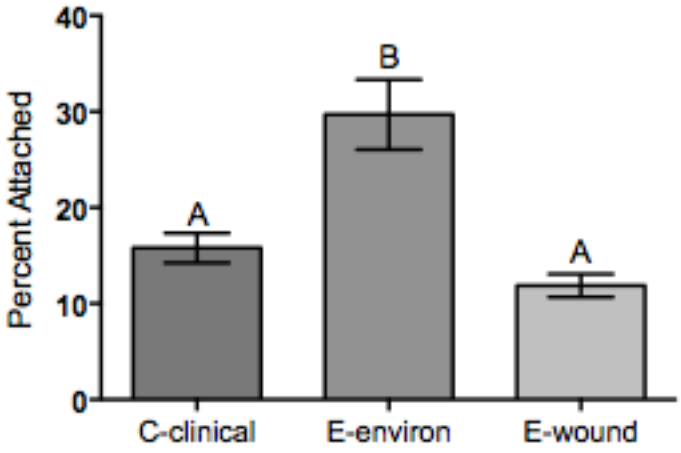

FIGURE 6: Effect of isolation source on *V*. *vulnificus* attachment to chitin. Attachment efficiencies of two clinically isolated C-genotypes (MO6-24 and CMCP6), two environmentally isolated E-genotypes (JY1701 and JY1305), and two E-genotypes isolated from wound infections (E64MW and LSU1657). Error bars represent the standard errors of the means for three replicates per strain. Clinical isolates (C-clinical and E-wound) attach to chitin significantly less than environmentally isolated E-genotypes. Different letters indicate statistically significant differences (One-way ANOVA; p=0.0002).

TABLE 1: Strains used in this study

Strain name	Source	Genotypes[a]	Lineage[b]	Genomic region XII[c]
JY1701	Environmental	A, E	II	-
JY1305	Environmental	A, E	II	-
E64MW	Human wound	A, E	UNK[d]	-
LSU1657	Human wound	A, E	UNK	UNK
CMCP6	Human blood	B, C	I	+
M06-24	Human blood	B, C	I	+
C7184[e]	Clinical	B, C	I	+
JDO1	*luxS* mutant	B, C	I	+

[a] – Genotype groupings according to polymorphisms within the 16s rRNA [55], and virulence correlated gene [7], respectively.

[b] – Lineage groupings based on MLST analysis of six housekeeping genes (Cohen *et al*. 2007)

[c] – +/- indicates the respective presence or absence of the 33-kb genomic island (region XII) [56].

[d] – Unknown

[e] – Parental wild-type strain of *luxS* mutant

TABLE 2. Primers used in this study

Gene	Primer target	Sequence (5’ – 3’)	Expected product (bp)
DNA gyrase subunit B	*gyrB* F	CTGAAGGGTCTGGATGCGG	97
	gyrB R	GTGCCATCATCTGTGTCCCC	
Type IV pilin protein subunit	*pilA* F	GCACAGCTCCAACCAGTAGT	57
	pilA R	TTGGCGGCACTTCAACAATG	
Type IV pilin protein prepilin peptidase	*pilD* F	TTGGCTTACTGGTAGGCAGC	128
	pilD R	GGTTTCTGTCGGTGGTGTGA	

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INTERSPECIFIC QUORUM SENSING MEDIATES THE RESUSCITATION OF VIABLE BUT NONCULTURABLE VIBRIOS

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Contributions by Tiffany C. Williams:

Experimental design, performed experiments, wrote and edited parts of manuscript, and response to reviewer critiques.

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Interspecific Quorum Sensing Mediates the Resuscitation of Viable but Nonculturable Vibrios

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Entry and exit from dormancy are essential survival mechanisms utilized by microorganisms to cope with harsh environments. Many bacteria, including the opportunistic human pathogen *Vibrio vulnificus*, enter a form of dormancy known as the viable but nonculturable (VBNC) state. VBNC cells can resuscitate when suitable conditions arise, yet the molecular mechanisms facilitating resuscitation in most bacteria are not well understood. We discovered that bacterial cell-free supernatants (CFS) can awaken preexisting dormant vibrio populations within oysters and seawater, while CFS from a quorum sensing mutant was unable to produce the same resuscitative effect. Furthermore, the quorum sensing autoinducer AI-2 could induce resuscitation of VBNC *V. vulnificus in vitro*, and VBNC cells of a mutant unable to produce AI-2 were unable to resuscitate unless the cultures were supplemented with exogenous AI-2. The quorum sensing inhibitor cinnamaldehyde delayed the resuscitation of wild-type VBNC cells, confirming the importance of quorum sensing in resuscitation. By monitoring AI-2 production by VBNC cultures over time, we found quorum sensing signaling to be critical for the natural resuscitation process. This study provides new insights into the molecular mechanisms stimulating VBNC cell exit from dormancy, which has significant implications for microbial ecology and public health.

At least 68 species of bacteria have been shown to enter a state of dormancy in which cells are viable but nonculturable (VBNC) (1), allowing them to evade harmful environmental stresses while maintaining the ability to return to an active state when the stress is alleviated (2). These cells are viable, as evidenced by their intact cell membranes and continued gene expression; however, they fail to replicate on routine laboratory media (2, 3). While in the VBNC state, these cells are able to resist a variety of normally fatal stresses, including antibiotic treatment (4), and they possess the ability to resuscitate within a host, potentially leading to disease initiation (5). Since these cells are not detectable by standard bacterial culture methods, the VBNC phenomenon is of significant public health and food safety concern. While there has been considerable growth in our knowledge of this dormancy state (1), very little is known regarding the molecular mechanisms for the resuscitation of VBNC cells.

Pathogenic vibrios exhibit seasonal fluctuations in abundance in water and in shellfish (6, 7), with their highest levels of culturability and incidences of infection observed during warm summer months (8, 9). The VBNC state is believed to be associated with this seasonal abundance in the environment, with culturability decreasing during winter months, when some cells enter the VBNC state, and increasing during warmer months, when these cells begin to resuscitate (10).

The opportunistic human pathogen *Vibrio vulnificus* is part of the normal microflora of the estuarine environment and is found concentrated within bivalves and other organisms that inhabit these waters (11, 12). *V. vulnificus* classically expresses its virulence after the ingestion of raw or undercooked shellfish (11), or by entry into open wounds (13), severely harming individuals with liver disease or immunodeficiency (14–16). With a case fatality rate of up to 50% (15), the majority (95%) of deaths associated with seafood in the United States are caused by this pathogen (7, 17). The VBNC state of *V. vulnificus* is easily studied *in vitro* by induction of dormancy using low-temperature incubation and of resuscitation by a simple temperature upshift (18), making it an ideal model organism for the study of this physiological state.

We previously found that upon addition of exogenous *V. vulnificus* or *Escherichia coli* cells to oysters, the culturability of the normal flora of *Vibrio* spp. within these oysters dramatically increased and remained high even after the added bacterial populations had been purged by the oysters (19). This indicated that preexisting and naturally present VBNC populations within these oysters resuscitated in response to the added metabolically active bacteria. In a related study, environmental water samples were enriched with the quorum sensing molecules CAI-1 and AI-2 to enhance the culturability of *Vibrio cholerae* (20). These findings led us to test the hypothesis that the increase in the culturability of *Vibrio* spp. upon the addition of exogenous metabolically active cells to oysters is due to quorum sensing signaling.

The phenomenon known as quorum sensing is the production of and response to signals (autoinducers) generated by bacteria of the same or other species in an effort to gauge the population density in a certain environment or to evaluate the cells' environmental location (21). This leads to global changes in gene expression (22) and has been shown to orchestrate virulence (23) and fitness factor production (24). For a more in-depth explanation of quorum sensing, the reader is directed to a recent review (25). Interestingly, some environmental factors that have been shown to affect quorum sensing, such as temperature, salinity, and pH, have independently been shown to play a role in VBNC dynamics

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(2, 26). Given these findings, we asked whether quorum sensing autoinducers mediate the resuscitation of VBNC *V. vulnificus*.

Here we demonstrate that the interspecific quorum sensing molecule AI-2 can resuscitate VBNC cells both *in vivo* and *in vitro*. Most importantly, we report for the first time that AI-2 is required by VBNC cells to resuscitate and that this quorum sensing-mediated process occurs naturally within VBNC cultures when environmental conditions are permissive, leading to resuscitation. Furthermore, we propose a molecular mechanism for the resuscitation of VBNC cells and show that it can be targeted to manipulate resuscitation.

MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study were *V. vulnificus* CMCP6, C7184, JDO1, and AH1, *V. parahaemolyticus* Sak-11, *V. harveyi* BB170, and *E. coli* K-12. All strains were stored at −80°C in Bacto Luria broth (LB) (BD, NJ) containing 20% glycerol. All strains were grown in Bacto heart infusion (HI) broth (BD, NJ) for 24 h at 30°C with aeration. Strains JDO1 and AH1 are *luxS* and *rpoS* mutants, respectively, and were derived from the parent strain C7184. JDO1 is a *luxS* mutant that is unable to produce 4,5-dihydroxy-2,3-pentanedione, an essential precursor to the autoinducer AI-2 (27), and AH1 is unable to produce the stress-related sigma factor RpoS (28). JDO1 and AH1 were grown with 2 and 15 μg/ml chloramphenicol, respectively. All bioluminescence-based quorum sensing assays were performed using autoinducer bioassay (AB) medium (29).

Resuscitation of dormant cells in oysters. *Crassostrea virginica* oysters were collected from Hoop Pole Creek, Atlantic Beach, NC, during September 2012. Oysters were maintained in 30-liter aerated aquaria containing 20‰ artificial seawater (ASW) (Instant Ocean; Aquarium Systems, Mentor, OH) and were fed Phyto-Feast daily (Reed Mariculture Inc., Campbell, CA).

Cell-free supernatants (CFS) were obtained from overnight cultures of *V. vulnificus* C7184 and JDO1 by filter sterilization (0.22-μm syringe-driven filter; Millipore, Tullagreen, Ireland). To confirm the sterility of CFS, 100-μl aliquots of CFS were spread plated onto HI agar. Oysters were placed in 3-liter tanks (4 oysters per treatment condition) of 20‰ ASW with aeration. CFS was added to oyster tanks at a 1:100 (vol/vol) ratio. After 24 h, oysters were shucked using a sterile knife and homogenized in 20‰ ASW (1:1 [wt/vol]), using sterile blender cups (Waring, Torrington, CT). Individual oyster homogenates were serially diluted in phosphate-buffered saline (PBS) and spread plated onto ChromAgar Vibrio (ChromAgar, Paris, France) to selectively quantify *Vibrio* species. This medium was employed to quantify total vibrios, as it has been demonstrated previously to indiscriminately allow for growth of all *Vibrio* species tested (30). Oysters which were incubated with CFS were compared to control oysters that were supplemented only with ASW. As an additional control, some oysters were supplemented with HI broth to ensure that nutrient carryover had no effect on vibrio levels.

Resuscitation of dormant cells in seawater. Natural seawater (NSW) was collected from Core Creek, Beaufort, NC, and stored at 20°C in sterile 50-ml conical tubes. Resuscitation experiments were performed within 24 h of collection. To eliminate nutrient carryover, 1 ml of overnight bacterial culture was washed twice with 15‰ ASW and incubated at 30°C for 24 h prior to collection of CFS by filter sterilization as described above. This nutrient-free CFS was added to the NSW at a 1:100 (vol/vol) ratio. The mixture was incubated at 20°C for 24 h before serially diluting and plating of the mixture on ChromAgar Vibrio. The fold change in CFU after 24 h in NSW supplemented with CFS was compared to that for controls that received only sterile 15‰ ASW. Experiments were performed in triplicate.

Production and resuscitation of *in vitro* VBNC cells. VBNC cultures were produced as previously described (18). Briefly, *V. vulnificus* was grown overnight in HI broth, added to fresh HI broth at a 1:100 (vol/vol) ratio, and grown to logarithmic phase (optical density at 610 nm [OD_{610}], 0.15 to 0.25). Cells were subsequently washed twice with 15‰ ASW to remove any nutrients, resuspended in 15‰ ASW at a 1:100 (vol/vol) ratio, and stored statically in a 4°C incubator. Cultures were quantified daily until *V. vulnificus* was no longer culturable on HI agar (<10 CFU/ml detectable).

To resuscitate VBNC cells, cultures were transferred from the 4°C incubator to room temperature (20°C). Cultures were immediately supplemented with nutrient-free CFS (collected as described above) from either *V. vulnificus*, *V. parahaemolyticus*, or *E. coli*, which are known to produce AI-2, at a 1:100 (vol/vol) ratio, or they were supplemented with 15‰ ASW at a 1:100 (vol/vol) ratio as a control. Additionally, we utilized nutrient-free CFS from a *V. vulnificus luxS* mutant strain (JDO1) unable to produce AI-2. These mixtures were serially diluted in PBS and plated at regular intervals onto HI agar to monitor resuscitation. Resuscitation curves were compared to those for control resuscitation cultures that received ASW. Three biological replicates were used for each treatment.

Use of synthetic autoinducer to induce resuscitation. VBNC cultures were prepared as described above. The synthetic autoinducer (*s*)-4,5-dihydroxy-2,3-pentanedione (DPD) (OMM Scientific, Dallas, TX) spontaneously forms the quorum sensing molecule AI-2 in liquid suspension (31). AI-2 was prepared by adding a 10-fold molar excess of boric acid to DPD, making a 50 μM DPD stock solution. To resuscitate VBNC cells, dormant cultures of C7184, JDO1, and AH1 were moved to 20°C, and AI-2 was immediately added to each culture, to a final concentration of 250 nM. Cultures were serially diluted and plated onto HI agar at regular intervals to monitor resuscitation. Resuscitation times were compared to those for control resuscitation cultures that received only ASW. Experiments were performed in triplicate.

Use of quorum sensing inhibitor to prevent resuscitation. Cinnamaldehyde is a quorum sensing inhibitor that has been shown to prevent binding of LuxR, the master regulator of quorum sensing, to DNA (32). We used a cinnamaldehyde concentration of 150 μM, which is noninhibitory to growth, as previously reported (32). Cinnamaldehyde-supplemented VBNC cultures (prepared as described above) were allowed to resuscitate by incubation at 20°C. Cultures were serially diluted and plated onto HI agar at regular intervals to monitor resuscitation. Resuscitation curves were compared to those for control resuscitation cultures that received an equal volume of ASW. Experiments were performed in triplicate.

Bioluminescence-based quorum sensing assay. The bioluminescence-based quorum sensing assay was performed as previously described (33), with slight modifications. Briefly, *Vibrio harveyi* strain BB170, which is bioluminescent only in response to AI-2 signals, was grown overnight on AB agar at 30°C. Cells were then removed using a sterile swab, diluted in AB broth to an OD_{610} of 0.7 to 0.8, and then incubated with shaking at 30°C until the OD_{610} reached 1.1. A 1:5,000 dilution of this culture in fresh AB broth was prepared to create the working solution. Nutrient-free CFS was added to the working solution at a 1:10 (vol/vol) ratio for a total of 1.5 ml and was incubated at 30°C with shaking for 4 h. After incubation, 200 μl of sample was transferred to a flat-bottom 96-well plate and read using a GloMax 96 microplate luminometer (Promega, Madison, WI). Background luminescence was assayed by quantifying control samples that contained 15‰ ASW and the working solution of BB170 at a 1:10 (vol/vol) ratio for a total of 1.5 ml. Data are presented as sample luminescence levels (arbitrary units) with the average background luminescence subtracted. Experiments were performed in triplicate.

Validation of resuscitation. To validate that during *in vitro* resuscitation experiments cells were truly resuscitating rather than growing from a small, undetectable population, theoretical generation times for resuscitating cells were calculated using equation 1:

$$\text{Theoretical generation time (min)} = \frac{60}{\left[\frac{log(C_f) - log(C_i)}{log2}\right]} \quad (1)$$

where C_i and C_f are the starting and ending CFU values, respectively, for

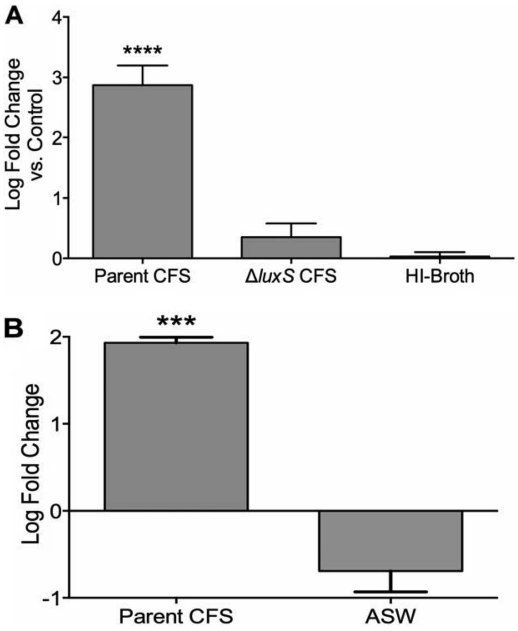

FIG 1 Increase in the culturability of *Vibrio* spp. in oysters and seawater upon addition of CFS. (A) Log fold change in culturability of *Vibrio* spp. in oyster tissues after 24 h of treatment with CFS from *V. vulnificus* C7184 (parent) or JDO1 (*luxS* mutant) relative to that of control oysters that received artificial seawater (ASW). Wild-type CFS significantly increased the culturability of *Vibrio* spp. in oysters (one-way ANOVA; $P < 0.0001$), whereas CFS from the *luxS* mutant did not. Since HI broth had little effect on culturability, nutrient addition did not affect the culturability of *Vibrio* spp. Error bars represent SD for 4 oysters. (B) Log fold change in levels of *Vibrio* spp. in NSW after 24 h (relative to that at 0 h) of treatment with either CFS from *V. vulnificus* C7184 or the ASW control (unpaired *t* test; $P = 0.0004$). Error bars represent SD for 3 NSW samples.

the 1-h time interval being analyzed. This method of validating resuscitation has been utilized by others (18, 20, 34) to demonstrate that the observed increase in culturability is indeed due to resuscitation rather than regrowth of a small culturable population.

Statistical analysis. Data were analyzed using unpaired Student's *t* test, or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons. Statistical significance was determined using an alpha level of 0.05. Error bars in all figures represent standard deviations (SD). All data were analyzed using GraphPad Prism (version 6.0; GraphPad Software Inc.).

RESULTS AND DISCUSSION

Addition of CFS increases culturability of vibrios in oysters and seawater. We showed previously that the addition of exogenous bacteria to oysters dramatically increased the culturability of the resident *Vibrio* spp. in oyster tissues, likely through the resuscitation of a dormant population (19). To evaluate whether this increase was in response to extracellular factors, CFS collected from *V. vulnificus* (C7184) was added to oysters, and the culturability of *Vibrio* spp. present among the members of the oyster microflora was monitored. We found that upon addition of CFS from AI-2-producing *V. vulnificus* cells to live oysters, the culturability of *Vibrio* spp. within these oysters increased significantly ($P < 0.0001$), indicating that an extracellular factor promoted *in vivo* resuscitation of *Vibrio* spp. (Fig. 1A). In contrast, the addition of nutrients (HI broth) to oysters did not result in a considerable increase in culturability (Fig. 1A). We also observed that a quorum sensing mutant lacking the ability to produce AI-2 was unable to produce the same effect (Fig. 1A), suggesting that the extracellular resuscitation-promoting factor was AI-2. A similar increase in culturability was observed when CFS was added to natural seawater (NSW) (Fig. 1B). These results provide evidence for the hypothesis that resuscitation of *V. vulnificus* from the VBNC state is a quorum sensing-related phenomenon.

Addition of CFS or AI-2 induces early resuscitation of *in vitro* VBNC cultures. If AI-2 mediates the resuscitation of VBNC cells present within oysters and seawater, then the addition of AI-2 to *in vitro* VBNC cultures would also be expected to induce early resuscitation. *V. vulnificus* can normally be resuscitated within 7 to 9 h after a temperature upshift from 4°C to 20°C (18). Therefore, we hypothesized that the addition of AI-2 to VBNC cultures immediately after a temperature upshift would cause resuscitation to occur much earlier. The addition of CFS from wild-type AI-2-producing *V. vulnificus*, *V. parahaemolyticus*, and *E. coli* bacteria induced resuscitation of VBNC cells in as little as 3 h, whereas CFS from a *V. vulnificus luxS* mutant deficient in AI-2 production was unable to induce a faster resuscitation than that of the control (~8 h) (Fig. 2A). Since CFS is composed of various components aside from AI-2, we tested the ability of synthetic AI-2 to induce resuscitation. Synthetic AI-2 was also able to induce early resuscitation of VBNC cells (Fig. 2A), indicating that AI-2 alone is able to induce the reactivation of dormant *V. vulnificus* cells during a temperature upshift.

Quorum sensing inhibitor delays resuscitation of *in vitro* VBNC cultures. To test whether the action of AI-2-mediated resuscitation was through the master quorum sensing regulator, SmcR (a LuxR homolog) (35), we employed an inhibitor of LuxR, hypothesizing that this would inhibit the resuscitation of VBNC cells. The LuxR inhibitor, cinnamaldehyde, was able to delay resuscitation by at least 40 h (Fig. 2B). This indicates that the resuscitation-promoting ability of autoinducers is effective, at least partially, through the quorum sensing regulatory pathways and that the inhibition of quorum sensing can hinder the resuscitation process.

Validation of resuscitation. To validate that the observed increase in culturability was in fact resuscitation rather than a regrowth of a small population of undetectable cells, we calculated the theoretical generation times required to yield such an increase if the cells were actually growing rather than resuscitating. In all cases, the generation times required to produce the observed increases in culturability were 13.9 min or less (Table 1). We determined *V. vulnificus* to have a generation time of 30.7 ± 0.17 min during logarithmic growth under nutrient-rich conditions (HI broth); thus, it is nearly impossible for this species to divide at a higher rate under the nutrient-depleted (ASW), nonaerated, room temperature conditions employed in our resuscitation studies. These resuscitation rates are much higher than normal rates of division would allow and therefore support our claim that these cells were truly resuscitating rather than growing from a small number of culturable cells.

Resuscitation occurs in response to increased AI-2. Since VBNC cultures artificially supplemented with autoinducers promoted resuscitation, it was speculated that this AI-2-mediated process might also occur naturally during a temperature upshift. To test this, the production of AI-2 and the culturability of cells during resuscitation from the VBNC state following a temperature upshift were monitored simultaneously. During resuscita-

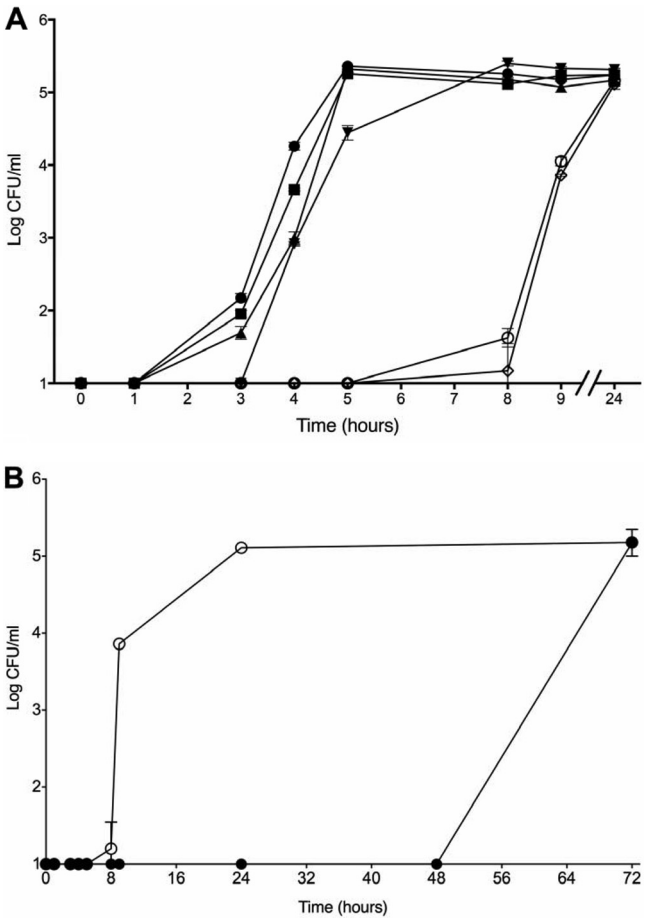

FIG 2 Resuscitation of VBNC *V. vulnificus* supplemented with CFS, synthetic AI-2, or cinnamaldehyde. (A) Culturability of *V. vulnificus* after temperature upshift and supplementation with CFS from *V. vulnificus* C7184 (parent; closed circles), JDO1 (*luxS* mutant; open circles), *V. parahaemolyticus* (closed squares), or *E. coli* (closed triangles) or with synthetic AI-2 (closed downward-facing triangles). Control cultures (open diamonds) received ASW. All wild-type CFS and synthetic AI-2 induced early resuscitation of VBNC cells, while CFS from the *luxS* mutant did not. Error bars represent SD for 3 replicates. (B) Culturability of *V. vulnificus* after a temperature upshift, with (closed circles) and without (open circles) 150 μM cinnamaldehyde. Error bars represent SD for 3 replicates.

tion, there was a rapid increase in AI-2 levels, reaching a maximum value 5 h after temperature upshift, even though initiation of culturability was not detected until ca. 7 h (Fig. 3). Interestingly, in repeated studies, resuscitation was detected only after AI-2

TABLE 1 Theoretical generation times required for the observed increase in culturability during *Vibrio vulnificus* resuscitation

Treatment	Theoretical generation time (min)
Vibrio vulnificus CFS	8.66
Vibrio parahaemolyticus CFS	10.57
Escherichia coli CFS	13.88
Vibrio vulnificus JDO1 CFS	7.48
Synthetic AI-2	11.77
Control[a] (no CFS)	7.01

[a] Untreated VBNC *Vibrio vulnificus* resuscitation.

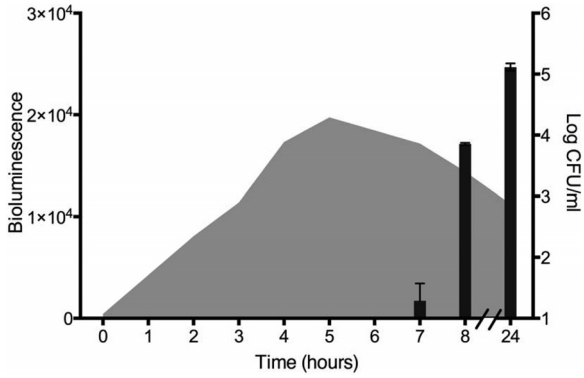

FIG 3 Production of AI-2 during temperature upshift-induced resuscitation from the VBNC state. The data depicted show the culturability of *V. vulnificus* cells (black bars) as they resuscitated from the VBNC state, with corresponding AI-2 levels (gray curve) in these cultures as measured by the AI-2 bioluminescence assay.

reached such a maximum, independent of the temporal occurrence of the maximum, suggesting that AI-2 levels must reach a threshold value before resuscitation can begin. It is interesting that AI-2 levels were able to increase before culturability could be detected following the temperature upshift. We postulate that either VBNC cells are able to produce AI-2 before they become culturable or a small, undetectable population signals to the dormant population to initiate resuscitation. A recent study by Buerger et al. (36) provided evidence for Epstein's (37) microbial scout hypothesis, which proposes that a small portion of a dormant population resuscitates stochastically (becomes "scouts"), independent of environmental signals. If environmental conditions are not permissive, these scouts are thought to die. However, if acceptable environmental conditions are sensed, then these scouts may signal to their dormant counterparts that growth-permissive conditions have been met (36). We propose that, at least for *V. vulnificus*, and likely other bacteria, quorum sensing autoinducers are the signals employed by the scouts to rouse the rest of the dormant population. This hypothesis is substantiated by our observed initial increase in AI-2 production without detectable culturability, indicating that scout cells, present at levels below our detection limits, had stochastically resuscitated and were signaling to the rest of the population, via quorum sensing, that acquiescent conditions had been reached. Since our studies have shown that resuscitation in response to autoinducers is not species specific, active cells may function as scouts for a dormant population of a different genus or species through AI-2 signaling. To confirm that resuscitation during a temperature upshift occurs in response to AI-2, the ability of VBNC cultures of the *V. vulnificus luxS* mutant to resuscitate was examined. The mutant was unable to resuscitate from the VBNC state during a temperature upshift unless the culture was supplemented with exogenous AI-2, again indicating that AI-2 is required for resuscitation to occur in *V. vulnificus* (Fig. 4A).

Resuscitation mechanism. It has previously been demonstrated that *V. vulnificus* cells in the VBNC state are nonculturable on laboratory media largely due to reduced catalase activity. This sensitizes the cells to the presence of reactive oxygen species and therefore prevents replication of these cells on media that routinely contain H_2O_2 (38). Furthermore, catalase production resumes upon resuscitation from the VBNC state, allowing renewed

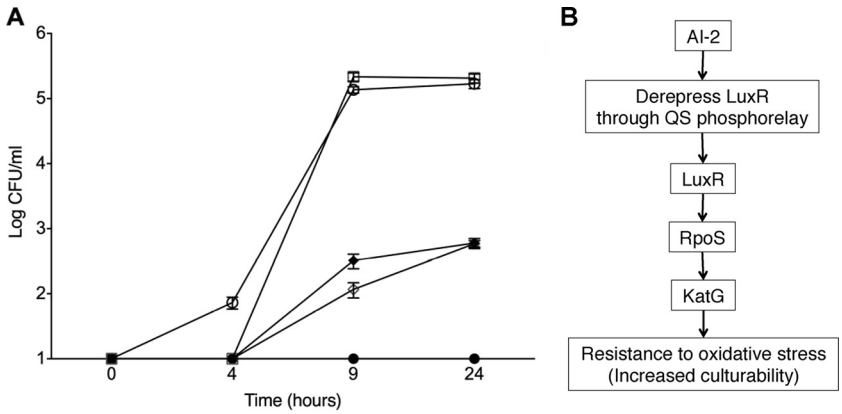

FIG 4 Resuscitation of *V. vulnificus luxS* and *rpoS* mutants during temperature upshift. (A) Culturability of *luxS* mutant (JDO1; closed circles), *rpoS* mutant (AH1; closed diamonds), and parent strain (C7184; open squares) after a resuscitation-inducing temperature upshift. JDO1 was unable to resuscitate within 24 h unless it was supplemented with exogenous AI-2 (open circles), indicating the requirement of AI-2 for the resuscitation of VBNC *V. vulnificus*. AH1 displayed a delayed ability to resuscitate, likely due to decreased survival at 4°C. Importantly, resuscitation of AH1 was not affected by the addition of AI-2 (open diamonds), indicating the importance of RpoS for AI-2-mediated resuscitation. (B) Proposed mechanism of resuscitation. An increase in AI-2 signaling causes the derepression of LuxR, a positive regulator of RpoS. Increased RpoS activity leads to an increase in production of catalase (KatG), which allows cells to be culturable on routine media containing H_2O_2.

culturability (39). The stress-related alternate sigma factor RpoS is known to be necessary for the production of catalase (40) and to play an important role in resuscitation from the VBNC state (41). Furthermore, Joelsson et al. (42) reported that LuxR enhances the expression of *rpoS*. Another study found that expression of *rpoS* significantly increased during resuscitation from the VBNC state (43). Here we found that a *V. vulnificus rpoS* mutant had a restricted ability to resuscitate within 24 h. Importantly, the addition of AI-2 to a VBNC culture of the *rpoS* mutant did not induce early resuscitation (Fig. 4A) like that observed with VBNC cells of the wild-type parent. This finding indicates that RpoS is an important factor in AI-2-mediated resuscitation. Combining the existing literature with our current findings, we propose a model of resuscitation in which increased AI-2 signaling stimulates expression of *rpoS* through the action of LuxR. This subsequently induces the expression of catalase (*katG*), thereby allowing cells to overcome the toxic properties of H_2O_2 in bacterial media and to return to a culturable state (Fig. 4B). However, it cannot be excluded that quorum sensing has an effect on other processes related to dormancy, such as toxin-antitoxin regulation, which has been studied extensively in persister cell formation, another type of bacterial dormancy (44).

Conclusions. A previous study demonstrated that artificially supplementing VBNC cells with quorum sensing autoinducers can resuscitate dormant *Vibrio cholerae* cells to improve their detectability (20). Here we provide evidence that interspecific quorum sensing autoinducers have the capability of resuscitating dormant bacterial populations both *in vivo* and *in vitro*. For the first time, we have elucidated the active interplay between autoinducer levels and VBNC dynamics in *V. vulnificus*.

Specifically, we found that VBNC cells resuscitate in response to a quorum sensing signal that is produced by cells within a dormant population when permissive conditions are reached. As proposed by Manina and McKinney (45), with the advent of real-time single-cell analysis technology, the role of quorum sensing in the resuscitation of dormant microorganisms may be studied with much greater resolution. This study may provide important insights for clinical intervention and for the development of innovative antimicrobial agents in the fight against drug-tolerant dormant cells. Overall, this study provides a greater understanding of the mechanisms employed by bacteria to exit from dormancy, which helps to explain the seasonality exhibited by bacterial populations in natural environments. These findings have important implications for microbial ecology and public health.

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Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

We declare no conflicts of interest for this article.

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BRIDGING THE GAP BETWEEN VIABLE BUT NON-CULTURABLE AND ANTIBIOTIC PERSISTENT BACTERIA

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Contributions by Tiffany C. Williams:

Literature review, wrote and edited parts of manuscript, and response to reviewer critiques.

Opinion

CellPress

Bridging the gap between viable but non-culturable and antibiotic persistent bacteria

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Microbial dormancy is a widespread phenomenon employed by bacteria to evade environmental threats including antibiotics. This intrinsic mechanism of antibiotic tolerance has drawn special attention to the role of dormancy in human disease, particularly in regards to recurrent infections. Two dormancy states, the viable but non-culturable state and bacterial persistence, both produce antibiotic-tolerant populations capable of withstanding prolonged lethal treatment. Currently described as two distinct forms of dormancy, they are rarely discussed in the same context. We argue here that these two dormant states are closely related phenomena which are part of a shared 'dormancy continuum'. This discussion is intended to stimulate discourse about these seemingly different but very similar dormant states.

Role of dormancy in antibiotic failure

The antibiotic resistance crisis has become a worldwide threat and is leading us into a 'post-antibiotic era' where common infections may lead to life-threatening diseases (http://www.who.int/drugresistance). The pervasive use of antibiotics has contributed to this problem since the first reported appearance of antibiotic resistance in 1940 [1]. However, a less conspicuous and possibly even more widespread phenomenon leading to antibiotic failure is bacterial dormancy (see Glossary).

In nature, microorganisms are threatened by environmental instability and must employ strategies that allow tolerance of harmful conditions that are detrimental to growth [2]. To cope with environmental stressors, many bacteria enter a transient state of dormancy that is characterized by a decrease in growth rate and metabolic function [3]. Dormant subpopulations can later resuscitate when the stress is alleviated or when cells encounter a signal indicating favorable environmental conditions [4,5]. Although this bet-hedging strategy has likely evolved for survival in uncertain environments, it is increasingly evident that dormancy plays a significant role in disease.

Entry into dormancy grants pathogens the ability to tolerate antibiotic treatment [6–8], which has been linked to recurrent infections caused by several bacteria [9–11]. In addition, because dormant cells are slow or non-growing, many are undetected by culture-based methods and thus pose a significant public health hazard [12]. The finding that dormant cells can resuscitate within a host and retain their virulence properties further corroborates this threat [13–16].

Two dormancy phenomena have been described in non-sporulating bacteria – the viable but non-culturable (VBNC) state and bacterial persistence. These two modes of existence are typically described as being distinct and are rarely discussed in the same context ([17,18] for up-to-date reviews). In the following discussion we compare the phenotypic characteristics and molecular foundations of these dormancy states and, based on the evidence, argue that these two states are not distinct but are closely related phenomena that are part of a shared 'dormancy continuum'. This simple connection opens up a great deal of previously overlooked research on both phenomena, allowing researchers to better understand bacterial dormancy with the goal of discovering superior methods of eradicating these multidrug-tolerant cells.

Dormancy in non-sporulating microbes

First described by Joseph Bigger [19], persister cells make up a multidrug-tolerant subpopulation within a growing culture. As opposed to antibiotic resistance, where a genetic factor leads to resistance to a particular antibiotic, persister cells are genetically identical to the non-persister population but exhibit a drug-tolerant phenotype [20]. Although persisters are viable, most studies have shown that they are in a non-growing state [21,22]. Notably, recent studies have

Glossary

Culturability: the ability of cells to form colonies after cells have been taken from a stressful environment and plated onto routine laboratory media.
Dormancy: significant reduction (but not complete loss) of metabolic activity.
Growth: the terms growth, growing, and non-growing refer here to the ability of a cell to divide while being subjected to a stress (antibiotics, temperature shock, etc.).
Persister: the multidrug-tolerant portion of a population of cells that gives rise to a susceptible population upon removal of antibiotics.
Viable but non-culturable (VBNC): refers to normally culturable cells that have lost their ability to grow on media but remain viable.

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found that persistence may be more complex than the simple lack of growth [23] because some mycobacteria persisters have been shown to grow within macrophages in the presence of Isoniazid antibiotic [24]. Recently, innovative methods for studying persisters at the single-cell level have led to some of the most important findings outlining the mechanisms underlying persistence [22]. These mechanisms are discussed and related to the VBNC state in more detail below.

The VBNC state was first described by Colwell and colleagues in 1982 [25]. It was found that *Escherichia coli* and *Vibrio cholerae* cells subjected to incubation in saltwater exhibited a decline in culturability, but a portion of the non-culturable population remained viable. Since then this dormancy state has been described in at least 85 species of bacteria, and the use of molecular techniques to assess metabolic activity and gene expression in VBNC cells has led to its acceptance as an important survival strategy against unfavorable environmental conditions [18]. As with persisters, VBNC cells are thought to be genetically identical to the rest of the population, exhibit a stress-tolerant phenotype, and to be in a non-growing state. Although the molecular mechanism for induction into the VBNC state is not fully understood, several genes shown to be important in the VBNC state have also been found to play integral roles in bacterial persistence.

In the following sections we relate these allegedly different dormancy states by describing their shared phenotypic characteristics and molecular mechanisms in more detail, and by providing published, but largely overlooked, evidence for their being part of the same dormancy phenomenon.

Shared phenotypic characteristics

Although discovered and studied independently, these dormancy phenomena share several characteristics that demonstrate their relatedness. We discuss here these important overlapping features and describe why such similarities lead to the idea that these dormant states are somehow related.

Antibiotic tolerance

Persister cells are antibiotic-tolerant by definition because they are isolated by treating cultures with high-dose antibiotics [20]. Owing to their antibiotic tolerance, it is thought that persisters may be a significant contributor to recurrent infections [26] such as those caused by *Mycobacterium tuberculosis*, *Salmonella*, *Chlamydia*, and pathogenic *E. coli*. The discovery of high-persistence mutants in cystic fibrosis patients undergoing repeated antibiotic therapy provided a stronger link between persisters and the recalcitrance of particular infections [11].

Recently, Nowakowska and Oliver [6] reported that VBNC cells of the human pathogen, *Vibrio vulnificus*, were resistant to killing by antibiotics, among other stresses (including heat, ethanol, acid, peroxide, and heavy metals). Moreover, it was shown that *E. coli* VBNC cells were present in a mouse urinary tract infection model after treatment with antibiotics, and that these cells resuscitated *in vivo* after antibiotic treatment ceased [16]. The recrudescence seen in *Helicobacter pylori* infections is thought to be a similar consequence of the VBNC state [8]. Others have shown that VBNC cells of *Vibrio parahaemolyticus, Vibrio alginolyticus, V. vulnificus*, and *Campylobacter jejuni* can resuscitate *in vivo* and retain virulence [13–15]. Furthermore, Lleo *et al.* [27] reported that 14–27% of culture-negative infections (i.e., infections in which bacteria were non-recoverable on media) were positive for pathogenic bacteria using PCR detection, demonstrating the clinical relevance of non-culturable bacteria. Together, these findings strongly suggest that VBNC cells, together with persisters, are likely to be a substantial source of antibiotic failure and persistent infections. However, this shared characteristic alone does not necessarily mean that these phenomena are related.

Formation of persisters and VBNC cells

Although persisters are thought to form stochastically as a result of fluctuations in gene expression [22], studies show that persistence is also induced by a variety of factors including starvation, carbon-source transitions, macrophages, indole signaling, oxidative stress, DNA damage, pH, and antibiotics [22,28–33]. Given that a variety of stresses induce persistence, it is likely that this capability has evolved as a general environmental stress response rather than for antibiotic tolerance alone. Similarly, it is well accepted that the VBNC state allows a subpopulation of cells to survive environmental stress. In fact, the VBNC state is commonly induced in a wide range of bacteria using stresses such as starvation, growth-inhibiting temperatures, low oxygen, non-optimal salinity, and stressful pH [4,34–37]. The fact that persistence and the VBNC state are induced by similar environmental stresses reinforces their relatedness.

Although VBNC and persistence states are induced by environmental stress, their existence in actively growing cultures has been demonstrated, indicating that both are likely formed stochastically. A recent study reported that a culture of *E. coli* in logarithmic phase contains ~100-fold more VBNC cells than persisters [38]. While this challenges the classical view that the VBNC state is induced by a stress, it supports the idea that both VBNC and persister cells are part of a bet-hedging strategy and are present even in ideal conditions as an 'insurance policy' against unanticipated encounters with lethal conditions. In light of these findings, it is possible that persistence is a transitory state leading to the VBNC state. This would cause an accumulation of VBNC cells with the presence of a small number of transitory persisters, as was found by Orman and Brynildsen [38].

Bacteria found in biofilms are particularly much more resistant to antibiotics than their planktonic counterparts [39]. This is likely due to the presence of stressful microenvironments that give rise to phenotypic heterogeneity within biofilms [40]. Environments deep inside biofilms tend to have less oxygen and nutrients, and they are more acidic owing to metabolic waste products [40]. Because these same stressors have individually been shown to induce both the VBNC and persister states, it is conceivable microenvironments within a biofilm could promote the formation of both antibiotic-tolerant persister and VBNC cells, possibly in a stratified manner. Figure 1 depicts the

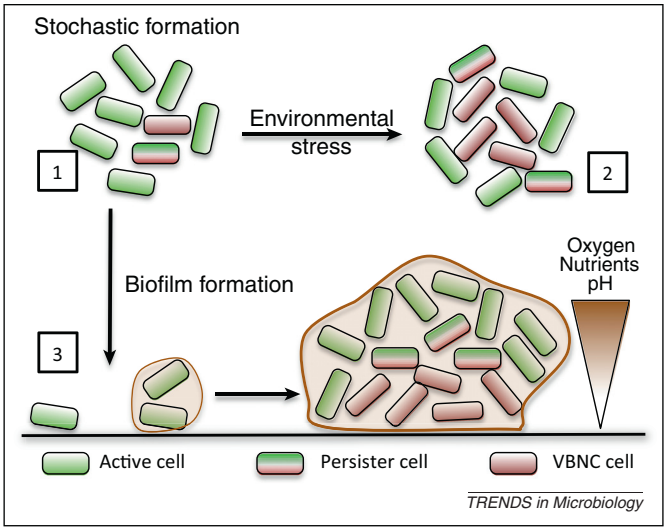

Figure 1. Factors stimulating VBNC cell and persister formation. (1) Persister and VBNC cells form stochastically owing to random fluctuations in gene expression. (2) Environmental stressors such as nutrient deprivation, low/high temperature, low/high pH changes, and oxidative stress induce cells to become VBNC or persistent. (3) Microenvironments within mature biofilms lead to phenotypic heterogeneity, which includes the formation of VBNC cells and persisters in areas of a biofilm that are most stressful (low oxygen, nutrients, high concentration of waste).

factors that stimulate the formation of VBNC and persister cells. These concepts warrant in-depth research into the role of biofilms on the VBNC state and persistence because biofilms play a significant role in the ecology and sometimes pathogenesis of many bacterial species.

For dormancy to be an effective survival strategy, cells must be able to recover in a process known as resuscitation. In the VBNC state, resuscitation is revealed when the inducing stress is removed and culturability is regained. A classic example can be seen in *V. vulnificus*, in which one environmental stress (low temperature) can induce a large population of cells to become VBNC, with simple reversal of the stress yielding resuscitation of anywhere between 0.1 and 100% of cells. For some species the formation and resuscitation of VBNC cells can be complex, with significant variations in the factors that induce and promote resuscitation from the VBNC state [18]. It is also important to recognize that, for some species shown to enter VBNC, the resuscitating promoting factors have not yet been identified [18]. For example, *Legionella pneumophila* requires a very specific condition (incubation with amoebae) to resuscitate [41].

Resuscitation in persisters is observed when the antibiotics are removed, allowing cells to regain the ability to form colonies on media. It is also hypothesized that the slow and steady decline in persister cells during long-term antibiotic treatment is indicative of persister resuscitation. A standard persister experiment produces the biphasic killing curve depicted in Figure 2A when a culture is exposed to antibiotics. The slope of the second phase of death reveals slower killing of persisters, and is indicative of cells that stochastically revert to a growing state (resuscitating) in the presence of antibiotics and which are therefore killed [17]. Interestingly, assessing the viability of cells during induction of the VBNC state produces a similar killing curve (Figure 2B). In addition, resuscitation from the VBNC state may involve a similar stochastic switch [5], a phenomenon known as the 'scout hypothesis' [42].

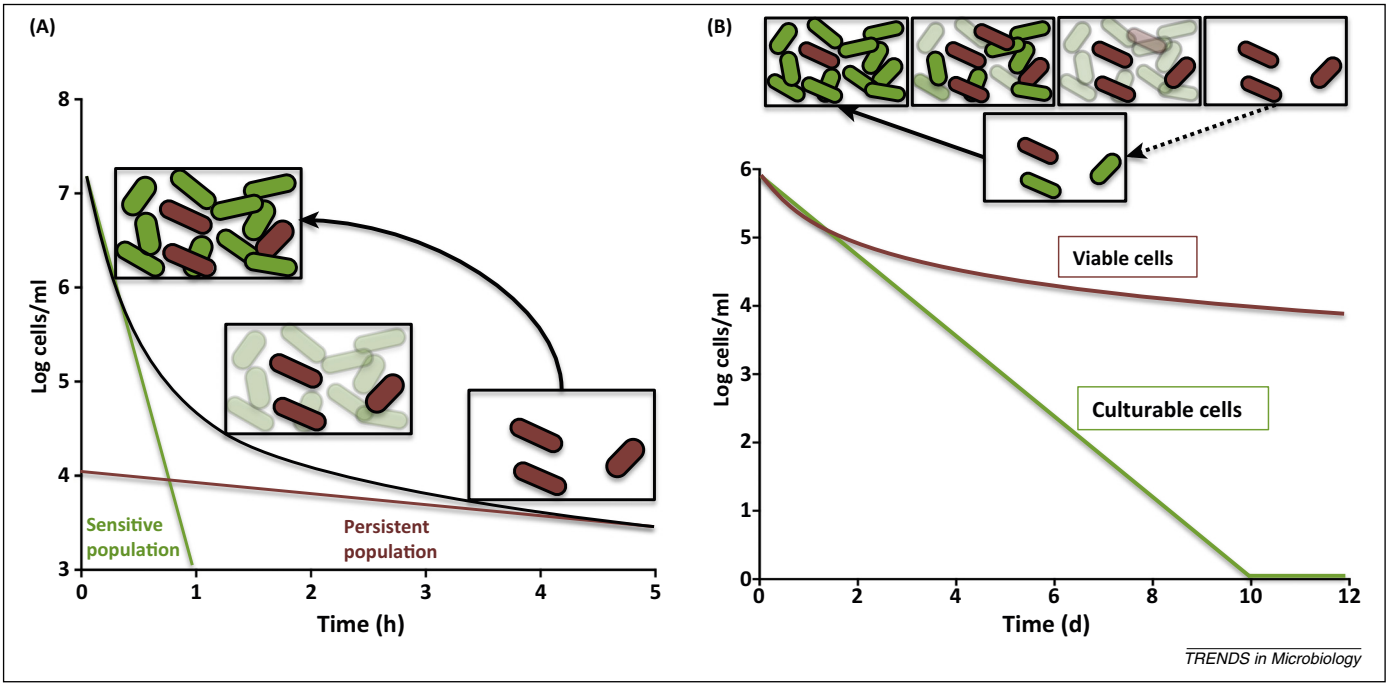

Figure 2. Dormancy dynamics of bacterial persistence and the VBNC state. **(A)** Persister cells are isolated by applying a lethal dose of antibiotics to a growing culture. This produces a characteristic biphasic killing curve in which the slope of the initial phase of killing (green line) represents the rapid death of the sensitive population and the slope of the second phase (red line) represents the much slower death of persisters. After removal of antibiotic (black arrow), persisters can regrow and give rise to antibiotic-sensitive cells that are genetically identical to the original population. Redrawn from [17]. **(B)** VBNC cells are isolated by applying a lethal stress (e.g., cold temperature) to a growing culture. During this process, cells become undetectable on nutrient media (green line); however, a proportion of the population remains viable (red curve) as can be determined by a variety of viability assays. When the inducing stress is removed the population becomes culturable after a resuscitation period (broken arrow). When cells are provided with nutrients they give rise to a population that is similarly tolerant to the inducing stress as the original population (unbroken arrow). Redrawn from [68].

Box 1. Observations implicating the persister formation mechanism in the VBNC state

(i) RelA
- *relA is* upregulated in VBNC *Vibrio cholerae* [42] (Figure I).

(ii) (p)ppGpp
- *Escherichia coli* mutants unable to produce (p)ppGpp are less likely to enter the VBNC state and subsequently resuscitate [43].

(iii) PPK
- PPK mutant *Campylobacter jejuni* exhibits decreased ability to enter the VBNC state [44].

(iv) Clp protease
- ClpP protein accumulates in the VBNC state of *Legionella pneumophila* [51].
- Deletion of C-terminal domain of ClpX in *S. enterica* delays entry into the VBNC state [52].

(v) Toxin–antitoxin
- HipB (antitoxin) mutant *E. coli* was non-culturable [53].
- Induction of RelE and ChpAK (MazF) toxins of *E. coli* produces cells that are VBNC [57].
- Ectopic expression of HigB toxin in *V. cholerae* produces VBNC cells [58].
- Overexpression of HipA toxin induces the VBNC state in *E. coli*. [59].
- Overexpression of VapC toxin in *Mycobacterium smegmatis* induces the VBNC state [60].

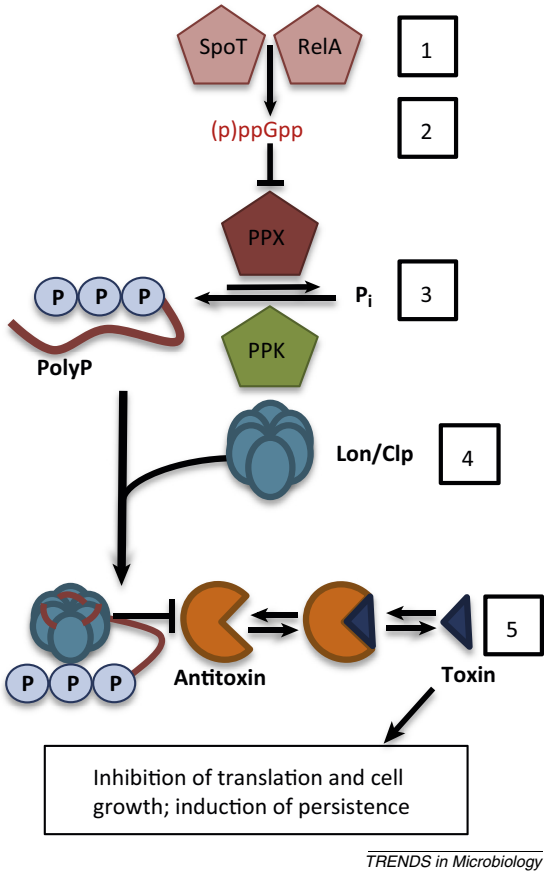

Figure I. Persister formation mechanism. Activation of RelA or SpoT causes an increase in intracellular (p)ppGpp levels, resulting in the inhibition of exopolyphosphatase (PPX), the enzyme that degrades PolyP. This results in accumulation of PolyP as a result of the constitutive activity of polyphosphate kinase (PPK). Lon protease is activated by PolyP and begins to degrade antitoxins. Free toxins inhibit translation and cause cells to enter growth arrest and persistence. The numbers in the box correspond to numbered processes in the figure. Mechanism redrawn from [17].

When compared, it becomes evident that persistence and the VBNC state are phenotypically similar, suggesting that these states share common molecular mechanisms.

Shared molecular mechanisms

The presence of persisters in unstressed cultures led to the hypothesis that they form stochastically. First proposed by Lewis and colleagues, type II toxin–antitoxin (TA) systems were found to be important in persister cell dynamics [43]. More recently, Gerdes and colleagues proposed a molecular mechanism for stochastic persister formation through the action of TA modules [22]; this is outlined in Box 1, Figure I (Box 2 gives a concise explanation of TA modules). The model is based on the activation of proteases that degrade antitoxins. This causes the liberation of their cognate toxins, which inhibit translation and thereby induce persistence. In the following sections we discuss this process in more detail and present evidence that implicates the molecular mechanism of persister formation in the VBNC state (summarized in Box 1).

Stringent response

The stringent response protein RelA synthesizes (p)ppGpp (or alarmones) during amino acid starvation [44], a stress that is known to induce both persistence and the VBNC state. Interestingly, persisters possess very high levels of ppGpp [22]. Because ppGpp inhibits exopolyphosphatase (PPX) [45], the enzyme that degrades polyphosphates (polyP) [46], this results in accumulation of polyP through the constitutive activity of polyphosphate kinase (PPK) [47]. PolyP activates Lon protease, which controls the activity of TA modules by degrading antitoxins, thereby freeing toxins to induce persistence. Similarly, studies have found that *relA* is upregulated during the VBNC state in *V. cholerae* [48], indicating that ppGpp may be an inducer of this state. Likewise, it has been shown that *E. coli* mutants unable to produce ppGpp were less likely to enter the VBNC state, whereas cells overproducing ppGpp more efficiently became VBNC [49]. A similar study in *C. jejuni* found that PPK mutants, deficient in accumulating polyP, exhibited a decreased ability to become VBNC [50]. These findings support the importance of the stringent response in both dormancy states.

Intracellular proteases and dormancy

Lon protease degrades all known type II antitoxins of *E. coli* and is important in persistence [51,52]; however, the role of Lon in the VBNC state has not yet been investigated. Interestingly, Clp, a protease that may function in the induction of persistence [17], shares substrate recognition domains with Lon [53], indicating that they may degrade similar proteins. Likewise, Clp protease degrades the MazE, P1 Phd, and Kis antitoxins of *E. coli* [54,55] and all known antitoxins of *Staphylococcus aureus* [56]. Studies have also shown that Clp protease may be important in the VBNC state. A study on *Legionella pneumophila* reported that ClpP accumulated in VBNC cells [57]. Another study on *Salmonella enterica* reported that a ClpX mutant exhibited a reduced rate of VBNC cell formation indicating that, as in persistence, proteolysis is an important mediator of the VBNC state [58].

Box 2. Type II toxin–antitoxin (TA) modules

Since the discovery of high-persistence mutants that were found to possess nucleotide substitutions in the *hipA* gene (a type II toxin), many studies have investigated the roles of a variety of TA modules in bacterial persistence. Type II TA loci encode a toxin protein that is neutralized by a protein antitoxin by direct interaction. Under specific conditions, cellular proteases can be activated to degrade type II antitoxins and consequently alter the level of free toxin within a cell. Several toxins, including RelE, MazF, VapC, HipA, and Doc, inhibit translation through a variety of mechanisms including mRNA and tRNA cleavage. Other toxins, such as CcdB (a MazF homolog) and ParE (a RelE homolog), inhibit DNA gyrase, thereby inhibiting DNA replication. Because the targets of toxins are crucial for cell growth and division, TA modules are thought to control persistence.

One difference between VBNC cells and persisters is their detectability on nutrient media. Whereas persisters are able to grow on media shortly after the antibiotic treatment, VBNC cells require a relatively prolonged resuscitation-promoting treatment before they can be cultured [18]. It is likely that continued degradation of antitoxins by proteases causes cells to become persisters and eventually VBNC cells. In accordance with our model that VBNC cells are further along a shared dormancy continuum than are persisters, we propose that the T:A ratio in VBNC cells is even higher than the ratio in persisters and, therefore, VBNC cells are in a deeper state of dormancy. A summary of this hypothesis is depicted in Figure 3. In fact, a study by Balaban and colleagues [59] demonstrated that cells lose the ability to divide once the amount of free toxin within a cell reaches a threshold, and the amount of free toxin determines the time that cells require to be resuscitated to the culturable state. It is thus likely that there is a free toxin threshold causing persistence, and a higher threshold that renders cells non-culturable. Accordingly, the fact that VBNC cells take longer to resuscitate may be due to the extended time required to replenish antitoxins to sequester the free toxins.

Consistent with this hypothesis, Conlon *et al.* [60] 'eradicated' *Staphylococcus aureus* persisters using rifampicin and a compound labeled ADEP4, which activates ClpP and causes the degradation of over 400 proteins. Because ClpP has been implicated in the VBNC state and in the degradation of antitoxins, ADEP4 treatment may induce the VBNC state by increasing free toxin levels. Unfortunately, the researchers used colony-forming units (CFU) as an indicator of viability, which is fundamentally unsuitable for the detection of non-growing but viable bacteria such as VBNC cells. When studying the eradication of dormant bacteria, viability assays such as reverse transcriptase PCR, propidium monoazide-PCR, flow cytometry, or direct viable counts must be employed [61] (see [62] for recent review on detection of viability).

Role of TA modules

TA systems have been proposed to be the main inducers of bacterial persistence [17]. The overproduction of TA proteins has been shown to inhibit cell growth and to increase antibiotic tolerance [51,63]. Similarly, the simultaneous deletion of 10 TA loci cumulatively reduced persister levels [51]. Recently it was shown that TA loci were induced in single cells that were antibiotic-tolerant, consistent with persistence [22]. Thus, there is strong evidence indicating TA systems to be key mediators of persistence.

There is also evidence for TA systems controlling the VBNC state. Gerdes and colleagues found that induction of the RelE and ChpAK toxins of *E. coli* produced cells that were 'viable but unable to proliferate', consistent with the definition for the VBNC state [8,64]. The Gerdes group also showed that ectopic expression of HigB-1 and HigB-2 toxins of *V. cholerae* caused a proportion of the population to lose culturability [65]. Rotem *et al.* [59] reported that *E. coli* mutants that lacked the HipB antitoxin but retained a functional HipA toxin were unable to form colonies, indicating that they may have been VBNC. Another study

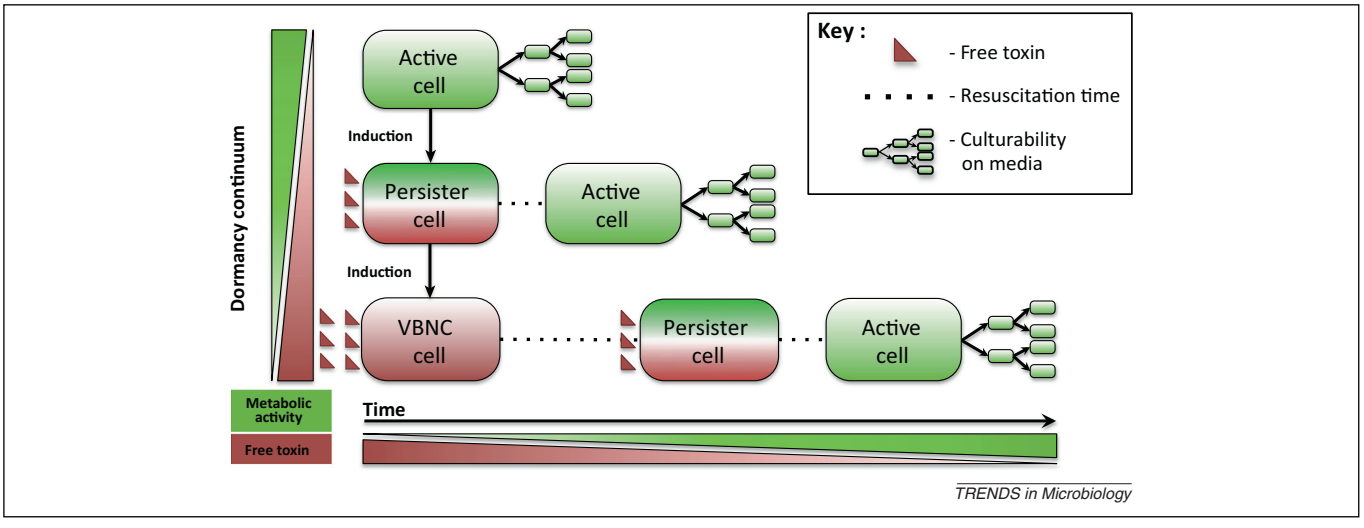

Figure 3. The microbial dormancy continuum hypothesis. Environmental stress induces cellular processes that lead to the degradation of antitoxins, causing the liberation of their cognate toxins (red triangles). This affects cellular metabolism and, consequently, growth. In the initial stages of dormancy, this produces antibiotic-tolerant non-growing cells (persister cells) that can quickly resuscitate upon removal of the stress. Resuscitated, or active cells, have relatively low free toxin levels and proliferate on media. However, if cells are exposed to prolonged stress, more free toxins accumulate, the extent of dormancy increases, metabolic activity further decreases, and cells require more time to resuscitate, rendering cells viable but nonculturable (VBNC cells). When VBNC cells are allowed to resuscitate after removal of the inducing stress, their free toxin levels slowly decrease while metabolic activity increases. At a particular free toxin threshold, cells are defined as persisters because they are non-growing, but can quickly resuscitate into active cells and become culturable on media.

Box 3. Outstanding questions

- Can evidence for overlapping VBNC and persister mechanisms be found in a standardized model?
- Do persisters become VBNC cells upon prolonged exposure to stress?
- In a characteristic persister experiment, is there a VBNC population present which is not detected?
- Do VBNC cells possess higher levels of free toxins compared to persisters?
- Is the VBNC state induced by a unique set of TA modules, or do VBNC cells employ the same toxins but require higher concentrations of free toxin?

found that overexpression of *hipA* in *E. coli* induced the VBNC state [66]. More recently it was found that overexpression of the VapC toxin caused *Mycobacterium smegmatis* to become VBNC, while overexpression of the VapB antitoxin prevented cells from entering the VBNC state [67]. These studies point to the importance of TA systems in the VBNC state and provide a strong connection between molecular mechanisms of persistence and the VBNC state.

Concluding remarks

There is compelling evidence that persistence and the VBNC state are intimately related phenomena. However, research in a standardized model must be conducted to further support these claims (Box 3). This is especially important because the evidence presented above is from a variety of species and may not apply to all bacteria that form persisters and VBNC cells. An attractive model for the simultaneous study of persistence and the VBNC state may be *V. vulnificus* given the ease with which VBNC and persister cells of this species can be induced and resuscitated, and in view of the extensive literature on the VBNC state in this pathogen [68]. In addition, efforts aimed at eradicating dormant cells should assay viability in conjunction with culturability, allowing the detection of VBNC and persister cells. The assumed dichotomy between persistence and the VBNC state, which is based on generalized definitions, has inhibited research on bacterial dormancy owing to the lack of recognition of important findings by those studying one or the other 'type' of dormancy. Because their physiologies are very similar, and appear to share molecular mechanisms, they should be studied in concert. In the effort to eradicate antibiotic-tolerant dormant cells to prevent recalcitrant infections, researchers must focus on cells ranging over the entire dormancy continuum. We hope that this discussion persuades those investigating both the VBNC state and persistence to consider both states and to overcome the stringency with which we have come to define these dormancy states.

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APPARENT LOSS OF *VIBRIO VULNIFICUS* FROM NORTH CAROLINA OYSTERS COINCIDES WITH A DROUGHT-INDUCED INCREASE IN SALINITY

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Contributions by Tiffany C. Williams:

Performed experiments and edited manuscript.

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Apparent Loss of *Vibrio vulnificus* from North Carolina Oysters Coincides with a Drought-Induced Increase in Salinity

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Despite years of successful isolation of *Vibrio vulnificus* from estuarine waters, beginning in 2007, it was extremely difficult to culture *V. vulnificus* from either North Carolina estuarine water or oyster samples. After employing culture-based methods as well as PCR and quantitative PCR for the detection of *V. vulnificus*, always with negative results, we concluded that this pathogen had become nearly undetectable in the North Carolina estuarine ecosystem. We ensured that the techniques were sound by seeding North Carolina oysters with *V. vulnificus* and performing the same tests as those previously conducted on unadulterated oysters. *V. vulnificus* was readily detected in the seeded oysters using both classes of methods. Furthermore, oysters were obtained from the Gulf of Mexico, and *V. vulnificus* was easily isolated, confirming that the methodology was sound but that the oysters and waters of North Carolina were lacking the *V. vulnificus* population studied for decades. Strikingly, the apparent loss of detectable *V. vulnificus* coincided with the most severe drought in the history of North Carolina. The drought continued until the end of 2009, with an elevated water column salinity being observed throughout this period and with *V. vulnificus* being nearly nonexistent. When salinities returned to normal after the drought abated in 2010, we were again able to routinely isolate *V. vulnificus* from the water column, although we were still unable to culture it from oysters. We suggest that the oysters were colonized with a more salt-tolerant bacterium during the drought, which displaced *V. vulnificus* and may be preventing recolonization.

Vibrio vulnificus is a halophilic bacterium capable of causing wound infections and fatal septicemia in humans (10, 24, 26). This organism is part of the normal flora in estuarine waters as well as in shellfish inhabiting those estuaries (26). Infections caused by *V. vulnificus* are the leading cause of seafood-borne deaths in the United States, typically resulting from the ingestion of oysters harboring the organism, and commonly result in primary septicemia with a fatality rate of >50%. Thus, *V. vulnificus* has the highest case fatality rate of any food-borne pathogen (2, 18, 25, 26).

The most important factors determining the *V. vulnificus* load in oysters are temperature and salinity. The temperature effect is easily seen in seasonal and experimental data (with temperatures of 13°C to 22°C being the most permissive to *V. vulnificus* survival). Salinity is also an important although less obvious factor affecting *V. vulnificus* levels (11, 13, 19, 20, 28, 30, 32).

Historically, *V. vulnificus* has been easily isolated from North Carolina and Gulf Coast estuarine waters and oysters (20, 27, 29, 30, 38–40), with plating onto selective media, such as cellobiose-polymyxin B-colistin (CPC) medium, with or without an enrichment step, being a commonly used procedure for the isolation of this organism from shellfish. Such *V. vulnificus*-specific media are used for primary isolation, but a confirmatory step employing molecular methods is typically used to verify the identification (8). However, beginning in the spring of 2007, colonies presumptively identified as *V. vulnificus* colonies on selective media could not be confirmed as this species. Coincidently, in 2007, North Carolina entered into the worst drought since recordkeeping began in 1895, significantly elevating estuarine salinity (22, 23). In the 6-year study that we report here, we describe the coincidence between extended extreme environmental changes and *V. vulnificus* oyster colonization and present a possible explanation for the continued lack of *V. vulnificus* isolation from North Carolina oysters despite a return of this species to North Carolina estuarine waters.

MATERIALS AND METHODS

Media. CPC$^+$ agar (a derivative of CPC agar) is both selective and differential for *V. vulnificus* (17, 39). Presumptive *V. vulnificus* colonies grown on CPC$^+$ medium were confirmed by subsequent PCR analysis, as described below (40). CHROMagar Vibrio (CHROMagar, Paris, France) is a chromogenic medium that distinguishes four *Vibrio* spp., including *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus* (7, 21, 43). As with CPC$^+$ medium, a confirmation step must be conducted to confirm the species of the isolates.

Oyster collection. More than 650 oysters (*Crassostrea virginica*) from several North Carolina sites were collected by hand from the intertidal zone between 2005 and 2010, with spring, summer, fall, and winter harvest dates. Oysters were either sampled at a laboratory near the collection site within 2 h of harvest or placed into coolers with ice packs and sampled within 6 h of collection. Oysters from a Gulf Coast site at Dauphin Island, AL, were shipped overnight with ice packs and sampled within 2 h of arrival.

Oyster sampling. Oyster tissue was aseptically removed and homogenized in 20‰ artificial seawater (ASW) at a 1:1 weight-to-volume ratio using sterile blender cups (Warring, Torrington, CT). After homogenization, samples were diluted in sterile phosphate-buffered saline (PBS) and spread onto both CPC$^+$ agar and CHROMagar Vibrio and incubated at 40°C and 37°C, respectively.

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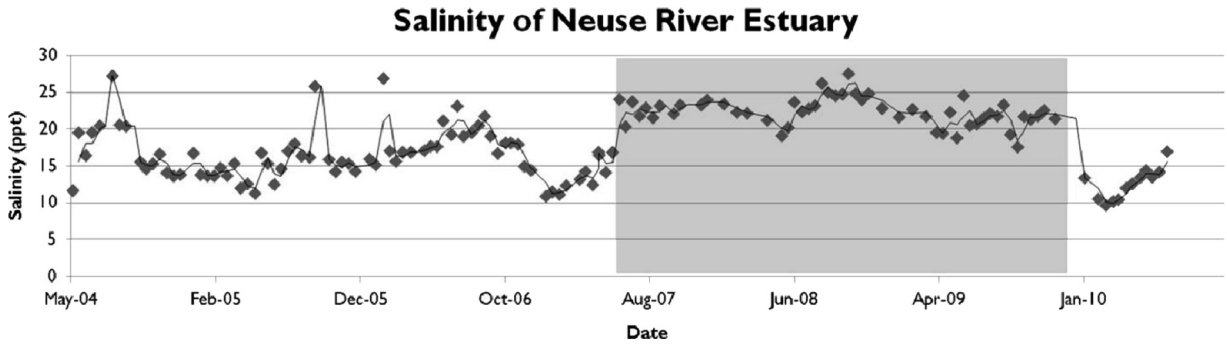

FIG 1 Biweekly salinity data from the Neuse River Estuary in North Carolina. The black line represents the monthly moving salinity average; the shaded area indicates the drought period.

Water sampling. Water samples (10 ml) were vacuum filtered onto 0.22-μm filters, which were aseptically placed onto the same media.

Species confirmation. Presumptive colonies were transferred onto heart infusion (HI) agar and grown overnight at 30°C. Each strain was subjected to PCR with primers amplifying the hemolysin gene *vvhA*, confirming the identification of the isolate as *V. vulnificus* (34, 40). Reactions were performed by using GoTaq polymerase (Promega, San Luis Obispo, CA) in a Techne Genius thermal cycler according to parameters suggested previously by Warner and Oliver (40). PCR products were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide.

Seeding of oysters with *V. vulnificus*. Oysters from the North Carolina coast were fed 24 h prior to being removed from maintenance tanks and placed into two separate tanks with artificial seawater with 20‰ salinity at 23°C. *V. vulnificus* cells (of the E genotype) were added to one tank at a final concentration of $\sim 10^4$ CFU per ml. Another tank served as a control (no *V. vulnificus* inoculum). Oysters were allowed to take up *V. vulnificus* cells for 24 h. Oysters from both tanks were then removed, shucked, and homogenized as described above.

PCR and quantitative PCR analyses of oyster homogenates. Diluted oyster homogenates (1 ml) from four control oysters and one artificially infected oyster were treated with the Wizard genomic DNA purification system (Promega), and isolated DNA was subjected to both PCR (described above) and quantitative PCR (Q-PCR). Q-PCR was performed by using the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) with a Lightcycler 2.0 instrument (Roche, Basel, Switzerland), employing E- and C-genotype-specific primers (40) in separate reaction mixtures. Reaction mixtures were heated to 95°C for 15 min to activate the polymerase. Reaction mixtures were then treated to 55 cycles consisting of denaturation at 94°C for 15 s, a 53°C annealing step for 30 s, and a 15-s extensions step at 72°C, followed by a single quantification read. Quantifications were calculated by using software bundled with the Lightcycler 2.0 instrument, using a standard curve generated by making seven 10-fold serial dilutions of target DNA.

Microbial identification. Colonies that presumptively appeared to be *V. vulnificus* positive on CPC$^+$ agar and/or CHROMagar Vibrio but which subsequently were confirmed not to be this species were subjected to genetic identification by PCR and by sequencing of the first 500 bp of the 16S rRNA gene (Accugenix, Newark, DE).

RESULTS

Sampling of North Carolina water and oysters. Estuarine salinity levels during the 2007-2009 drought (22.4‰ ± 1.9‰) were significantly higher ($P < 0.001$, determined by Student's *t* test) than those during nondrought periods (15.9‰ ± 3.5‰) (Fig. 1). Natural oyster samples plated onto CPC$^+$ medium resulted in 3,990 presumptive *V. vulnificus* isolates, which were subjected to PCR confirmation. In both 2005 and 2006, 40.7% of the presumptive isolates were positively confirmed to be isolates of this species, whereas in the drought period of 2007 to 2010, the rate of confirmation dropped to 0.7% or lower (Table 1). To ensure that the lack of *V. vulnificus* recovery was not due to deficiencies in the CPC$^+$ medium, we began utilizing an additional medium, CHROMagar Vibrio, in 2010 to collect bacteria from oyster samples. Only 4% of 456 presumptive colonies isolated on this medium were confirmed to be *V. vulnificus* isolates (Table 1). There was a highly significant difference between the number of presumptive isolates confirmed in drought years and the number confirmed in predrought years ($P < 0.001$ using chi-square analysis with a Yates correction for continuity). In addition, between 2006 and 2010, water samples were taken from the same North Carolina estuaries from which the oysters were harvested and plated onto CPC$^+$ agar. This generated a total of 2,404 presumptive *V. vulnificus* isolates. In 2006, before the drought began, 45.7% of isolates from these water samples were determined to be *V. vulnificus* isolates. In 2007, none of the isolates were confirmed to be *V. vulnificus* (although the sample size was small), and in 2008, only 2.4% were determined to be *V. vulnificus*. In the last part of 2009 and in 2010, after the drought period ended, the percentages of confirmed *V. vulnificus* isolates in water samples were 38.1% and 42.4%, respectively (Table 2), returning to predrought values.

Of the samples taken from oysters seeded with *V. vulnificus*, homogenized, and plated onto CPC$^+$ agar, 79 of 80 (99%) presumptive isolates were confirmed to be *V. vulnificus* isolates. Homogenates from oysters harvested from Gulf Coast waters between 2008 and 2010 and plated onto CPC$^+$ agar generated 131

TABLE 1 Presumptive *V. vulnificus* isolates, obtained from North Carolina oysters using either CPC$^+$ agar or CHROMagar Vibrio, confirmed as *V. vulnificus* following PCR analysis[a]

Isolation medium	Yr	No. of isolates tested	% confirmed *V. vulnificus* isolates
CPC$^+$ agar	2005	166	40.7
	2006	201	40.7
	2007	1,041	0.6
	2008	1,428	0.6
	2009	404	0.7
	2010	750	0.7
CHROMagar Vibrio	2010	456	4.0

[a] The shaded area indicates data from the drought period.

TABLE 2 Presumptive *V. vulnificus* isolates, obtained from North Carolina estuarine waters using CPC$^+$ agar, confirmed as *V. vulnificus* following PCR analysis[a]

Yr of isolation	No. of isolates tested	% confirmed *V. vulnificus* isolates
2006	138	45.7
2007	45	0
2008	245	2.4
2009 (September–December)	425	38.1
2010	1,551	42.4

[a] The shaded area indicates data from the period of drought.

TABLE 4 Molecular identification of false-positive isolates from CPC$^+$ agar and CHROMagar Vibrio using 16S rRNA gene sequencing[a]

Colony type	Sequence match (% alignment difference)
Presumptively positive on CPC$^+$ agar	*V. coralliilyticus* (1.3)
	V. nereis (1.4)
	V. tubiashii (1.7)
	V. sinaloensis (1.8)
Presumptively positive on CPC$^+$ agar and CHROMagar Vibrio	*V. mediterranei* (1.0)

[a] The species listed are hits from the proprietary Accugenix sequence library that aligned to our unknown sequences with less than a 2% difference.

presumptive *V. vulnificus* isolates, with an additional 10 isolates being obtained with CHROMagar Vibrio. While North Carolina oysters during this period yielded <1% confirmed *V. vulnificus* isolates, Gulf Coast isolates yielded >96% positive confirmation of *V. vulnificus* isolates (Table 3).

PCR and Q-PCR detection of *V. vulnificus* in oyster homogenates. Oysters (both natural and those seeded with *V. vulnificus*) were homogenized, and total DNA was extracted. PCR analysis of control oysters detected no *V. vulnificus* cells, while the spiked oyster homogenates produced PCR amplicons for the *V. vulnificus*-specific *vvhA* (hemolysin) and *vcgE* (virulence-correlated) genes (data not shown).

These same DNA extracts were also subjected to quantitative PCR analysis with primers specific for the E and C genotypes of *V. vulnificus* (40). The numbers of copies of the *V. vulnificus* C-genotype-specific gene (*vcgC*) were undetectable in all tested oysters (control and seeded). In contrast, the oysters seeded with E-genotype *V. vulnificus* cells contained enough *V. vulnificus* DNA to be detected by E-type-specific probes, yielding 1.3×10^4 copies per μl of concentrated sample (data not shown).

Sequence-based identification. Two false-positive (PCR-negative) isolates on CPC$^+$ agar were identified to the genus level based on 16S rRNA gene sequencing. Neither isolate was identified as *V. vulnificus* (>7% sequence mismatches). Differing by <2% sequence alignment (the top matches) were *V. coralliilyticus*, *V. mediterranei*, *V. nereis*, *V. tubiashii*, and *V. sinaloensis* (Table 4).

DISCUSSION

The isolation of *V. vulnificus* from the oysters and water of North Carolina estuaries has been routinely accomplished by our laboratory and others (1, 4, 20, 27, 29, 32, 41, 42). Historically, by use of CPC$^+$ agar, designed for the isolation of *V. vulnificus* from environmental samples (39), the organism has been easy to collect and identify from oysters in North Carolina. In 2007, the isolation of confirmed *V. vulnificus* colonies was extremely difficult, even though a large number of samples was tested. Concerned about a possible deficiency in the isolation medium, we further employed CHROMagar Vibrio, but this medium performed only slightly better. Both media yielded colonies that appeared to be *V. vulnificus* colonies, but very few were confirmed by molecular testing. The phenomenon of *V. vulnificus*-specific media losing specificity when samples contain a large number of competing *Vibrio* spp. was reported previously by Macian et al. (16), offering a possible explanation for the presence of false-positive *V. vulnificus* colonies on these typically reliable media. Due to the inability to isolate *V. vulnificus* using typically applied culture-based methods, we tested molecular methods of detection, including PCR and Q-PCR, on DNA extracted from oyster tissue. No *V. vulnificus* could be detected by either method.

TABLE 3 Presumptive *V. vulnificus* isolates, obtained from Gulf Coast oysters using either CPC$^+$ agar or CHROMagar Vibrio, confirmed as *V. vulnificus* isolates following PCR analysis

Isolation medium	Yr	No. of isolates tested	% confirmed *V. vulnificus* isolates
CPC$^+$ agar	2008	80	96
	2009	31	98
	2010	20	100
CHROMagar Vibrio	2010	10	100

To confirm that the isolation and confirmation techniques were sound, oysters were seeded with *V. vulnificus*. These oysters yielded confirmed *V. vulnificus* cells by culture and Q-PCR detection methods, providing evidence that the media and techniques were working correctly. As further verification of the methodology, Gulf Coast oysters were obtained and processed in the same fashion as North Carolina oysters, with confirmed *V. vulnificus* cells being easily recovered.

Having established that North Carolina oysters harbored extremely reduced numbers of *V. vulnificus* cells, we investigated potential events that might cause such a sudden and significant loss. The drought that occurred during our study period was the most severe since recordkeeping began in 1895 (23). These conditions, which began in the middle of 2007 and persisted until the end of 2009 (22), resulted in a long-term (>2 1/2 years) increase in the average salinity of the estuary. While previous increases of salinity to this level had occurred, these were short-term increases and unlike the extended drought that occurred during this study (R. T. Noble and H. Paerl, unpublished data).

Kaspar and Tamplin (11) determined previously that the survival of *V. vulnificus* decreased in seawater with salinity greater than 25‰. These findings were supported by *in situ* data reported by Motes et al. (20), showing that increases in salinity in Apalachicola Bay were linked with declines in rates of *V. vulnificus* recovery from oysters, and by Wetz et al. (42), who found that salinity-lowering storm events resulted in an increased rate of recovery of *V. vulnificus*. Consistent with such observations, Jones (S. H. Jones, presented at the Proceedings of the 1994 *Vibrio vulnificus* Workshop, Washington, DC) found that oysters moved to water

with elevated (25‰) salinity were cleared of *V. vulnificus*, and Motes and DePaola (19) reported similar results for oysters relayed from estuarine to offshore (32‰) sites. Such studies, however, have not examined the presence of *V. vulnificus* in high-salinity waters for periods lasting longer than 21 days (1, 9, 11, 13, 15, 19, 20, 32, 33, 36, 37, 44; S. H. Jones, presented at the Proceedings of the 1994 *Vibrio vulnificus* Workshop, Washington, DC), while our study suggests that long-term-elevated salinity (even less than 25‰, considered to be the upper limit of the *V. vulnificus* salinity preference) could negatively impact oyster colonization by *V. vulnificus*.

It is possible that other environmental changes, unaccounted for in this study, could have contributed to the loss of *V. vulnificus*. That considered, it is conceivable that the lengthy drought and shift in estuarine salinity either induced *V. vulnificus* to abandon the oyster habitat or had outright bactericidal effects. Either possibility would lead to a loss of *V. vulnificus* in oysters, leaving an empty niche for an organism with similar physiological characteristics but able to endure elevated salinities.

After the drought eased at the end of 2009, the salinity of the North Carolina estuary returned to normal. The number of presumptive *V. vulnificus* isolates from water samples that were confirmed to be isolates of this species quickly increased to pre-drought levels. Nevertheless, oysters harvested from these waters in 2009 and 2010 still contained extremely low numbers of *V. vulnificus* cells.

It is possible that by using enrichment, we would have been able to detect low numbers of *V. vulnificus* cells in the environment, but with the molecular and culture-based methods that we employed, we found levels in oysters to be nearly nondetectable. It is not known why *V. vulnificus* again became detectable in estuarine waters by our methods yet concentrations of *V. vulnificus* in the oysters that inhabit these waters remain mostly nondetectable. We speculate that the answer may lie in results obtained by our laboratory and many others over the last 30 years. Studies examining the uptake and depuration of *V. vulnificus* in seeded oysters have all reported that *V. vulnificus* cells are rapidly taken up but are not retained and are quickly depurated (3, 5, 6, 12, 14, 31, 35). If the oyster microflora is firmly established during the early stages of oyster development, transient bacterial cells acquired through gill filtration could be unable to establish residency in the oyster gut. The "original" population would likely be displaced only by extreme events, such as large and acute shifts in salinity, which occur when oysters are relayed to waters with much higher salinity (19, 20), or as in the moderate yet chronic salinity increases described in our study. If this is correct, the reemergence of a significant *V. vulnificus* population in adult North Carolina oysters may be observed only when oyster larvae produced after the drought conditions eased (i.e., after 2009) develop into adults, a period of ca. 2 years. This is a testable hypothesis which we intend to pursue over the next several years.

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A NEW CULTURE-BASED METHOD FOR THE IMPROVED IDENTIFICATION OF *VIBRIO VULNIFICUS* FROM ENVIRONMENTAL SAMPLES, REDUCING THE NEED FOR MOLECULAR CONFIRMATION

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A new culture-based method for the improved identification of *Vibrio vulnificus* from environmental samples, reducing the need for molecular confirmation

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ABSTRACT

Vibrio vulnificus is an opportunistic human pathogen responsible for 95% of seafood related deaths in the US. Monitoring the presence of this bacterium in estuarine waters and shellfish is of medical and economic importance due to its ability to cause severe wound infections and fulminant septicemia. Current methods for isolating *V vulnificus* from environmental samples typically employ an initial selective medium which requires subsequent molecular confirmation of presumptive *V vulnificus* isolates. Although culture-based methods are accessible and inexpensive, they lack the specificity needed to definitively identify *V. vulnificus*. The goal of this study was to develop a more accurate, culture-based method for the initial detection of *V vulnificus,* thereby decreasing or eliminating the requirement for confirmatory molecular tests. Colony color characteristics of a variety of *Vibrio* species were determined on three commonly employed media to identifY those which present as false-positive isolates for *V vulnificus.* We subsequently developed a triple-plating method which utilizes three media in combination to greatly decrease the number of false positive isolates. The number of isolates positively identified as *V vulnificus* using the triple-plating method were compared to a typical single-plating method and revealed over a 2-fold increase in ability to accurately predict *V vulnificus* isolates. We suggest that this new method will enhance the predictive power of culture-based methods, reduce the cost and time spent on additional detection methods, and may be a valuable alternative when molecular methods are not available or unaffordable.

1. Introduction

Vibrio *vulnificus* is ubiquitous in estuarine environments and has been isolated from water, sediments, fish, and shellfish (Daniels, 2011; Oliver, 2006a). This bacterium is a medically relevant pathogen due to its ability to cause fulminant and potentially fatal systemic infection when ingested, usually via raw or undercooked shellfish (Oliver, 2006b). In an analysis of data provided by the U.S. FDA between 1992 and 2007 in which 459 cases were examined, this bacterium was documented to have a 51.6% fatality rate Uones and Oliver, 2009; Oliver, 2006b), the highest of any food-borne disease (Oliver, 2012a). Furthermore, this water-borne pathogen can cause severe wound infections such as necrotizing fasciitis, although with a lower mortality rate (ca. 25%) than systemic disease (Oliver, 2006b). Wound infections caused by \I. *vulnificus* are the predominant form of disease by this organism in Europe, with significantly increasing rates of infection being seen in Baltic countries (Baker-Austin et al., 2013). Considering the public health hazard presented by this pathogen, it is of extreme importance to be able to isolate and correctly identify this organism from its natural environment, particularly in countries where shellfish is consumed (Oliver and Kaper, 2007).

\I. *vulnificus* strains are phenotypically and genetically diverse and can be categorized into 3 biotypes based on biochemical characteristics in which biotype 1 (BT1) strains are primarily responsible for the majority of human infections, whereas biotype 2 (BT2) strains are more frequently associated with disease in eels (Bisharat et al., 1999; Sanjuan et al., 2009; Tison et al., 1982). Biotype 3 (BT3) strains have distinct phenotypic and molecular patterns that indicate the occurrence of a recent genome hybridization event between BT1 and BT2 (Bisharat et al., 1999, 2005, 2007). To date, documented isolates of this biotype have been geographically limited to aquaculture facilities in Israel (Bisharat et al., 2005). Previous genetic analyses of BT1 strains have revealed the presence of genetic polymorphisms that highly correlate with the source of isolation (Chatzidald-livanis et al., 2006; Nilsson et al., 2003; Rosche et al., 2010; Rosche et al., 2005; Sanjuan et al., 2009; Senoh et al., 2005; Vickery et al., 2007). Thus, we further sub-type BT1 strains into two genotypes: C-genotypes (clinical)

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and E-genotypes (environmental), which can be differentiated by PCR analysis of the virulence-correlated gene (vcg) (Warner and Oliver, 2008). Additional genetic distinctions amongst these two genotypes have also been established (Cohen et al., 2007; Froelich and Oliver, 2011; Gulig et al., 2010; Morrison et al., 2012).

Various selective and/or differential media are employed to isolate *Vibrio* species from environmental samples (Oliver, 2012b). These media are typically useful in permitting the growth of select *Vibrio* species while excluding the growth of other vibrios and closely related genera. However, due to the phenotypic variability of Vibrio species, all of these media require an additional molecular step, such as PCR, to confirm the identification of the organism of interest (Harwood et al., 2004). Thiosulfate-citrate-bile salts-sucrose agar (TCBS) was one of the first selective media used for the isolation and purification of vibrios (Oliver, 2012b). On this medium, strains able to ferment sucrose form yellow colonies whereas sucrose non-fermenters, such as V. *vulnificus,* appear green (Thompson et al., 2004). This medium has been widely employed for isolation of *Vibrio cholerae,* Vibrio *parahaemolyticus,* Vibrio *fluvialis,* and V. *vulnijicus* from clinical specimens as well as from the aquatic environment, yet studies have demonstrated batch-to-batch and brand-to-brand variations of this medium, with the level of recovery of vibrios varying greatly (Bolinches et al., 1988; West et al., 1982). Additionally, despite the selectivity for vibrios, other genera such as *Staphylococcus, Flavobacterium, Pseudoaltermonas,* and *Shewanella* can grow on this medium as well (Thompson et al., 2004). Thus, TCBS is not typically used as the primary method for isolation of V. *vulnificus* from environmental samples.

Colistin/Polymyxin B/Cellobiose agar (CPC) and CPC+ (a modified version of CPC) have been used by labs worldwide to isolate *V. vulnificus* from environmental samples (Massad and Oliver, 1987; Sun and Oliver, 1995; Warner and Oliver, 2007). Due to its ability to ferment D-cellobiose, *V. vulnificus* yields characteristic yellow colonies with a darker center and yellow halo, whereas *V. cholerae* and other vibrios typically do not grow on this medium, or grow as green colonies with a purple halo. As demonstrated by Warner and Oliver (Warner and Oliver, 2007), CPC + is highly effective in the isolation of V. *vulnificus,* without the need for enrichment, and does not exhibit genotypic bias. Presumptive V. *vulnificus* colonies on CPC + can be confirmed by genetic testing (Rosche et al., 2005; Warner and Oliver, 2008).

CHROMagar Vibrio (CHROMagar; Paris, France), here denoted as CaV, is a proprietary medium that uses chromogenic technology to allow for isolation and detection of V. *vulnificus, V. parahaemolyticus,* V. *cholerae,* and V. *alginolyticus* based on colony color development. On this medium V. *vulnificus* colonies appear turquoise, likely due to (»-galactosidase activity (Monget and Robichon, 2011). The ability to isolate and identify four pathogenic vibrios with one medium is highly advantageous, and such a medium provides a considerable amount of information about the population structure of an environment being examined. In the current study, we demonstrate that direct recovery of V. *vulnificus* on CaV is comparable to that of CPC +. However, this medium also must be used in conjunction with a molecular method in order to confirm the identity of presumptive colonies.

Despite the usefulness of CPC + and CaV in isolating V. *vulnificus* from environmental samples, other organisms have the ability to grow on these media, sometimes appearing identical to colonies of V. *vulnificus.* This appears to be particularly the case when *V. vulnificus* populations are diminished, a phenomenon reported by several investigators (Arias et al., 1999; Froelich et al., 2012; Macian et al., 2000; Staley et al., 2013). Studies by our lab have indicated that certain environmental conditions, *e.g.* long-term drought leading to a significant increase in estuary salinity can, result in the ability of other Vibrio species (likely V. coralliilyticus and *V.* mediterranei) to outcompete V. *vulnificus* in the environment (Froelich et al., 2012). Staley et al. (2013) recently documented a similar phenomenon in which V. *vulnificus* appeared to be outcompeted by *V. sinaloensis* in warmer estuarine waters (Staley et al., 2013). Under these circumstances, the number of false positive isolates can greatly exceed the number of true *V. vulnificus* isolates using the aforementioned media. This can lead to a significant loss of time and resources when attempting to isolate and identify this species in an environmental sample. For example, a recent study in which 1041 V. *vulnificus* presumptive isolates from CPC + were examined, reported only 0.6% could be confirmed to be this species (Froelich et al., 2012). That study resulted in an expense of over $1200 in order to positively identify only six isolates of *V. vulnificus*.

To better characterize the growth of Vibrio species and strains on CPC+, TCBS, and CaV, we examined colony color reactions for 60 Vibrio strains representing 17 Vibrio species and identified strains which appeared identical to *V. vulnificus.* We sought to eliminate these false positive isolates by developing an improved culture-based method utilizing all three media. In this case, presumptive colonies of *V. vulnificus* initially grown on CaV were subsequently plated onto both CPC + and TCBS allowing for the identification of "triple-positive" isolates which were predicted to be V. *vulnificus*. We compared the performance of this new method to the conventional single-plating method, and validated its utility in vitro and in situ using molecular confirmation. Cross-plating *V. vulnificus* presumptive isolates on all three media was shown to significantly reduce the number of false positive isolates, subsequently reducing the number of isolates that require molecular confirmation. We suggest this new and simple triple-plating technique will provide a more accurate, more time efficient, and more cost effective method for the initial detection of V. *vulnificus.* This method would be particularly useful when molecular methods are not available or too costly.

2. Methods

2.1. Characterizing growth and colony color of Vibrio species/strains on each medium:

The twenty-five strains of V. *vulnificus* used in this study (including all three biotypes and both C- and E-genotypes) are listed in Table 1. Thirty-five additional Vibrio strains, representing 16 Vibrio species, were characterized on each medium, and are listed in Table 2. Each strain was grown overnight in Heart Infusion (HI) broth, diluted in phosphate buffered saline (PBS), and plated onto TCBS, CaV, CPC+, and HI agar plates. HI and TCBS were incubated at 30 °C, CaV at 37 °C and CPC+ at 37 °C (pure culture; laboratory studies) or 40 °C (environmental studies). Each medium was then evaluated for the ability of each organism to grow, along with colony color developments. Strains that grew identically to *V. vulnificus* on each medium were noted.

22. Comparing direct recovery of V. vulnificus on CaV and CPC +

To evaluate the use of CaV as an initial selective medium, overnight and starved cultures of V. *vulnificus* cells were plated onto CaV and compared to direct recovery on CPC+ and HI. V. *vulnificus* isolates (of both C- and E-genotypes) were prepared by growing strains from freezer stocks in HI for 24 h. Serial dilutions were made and plated onto HI, CPC +. and CaV and incubated at appropriate temperatures overnight. To demonstrate that physiologically starved cells could also be recovered on CaV, an aliquot of overnight cells were washed twice with PBS to remove residual nutrients, inoculated into a basal salt medium (BSM) lacking any carbon source at a final cell concentration of 2e8 CFU/ml, and incubated standing at room temperature for six months. These starved cultures were then diluted into PBS, plated onto HI and selective media and incubated at appropriate temperatures overnight. Growth on CaV and CPC + was measured in CFU/ml and compared to growth on HI, with the resultant ratio calculated as percent recovery. V. *vulnificus* strains used for this study are specified in Table 1. Results were statistically analyzed using a 2-way ANOVA

Table 1
V. vulnificus reference strains used in this study, along with biotype, genotype, and their respective colony color reactions on each medium.

Strain	Biotype	Genotype	CPC+	CAV	TCBS
CMCP6[‡,§]		C	Yellow	Turquoise	Green
YJ016[‡,§]		C	Yellow	Turquoise	Green
M06-24		C	Yellow	Turquoise	Green
C7184k2[‡,§]		C	Yellow	Turquoise	Green
LSU1866[¶]		C	Yellow	Turquoise	Green
NR02-232[‡,§]		C	Yellow	Turquoise	Green
UNCC913		C	Yellow	Turquoise	Green
UNCC1002		C	Yellow	Turquoise	Green
JY170I[‡,§]		E	Yellow	Turquoise	Green
JY1305[‡,§,0]		E	Yellow	Turquoise	Green
ENVI[‡,§]		E	Yellow	Turquoise	Green
LSU549[¶]		E	Yellow	Turquoise	Green
LSU2098[‡]		E	Yellow	Turquoise	Green
BC478[‡]		E	Yellow	Turquoise	Green
E64MW		E	Yellow	Turquoise	Green
CEIT4174[¶]	2	E	Yellow	Turquoise	Green
CEIT4601[¶]	2	E	Yellow	Turquoise	Green
CEIT4607[¶]	2	E	Yellow	Turquoise	Green
33149[¶]	2	E	Yellow	Turquoise	Green
CEIT4866[¶]	2	E	Yellow	Turquoise	Green
RIU-2[¶]	2	E	Yellow	Turquoise	Green
94-8-112[¶]	2	E	Yellow	Turquoise	Green
32[¶]	3	E	Green/Purple	White	Green
1033[¶]	3	E	Green/Purple	White	Green
11028[¶]	3	E	Green/Purple	White	Green

[¶] Unless otherwise noted, all strains were from UNC Charlotte, Charlotte, NC.
[‡] Strains used for comparing direct recovery of *V. vulnificus* cells on CAV and CPC+ (Section 2.2).
[¶] Strains used for comparing direct recovery of starved *V. vulnificus* cells on CAV and CPC+ (Section 2.2).
[0] Strains used for *in vitro* evaluation of triple-plating method in artificially infected oysters (Section 2.4).
[¶] Coleecion Espanola de Cultivos Tipo, Spain.
[¶] Courtesy of carmen Amaro, Universidad de Valencia, Valencia, Spain.

Table 2
Other *Vibrio* species and strains used in this study, along with their respective colony color reactions on each medium.

Species	Strain name	CPC+	CAV	TCBS
V. parahaemolyticus	NY477	NG	Purple	Green
V. parahaemolyticus	F11.3A	NG	Purple	Green
V. parahaemolyticus	SAK11	NG	Purple	Green
V. parahaemolyticus	SPRC1019	NG	Purple	Green
V. parahaemolyticus	AQ3815	Green/purple	Purple	Green
V. parahaemolyticus	WRl	Yellow	Purple	Green
V. cholerae	2633-78	NG	Light blue	Yellow
V. cholerae	2741-80	Green/purple	Light blue	Yellow
V. cholerae	2076-79	Green/purple	Light blue	Yellow
V. cholerae[¶]	C6706	Green/purple	Light blue	Yellow
V. cholerae	2690-79	Green/purple	Light blue	Yellow
V. cholerae	924- 79	Green/purple	Turquoise	Yellow
V. cholerae	Q2052313	Green/purple	Turquoise	Yellow
V. alginolyticus[¶]	08653	Yellow	White	Yellow
V. alginolyticus[¶]	2208-1B	NG	White	Yellow
V. alginolyticus[¶]	0-04-01	Green/purple	White	Yellow
V. harveyi[¶]	35084	Yellow	White	Yellow
V. harveyi[¶]	BB120	Yellow	White	Green
V. anguillarum[¶]	ATCC 19264	NG	White	Yellow
V. anguillarum	VIB1	NG	White	Yellow
V. anguillarum	VIB275	NG	White	Green
V. anguillarum	VIB2	NG	White	Yellow
V. fluvialis	UNK	Green/purple	Turquoise	Yellow
V. mimicus	UNK	NG	Turquoise	Green
V. hollisae[¶]	8391	NG	Turquoise	Green
V. hollisae[¶]	2039	NG	Purple	Green
V. furnissii[¶]	1955-83	NG	Purple	Yellow
V. furnissii	8760	NG	White	Yellow
V. metschnil<ovii[¶]	ATCC 7708	NG	White	Green
V. natriegens[¶]	ATCC 14048	NG	Purple	Yellow
V. nigripulchritudo[¶]	ATCC 27043	NG	White	Green
V. proteolyticus[¶]	ATCC 15338	NG	White	Green
V. pelagius[¶]	ATCC 25916	NG	White	Green
V. aestruianus	OSU-65	NG	Turquoise	Yellow
V. leiognathi	B474	NG	White	Green

[¶] Courtesy of Food and Drug Administration, Gulf Coast Seafood Laboratory, Dauphin Island, AL.
[‡] Courtesy of Bonnie Bassler, Princeton University, Princeton, NJ.
[¶] ATCC — American Type Culture Collection, USA.

2.3. Triple-plating method

Environmental (water or oyster) samples were prepared as described by Froelich *et al.* (Froelich et al., 2012), plated onto CaV, and incubated at 37 °C overnight. Presumptive *V. vulnificus* (turquoise colored) colonies were isolated using sterile toothpicks, picked onto CPC+, TCBS, and HI using a 48-square colony grid template (Epicentre Biotechnologies, Madison, Wisconsin), and incubated overnight at the temperatures listed in Section 2.1. Each isolate's growth pattern (i.e. appearance on all three media) was documented. Isolates that produced the *V. vulnificus* growth pattern (turquoise on CaV, yellow on CPC+, and green on TCBS) were referred to as "triple positive isolates" and were predicted to be *V. vulnificus*. Isolates that did not produce the *V. vulnijicus* growth pattern were considered to be false positives, but were still subjected to molecular testing for confirmation.

To validate the methodology, all isolates (triple positives and presumptive false positives) were picked from the **HI** plate and grown overnight in 1 ml of HI broth at 30 °C with shaking. DNA was extracted from these samples by boiling the liquid culture for 10 min followed by centrifugation at 10,000 xg for 10 min and discarding the pellet. All samples were subsequently subjected to genetic testing using PCR, probing for *vvhA* (hemolysin) and *vcg* (virulence correlated) genes, as previously described (Warner and Oliver, 2008). The percentage of isolates correctly predicted to be *V. vulnificus* using one medium (CaV alone) was compared to the accuracy of using three media in tandem (the triple-plating method). A schematic illustration of the steps of the triple-plating method is provided in Fig. 2 for reference.

2.4. *In* vitro *evaluation of triple-plating method* in *artifidally infected oysters*

To evaluate the performance of the triple-plating method, oysters held in aquaria with 20‰ artificial seawater [ASW, Instant Ocean, Aquarium Systems, Mentor, OH] at 23 °C were seeded with $Sx10^4$ CFU/ml *V. vulnificus* (final concentration) for 24 h. Additionally, a set of control oysters not seeded with exogenous *V. vulnijicus* were included in the study. Oysters were removed from tanks, then aseptically shucked and homogenized as previously described (Froelich et al., 2012). Oyster homogenates were diluted in PBS and 100 µl were spread onto CaV agar plates and incubated at 37 °C overnight. A total of 92 presumptive *V. vulnificus* isolates from CaV were selected and plated onto CPC+, TCBS, and HI and incubated overnight at appropriate temperatures. All92 isolates were subjected to molecular testing using the methods described above to confirm *V. vulnificus* isolates. The accuracy of positively predicting *V. vulnijicus* isolates using the new triple-plating method was then compared to the accuracy of using CaV alone. *V. vulnijicus* strains used for this study are specified in Table 1.

2.5. *In* situ *evaluation of triple-plating method for environmental* isolates

To further validate the utility of the triple-plating method for environmental sampling, oysters and water samples were collected from several estuarine sites along eastern N.C. over a six month period. Each water and oyster sample was prepared as previously described (Froelich et al., 2012), spread onto CaV, and incubated at 37 °C overnight. Presumptive *V. vulnificus* isolates from CaV (turquoise colonies)

were selected and cross plated onto CPC+, TCBS, and HI followed by overnight incubation at appropriate temperatures. Colony color was documented on a total of 152 isolates which were then probed for *V. vulnificus* specific genes as outlined in Section 2.3. The accuracy of positively predicting *V vulnijicus* isolates using the new triple-plating method was compared to the accuracy of using CaV alone.

3. Results and discussion

3.1. Characterization ofV. vulnijicus strains on selective media

Considering the genotypic heterogeneity displayed by *V vulnificus* strains, we characterized the growth and colony color development of a variety of *V vulnificus* strains on CPC +. CaV and TCBS (Table 1). Of the 25 *V vulnificus* strains examined, all BT1 and BT2 strains produced the expected *V vulnificus* growth pattern, however BT3 strains appeared white on CaV, and green/purple on CPC+, indicating that the triple plating method is not suitable for this biotype. This finding is supported by a previous study which found BT3 strains to be incapable of sucrose and cellobiose fermentation, and negative for ('>-galactosidase activity (Bisharat et al., 1999). Thus, it is important to note that the triple-plating method developed in this study pertains specifically to BT1 and BT2 strains. Due to the current geographical isolation ofBT3 strains (Bisharat et al., 1999, 2005; Paz et al., 2007) the inability to conform to the currently designed triple plating method does not present a significant limitation. Nonetheless, BT3 strains displayed a unique growth pattern (i.e. no other strain grew white on CaV, green/purple on CPC +. *and* green on TCBS), indicating that the triple-plating method could possibly be utilized to detect these strains in environmental samples when desired. Further studies are warranted to support this hypothesis.

3.2. Characterization of other Vibrio species on selective media

In this study, several Vibrio species were able to grow like *V vulnificus,* consequently appearing as false-positive isolates on each medium (Tables 2 and 3). TCBS was the least selective of the three media, with all 60 strains tested being able to grow, 46% of which produced green (non-sucrose fermenting) colonies alike *V vulnijicus* (Tables 1 and 2). Previous studies have demonstrated the ability of CPC + to inhibit or differentiate the growth of a number of non-vulnijicus Vibrio species (Warner and Oliver, 2007). In the current study, CPC + was the most selective media, with 63% of non-*V. vulnijicus* strains being unable to grow however 11% of strains tested were able to ferment cellobiose resulting in yellow colonies similar to *V vulnijicus.* The selectivity of CaV was not exclusive to the four pathogenic vibrios for which it was developed as all Vibrio strains tested were able to grow on CaV, resulting in one of the four colors characteristic of this medium, of which 17% of non-*V vulnificus* strains appeared similar to this species.

Table 3
Vibrio species which appear indistinguishable from *V. vulnificus* on each medium.

CPC+ (19%)*	CAV (31%)	TCBS (62.5%)
V. parahaemolyticus	*V. cholerae*	*V. parahaemo!}!ticus*
V. alginolyticus	*V. hollisae*	*V. harveyi*
V. harveyi	*V. mimicus*	*V. anguillarum*
	V. fluvialis	*V. mimicus*
	V. aestruianus	*V. hollisae*
		V. metschnil<ovii
		V. nigripulchritudo
		V. proteo!}!ticus
		V. pelagius
		V. leiognathi

* Percentage of non-V. *vulnificus Vibrio* species (total of 16) in which at least one strain appeared identical to *V. vulnificus.*

Table 3 lists Vibrio species of which at least one strain grew identical to *V vulnificus* (BT1 and BT2) on each individual medium. Despite the presence of *V vulnijicus* false positives on a single medium, *V. vulnificus* strains displayed a unique growth pattern across all three media (i.e. no other strain appeared turquoise on CaV, yellow on CPC +.*and* green on TCBS). This led to the hypothesis that these media could be used in tandem to eliminate false-positive isolates and enhance the selectivity of culture-based methods for the initial detection of *V vulnificus.*

3.3. Comparing direct recovery ofV. vulnificus on CaV and CPC +

The triple-plating method requires the use of an efficient initial selective medium to guarantee ample recovery of *V vulnijicus* from environmental samples when the bacterium is present. CPC + was shown here to be the most selective in vitro (Table 2), and it has previously been demonstrated to permit greater recovery of physiologically starved *V vulnificus* cells compared to other media (Warner and Oliver, 2007). However, a recent study found CaV to be slightly more selective compared to CPC + when used for environmental sampling (Froelich et al., 2012). To compare the plating efficiencies of CaV and CPC + we examined the percent recovery of *V vulnificus* on these selective media relative to HI but found no significant difference between the media, as well as no selectivity between C- and E-genotypes ($p > 0.05$) (Fig. 1A).

To more closely simulate environmental conditions and demonstrate that physiologically starved cells could also be recovered on CaV, we examined the percent recovery of starved *V vulnificus* cells

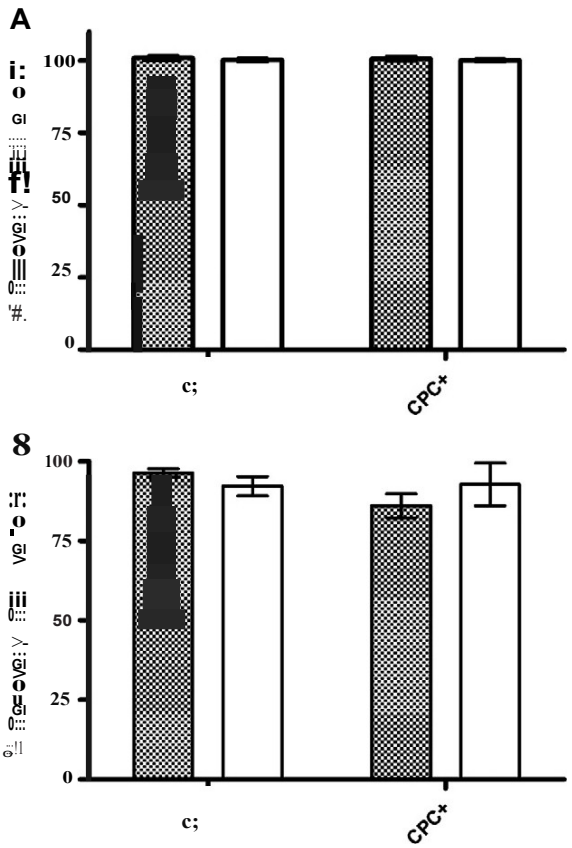

Fig. 1. Comparison of percent recovery of (A) overnight and (B) starved cultures of *V. vulnificus* on CAV, and CPC+ relative to HI (C-genotype, gray bar; £-genotype, white bar). No statistical difference ($p > 0.05$) was observed between media or C-genotype using a 2-way ANOVA

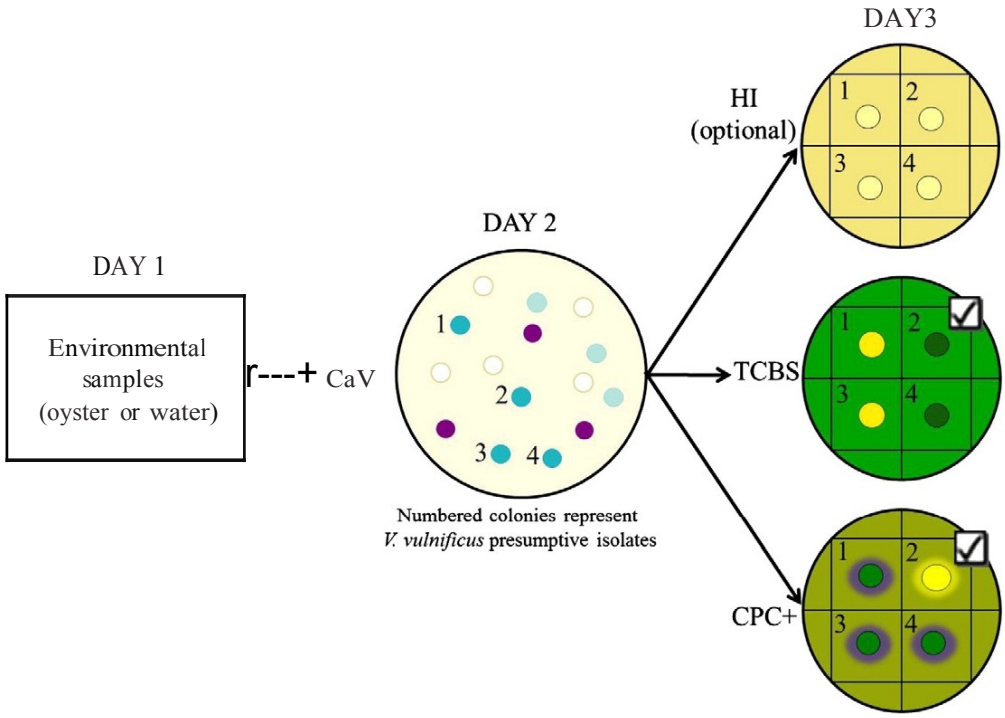

Fig. 2. Schematic illustration of the triple-plating method for the improved culture-based detection of *V. vulnificus*. On day 1, environmental samples are initially spread onto CaV and incubated overnight at 37 °C. On day 2, isolates that appear turquoise (presumptive for *V. vulnificus;* colonies numbered 1-4) are picked from CaV, plated onto HI (optional), TCBS, and CPC+ (using a grid template), and incubated at their appropriate temperatures (see Section 2.1). On day 3, isolates that appear green on TCBS and yellow on CPC+ are referred to as triple-positives and are predicted to be *V. vulnificus* (indicated by the checkmark on isolate #2), whereas all other isolates are non-*V. vulnificus* false-positives on CaV (colonies #1, 3, and 4). Optional molecular confirmation can be performed using isolates plated onto HI (see Section 2.3 for details). Note: figure has been simplified: grid templates can allow for up to 48 isolates per plate.

on CPC + and CaV. Again, statistical analyses revealed no significant difference between the two media or genotypes ($p > 0.05$) (Fig. 1B). For the development of the triple-plating method, CaV was chosen as the initial selective agar due to its potential to differentiate between the four medically and economically relevant pathogens however results indicate that these media have comparable efficiencies for the recovery of *V vulnificus*.

3.4. *In* vitro *and* in situ *evaluation of triple-plating method*

Considering the number of Vibrio species and strains that mimic *V vulnificus* on routine culture media, we implemented a new triple-plating method to greatly reduce the number of false positive colonies that appear when attempting to isolate and identify this species from water and oysters (Section 2.3 and Fig. 2). We evaluated the efficiency of the new triple-plating method for detecting *V. vulnificus* by comparing it to the conventional single-plating method. To do this in vitro, we seeded laboratory-maintained oysters (determined to possess non-V. *vulnificus* endogenous flora that grew turquoise on CaV) with exogenous *V vulnificus*. Using the single-plating method, 92 presumptive (turquoise) *V. vulnificus* isolates were subjected to PCR confirmation of which 43.5% were confirmed to be *V.* vulnificus.

Table4
Comparison of culture-based methods for the accurate identification of *V. vulnificus* isolates in laboratory maintained oysters seeded with *V. vulnificus*.

	Single-plating method	Triple-plating method
No. of CaV turquoise isolates	92	92
No. predicted to be *V. vulnificus*	92	40
No. confirmed by PCR	40	40
% Correctly identified	43.5%	100%

Using the triple-plating method, 40 of these 92 isolates were identified as "triple positives" (presumptive *V vulnificus* on all three media), of which 100% were confirmed to be this species using PCR (Table 4). The remaining 52 non-triple positive isolates were determined to not be *V vulnificus* and were concluded to be false-positive isolates on CaV. Thus, the new triple-plating method was able to discriminate between CaV turquoise colonies present as the oyster's endogenous non-V. *vulnificus* population, and the population of *V vulnificus* that was artificially added to these oysters. Compared to single plating, this new method reduced the amount of molecular testing needed by 56.5% and increased our ability to accurately identify *V vulnificus* (using simply a culture-based method) by 2.3-fold.

To validate the performance of the triple-plating method using environmental samples, we collected 152 CaV presumptive isolates from water and oyster samples (Table 5). The triple-plating method reduced the number of presumptive *V vulnificus* isolates from 152 to 49, of which 46 were confirmed to be *V vulnificus* by PCR. As a

Table 5
Comparison of culture-based methods for the accurate identification of *V. vulnificus* isolates collected from environmental (oyster and water) samples.

	Single-plating method	Triple-plating method
No. of CaV turquoise isolates	152	152
No. predicted to be *V. vulnificus*	152	49
No. confirmed by PCR	54	46
% Correctly identified	35.5%	92.8%
No. false-positive isolates	98	3
No. false-negative isolates	0	8
Estimated cost of testing (with PCR)	$178	$60
Estimated cost of testing (without PCR)	Not applicable•	$8

• Not applicable — too inaccurate and unreliable to use this method without PCR.

result, the percentage of *V vulnificus* accurately predicted on plating media increased from 35.5% to 92.8%, reducing the number of false-positive isolates from 64.5% to 2%.

Despite the apparent benefits of the triple-plating method, it is important to note the limitations of any such study, particularly regarding the existence of false-negative *V vulnificus* isolates which would remain undetected using this method. In this study, eight *V vulnificus* environmental isolates were sucrose positive (i.e. formed yellow colonies on TCBS), all of which were genetically confirmed to be E-genotypes. While *V vulnificus* is referred to as a sucrose-negative species (Farmer, 1979; Oliver, 2012b; Tantillo et al., 2004), there has been previous documentation of sucrose-positive *V vulnificus* isolates (Arias et al., 1998; Farmeret al., 1991; Wrightet al, 1993).Although this phenotype is considered to represent a minority (~3-20%) of all *V vulnificus* strains, they would inevitably be missed using the triple-plating method. Future studies are required to more thoroughly investigate the abundance of sucrose-positive *V. vulnificus* isolates in the environment.

We compared the cost expenditure to perform the single and triple-plating methods followed by confirmatory PCR. Cost analyses for each method were calculated by obtaining current (non-discounted) costs for culture-based and molecular-based methods. As shown in Table 5, the single-plating method required PCR confirmation of all 152 isolates, resulting in an estimated expense of $178, whereas the triple-plating method reduced the cost to approximately $60, representing a 66.3% decrease in cost Considering the remarkable accuracy of the proposed triple-plating method, it is possible to remove the molecular component entirely when isolating and identifYing *V vulnificus* from environmental samples. In this case, testing 152 isolates would cost less than $8, representing a 95.5% decrease in cost Thus, this new and simple cross-plating technique is more time efficient, more cost effective and could prove to be a suitable option for those who are not able to routinely employ molecular methods.

3.5. Conclusions

Elucidating the population structure of *V vulnificus* in its natural environment is essential for our understanding of the ecology and prevalence of this opportunistic human pathogen. Currently used culture-based methods for the isolation and detection of *V vulnificus* involve the use of a single medium in which *V. vulnificus* presumptive isolates are typically confirmed using molecular techniques. To date, there exists no single medium that possesses the specificity and selectivity required to accurately detect *V. vulnificus* in the environment, particularly when in low numbers (Harwood et al., 2004; Warner and Oliver, 2007). In our study three commonly used selective and differential media (CPC+, CHROMagar Vibrio, and TCBS) were all shown to permit growth of *V vulnificus* false-positive isolates. This finding is supported by previous environmental studies in which *V vulnificus* presumptive isolates from each medium sometimes failed confirmation using molecular-based methods (Arias et al., 1998; Froelich et al., 2012; Macian et al., 2000; Staley et al., 2013). This has the potential to confound results if *V vulnificus* populations are low in an environment harboring other *V* vulnificus-like species. Indeed, when *V vulnificus* populations were diminished in North Carolina oysters and waters due to a period of prolonged and severe drought, the ability of CaV or CPC + to accurately detect *V. vulnificus* was reduced to 4% and <1%, respectively. (Froelich et al., 2012).

From the results of this study, we suggest a new and simple cross-plating technique in which only isolates that phenotypically appear as *V vulnificus* on all three media (referred to as triple-positive isolates) proceed to molecular testing (Fig. 2). While the triple-plating method is not completely exempt from false-positive and false-negative isolates, it considerably increases the efficiency of culture-based presumptive identification ofV *vulnificus* from environmental samples, while also reducing the time and resources required for confirmatory molecular methods. Indeed, our results indicate that molecular confirmation of *V vulnificus* isolates may not be required following this new culture-based method. Furthermore, it is anticipated that a similar cross-plating method could be developed to improve culture-based identification of other vibrio pathogens such as *V. parahaemolyticus*. Substantially reducing false-positive isolates not only enhances the predictive power of culture-based methods, but also reduces the need for expensive laboratory equipment, costly molecular reagents, and complicated experimental procedures. Many laboratories may not have access to the required resources and in these cases this inexpensive, efficient, culture-based technique would be economically invaluable.

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