GENETIC MECHANISMS OF PERSISTENCE OF THE CULTURABLE *Vibrio cholerae* IN AQUATIC ENVIRONMENT

By

MOHAMMAD JUBAIR

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF DHAKA
2015

To my family and friends who gave me the strength to persevere

ACKNOWLEDGMENTS

I am very thankful for my family members for their continued support, inspiration, and positive feedback during the 4 plus years of challenging work needed to complete my Ph.D. thesis, a milestone and ultimate goal of my life. My special thanks go to my beloved wife (Shareen Haider Bushra) who not only reminded me on the importance of my highest degree but also contributed patiently in all aspects of my study, including her understanding and sharing her thoughts whenever it was necessary. I am eternally grateful for her love and her support.

I am grateful to Dr. Mahmuda Yasmin, Professor, Department of Microbiology in the University of Dhaka (DU), Bangladesh, who considered me as her PhD student. I am grateful to her for her continued support in completing the administrative tasks in DU. It was possible to complete this work as a sandwich PhD program between DU, Bangladesh and Emerging Pathogens Institute (EPI), University of Florida (UF) at Gainesville, FL, USA, wherein she contributed enormously in every aspect in order to complete my dissertation. She always inspired me over phone when I was working in USA.

I am grateful to Dr Afsar Ali, Research Associate Professor of Environmental & Global Health Department at UF, FL, USA for accepting me as a research scholar in his laboratory in the Emerging Pathogens Institute at UF. During my research in his laboratory, Dr. Ali provided me a tremendous research opportunity, material support and day to day supervision of my research work without which this thesis would not have been possible. It is his laboratory I gained an appreciation for the complexity and wonder of environmental microbiology and molecular genetics;Dr. Ali also taught me what it takes to become a successful researchers and scientist. I

am truly grateful for all the members (both past and present) in Dr. Ali laboratory for their positive attitude and mutual respect to each other. Each one will always have a special place in my heart. I am particularly grateful to Thomas A. Weppelmann, Meer T. Alam, Srestha S. Roy, Mustafizur Rahman PhD and Huang Xianing PhD as my colleagues and dearest friends. They sincerely assisted to the completion of my work through experimental design, troubleshooting, presentation development, and manuscript preparation.

I would like to thank Bonnie Bassler of Princeton University for donating the *V. cholerae* quorum sensing reporter strain MM920. I also am grateful to Mattia Prosperi of EPI, UF for his help with the preparation of figures and Mohammed H. Rashid of EPI, UF for his technical support. I am grateful to Judith A. Johnson of EPI, UF and James B. Kaper of University of Maryland at Baltimore for their critical review of manuscripts submitted to the journals. I thank Byung-Ho Kang and Karen Kelly of Interdisciplinary Center for Biotechnology Research (ICBR), UF for helping us with Scanning and Transmission electron microscopy. I am grateful to Meer T. Alam of EPI, UF for his help with antibiotic susceptibility assay. I would also like to thank Yang Yang and Thomas A. Weppelmann of Department of Biostatistics and Environmental and Global Health, respectively, of EPI, UF for their help with Statistical analyses.

Finally and most importantly, I am thankful to Allah, the ultimateand true mentor. He provided many answers to redeem failing experiments and he was always there to provide me an outstretched hand to keep me from failing. I am grateful for my faith in Him because I am nothing without Him.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure page

LIST OF ABBREVIATIONS

VPI	Vibrio pathogenicity island
tcp	Toxin co-regulated pilus gene
ctx	Cholera toxin gene
rst	Resistance gene
VBNC	Viable but non-culturable
EPS	Exopolysaccharide
GASP	Growth advantage in stationary phase
PP	Persister phenotype
SEM	Scanning electron microscopy
CAI	Cholera auto inducer
APW	Alkaline peptone water
tox	Toxin gene
omp	Outer membrane protein gene
fla	Flagella gene
vps	Vibrio polysaccharide gene
SacII	Restriction endonuclease enzyme
XbaI	Restriction endonuclease enzyme
BamHI	Restriction endonuclease enzyme
cDNA	Complimentary DNA
pho	Phosphate transport system gene
pst	Phosphate-specific transport gene
TEM	Transmission electron microscopy

NaCl	Sodium chloride
Flr	Flagella regulatory gene
mot	Motility gene
SCLM	Scanning confocal laser microscopy
H_2O_2	Hydrozen peroxide
ORF	Open reading frame
GASP	Growth advantage in stationary phase
PP	Persister phenotype
SEM	Scanning electron microscopy
CAI	Cholera auto inducer
APW	Alkaline peptone water
tox	Toxin gene
omp	Outer membrane protein gene
fla	Flagella gene
vps	Vibrio polysaccharide gene

Abstract of Dissertation Presented to the Graduate School of the University of Dhaka in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

GENETIC MECHANISMS OF PERSISTENCE OF THE CULTURABLE *Vibrio cholerae* IN AQUATIC ENVIRONMENT

By

MOHAMMAD JUBAIR

January 2015

Supervisor:Mahmuda Yasmin PhD, Professor, Department of Microbiology, University of Dhaka, Dhaka, Bangladesh
Co-supervisor:Afsar Ali PhD, Research Associate Professor, Department of Environmental & Global Health, University of Florida, Gainesville, FL, USA
Department: Department of Microbiology, University of Dhaka

Cholera, an ancient disease caused by toxigenic strain of *Vibrio cholerae*, remains a human scourge killing millions of people worldwide in the past through pandemics and epidemics, and still continues to be a major public health threat to countries where sanitation and clean drinking water are not optimal. According to World Health Organization (WHO), 48 countries (mostly from Africa, Asia and South Americas) reported 2-3 million cases of cholera with 100-120 thousand deaths. Although *V. cholerae* is autochthonous to aquatic environments, the physiologic and genetic basis of persistence of the organism is not clearly understood despite decades of research. In other bacterial species, it has been reported that a subpopulation of a bacterium can shift to a “persister” phenotype by stochastic mechanism in response to environmental stressors, including antibiotic and nutrient-poor/limitation stresses. Furthermore, reports are also available that bacteria persisting in stationary phase can assume a growth advantage stationary phase (GASP) where a subpopulation of bacterium sustains positive selection mutation. Both persister and GASP phenotype are shown to play major role in

the persistence of bacteria in adverse survival conditions. I hypothesis that, like other bacterial species, *V. cholerae* can assume both persister and GASP phenotypes and that such phenotypes can promote environmental persistence of *V. cholerae*. As microcosms mimic environmental conditions, particularly relative to nutrient-poor environmental conditions, I used filtered sterilized lake water microcosms (FSLW) to test my hypothesis. My data presented in this work have indeed proved that a subpopulation of *V. cholerae* can assume persister and GASP phenotype and that both phenotypes can response to supplementation of complex nutrients, including chitin and phosphate. My data also exhibited that GASP phenotype promotes a biofilm specific to FSLW but not in nutrient rich L-broth. In summary, I demonstrate that *V. cholerae* can survive in culturable form for years in nutrient-poor conditions and that, persister and GASP phenotypes underwent many changes, including morphologic, genetic and physiologic changes that presumably contributed to their persistence in microcosms.

CHAPTER 1

BACKGROUND

Persistence of cholera in environment: History and Impact

Introduction

Cholera, characterized by profuse diarrhea with rice water stools, vomiting and severe dehydration, is an ancient disease that continues to pose a major public health threat globally , particularly in countries where safe drinking water, adequate sanitation and hygiene are suboptimal [1, 2]. According to World Health Organization (WHO), Cholera represents an estimated burden of 1.4 to 4.3 million cases, and 28,000 to 142,000 deaths per year worldwide [3]. In 2013, 47 countries reported a total of 129,064 cases of cholera including 2102 deaths, giving a case-fatality rate (CFR) of 1.63%[4]. This number is vastly underreported as many developing countries do not report any case of cholera because of trepidation from loss of international business opportunities and tourism [5, 6].

The causative agent of cholera is *Vibriocholerae* which is a gram-negative bacterium with curved rod and a singular polar flagellum. Of the 220 known serogroups of *V. cholerae*, only serogourps O1 and O139 are capable of causing deadly diarrhea[7,8, 9]. Serogroup O1 has two biotypes, including classical and El Tor biotypes[10]. The genomes of both serogroups O1 and O139 carry a lysogenized phage that encodes a potent enterotoxin known as cholera toxin eliciting cholera[8]. Furthermore, the genomes of these serogroups also carry a genomic island termed asvibrio pathogenicity island (VPI) that encodes, among many genes, a *tcpA* gene encoding a TcpA protein required for the colonization of the microorganisms into human intestine [5]. Non-O1 strains of *V. cholerae* rarely carry ctx phage and TcpA protein making

them unable to cause cholera; however, they can cause extraintestinal infections, including mild form of gastritis, wound infections and cellulitis [8]. Differences in the sugar composition of the heat-stable somatic "O" antigen are the basis of the serological classification of *V. cholerae* [11]. All strains of *V. cholerae* that did not agglutinate with "O" antiserum are referred to as non-O1 *V. cholerae*[12, 13]. Each biotype of *V. cholerae* is characterized into two major serotypes-Ogawa and Inaba. While strains of the Ogawa serotype express the A and B antigens and a small amount of C antigen, Inaba strains express only A and C antigens. In addition to Ogawa and Inaba serotypes, a rare third serotype (Hikojima) expressing all three antigens have also been identified [5,6](Figure 1-1).

History of cholera pandemics

Historically, since 1817, there have been seven cholera pandemics with first six pandemics is reported to be originated in the Bay of Bengal while the current and seventh pandemic has heralded in the 1961 in Indonesia and is continuing[8, 14, 15].While pandemics 5^{th} and 6^{th} were caused by classical biotype, it is widely believed that classical biotype was also responsible for causing cholera pandemics 1-4^{th}[5, 9]. The El Tor biotype is responsible for 7^{th} pandemic; furthermore, serogroup O139 originated initially in early 1990s in the Bay of Bengal region was thought to have potential to cause 8^{th} pandemic which is yet to be materialized[1].

In recent years, *V. cholerae* O1 strains exist that cannot be classified into classical or El Tor biotypes, termed as an 'atypical El Tor' which appear to have a mix of both biotypes[16,17,8]. El Tor strains isolated before 2001 in Bangladesh carried the *ctxB3* allele which

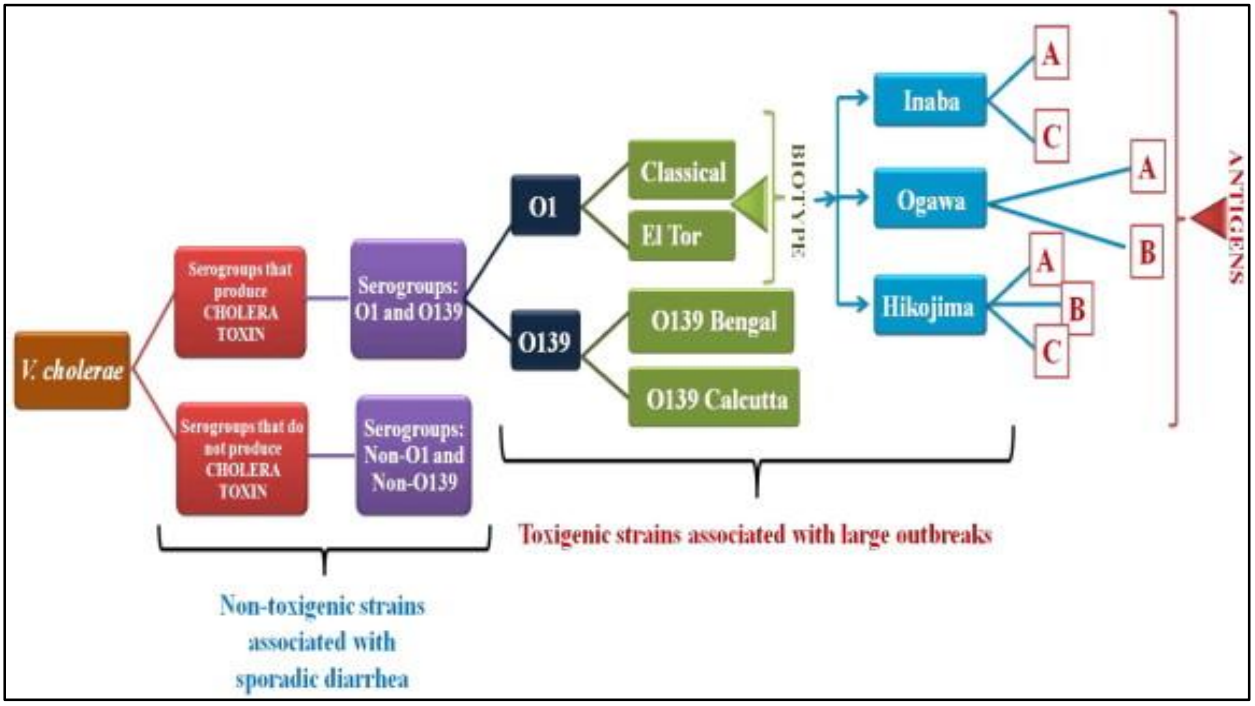

Figure 1-1: Classification of *Vibrio cholerae* serogroups with toxigenic and non-toxigenic group. The respective biotypes within a serotype are also represented [5].

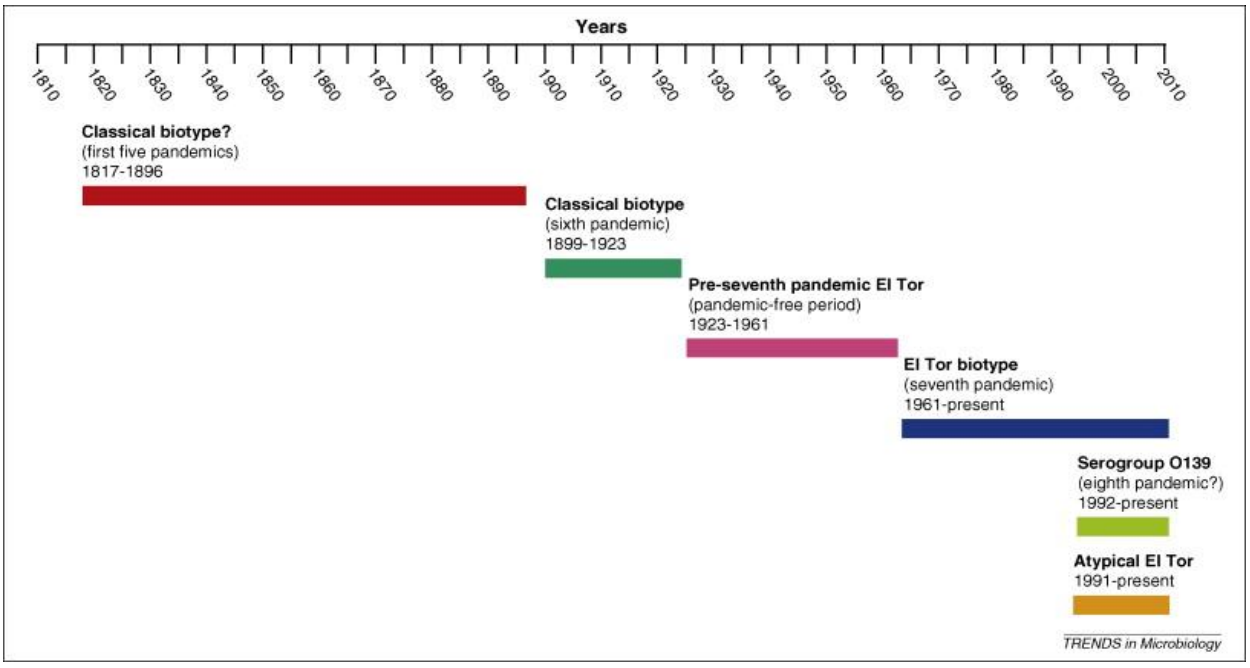

Figure 1-2: Key evolutionary events in cholera epidemiology since 1817. The first five pandemics were caused by the classical biotype, whereas the classical strains appear to have caused the sixth pandemic. Between the sixth and seventh pandemics, there was an intervening period of 38 years during which cholera only occurred as local outbreaks caused by El Tor strains [8].

produces CT of the typical El Tor biotype. However, all El Tor strains isolated after that date in Bangladesh harbor*ctxB1*, producing CT of the classical biotype[8]. These atypical El Tor strains have been designated 'altered El Tor' in the recent literature. Several El Tor strains isolated between 1991 and 2004 also harbor the classical CT allele *ctxB1*, designated as 'hybrid El Tor' [8](Figure 1-2). Continued investigation revealed that several of these strains carry both $rstR^{cla}$and $rstR^{el}$, indicating the presence of two different copies of ctx$^{\Phi}$, either as a tandem array or located on different chromosomes [8].

Reservoirs of *V. cholerae*

V. cholerae is a natural and autochthonous component of aquatic reservoirs, including fresh, riverine, estuarine and marine environments [18, 19]. Humans, a transient host of toxigenic *V. cholerae*, acquire the bacterium upon the consumption of food and water contaminated with the bacterium[20]. In the aquatic environments, *V. cholerae* can survive either in planktonic form or in a sessile mode (in which the baterium forms biofilms) is protected from environmental stressors and predation[2, 21]. Suggestions are made that *V. cholerae* can attach to surfaces provided by plants, blue green algae, copepods (zooplankton), crustaceans, chirmoid egg mass and insects [18,22]. Although, some authors argued that there is a direct correlation of cholera outbreaks and the seasonal occurrence of algal blooms [18, 19, 23], there is no direct evidence that such events produces more toxigenic *V. cholerae* strains responsible for cholera epidemics. Environmental and climatic factors positively correlated with environmental blooms of toxigenic *V. cholerae*, includes, increased temperatures, rainfalls, internal and external displacement of human movements, and human fecal contamination of surface and ground waters[24, 25]. Recently, it has increasingly being recognized that lytic

phages of *V. cholerae* influence the dynamics of the occurrence and distribution of environmental *V. cholerae* and modulate the evolution of the pathogen, particularly in cholera endemic countries[26, 27].

Environmental reservoirs and mechanisms of persistence

In addition to attachment to aquatic plants and crustaceans, *V. cholerae* can adapt to harsh and perpetual changing environmental conditions, including nutrient-poor/limited environment, cold temperature, predation and against other environmental stressor(s)[20]. In this context, there is a body of literature suggesting that, in response to nutrient-poor condition and in cold temperature, *V. cholerae* can assume a viable but non-culturable state (VBNC)[28, 29] that is unable to grow in nutrient-rich media but can be detected via molecular based assays[30, 31]. It is also hypothesized that, upon return to favorable survival condition(s), VBNC can render to culturable form[22, 32]. However, the resuscitation of VBNC and its role in cholera pathogenesis remain highly debated. Indeed Nelson et al. reported that, VBNC may have very limited or no role in cholera disease[26].

V. cholerae can also switch from its smooth colony phenotype to a wrinkled phenotype in response to nutrient-poor/limited conditions[33]. The rugose phenotype is characterized by its characteristic production of copious amounts of exopolysaccharide (rEPS) that confers resistance to chlorine, UV light, osmotic and oxidative stresses and predation[33-35]. However, about 50% of *V. cholerae* strains of clinical and environmental origins are able to switch to the rugose phenotype even under a condition that promote the very high-frequency rugose exopolysaccharide production[36]. These observations suggest that *V. cholerae* may adopt

other survival strategies to persist in aquatic environments while regularly causing cholera outbreaks and epidemics in cholera endemic countries.

Bacterial persister cells

In response to diverse stressors, including host mediated immunity, antibiotic treatment, predation and nutrient-poor/limited conditions, many bacterial pathogens can assume "persister" (dormant) phenotype by stochastic mechanism[37-41]. In contrast to VBNC, persister cells though remain dormant during the stressful growth conditions, become culturable once the stressors are eliminated and growth conditions are turned into permissive state for normal growth[42]. Toxin-antitoxin model is attributed to persister phenotype and many toxin antitoxin genes have been identified from diverse pathogens contributing persister phenomenon[37, 43, 44]. High persister (*hip*) mutants of *Pseudomonas aeruginosa* are selected in patients with cystic fibrosis[37, 45]. Similarly, *hip* mutants of *Candida albicans* are reported in patients with an oral thrush biofilm[46]. Persisters are likely responsible for the recalcitrance of chronic infectious diseases to antimicrobial therapy [37]. In keeping with this persister model, I hypothesize that a subpopulation of *V. cholerae* cells switches to a culturable phenotype or dormant state in response to nutrient starvation upon its release into aquatic environments [42].

Growth advantage in stationary phase (GASP) phenotype

The bacterial life cycle consists of three or four phases according to some standard textbooks. However, in the laboratory there are actually five phases: lag phase, exponential or logarithmic phase, stationary phase, death phase and extended or long-term stationary phase[47]. After death phase, *E. coli* can be maintained in batch culture for long periods of time

without addition of nutrients. Unlike early stationary phase, in which there is little cell division, long-term stationary phase is a highly dynamic period in which the birth and death rates are balanced [47,48]. In addition to many physiological, morphological and gene-expression changes that occur as cells enter long-term stationary phase, potentially the most significant changes are associated with genetic alterations that occur in most cells incubated during long-term stationary phase in batch culture[47]. The signal phenotype associated with these changes is the appearance of the growth advantage in stationary phase (GASP) phenotype [47, 48]. Initial models of the GASP phenomenon described a system in which one GASP mutant would sweep through the population, displacing all other cells until it was itself displaced by the next mutant of increased fitness [49,50]. It is established now that these cultures are far more diverse, with many subpopulations (distinct genotypes) present simultaneously [47,48]. In my study, I provide evidence that glycerol stored persister *V. cholerae* (700 days-old cells) had transitioned to what appeared to be a GASP phenotype. Further genetic investigations of my GASP cells appeared to sustain mutations to select GASP mutants as seen with other organisms [47]and thereby promoting competitive environmental fitness and adaptation.

Objectives of the study

While major progress has made on the pathogenesis of the disease cholera, very little is known about the persistence of *V. cholerae* in its aquatic environment despite the fact that *V. cholerae* is a natural component of aquatic environment[42]. There are two schools of thoughts as to the mechanism of persistence of *V. cholerae* in its aquatic environment. Dr. Colwell and her colleagues have proposed that *V. cholerae* can enter into a VBNC state in response to cold temperature and nutrient stress [32, 51]. However the role of VBNC in cholera transmission

remains highly controversial, and is a subject of ongoing debate [30, 31]. Another group of investigators argued that *V. cholerae* persists by encasing itself in the matrix of exopolysaccharide (biofilm) [26]. However, the failure of mass-scale isolation of culturable *V. cholerae* even during ongoing epidemics in cholera endemic countries such as Bangladesh does not support the hypothesis that biofilm exclusively serves as the seed of index case of *V. cholerae* during cholera outbreaks[33, 34]. Furthermore, not all *V. cholerae* isolates can switch from smooth to exopolysaccharide-producing rugose phenotype (*in vitro* studies) limiting the role of rugose phenotype as central environmental persisters[33]. I hypothesized that *V. cholerae* can assume other phenotypes to promote its persistence in aquatic reservoirs in order to counter environmental stressors. To test my hypothesis, I used nutrient-poor lake water microcosms that mimic the environmental reservoirs particularly with regard to nutrient limiting condition.

Aim 1: To Characterize the mechanism of survival of *V. cholerae* in nutrient-poor environments

Available data suggest that the *V. cholerae* survives between epidemics in aquatic reservoirs (including fresh, marine and estuarine waters) with environmental triggers causing seasonal increases in counts, followed by "spill-over" into human populations [1]. This model is complicated, however, by the observation that these environmental reservoirs are often nutrient-poor with extremely low or non-detectable *V. cholerae* cell counts between epidemics or even during epidemics [22, 52]. I still do not have a good understanding of the cellular mechanisms underlying environmental persistence of the microorganism, or of the environmental triggers that stimulate cell growth. Therefore, the objective of Aim 1 (Chapter 2)

is to explore an acceptable model which best explains the persistence of *V. cholerae* in the nutrient-limited environments for years and the effect of nutrients on the growth of *V. cholerae* persisting in laboratory based microcosms.

Aim 2: To assess the roles for motility and biofilm formation for the long-term surviving *V. cholerae* in nutrient-poor microcosms

Bacteria persisting in stationary phase can assume a growth advantage stationary phage (GASP) after sustaining beneficial mutation which can appear as a major cellular population promoting bacterial persistence[38, 40]. I hypothesize that *V. cholerae* persisting in lake water microcosms can sustain beneficial mutation to generate a cell population that promotes environmental persistence. Indeed I observed that, a subpopulation of *V. cholerae* persiting for 700 days in lake water microcosms lost flagella. In Aim 2 (Chapter 3), I demonstrated that non-flagellated (non-motile) *V. cholerae* can produce exopolysaacharide specific to FSLW that confers resistance to oxidative stresses.

Aim 3: Comparative gene-expression profiles between wild-type and GASP *V. cholerae* persisting for 700 days in FSLW

To adapt to hostile and stressful survival conditions, bacterial species employ diverse phenotypic changes either by stochastic mechanisms or by adopting a “growth advantage stationary phase” (GASP) phenotype[48-50, 53]. In addition to many physiological, morphological and gene-expression changes that occur as cells enter long-term stationary phase, potentially the most significant changes are associated with genetic alterations that

occur in most cells incubated during long-term stationary phage in batch culture [47]. In aim 3 (Chapter 4), I compared the whole genome-based gene expression profiles between wild-type and GASP phenotype of *V. cholerae* using a custom made microarray platform.

CHAPTER 2

Survival of *Vibrio cholerae* in nutrient-poor environments is associated with a novel "persister" phenotype*

INTRODUCTION

Cholera, an ancient human disease, continues to be a major public health threat worldwide, particularly in countries where sanitary conditions and hygiene are suboptimal [1]. *V. cholerae* strains producing cholera toxin cause the disease cholera, which tends to occur in seasonal epidemics in cholera-endemic regions [2, 21]. Available data suggest that the microorganism survives between epidemics in aquatic reservoirs (including fresh, marine and estuarine waters), with environmental triggers causing seasonal increases in counts, followed by "spill-over" into human populations [1]. This model is complicated, however, by the observation that these environmental reservoirs are often nutrient-poor with extremely low or non-detectable *V. cholerae* cell counts between epidemics or even during epidemics [22, 52]. I still do not have a good understanding of the cellular mechanisms underlying environmental persistence of the microorganism, or of the environmental triggers that stimulate cell growth.

It has been shown that *V. cholerae* enters into a viable but non-culturable state (VBNC) in response to nutrient starvation and cold temperature [32, 51]; however, the resuscitation of VBNC, at least under laboratory conditions, is inconsistent, raising questions about the role of the VBNC state in cholera epidemiology [30, 31]. *V. cholerae* can also switch from a smooth

---

*Reprinted with permission

.Jubair M, Morris JG Jr, Ali A (2012) Survival of *Vibrio cholerae* in Nutrient-Poor Environments Is Associated with a Novel "Persister" Phenotype. PLoS ONE 7(9): e45187.

colonytype to a “rugose” (wrinkled) colony variant [54, 55]. The rugose variantcharacteristically produces copious amounts of an exopolysaccharide that confers resistance to chlorine, acid pH, and oxidative and osmotic stresses[33, 35,56].Thus, because of the superior survival ability of rugose variant (particularly in stressful environments), compared to their smooth counterpart, my group [33] and other investigators [34] have hypothesized that the rugose variant is a survival phenotype of *V. cholerae*. However, that idea is controversial because (i) in my group’s own studies in Bangladesh, the efforts to isolate rugose variants of *V. cholerae* from the bacterium's aquatic reservoirs have generally not been successful (Ali et al; unpublished observations), and (ii) a previous study [33], using a medium that promotes high-frequency rugose production, found that a majority of the *V. cholerae* strains tested were unable to shift to the rugose state.

For other bacterial pathogens, exposure to adverse growth conditions/antibiotics can promote the emergence of a sub-population of what have been termed “persister” cells, which remain viable for extended periods of time under stress conditions via a variety of mechanisms [37-41]. In keeping with this persister model, I hypothesize that a subpopulation of *V. cholerae* cells switches to a culturable persister phenotype (PP) or dormant state in response to nutrient starvation upon its release into aquatic environments. In this chapter, I present evidence that *V. cholerae* is able to survive in a culturable form for over two years with no added nutrients in a fresh water lake microcosm; that cells in this environment undergo unique morphologic changes, with reversion back to “normal” *V. cholerae* morphology when placed back in nutrient-rich conditions; that they elicit quorum sensing responses, in keeping with prior

reports regarding *Pseudomonas aeruginosa* persisters [57, 58]; and that growth is enhanced when strains are exposed to chitin or phosphate.

MATERIALS AND METHODS

Preparation of nutrient-poor microcosms

Fresh water used to prepare microcosms was collected from a 30.2-acre natural lake (Wauburg Lake) in Gainesville, Florida. At thetime of collection, the pH and the salinity of the water were determined using a portable HACH pH and conductivity meter (model D0175). The pH of collected water ranged from 7.2-8.3. Nutrient composition was determined on two occasions, and was virtually identical both times; Table 2-1 shows results from one determination, compared to L-broth.The low nutrient content found in my lake water is similar to what has been seen in pond waters obtained from Bangladesh and other regions of the United States [30].Aliquots (500-ml) of lake water were sterilized using Nalgene 0.22 µm membrane filter units (Nalgene), and microcosms prepared by transferring 50 ml to sterile 250-ml Erlenmeyer flasks. Inocula were prepared by inoculating aliquots (3-ml) of L-broth with a single colony from an overnight plate culture of *V. cholerae* strain N16961. After overnight incubation, cells in L-broth were washed in saline (0.85% NaCl), appropriately diluted, and 100 µl of diluted culture was inoculated into the microcosm flasks. As confirmed by plate counts (Figure 2-1A), initial *V. cholerae* concentrations in the microcosms ranged from 10^4 to 10^6 cfu/ml (Figure 2-1A and; Table 2-2).

Maintenance of microcosms

Microcosms were maintained at room temperature unless otherwise indicated. The number of persisting culturable *V. cholerae* (cfu/ml) from each microcosm was determined using a standard plate count method; samples for culture were collected daily for the first 8 days after inoculation, and every 15 days thereafter for as long as the microcosm was maintained. Before plating, each microcosm flask was hand-swirled for three minutes to disperse any bacterial cell aggregates that might have been present.

Microcosms in which bacteria were not detected were re-examined three consecutive days by plating aliquots (100-µl) of undiluted samples on L-agar (10 plates). If no colonies were identified, a final aliquot of 100 µl from each of the microcosms was inoculated into fresh 3-ml L-broth, incubated at 37 and 25^{o}C in both shaking and static growth conditions, and then plated on L-agar with overnight incubation at 37^{o}C. If all plates were negative, I concluded thatculturable bacteria were not present in the microcosm. Studies were terminated after 770 days (Figure 2-1A).

Transfer (daughter) microcosms

Twelve “daughter” microcosms, prepared as described above, were inoculated with an aliquot of 1 ml from an original (seed) microcosm (microcosm M4; see Figure 2-1A, Table 2-2) at day 96 of incubation. Samples were collected for plate counts using methods and time intervals as described above for the original microcosms. Studies were terminated at 630 days (Figure 2-1B, Table 2-3).

Scanning electron microscopy (SEM)

*V. cholerae*grown either in L-broth or persisting in microcosms at room temperature was fixed with Trumps buffer and deposited onto 0.4 μm polycarbonate membrane filters. The fixed cell samples were washed three times with phosphate buffered saline (PBS; pH 7.24), post fixed with 2% osmium tetroxide, rinsed with deionized water, and dehydrated with ethanol series 25%, 50%, 75%, 95%, 100%. Fixation, rinsing, and dehydration were performed with a Pelco BioWave Pro laboratory microwave (Ted Pella, Redding, CA, USA). Ethanol was removed from the samples by critical point drying (Autosamdri-815, Tousimis Research Corp, Rockville, MD, USA). Dried samples were mounted on carbon adhesive tabs on aluminum specimen mounts and coated with Au/Pd with a sputter coater (DeskV Denton Vacuum, Moorestown, NJ, USA). Micrographs of bacterial cells were acquired with field-emission scanning electron microscope (S-4000, Hitachi High Technologies America, Inc. Schaumburg, IL, USA).

Quorum sensing assay

Quorum sensing assays were performed as described by Miller et al [59].Briefly, cell-free spent cultures (filtered through a 0.22 μm disposable syringe filter) of *V. cholerae* (ca.10^3 cfu/ml) persisting in FSLW microcosms at room temperature were used as a source of CAI-1 activity. Supernatants were derived from microcosms persisting for 24 h, 180 days, and 700 days. As a positive control, a single colony of *V. cholerae* N16961 was grown statically overnight in L-broth at 25^{o}C. Control cultures were standardized to ca. 10^3 cfu/ml by diluting with L-broth, and filter sterilized as described above. Cell-free culture supernatants were tested for the presence of CAI-1 activity by inducing light production in the *V. cholerae* reporter strain MM920

(a kind gift of Bonnie Bassler, Princeton University, PA) containing the cosmid pBB1, which carries the *V. harveyi lux* operon [59]. This reporter strain neither produces CAI-1 nor responds to AI-2. The reporter strain was grown overnight in L-broth media with shaking at 30^{o}C, diluted 1:10 in fresh L-broth medium, and 70 µl aliquots transferred to an opaque wall 96-well microtiter plate. Thirty µl cell-free supernatant was added to microtiter wells containing 70 µl MM920 culture to obtain a final culture volume of 100 µl. The plates were incubated at 30^{o}C with agitation and light production was measured at 30 min intervals in a BioTek Synergy 2 plate reader (Biotek Instruments, Winooski, VT). Data were reported as peak fold light induction compared to sterile L-broth and lake water controls.

Supplementation of major nutrients to microcosms

A series of transfer studies were conducted monitoring growth/survival of *V. cholerae* in nutrient-supplemented microcosms. Briefly, an aliquot of 5 µl of *V. cholerae* was directly inoculated into 3-ml of filter sterilized lake water (final concentration of ca. 40-60 cfu/ml) supplemented with different nutrients, as described below. Transfer microcosms were monitored daily for 8 days after inoculation, using inocula from microcosm M4 at day 180 and 700+. Transfer microcosms were supplemented (prior to adding the *V. cholerae* inoculum) with: (i) readily available carbon sources (0.5, 1 and 2% sucrose), (ii) complex carbon sources (alkaline peptone water [APW][a mixture of 1% Peptone and 0.5% Yeast extract, pH 8.6] and chitin [0.05, 0.1 and 0.15 %][Sigma-Aldrich, St. Louise, MO]), (iii) nitrogen sources (ammonium bicarbonate [0.5, 1.0 and 1.5 mM] or (iv) phosphate [0.5, 1.0 and 1.5 mM K_2HPO_4].The cultures were incubated statically at room temperature or at 37^{o}C as required and subsequently plated on L-

agar. In the initial experiments, I did not find any significant differences in response based on differences in nutrient concentrations. Consequently, I restricted my studies to a single final concentration of representative compounds: (i) sucrose (1%); (ii) 1X APW; (iii) chitin (.05%); (iv) ammonium bicarbonate (1.0mM); and (v) phosphate (1.0 mM).

RESULTS

Persistence of culturable *V. cholerae* in lake water microcosms as "persister phenotype"

I prepared fifteen original independent microcosms. Eight of the 15 microcosms were culture-negative within 9 days of inoculation; 5 become culture negative within 120 days (Table 2-2), while in two instances culturable *V. cholerae* were detectable for over 700 days (Figure 2-1A). To explore the persister phenotype's response and adaptation to fresh FSLW, 1 ml inocula were transferred on day 96 from microcosm M4 to twelve fresh 50 ml FSLW microcosms. *V. cholerae* in these transfer ("daughter") microcosms showed an initial increase in counts, from ca. 10^2 to ca. 10^4 cfu/ml (Figure 2-1B and Table 2-3) suggesting that starved *V. cholerae* in microcosms can response to miniscule amounts of nutrients contained in FSLW. Subsequent patterns of survival in these daughter microcosms were similar to those seen with the initial microcosms, with 2 of the 12 showing death of the microcosm within 8 days, 7 of the 12 exhibiting death between 9 and 220 days (Table 2-3), and 3 having culturable *V. cholerae* for periods in excess of 600 days (Figure 2-1B). Microorganisms recovered from all microcosms were confirmed as *V. cholerae*, based on serologic and genetic analysis, including screening for *toxR* and *ompW* (*V. cholerae* species-specific genes) and *tcpA* and *ctxB* (*V. cholerae* virulence genes).

Morphology of *V. cholerae* persisting in microcosms.

As *V. cholerae* persisted for over 700 days in microcosms, I examined the cellular morphology of the bacterium at multiple survival points. When examined by scanning and transmission electron microscopy, material from microcosms showed evidence of morphologic changes in cells within 24 h of introduction into the nutrient-poor environment: this includes what appeared to be stressed *V. cholerae* cells with a single polar flagellum, and elongated and helical cells with predominantly bipolar flagella with evidence of cell division in elongated cells (Figure 2-3, panel B). Material from microcosms that retained culturability at 180 days showed (i) no evidence of cell division, (ii) formation of predominantly helical and spiral cells with a very limited number of coccoid cells, including cells producing and disseminating what appeared to be numerous outer membrane vesicles and buds [60], (iii) a high degree of pilliation, (iv) replacement of a singular polar flagellum with bipolar and peritrichous flagella, and (v) non-flagellated curved and rod shaped cells (Figure 2-3, panel C). At 700 days, the majority of cells were very small in size, with a high degree of aggregation associated with pleotrophic flagella emanating virtually from all sides of the cells (Figure 2-3, panel D). Direct transfer into L-broth of microcosm material (100 µl) from each of these time points resulted in reversion to a cell morphology (Figure 2-3, frame at far right of panels B, C, and D) indistinguishable from that of the original inoculum (Figure 2-3, panel A). Material from "dead" microcosms, from which *V. cholerae* could not be isolated, showed only cellular debris, with no evidence of intact cells, including no evidence of the small, coccoid cells that have been associated with the viable but non-culturable phenotype [32]. When material from the 700+ day microcosms was directly plated on L-agar, *V. cholerae* colonies showed a reduction of 75% in colony size , as compared

with colonies recovered from samples collected from the original inoculum and from microcosms through about day 300 after inoculation. These small colonies reverted to normal colony size when passed a second time on L-agar (Figure 2-2).

Quorum Sensing

Formation of persister cells in *Pseudomonas aeruginosa* has been linked with a quorum sensing mechanism [57, 58]. To determine if *V. cholerae* persister cells produce quorum sensing signals in adapting to nutrient stressed microcosm condition, I measured CAI-1 signaling molecules in cell-free spent microcosm materials using *V. cholerae* reporter strain MM920, as previously described [59]. Cell-free supernatants obtained from microcosms at 24 h, 180 days, and 700 days all showed >2-fold increase in activity as compared with sterile spent culture media from L-broth stationary cultures (Figure 2-4).

Effect of nutrients on the growth of *V. cholerae* persisting in microcosms

When either chitin or potassium phosphate was added to the FSLW prior to the inoculation of materials from the original microcosm (inocula taken from both 180 and 700+ days), *V. cholerae* showed an increase in counts (Figure 2-5A and 2-5B, respectively). Although both chitin and phosphate promoted the growth of nutritionally stressed *V. cholerae* in microcosms, there were differences in response when inocula from 180 and 700+ days were compared. Bacteria from 180 days (still showing typical large colony morphology; Figure 2-2) showed the most striking response to chitin (Figure 2-5A). In contrast, bacteria from 700+ day microcosms (which had a small colony morphology; Figure 2-2) exhibited much more prominent response to phosphate (Figures 2-5B). Interestingly, a more readily available carbon

source (sucrose) was able to only poorly promote the growth of *V. cholera*e. In contrast, complex organic material present in APW promoted the robust growth of the organism at all experimental conditions. My results suggest that once *V. cholerae* senses a nutrient-poor environment, it immediately turns off pathways needed to metabolize inorganic simple carbohydrate, while turning on genetic mechanisms required to metabolize complex carbohydrate. I, however, cannot rule out the possibility that, in addition to complex carbohydrate, proteins, minerals, and vitamins in APW contributed the robust growth of the bacterium.

I was unable to induce culturable *V. cholerae* cells from apparently dead microcosms when the microcosms were supplemented with nutrients used in my experiments. My results corroborate the earlier report that once culturable *V. cholerae* becomes non-detectable in microcosms, it cannot be resuscitated into a culturable form using methods employed in previous study [61].

DISCUSSION

The key question asked in this study was: Does *V. cholerae* retain a culturable phenotype in response to nutrient starvation, and if so what morphological and physiological changes occur in association with this phenotype? As monitoring of *V. cholerae* survival in the natural environment is difficult (as reservoirs may differ widely in nutrient content, temperature, and a range of other variables), I used a lake water microcosm model that permitted careful monitoring of survival across time, in a way that would not be possible outside of the laboratory. At the same time, it may also limit the range of responses: under

different environmental conditions, *V. cholerae* may respond in different ways (including elicitation of the viable but non-culturable phenotype, which I did not observe). Consistent with my hypothesis, I demonstrated that in response to nutrient limitation, *V. cholerae* promotes and selects a phenotype that fits the classic definition of the persister model seen with other bacteria [37, 38]. The cells described here survived for over 700 days in lake water microcosms without loss of culturability. In contrast to my findings, in a microcosm study reported by other investigators, culturable *V. cholerae* was detected for 85 days, with viable but non-culturable (VBNC) cells identified for an additional 495 days, after which the VBNC cells became undetectable [61]. It remains to be seen how these different mechanisms contribute to the seasonal patterns of epidemic cholera seen in endemic regions.

In keeping with a "persister" conceptual framework [37, 38], which implies a stochastic origin, cells transferred from persisting microcosms into new nutrient-poor FSLW showed patterns of survival that mirrored those seen among the initialmicrocosms. I did see evidence that persisting cells underwent morphologic changes, beginning as early as 24 h after introduction into a nutrient-poor environment, and progressing across time to novel morphologic types characterized by small cell size, aggregation, and pleotrophic flagella by 700 days. However, when persisting cells were placed back into rich media, they regained a "normal" microscopic appearance. Similarly, microcosm material directly plated on L-agar had a small colony morphology, which reverted to a normal morphology when re-plated. The genetic basis and drivers for these morphologic changes remains to be determined: among other possibilities, it may reflect action of a two-component genetic regulatory system, genetic switching, epigenetic mechanisms, or even a single nucleotide mutation. Indeed, a study using

an advanced genetic screening mechanism and subsequent knock out mutation demonstrated that global regulators, including DksA, DnaKJ, HupAB, and IhfAB were involved in the persister formation in *Escherichia coli*[62].

My data suggest that quorum sensing plays a role in this process, as has been described for *Pseudomonas aeruginosa* strains having a persister phenotype [57]. Although I have seen a significant increase (2-fold) in quorum sensing molecules in *V. cholerae* persisting in microcosms compared to that of in L-broth grown cells, I observed no difference in signaling molecules when microcosm materials were examined at 1, 180, and 700 days post inoculation.This may reflect continuous production of controlled amounts of quorum sensing molecules or alternatively, the CAI-1 molecules are highly stable in under conditions present in the microcosm, reducing the rate of decline once produced by *V. cholerae* in the early phase of persistence. Interestingly, quorum sensing mechanisms/signaling molecules increased the persistence of *Pseudomonas aeruginosa*[58, 63]. Again, further molecular studies will be needed to confirm and define the mechanisms involved, and the role of quorum sensing in the persister state.

In environmental studies conducted by my group and others, there is clear evidence that seasonal cholera epidemics are preceded by increases in *V. cholerae* in environmental reservoirs [64]. I did find that growth of persisting cells was differentially facilitated by chitin (a complex carbon source), phosphate, and the presence of complex carbohydrates. Chitin is well recognized as a key trigger for natural competency and biofilm formation in *V. cholerae*[65]. The link with chitin would be in keeping with prior work suggesting that occurrence of seasonal

epidemics is triggered by blooms of phytoplankton, followed by zooplankton such as copepods, events which would increase exposure to chitin[52]. Similarly, environmental phosphate concentrations tend to be driven by variables such as rainfall (linked with agricultural runoff), and/or dry season conditions that decrease water levels in ponds and other potential reservoirs[66, 67]; both events are variables that have been linked with initiation of epidemics[66].

Table 2-1. Comparison of the major nutrient components of L-broth and filter sterilized lake water.

Component	L-broth (mg/L)[a]	FS LW (mg/L)[b]
Total carbohydrate	1,645	14.3
Total nitrogen	1,845	1.55
Ammonia nitrogen	820	0.28
Sodium	731	8.55
Chloride	642	15.0
Calcium	2.0	7.48
Iron	0.2	1.0
Potassium	183	1.43
Phosphate	430	0.06
Magnesium	120	1.78

[a]Analysis were performed by Becton and Dickenson (sparks, MD). The data represent the total amount of each component contained in the Bacto Trypton and Yeast extract used to prepare L-broth. The sodium chloride values also include the amount of NaCl used to prepare L-broth.
[b]The FSLW used in the microcosm assay was analyzed by Advanced Environmental Laboratories, Inc., (Gainesville, Florida, USA)

Table 2-2. Persistence of *V. cholerae* strain N16961 in filter sterilized lake water microcosm (original microcosms)

Microcosm (M)	log_{10}cfu/ml[a]	Persistence (in days)[b]
M1	5.14	9
M2	4.83	8
M3	5.24	8
M4	5.6	770
M5	4.6	4
M6	4.48	3
M7	4.34	4
M8	5.57	96
M9	5.05	120
M10	5.23	120
M11	4.96	120
M12	5.62	770
M13	5.15	6
M14	4.85	5
M15	5.2	22

[a]Number of culturable *V. cholerae* colony from each individual microcosm was determined using the standard plate count immediately after adding the inoculum to the microcosm.
[b]Days after which the culturable *V. cholerae* in microcosm became non-detectable.

Table 2-3. Persistence of *V. cholerae* strain N16961 in transfer (daughter) microcosm (TM).

Transfer microcosm (TM)	log_{10}cfu/ml[a]	Persistence (in days)[b]
TM1	2.64	29
TM2	2.61	3
TM3	2.36	630
TM4	2.34	111
TM5	2.3	630
TM6	2.41	630
TM7	1.3	98
TM8	1	7
TM9	2.08	71
TM10	2.11	33
TM11	1.3	33
TM12	1	220

[a]Number of culturable *V. cholerae* colony from each individual microcosm was determined using the standard plate count immediately after adding the inoculum from original microcosm (M4) to freshly prepared FSLW.
[b]Days after which the culturable *V. cholerae* in microcosm became non-detectable.

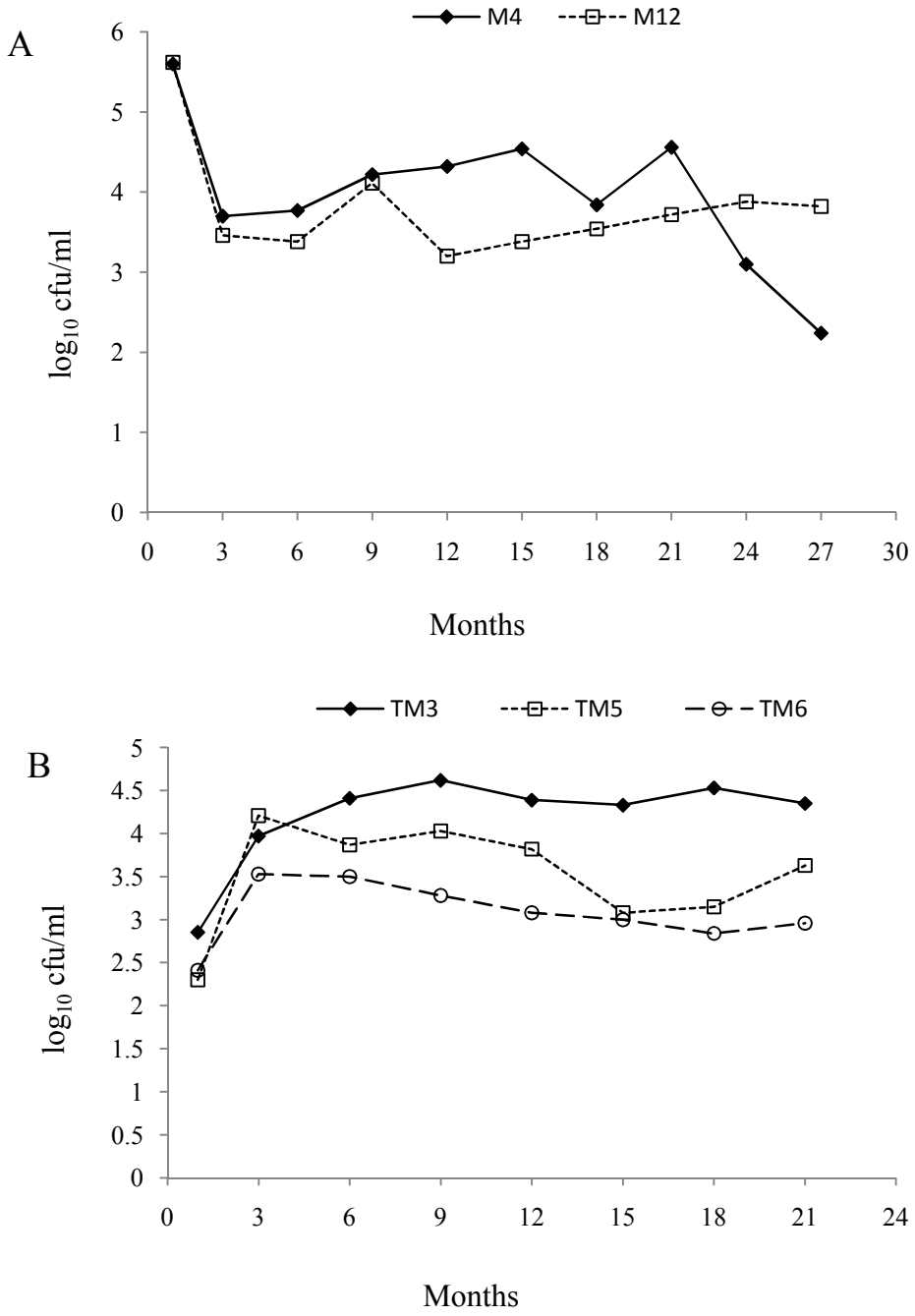

FIGURE 2-1. Persistence of epidemic strain of *V. cholerae* N16961 in filter sterilized lake water (FSLW) microcosms stored statically at room temperature: (A) persistence of two microcosms (M4 and M12) for >700 days. (B) persistence of *V. cholerae* in transfer (daughter) microcosms. Transfer microcosm (TM) was prepared by transferring one ml culture from original microcosm (M4, Figure 2-1A) to fresh FSLW (49-ml) at day 96. The microcosms were stored statically at room temperature. Data represent the persistence of three microcosms in excess of 600 days.

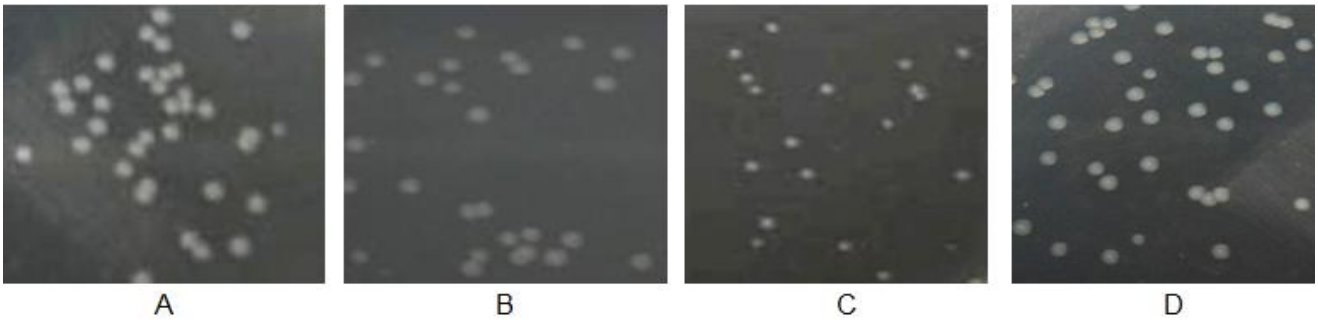

FIGURE 2-2. Photographs of *V. cholerae* N16961 colonies on L- agar plates incubated overnight at 37°C. (A) *V. cholerae* colonies on L-agar (colony diameter ranges from 2 to 2.5 mm), (B) *V. cholerae* colonies on L-agar after persisting in 180 days in microcosm (colony diameter ranges from 2 to 2.5 mm), (C) *V. cholerae* colonies growing on L-agar after persisting in 700 days in microcosm (colony diameter ranges from 0.5 to 0.6 mm), (D) an aliquot (100 µL) of 700 days microcosm was transferred into 3 ml of L-broth and incubated at room temperature. Subsequently the culture was plated on L-agar (colony diameter ranges from 2 to 2.5 mm).

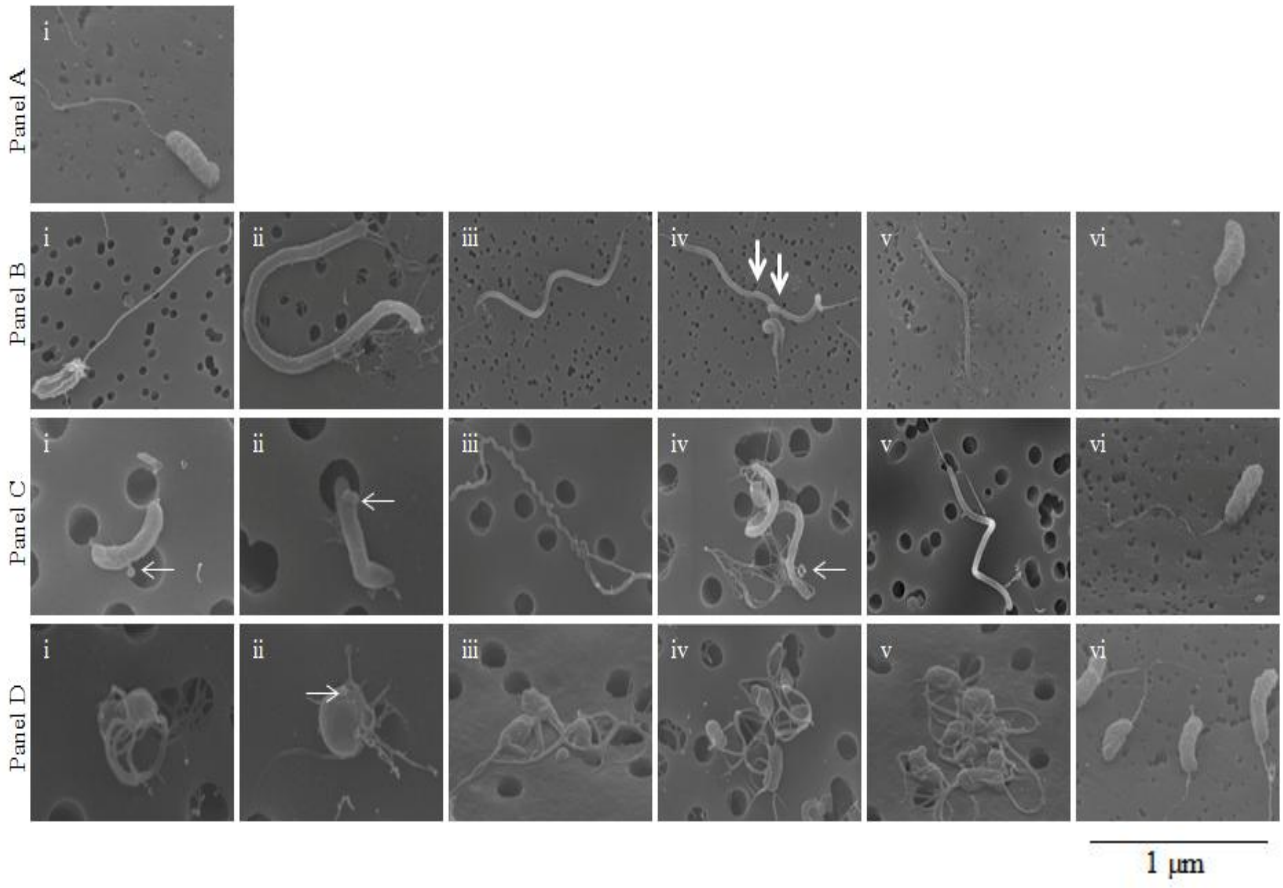

FIGURE 2-3. Scanning electron micrographs of *V. cholerae* strain N16961. Panel A: Scanning electron microscopy (SEM) image of *V. cholerae* grown statically overnight at room temperature in L-broth. Panel B: Images obtained with SEM from *V. cholerae* grown overnight statically at room temperature in FSLW microcosm. Images i to v exhibit diverse *V. cholerae* morphologies. Image vi obtained after an aliquot of 100 µl of the 24 h old microcosm was transferred to L-broth and incubated statically overnight at room temperature before SEM was performed. Panel C: Images obtained with SEM from *V. cholerae* persisting statically at room temperature in microcosm for 180 days. Images i through v exhibit different *V. cholerae* morphologies. Image vi obtained after an aliquot of 100 µl of the 180 days old microcosm was transferred to L-broth and incubated overnight at room temperature before SEM was performed. Panel D: Images obtained with SEM from *V. cholerae* persisting statically at room temperature in microcosm for 700 days. Images i through v exhibit different *V. cholerae* morphologies. Image vi obtained after an aliquot of 100 µl of the 700-day old microcosm was transferred to L-broth and incubated overnight at room temperature before SEM was performed. (scale bar, 1 µm; thick arrows indicate evidence of cell division; thin arrows indicate bud and OMVs formation).

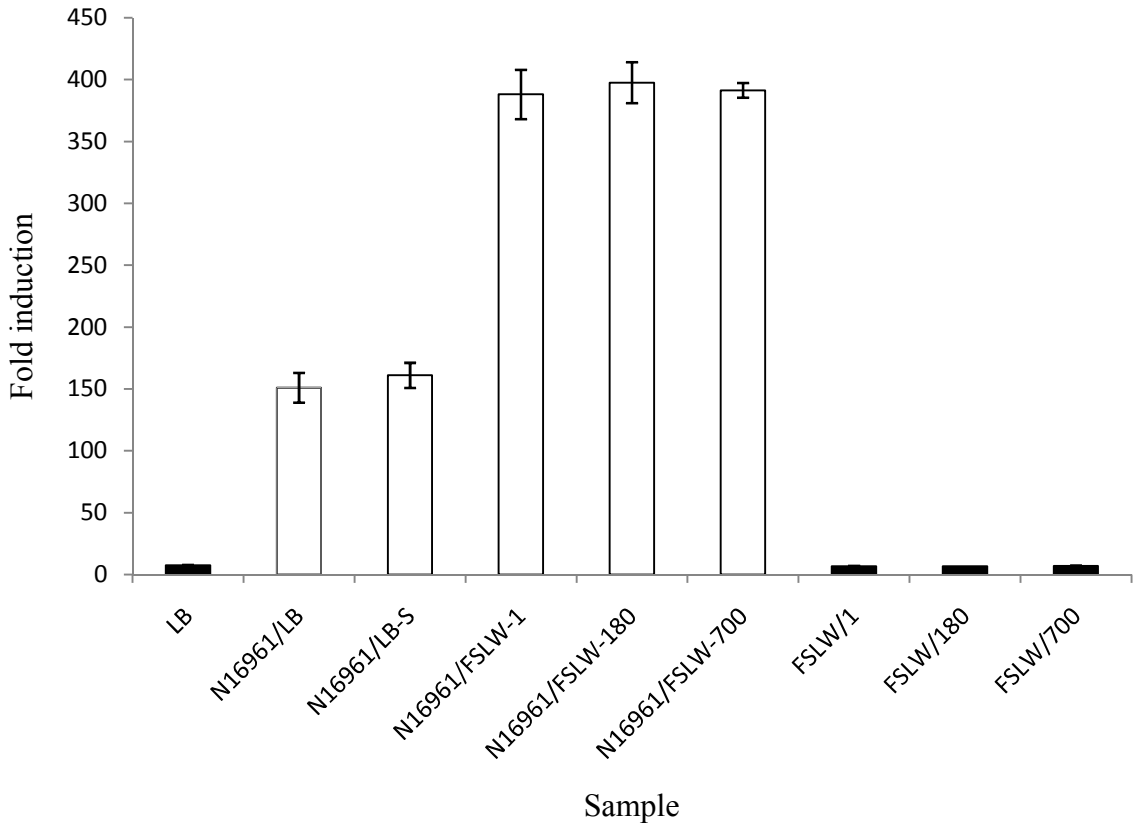

FIGURE 2-4. Effect of quorum sensing mechanism(s) on the persistence of *V. cholerae* in FSLW microcosm. Measurement of CAI-1 activity in *V. cholerae* grown either overnight in L-broth at 37^{o}C with a shaking speed of 250 rpm or in *V. cholerae* persisting in FSLW statically at room temperature for 1 day (24 h), 180 days and 700 days. Data represent the average results obtained from 6 independent experiments. Cell-free spent media were used as sources of CAI-1 molecules that induced light production in *V. cholerae* strain MM920 as described previously [59]. LB, sterile L-broth without reporter strain, MM920; N16961/LB, overnight grown *V. cholerae* in L-broth was diluted appropriately (to obtain ca. 10^3 cfu/ml) in L-broth and the diluted sample was filter sterilized and used, N16961/LB-S; cell-free spent medium of *V. cholerae* grown overnight in L-broth was diluted in L-broth appropriately and used; N16961/FSLW-1, *V. cholerae* (ca. 10^3 cfu/ml) was grown overnight statically at room temperature in FSLW microcosm; N16961/FSLW-180, cell-free supernatant was obtained from *V. cholerae* persisting for 180 days in FSLW microcosm; N16961/FSLW-700, cell-free supernatant was obtained from *V. cholerae* persisting for 700 days in FSLW microcosm; FSLW/1, 100 µl FSLW without reporter strain; FSLW/180, 100 µl sterile spent media obtained from 180 days old microcosm without reporter strain inoculation; FSLW/700, 100 µl sterile spent media obtained from 700 days old microcosm without reporter strain inoculation. Bar indicates standard error.

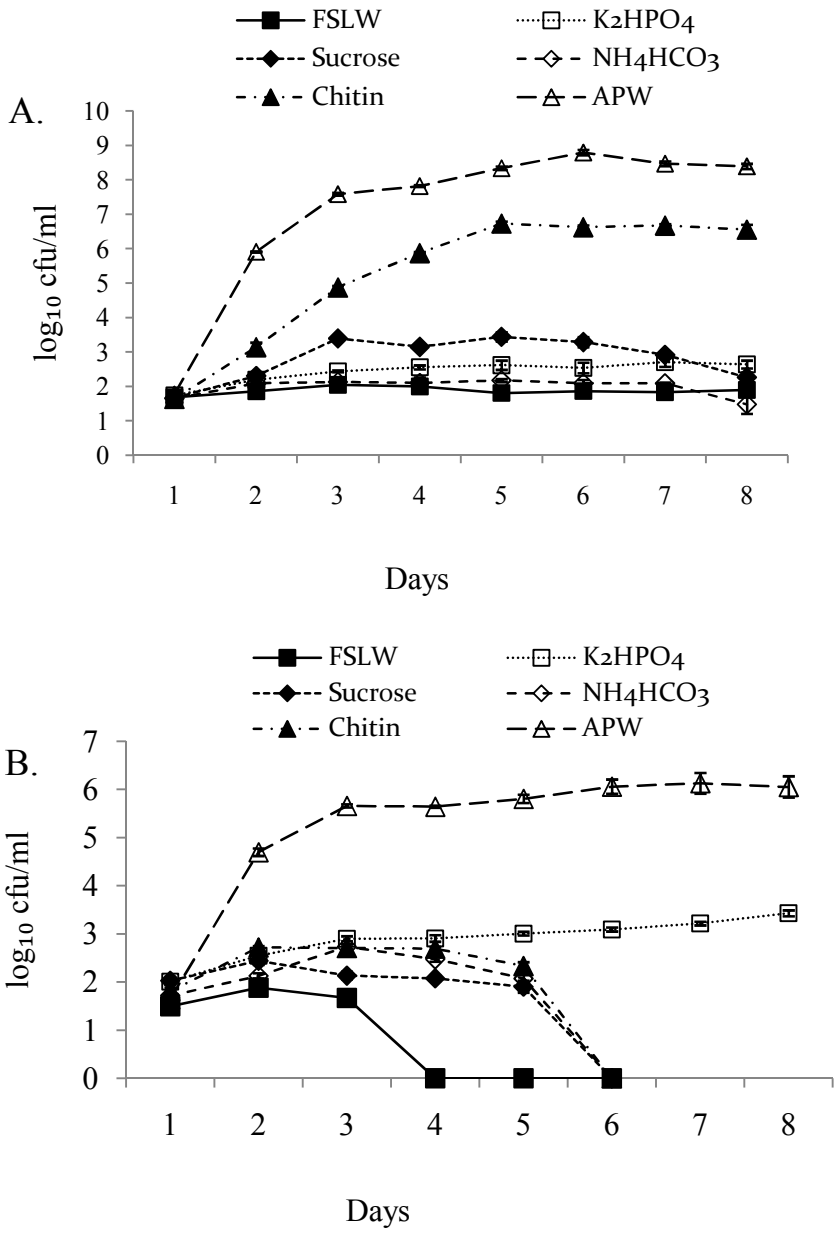

FIGURE 2-5. Effect of fresh FSLW supplemented with nutrients on the growth and persistence of *V. cholerae* surviving in microcosms incubated statically at room temperature for 180 and 700 days. Data represent the average result obtained from six independent microcosms: (A) Five µl inocula (ca. 40-60 cfu/ml) from microcosms persisting for 180 days were directly transferred to 3 ml fresh FSLW supplemented with indicated nutrients. Counts (cfu/ml) of culturable *V. cholerae* were determined for 8 consecutive days using standard plate count, and (B) indicated nutrients were added to the fresh FSLW prior to transfer of 5 µl inocula (ca. 40-60 cfu/ml) from microcosm persisting for 700 days. Counts (cfu/ml) of culturable *V. cholerae* were determined for 8 consecutive days using standard plate count. Bar indicates standard error.

CHAPTER 3

Vibrio cholerae Persisted in Microcosm for 700 days Inhibits Motility but Promotes Biofilm Formation in Nutrient-poor Lake Water Microcosms*

INTRODUCTION

Cholera is a major public health threat worldwide, particularly in countries where safe drinking water, adequate sanitation and hygiene are suboptimal [1]. Cholera toxin (CT)-producing *V. cholerae* strains, generally in serogroups O1 and O139, are the cause of epidemic cholera. *V. cholerae* has two life styles, including transient passage through the human intestine where it causes profuse diarrhea (i.e. cholera), and a second existence in aquatic environments, including fresh, estuarine and marine environments [1, 2, 21]. In aquatic reservoirs, the microorganism can survive either in planktonic (free-living) form or in biofilms [2, 21]. Available data suggest that the bacteria survive between epidemics in these aquatic reservoirs, with environmental triggers causing seasonal increases in counts, followed by "spill-over" into human populations [1]. The genetic and physiologic basis of persistence of *V. cholerae* in the environments, particularly during inter-epidemic period, is poorly understood.

In this context, it has been suggested that *V. cholerae* can enter into a viable but non-culturable state (VBNC) in response to nutrient starvation and/or cold temperature [32, 51];

---

*Reprinted with permission.
Jubair M, Atanasova KR, Rahman M, Klose KE, Yasmin M, et al. (2014) *Vibrio cholerae* Persisted in Microcosm for 700 Days Inhibits Motility but Promotes Biofilm Formation in Nutrient-Poor Lake Water Microcosms. PLoS ONE 9(3): e92883.

however, the resuscitation of VBNC, under laboratory conditions, is inconsistent, raising questions about the role of the VBNC state in cholera epidemiology [30, 31]. *V. cholerae* can also switch from a smooth colony type to a "rugose" (wrinkled) variant characterized by copious production of exopolysaccharide conferring resistance to chlorine, osmotic and oxidative stresses [33-35]. However, the role(s) of rugose variant of *V. cholerae* in epidemic cholera is limited because not all *V. cholerae* strains are capable of switching to rugose variant even in a medium promoting high-frequency rugose production [33].

Amid this conundrum, I recently reported that a subset of culturable *V. cholerae* assume what I have termed a "persister" phenotype in a "filter sterilized" lake water (FSLW) microcosm model [42]. In that study I found that only 13% of the microcosms yielded cells that persisted in excess of 700 days while 87% of the microcosms resulted in the death of cells by 120 days. Furthermore, I observed that persisting cells in 700-day old microcosms expressed a small colony phenotype associated with very small rod shaped cells with peritrichous flagella and a high degree of cell aggregation. In contrast, cells persisting in microcosms for 24 h exhibited normal colony phenotype with heterogeneous mixtures of cells with predominantly long helical cells with bipolar flagella [42]. A "growth advantage in stationary phase" (GASP) phenotype describes microorganisms that survive long-term in a stationary growth phase under stressful conditions [47, 48, 53]. For further analysis of 700 day-old cells, I subcultured the cells from microcosms onto L-agar and subsequently stored them in glycerol broth at -80^{o}C. As I was not certain if 700 day-old persister cells of microcosm origin will retain their genetic and phenotypic traits unchanged upon storage in glycerol broth, for my convenience, I refer this glycerol-stored cells to GASP-700D phenotype; in contrast, wild-type *V. cholerae* N16961S strain grown

overnight statically in FSLW at room temperature will be henceforth termed as N16961S-24 (Table 3-1).

Persister cells in other human pathogens exhibited biofilm formation conferring resistance to environmental stresses [38, 39, 41, 58]. In *V. cholerae* the positive association of polar flagellum to biofilm formation has been demonstrated [68]. To better understand the GASP-700D phenotype of *V. cholerae* and to compare the differences, if any, between N16961S-24 and GASP-700D, I investigated the role(s) of novel flagella elicited by N16961S-24 and GASP-700D, respectively [42], in motility and biofilm formation. Here, I provide evidence that GASP-700D showed no motility in soft agar; produced biofilm only in nutrient-poor FSLW; and conferred resistance to oxidative stress when compared to N16961S-24.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains, including *V. cholerae* wild-type strain N16961S and its isogenic mutants (obtained either natural selection and/or created by defined genetic mutations) used in this study are listed in Table 3-1. As reported earlier, I generated *V. cholerae* N16961 persister cells (in excess of 700 days) in "filter sterilized" lake water microcosm model. Briefly, aliquots (500 ml) of lake water were sterilized using Nalgene 0.22 µm membrane filter units (Nalgene), and the microcosms were prepared as follows: 50 ml of "filter sterilized" lake water (FSLW) was transferred into a sterile 250 ml Erlenmeyer flask; for inoculum preparation a single colony of *V. cholerae* N16961strain, obtained from L-agar grown overnight at 37^{o}C, was inoculated into 3 ml of L-broth. The culture was incubated overnight at 37ºC with a shaking speed of 250 rpm, spun

down and the resulting pellet was washed 2X in saline (0.85% NaCl), reconstituted in 3 ml saline, appropriately diluted, and 50 µl of diluted culture was inoculated into the microcosm flasks containing 50 ml FSLW. As confirmed by plate counts, initial *V. cholerae* concentrations in the microcosms ranged from $10^4$to 10^6 cfu/ml. The culturable cells from microcosm were determined at intervals using standard plate counts. The 700 day-old cells were subcultured on L-agar and stored in glycerol broth at -80^{o}C. While I cannot be certain that this is true for all GASP-700D cells, I observed that GASP-700D exhibited small colony phenotypes on L-agar for at least four consecutive days of subculture both at room temperature and at 37^{o}C. However, when the cells were inoculated into L-broth and incubated overnight at 37^{o}C with a shaking speed of 250 rpm, a mixture of small and large colonies were observed on L-agar upon plating. All the strains used in this study were subcultured from glycerol stock at -80^{o}C onto L-agar and incubated overnight at 37^{o}C before being used for specific experiments. As needed, antibiotic was added to the bacterial cultures as follows: ampicillin (100 µg/ml) and polymyxin B (50 U/ml).

Genetic manipulations

A Δ*flaA* mutant (AA212; Table 3-1) was created in the background of N16961S strain (Table 3-1) using a Δ*flaA* gene targeting vector described previously [69]. For creating in-frame mutation in *hydG*/*vpsR*[34, 70] and in a rugosity-associated gene, *vpsA* (VC0917, encoding UDP-N-acetylglucosamine 2-epimerase [*wecB*]) [71] in the back ground of N16961S, N16961R and GASP-700D (Table 3-1), a two-step PCR cloning strategy was used. Briefly, two PCR products flanking an internal deletion (420-bp) in *vpsR* were engineered. Each PCR product carries a

restriction endonuclease site at its 5' end; however, 3'-ends of the forward and reverse PCR products carried a common restriction site to facilitate deletion mutation. For *vpsR*, *SacII* and *XbaI* sites were introduced at 5' and 3' ends, respectively, of the forward PCR amplicon (560-bp) while 5' and 3' ends of reverse PCR product (520-bp) were introduced with *XbaI* and *BamHI* sites, respectively. Primers aa212 and aa213 (Table 3-2) were used to amplify forward PCR fragment using N16961S chromosomal DNA as a template with standard PCR conditions. The PCR product was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA). The purified PCR product was digested with *SacII* and *XbaI,* the digested product was purified, and the PCR product was ligated with a similarly digested vector, pWSK29, [72] resulting in a plasmid (pAA69). The plasmid was transformed into *Escherichia coli* DH5α as described previously [73]. Next, two convergent PCR primers, including aa214 and aa215 (Table 3-2) were used to amplify the reverse PCR product; the amplicon was purified and digested with *XbaI* and *BamHI*. The digested products were purified and ligated into a similarly digested plasmid (pAA69), resulting in a plasmid pAA73 containing a 420-bp internal deletion of *vpsR*. The plasmid was transformed into DH5α. Subsequently, plasmid pAA73 was digested with *SacI* and *SalI* to retrieve a 1080-bp fragment and the fragment was gel purified. The purified fragment was ligated into a similarly digested suicide vector, pCVD442, [74] and transformed into an *E. coli* S17 λ *pir* resulting in a plasmid pAA78 (Table 3-1). *E. coli* S17 λ *pir* carrying pAA78 was conjugated to *V. cholerae* N16961S, N16961R and GASP-700D. Selection of transconjugants, counter selection, and chromosomal mutation using homologous recombination of *vpsR* was performed as described previously [70, 73]. Mutants sustained an internal in-frame deletion in *vpsR* (SΔ*vpsR,* mutation in smooth background [AA215, Table 3-1], RΔ*vpsR*, mutation in rugose background [AA216] and

GASP-700DΔ*vpsR,* mutation in GASP-700D background [AA217]) were verified by PCR and DNA sequencing as described previously [73]. A similar approach was also used for creating a null mutation in the *vpsA* gene in the background of N16961S, N16961R and GASP-700D, resulting in the mutants AA218, AA219 and AA220, respectively. Primers (aa264 and aa265, aa266 and 267) used to create null mutation in *vpsA* are listed in Table 3-2.

Motility assay

Motility of *V. cholerae* strains was determined using motility agar plates as described previously [73] with minor modifications. The experiment was performed with cells grown both in L-broth and FSLW. Briefly, N16961S, N16961R, GASP-700D and Δ*flaA* mutant were grown in L-broth and incubated overnight statically at room temperature. As for FSLW, the strains were first subcultured onto L-agar; a single colony from L-agar was grown in 3 ml L-broth and incubated overnight statically at room temperature. Subsequently, the cultures were spun down at 7,000 rpm for 5 min in a table top centrifuge; the pellet was washed 2X with FSLW and suspended into 3 ml FSLW and the culture was incubated overnight statically at room temperature. An inoculating wire was dipped into each culture and then stabbed into the motility agar plate. The plates were incubated for 8 h and overnight at 37^{o}C. Zones of migration of bacterial strains around the inoculating sites were measured at 8 h and after overnight incubation of the plates. If no zone was detected, a block of agar was cut around the inoculating site, homogenized in saline (0.85% NaCl), appropriately diluted in saline, and then plated on L-agar to determine if any culturable cells were survived in the inoculation site.

Quantitative real-time reverse transcription PCR (qRT-PCR)

For qRT-PCR, *V. cholerae* strains, including N16961S-24, N16961R-24 and GASP-700D (Table 3-1) were grown in FSLW overnight statically at room temperature. Total RNA was extracted and purified from each culture using the RNeasy kit (Qiagen, Valencia, CA); the contaminating DNA in the preparation was eliminated on-column by DNase digestion. Total RNA (10 ng) was converted to cDNA, and the RT-PCR assay were performed using iScript one-step RT-PCR kit with SYBR green (Bio-Rad, Hercules, CA) and CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) following manufacturer's instructions. Primers used in this study are listed in Table 3-2. For each sample, the mean cycle threshold of the test transcript was normalized to that of *toxR* (*toxR* was equally expressed both in L-broth and in FSLW) and presented relative to *V. cholerae* N16961S-24 strain that has arbitrarily been taken as 1 (Figure 3-2). Values above 1 or less than 1of a selected gene indicate that the transcript was present in higher or lower numbers, respectively, than that of control strain. Data are based on three independent experiments. Previous report using qPCR demonstrated that *V. cholerae* expressed *phoB* and Pst-system genes while repressed *tcp* genes when grown in "filter sterilized" pond water microcosms compared to its growth in nutrient-rich L-broth [30]. To validate my qRT-PCR data, I compared the differential gene expression by growing *V. cholerae* N16961S strain in nutrient-rich L-broth incubated overnight statically, and in nutrient-deficient FSLW under identical growth conditions. Expression of transcripts was determined as described above except that the threshold of transcript was presented relative to *V. cholerae* N16961S strain grown in L-broth.

Biofilm assays

Quantitative assessment of biofilm produced by *V. cholerae* strains grown both in L-broth and in FSLW was measured as described previously [68] with modifications. Twenty-four well polystyrene plastic plates (Corning Incorporated, Corning, NY) were used as the surface for bacterial attachment. For assessment of biofilm produced in L-broth, *V. cholerae* strains, including N16961S, SΔ*vpsR*, SΔ*vpsA*, N16961R, RΔ*vpsR*, RΔ*vpsA*, GASP-700D, GASP-700D Δ*vpsR* and GASP-700DΔ*vpsA* (Table 3-1) were examined. Biofilm assay was performed as described previously [68]. For measurement of biofilm produced in FSLW, *V. cholerae* strains, including N16961S-24, SΔ*vpsR*, SΔ*vpsA*, N16961R-24, RΔ*vpsR*, RΔ*vpsA*, GASP-700D, GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA* (Table 3-1) were investigated. Briefly, a single colony of each strain grown overnight on L-agar was inoculated into 3 ml L-broth and the cultures were incubated overnight with shaking (250 rpm) at 37^{o}C. The culture was spun down and the pellete was washed 2X with FSLW and subsequently reconstituted into 3 ml FSLW. Fifty µl culture was then mixed to 450 µl fresh FSLW (ca. 10^{8} cfu/ml) in a well of plastic plate; the culture was incubated overnight statically at room temperature. Following overnight incubation the cultures were discarded, and the wells were rinsed vigorously with distilled water to remove non-adherent cells, filled with 600 µl of a 0.1% crystal violet solution (Sigma, St. Louis, MO), allowed to incubate for 30 min at room temperature, and the wells were again rinsed vigorously with water. Quantitative biofilm formation was determined by measuring the optical density at 570 nm (OD 570) of a solution produced by extracting cell-associated dye with 600 µl of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO).

Confocal microscopy

To perform confocal microscopic analysis on possible biofilm formation by *Vibrio cholerae* strains, including N16961S-24, SΔ*vpsR*, SΔ*vpsA*, N16961R-24, RΔ*vpsR*, RΔ*vpsA*, GASP-700D, GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA* (Table 3-1) were grown (ca. 10^8 cfu/ml) in 4-well cell culture plates (Thermo Scientific Nunc, Pittsburgh, PA) containing 500 µl FSLW. To provide bacterial attachment platform, a 12 mm round glass cover slip (Warner Instruments, Hamden, CT) was dipped into each culture well, and the cultures were incubated overnight statically at room temperature. Next day, the cover slips were washed three times with Dulbecco's PBS (DPBS) (HyClone Laboratories, Logan, UT), and fixed in 10% neutral buffered formalin solution (Sigma-Aldrich, St Louis, MO). They were washed again with DPBS and stained using 300 µl/well of 1:1000 SYTO 9 dye (LIVE/DEAD BacLight Bacterial Viability kit, Invitrogen, Grand Island, NY). Following three consecutive DPBS washings, glass cover slips were mounted onto 75 X 25 mm microscopic slides (Corning Inc., Corning, NY). The cover slips were analyzed on a confocal microscope (Leica Microsystem, Buffalo Grove, IL) with an excitation and emission wavelengths of 484 and 500 nm, respectively. The biofilm thickness was measured as an average of 5 non-overlapping fields per slide with a 20X HCX PL APO lambda blue magnifying objective. Images were digitally reconstructed with z-projections of x-y sections using Leica Application Suite Advanced Fluorescence (Leica Microsystem, Buffalo Grove, IL) and DAIME softwares [75]. The volumes of biofilms were calculated as follows: the x-y areas of each z-section were measured using ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA) and were multiplied by the value of the z-step to obtain the volume of the biofilm at each section. Total biofilm volumes were calculated as a sum of the separate volumes of the z-sections as described previously [76]. At least two biological replicates were used in the imaging processes.

Transmission electron microscopy (TEM)

For transmission electron microscopic (TEM) analysis, *V. cholerae* strains N16961S-24, N16961R-24 and GASP-700D (Table 3-1) were grown in 3 ml FSLW and the cultures were incubated overnight statically at room temperature. Material from each culture was subjected to ruthenium red staining and the stained cells were examined using TEM for the presence of bacterial exopolysaccharide as described previously [33]. In brief, the cultures were fixed in a solution of 2% glutaraldehyde-50 mM lysine-500 ppm ruthenium red in 0.1 M cacodylate buffer (pH 7.2) for 1-hr at room temperature followed by an overnight incubation at 4^{o}C. Samples were then washed twice in 0.1 M cacodylate buffer, pelleted and encapsulated in 3% low-temperature gelling agarose type VII (Sigma-Aldrich, St. Louis, MO). The following steps were processed with the aid of a Pelco BioWave Pro laboratory microwave (Ted Pella, Redding, CA, USA). Fixed cells were post-fixed with 1% buffered osmium tetroxide one minute in hood, 45 seconds at 100W under vacuum three minutes in hood, water washed, dehydrated in a graded ethanol series 25%, 50%, 75%, 95%, 100% followed by 100% acetone, 1X each 45 sec at 180W. Dehydrated samples were infiltrated in a graded acetone/spurr's epoxy resin 30%, 50%, 70%, 100%, 100%, 1X each, three minutes at 220W under vacuum followed by 10 minutes on bench top. Resin infiltrated cells were cured at 60^{o}C for 2 days. Cured resin blocks were trimmed, thin sectioned and collected on formvar copper 100 mesh grids, post-stained with 2% aq. Uranyl acetate and Reynold's lead citrate. Sections were examined with a Hitachi H-7000 TEM and digital images were acquired with Veleta camera and iTEM software.

Stress resistance assay

Stress resistance of GASP-700D, includingboth oxidative and osmotic stresses, and stress to chlorine was assessed as described earlier [33, 35]. N16961R-24 and N16961S-24 were used as positive and negative controls, respectively. Bacterial inoculum (ca. 10^8 cfu/ml) was inoculated in 3 ml FSLW and incubated as described above. For oxidative stress, 20 mM H_2O_2 (final concentration)(hydrogen peroxide 3%, Ricca Chemical, Arlington, TX) was added to each culture and the resistance of each culture to H_2O_2 was recorded for every 5 min for 15 min. The culturable bacteria survived the stress were determined using standard plate count at each experimental time point. Similarly, for osmotic and chlorine stresses, *V. cholerae's* culture in FSLW was exposed to 2.5 M NaCl (at final concentration) (Avantor Performance Materials, Center Valley, PA) and 3 mg free chlorine per liter [3 ppm] (sodium hypochlorite, Sigma, St Louis, MO), respectively. Resistance of each *V. cholerae* strain to osmotic and chlorine stresses was determined by measuring the culturable bacteria present (i) for every 15 min for one h [35], and (ii) for every 5 min for 15 min [33], respectively. Percent survival of the bacteria was calculated by dividing the number of bacterial colonies counted at a given time by the number of colonies added to the culture before supplementing the culture with stress ingredient, and then multiplying the result by 100.

Statistical analysis

One-way ANOVA was performed in STATA v 12 (StataCorp®, College Station Texas, USA) to determine the significant differences in diverse traits assessed in the study. Equal variance

within groups was assessed using Barlett's test, and a Bonferroni correction was implemented to control type I error for multiple comparisons between the wild-type and its isogenic mutants or variants. A p-value of <0.005 was considered as statistically significant.

RESULTS

Comparison of motility between N16961S-24 and GASP-700D of *V. cholerae*

V. cholerae carries a single polar flagellum required for its motility. Since I am the first to describe that *V. cholerae* can switch, in response to nutrient-starvation in FSLW, from a canonical single polar flagellum to bipolar and peritrichous flagella in N16961S-24 and GASP-700D , respectively[42], I was interested to investigate the role(s) of bipolar and peritrichous flagella, if any, in motility using motility agar. I also included a *ΔflaA* mutant strain that is non-motile because it lacks the major flagellin subunit [69], and a (motile) rugose variant of *V. cholerae* (N16961R). When the bacterial strains were grown in L-broth before inoculating into motility agar, both N16961S (smooth variant) and N16961R (rugose variant) were motile (Figure 3-1, #1 and #2), with the rugose variant exhibiting approximately 2.5-fold reduced motility, which is consistent with previous reports described by my group and others [33, 77]. To my surprise GASP-700D was non-motile (Figure 3-1, #3). As expected, the Δ*flaA* mutant was non-motile (Figure 3-1, # 4). When grown in nutrient-poor FSLW, both N16961S-24 and N16961R-24 strains demonstrated motility, with N16961S-24 exhibiting increased motility compared to the rugose variant (Figure 3-1, # 5 and #6) further corroborating that the rugose variant is less motile than its smooth counterpart. Interestingly, GASP-700D, in contrast to N16961S-24, did not move from the point of inoculation, even after 24 h of growth in motility agar (Figure 3-1, #

7). As expected, the Δ*flaA* mutant was non-motile (Figure 3-1, # 8). My data suggest that unlike the bipolar flagella of N16961S-24, GASP-700D did not facilitate productive motility both in L-broth and FSLW. To ensure that GASP-700D was viable at the inoculation site, I examined a block of agar consisting of the entire inoculation site as described in methods section. I obtained ca. 1 $X10^6$ cfu, confirming that GASP-700D was surviving inside the agar but defective in motility.

As GASP-700D exhibited no motility in soft agar, I further investigated using qRT-PCR to determine whether flagellar genes, including *flaA* (encodes critical flagellin), *flrC* (encodes regulator of Class III flagellar genes), *motY* and *motB* (encode flagellar motor) and *flrA* (encodes master regulator of all flagellar genes) were repressed in GASP-700D relative to N16961S-24. A previous study reported that *phoB* and Pst system genes of *V. cholerae* were expressed in nutrient-poor FSLW compared to nutrient-rich L-broth, whereas *ctxA* and *tcp* genes were repressed under the same conditions [30].

I first compared the relative expression of *phoB*, Pst-system genes, *ctxA* and *tcp* genes by N16961S-24 and GASP-700D grown in nutrient-poor FSLW to that of wild-type *V. cholerae* N16961S grown in nutrient-rich L-broth in otherwise identical growth conditions (Figure 3-2). The *phoB* and Pst-system genes were highly expressed, while *tcp* genes and *ctxA* were repressed, by N16961S-24 and GASP-700D grown in FSLW relative to N16961S grown in nutrient-rich L-broth, confirming the results of the previous study[30]. Additionally, expression of the flagellar genes, except *flrC*, was also down-regulated in GASP-700D, as well as in the rugose N16961R-24 variant, compared to their expression in N16961S-24 when grown in FSLW.

Strikingly, *flrA*, the master flagellar regulatory gene, was 1,000-fold down regulated ($p<0.005$) in GASP-700D compared to N16961S-24, suggesting that flagellar synthesis is down-regulated in GASP-700D (Figure 3-3). Taken together, my results suggest that GASP-700D may have lost peritrichous flagella and/or some flageller gene(s) might have sustained mutation(s) in GASP-700D resulting in the defect of productive motility. Indeed, microorganisms surviving for long-time in stressful stationary growth cultures can result in the selection of mutants that express GASP phenotype [47].

Comparative assessment of biofilm formation between N16961S-24 and GASP-700D of *V. cholerae*

I previously reported that 700 days-old persister cells showed a high degree of cell to cell aggregation compared to N16961S-24 [42]. Furthermore, the flagella of N16961S-24 allow motility, whereas GASP-700D does not facilitate productive motility. Because *V. cholerae* motility and the polar flagellum contribute to biofilm formation [68, 78], I was interested in determining the role(s) of the novel bipolar and possible non-productive/deleted peritrichous flagella elicited by N16961S-24 and GASP-700D, respectively, in biofilm formation when grown in nutrient-poor FSLW. As *V. cholerae* biofilm is produced and positively regulated by *vps* genes and *vpsR* gene, respectively, I created *vpsR* and *vpsA* in-frame deletion mutations in the background of N16961S, N16961R and GASP-700D. As expected, *vpsR* and *vpsA* mutants inhibited rugose colony phenotype (Figure 3-4A)[34, 73]. I initially measured biofilm production by *V. cholerae* strains, including N16961S, N16961R, GASP-700D and in-frame deletion mutants of the *vpsR* and *vpsA* biofilm genes, in the background of N16961S (SΔ*vpsR* and SΔ*vpsA*),

N16961R (RΔ*vpsR* and RΔ*vpsA*) and GASP-700D (GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA*) in nutrient-rich L-broth incubated overnight statically at room temperature. In L-broth, the rugose N16961R variant produced about 40- and 120-fold more biofilm ($p<0.005$) than its smooth counterparts N16961S and GASP-700D respectively; as expected, the N16961R mutants, RΔ*vpsR* and RΔ*vpsA* were defective for biofilm formation (Figure 3-4B). My data are consistent with earlier reports that demonstrated a requirement for *vpsR* and *vpsA* for biofilm formation in nutrient-rich L-broth [71, 79].

I next examined comparative biofilm forming abilities among the *V. cholerae* strains by growing them in nutrient-poor FSLW. Interestingly, the rugose N16961R-24 variant produced 36-fold less biofilm ($p<0.005$) under nutrient-poor conditions (FSLW) compared to that of under nutrient-rich conditions (L-broth) (Figures 3-4B and 3-4C), demonstrating that nutrient-rich conditions favor increased *vps*-mediated biofilm formation. Interestingly, GASP-700D produced about 4-fold ($p<0.005$) and 1.5-fold ($p=0.24$) higher biofilm compared to N16961S-24 and N16961R-24 in FSLW, respectively. In contrast to biofilm production in nutrient-rich L-broth, the Δ*vpsR* mutants (except for SΔ*vpsR*) and Δ*vpsA* mutants produced increased biofilm formation ($p<0.005$) in FSLW compared to N16961S-24. My observations suggest that GASP-700D and *vpsR* and *vpsA* mutants produced biofilm in response to nutrient stress (in FSLW), and that this biofilm is somewhat independent of VPS-mediated biofilm formation as described previously [34].

To gain further insights into the three dimensional and architectural appearances of biofilms, I examined biofilm produced by *V. cholerae* strains in FSLW described above using

scanning confocal laser microscopy (SCLM). As shown in Figure 3-5A and -3-5B, the GASP-700D produced a highly-developed coalesced biofilm with 81 µm high pillars and columns filled with fluids. In contrast, the smooth variant N16961S-24 displayed a less-developed biofilm (mostly monolayer) with 17 µm pillars, and, as expected, the rugose variant produced scattered and developed biofilm with 64 µm pillar. GASP-700D appeared to be densely aggregated rather than dispersed, as in the N16961R-24 biofilms. Except SΔ*vpsR*, all Δ*vpsR* and Δ*vpsA* mutants examined exhibited patchy biofilms in FSLW with GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA* displayed much higher patchy biofilms (Figure 3-5A). Specifically, SΔ*vpsR,* SΔ*vpsA,* RΔ*vpsR,* RΔ*vpsA,* GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA* strains formed patchy biofilms with the pillars' heights of 15, 56, 36, 52, 61 and 64 µm, respectively (Figure 3-5B). Figure 3-5C shows the quantitative analysis of biofilm formation which indicates that all strains except SΔ*vpsR* produced increased biofilm ($p<0.005$) compared to N16961S-24.

I stained biofilms formed by N16961S-24, N16961R-24 and GASP-700D in FSLW with ruthenium red, and examined the biofilm matrix using transmission electron microscopy (TEM) (Figure 3-6). Copious amounts of exopolysaccharide matrix could be detected surrounding the N16961R-24 cells, whereas very little exopolysaccharide matrix could be seen in the biofilm of N16961S-24. Likewise, GASP-700D biofilms appeared to contain very little exopolysaccharide matrix, suggesting that GASP-700D forms VPS-independent biofilms. Taken together, my data support the idea that GASP-700D produced biofilm specific to FSLW and that this biofilm is independent of VPS-mediated biofilm.

Stress resistance

My group and others have previously reported that *V. cholerae* rugose variants, that produce copious amounts of exopolysaccharide and biofilm, can resist chlorine, oxidative, and osmotic stresses [33-35]. As GASP-700D produced FSLW-specific biofilm, I investigated whether this phenotype, like rugose phenotype can resist diverse stresses [80, 81]. To this context, I subjected GASP-700D to H_2O_2, chlorine, and NaCl stresses. I note that there were no obvious growth differences among *V. cholerae* strains grown in L-broth and examined in this study (data not shown). Interestingly, I observed that, like N16961R-24, GASP-700D was more resistant to H_2O_2 in FSLW ($p<0.005$) compared to N16961S-24 (Figure 3-7). However, unlike N16961R-24, GASP-700D was as susceptible as N16961S-24 when exposed to chlorine and osmotic stresses (data not shown).

DISCUSSION

Recently, I reported a *V. cholerae* "persister" phenotype which is a key step in the understanding of the long-term survival of *V. cholerae* in the environment[42]. However, substantial work still needs to be done to understand this phenotype, and to assess its role in cholera transmission. In the current study, I provide evidence that glycerol stored persister cells (700 days-old cells) have transitioned to what appeared to be a growth advantage in stationary phase (GASP) phenotype. Compared to its wild-type strains (N16961S-24 and N16961S), GASP-700D phenotype of *V. cholerae* exhibited: (i) non-motile phenotype, (ii) enhanced exopolysaccharide production and biofilm formation that are specific to FSLW, and independent of *vps*, (iii) resistance to oxidative stress, and (iv) small colony phenotype. The

storage and subculture of persister cells in glycerol broth at -80^{o}C may have influenced the observed phenotype seen with GASP-700 as described above.

I hypothesize that, during long-term survival (700 days) in stressful stationary culture, *V. cholerae* may have adopted two responses, including: (i) assume "persister" phenotype [42], and (ii) select GASP mutants that successfully adapt to stressful growth conditions[47]. Although I currently have no supporting evidence to conclude that GASP-700D genome has any mutation, I did observe that GASP-700D is defective in productive motility implying that GASP-700D may have possible mutation(s)/alteration in its genome. I propose that GASP-700D represents a GASP phenotype. Indeed, previous reports demonstrated that GASP phenotypes with genetic mutations are common in microorganisms surviving long-term in stressful and stationary growth phase.

The nutrient-poor growth conditions in FSLW affect the motility of *V. cholerae* even before its transition to GASP-700D in a phase-dependent manner. The smooth variant exhibited reduced motility in soft agar after 24 h growth in FSLW. In contrast, the rugose variant, which normally shows reduced motility in comparison with the smooth variant, was unaltered for motility after 24 h growth in FSLW. Once the bacteria have transitioned into GASP-700D, however, they appear non-motile in this assay (Figure 3-1). qRT-PCR revealed a dramatic down regulation (1000-fold) of *flrA* expression in GASP-700D (Figure 3-3). FlrA is the "master regulator" of the flagellar transcription hierarchy [82]. It is the sole Class I flagellar factor that activates σ^{54}-dependent transcription of Class II flagellar genes, thus initiating flagellar synthesis. It is not known how *flrA* transcription is itself controlled in *V. cholerae*, but

expression of the FlrA homologue FleQ in *Pseudomonas aeruginosa* has been shown to be negatively regulated by the alternate sigma factor AlgT, which results in loss of motility that is simultaneous with increased polysaccharide expression and biofilm formation [83]. It is not clear whether the reduction in *flrA* transcription is responsible for the non-motile phenotype, because interestingly, transcription of other flagellar genes within the transcription hierarchy, including the Class III regulator *flrC*, the motor genes *motB* and *motY*, and the major core flagellin, *flaA,* were not dramatically reduced in GASP-700D. It has been shown previously that mutation of *flhG* leads to the expression of multiple polar flagella, and the *flhG V. cholerae* strain appears non-motile in soft agar, possibly due to an inability to effectively coordinate flagellar function[84].

Previous studies of *V. cholerae* biofilm formation have mostly focused on nutrient-rich growth conditions either in static and/or in flow-cell methods [33, 85]. Under these conditions, the rugose variant produces robust biofilms with three dimensional pillars and columns [85]. Here, I studied biofilms formed in nutrient-poor FSLW conditions that more closely mimic the natural environment of *V. cholerae*[32, 86]. I found that nutrient-poor conditions promote much less biofilm formation than the nutrient-rich conditions; even with the rugose variant (Figures 3-4B and 3-4C). My group's previous study demonstrated that a number of sugars, including sucrose and glucose, inhibited *V. cholerae* exopolysaccharide expression [87]. In contrast, glucose promoted biofilm production by *Staphylococcus aureus*[88, 89]. My observations suggest that physical and chemical parameters, including nutrient composition, pH, and attachment surfaces, can influence the outcome of biofilm formation by *V. cholerae*.

GASP-700D produces a well-developed biofilm in FSLW that appears predominantly coalesced rather than scattered. In contrast, the rugose variant forms well-developed but scattered biofilms (Figure 3-5A). However, in the absence of the VPS genes Δ*vpsR* or Δ*vpsA*, the rugose variant forms biofilms with similar coalesced characteristics to GASP-700D in this medium, as does a SΔv*psA*, GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA* mutants (Figures 3-5A and 3-5B). This suggests that GASP-700D and the strains lacking *vps* genes form biofilms that are independent of VPS, and that *vps* genes may negatively affect the expression of the alternative biofilm matrix. Ruthenium red staining failed to detect exopolysaccharide in the GASP-700D biofilms in FSLW (Figure 3-6), in contrast to the abundant exopolysaccharide in the rugose variant biofilms, suggesting that the GASP-700D biofilms may contain yet to be defined biofilm matrix. Such a putative extracellular matrix could drive the development of the alternative, coalescing biofilms seen in the GASP-700D which is more resistant to oxidative stress than either smooth or rugose variants. Oxidative stress resistance may be due to the alternative biofilms formed under these conditions, or alternatively, to enhanced expression of resistance factors. Further genetic investigations of GASP-700D is needed in order to enhance my understanding if *V. cholerae* GASP-700D sustained mutation(s) to select GASP mutants as seen with other microorganisms [47] and thereby promoting competitive environmental fitness and adaptation.

Table 3-1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Reference
V. cholerae strains		
N16961S	A wild-type, smooth, O1 El Tor strain isolated in Bangladesh in 1971	[33]
N16961S-24	A growth of N16961S in nutrient-poor "filter sterilized" lake water incubated overnight statically at room temperature	This study
N16961R	A rugose variant of N16961S strain	[73]
N16961R-24	A growth of N16961R in nutrient-poor "filter sterilized" lake water incubated overnight statically at room temperature	This study
GASP-700D	700 days-old N16961S culture persisting in nutrient-poor FSLW was grown on L-agar and subsequently stored in FSLW supplemented with 30% glycerol at -80^{o}C	[42]
AA212	A Δ*flaA* null mutation in the background of N16961S strain	This study
AA215	A SΔ*vpsR* (420 bp internal in-frame deletion) created in the N16961S strain	This study
AA216	A RΔ*vpsR* (420 bp internal in-frame deletion) created in the N16961R	This study
AA217	A GASP-700DΔ*vpsR* (420 bp internal in-frame deletion) created in the background of GASP-700D strain	This study
AA218	A SΔ*vpsA* (VC_0917) in-frame null mutation was created in N16961S strain	This study
AA219	A RΔ*vpsA* (VC_0917) in-frame null mutation was created in N16961R strain	This study
AA220	A GASP-700DΔ*vpsA* (VC_0917) in-frame null mutation was created in the background of GASP-700D strain	This study
E. coli strains		
DH5α	*recA* Δ*lac*U169 ϕ80d *lacZ*ΔM15	Gibco, BRL
S17-1 λ *pir*	*Pro hsdR hsdM*$^{+}$ Tmp^{r} Str^{r}	[90]
Plasmids		
pWSK29	Low-copy-number vector, Amp^{r}, *ori* pSC101	[72]

pCVD442	Suicide vector, *ori* R6K, Amp^r, *sacB*	[91]
pKEK93	Δ*flaA*::Cm in pCVD442	[69]
pAA69	A 560-bp PCR fragment (*SacII/XbaI)* containing the 5'-end of *vpsR* gene of N16961 cloned into similarly digested pWSK29, Amp^r	This study
pAA72	A 540-bp PCR fragment (*SacII-SpeI)* fragment upstream of *vpsA* gene of N16961 cloned into similarly digested pWSK29, Amp^r	This study
pAA73	A 520-bp PCR fragment (*XbaI-BamH1)* was cloned into similarly digested pAA69, resulting in a plasmid (pAA73) containing 420-bp internal in-frame deletion. Amp^r	This study
pAA74	A 360-bp PCR fragment (*SpeI-EcoR1)* downstream of *vpsA* was cloned into similarly digested pAA72, resulting in a plasmid (pAA74). Amp^r	This study
pAA77	A 900-bp PCR fragment (*SacI-SalI)* from pAA74 was cloned into similarly digested pCVD442, Amp^r	This study
pAA78	A 1080-bp PCR fragment (*SacI-SalI)* from pAA73 was cloned into similarly digested pCVD442, Amp^r	This study

Table 3-2. Oligonucleotide primers used in this study

Primer types	Primer name	Direction	Target Gene	Sequence (5' to 3')[a]
PCR				
	aa212	Forward	*vpsR*	TCC CCG CGG ATG AGC ACT CAA TTC CG
	aa213	Reverse	(1st fragment)	GCT CTA GAC GAG CCA AAA TGT GGC C
	aa214	Forward	*vpsR*	GCT CTA GAT CAG ACA TCG AAA AAG C
	aa215	Reverse	(2nd fragment)	CGG GAT CCT TAG AAG TTT TCA TCG G
	aa264	Forward	*vpsA*	TCC CCG CGG GCA AGG CGA ATC GAC AAG
	aa265	Reverse	(1st fragment)	GGA CTA GTC ACT TCC CCA CAT CCT CT
	aa266	Forward	*vpsA*	GGA CTA GTG CGA AAT AGA CTC ATC AGG GG
	aa267	Reverse	(2nd fragment)	GGA ATT CGC CAT TGC GCC AAT TTT TCG G
Real time PCR				
	aa125	Forward	*flaA*	TAA CAG TGC AAA AGA TGA CG
	aa126	Reverse		GTT TGA GCA ATC GAA ATA CC
	aa127	Forward	*flrC*	AAG CAA AGT CTT AAT CGT AG
	aa128	Reverse		ACA CTG TGT GAT TTG AGT TT
	aa133	Forward	*phoB*	ATG TCT AGA AGG ATT CTG GT
	aa134	Reverse		GAA TCA TAA TCT TCA GCC TC
	aa135	Forward	*pstB2*	TGA ATC GTA TGA ATG ATC TC
	aa136	Reverse		TCA TAA ATA CTC ATT GGG AA
	aa137	Forward	*pstS*	CAC TTA TAG GAA ATT CGT GA
	aa138	Reverse		ACT GCT GAG ATT GTC TCT TT
	aa139	Forward	*tcpA*	CAA ACT TAT CGT AGT CTT GG
	aa140	Reverse		CAT AGC TGT ACC AGT GAA AG
	aa141	Forward	*tcpP*	GAA TGA ATG CAC TAA TCA AG
	aa142	Reverse		ATT ATT TGA TCA TTT GGA CA
	aa143	Forward	*toxR*	ATA TCG ATG AGT CAT ATT GG
	aa144	Reverse		TAA TCG AAT GAT CTC TTC AC
	aa180	Forward	*tcpH*	GTG TAA CGA TCA TCG CAC TC

aa181	Reverse		ATA GGT TAC AAA CCG AAT GG
aa254	Forward	*motB*	GAA CAA CAA TGT AAA TGT CC
aa255	Reverse		TAA ATT TCA GTA CGT CCA TC
aa256	Forward	*motY*	CCC TAC AAT GAG TTT AGT GA
aa257	Reverse		GCT TTA TTC AAC TCA ACA CT
aa258	Forward	*flrA*	GAG TTT AGC GAA ACT ACT TG
aa259	Reverse		TAG ACT CAA TGA CCT CAC AC
aa260	Forward	*ctxA*	TCT AGA CCT CCT GAT GAA AT
aa261	Reverse		AAG GTT GAT ATT CAT TTG AG

[a]Underline sequences represent restriction sites

Figure 3-1. Swimming behavior of *V. cholerae* strains in motility agar. The bacterial strains were grown either in L-broth (L) or in FSLW (LW) and incubated overnight statically at room temperature before inoculating into motility agar. After inoculation, the plates were incubated at 37^{o}C for 8 h before obtaining the images. 1, N16961S (L); 2, N16961R (L); 3, GASP-700D (L); 4, SΔ*flaA* (L); 5, N16961S (LW); 6, N16961R (LW); 7, GASP-700D (LW); 8, SΔ*flaA* (LW).

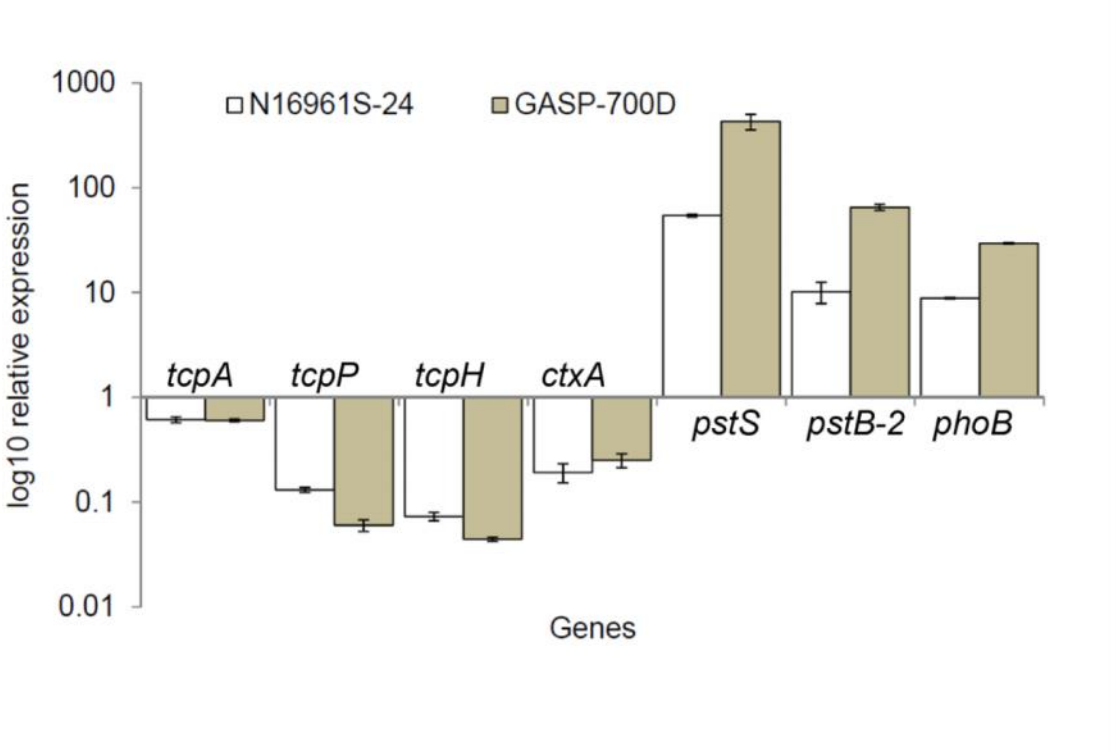

Figure 3-2. Comparative analysis of the differential gene expression among *V. cholerae* strains N16961S and GASP-700D using qRT-PCR. N16961S was grown both in nutrient-rich L-broth and in nutrient-poor FSLW (N16961S-24) (ca. 108 cfu/ml), and the cultures were incubated overnight statically at room temperature. GASP-700D was grown (ca. 108 cfu/ml) in FSLW only. Expression of each gene was normalized to that of toxR, and subsequently compared to that of the wild-type N16961S grown in L-broth. Data represent the average results of three independent experiments and error bars indicate as means ± standard deviation (SD).

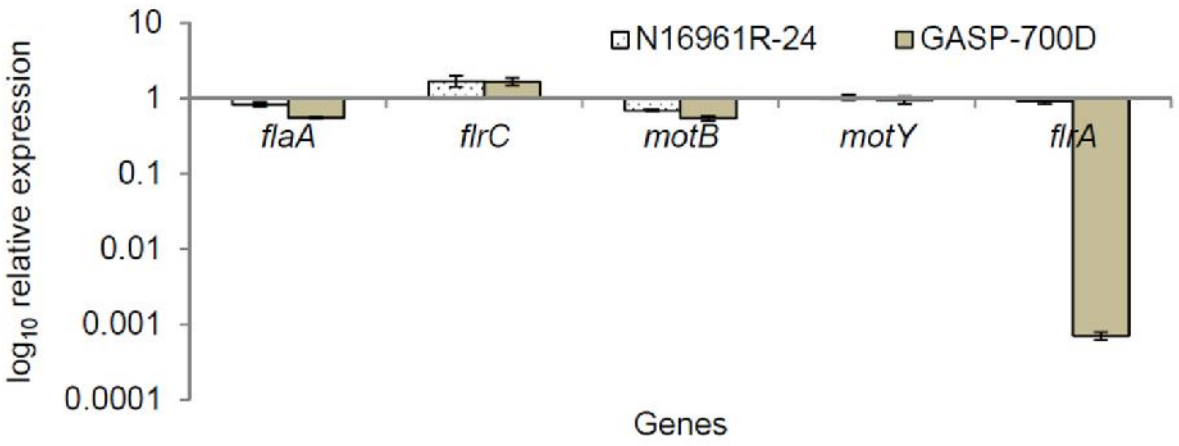

Figure 3-3. Comparative expression analysis of selected flagellar genes as measured by qRT-PCR among *V. cholerae* strains, including N16961S-24, N16961R-24 and GASP-700D. Each strain (ca. 10^8 cfu/ml) was grown in nutrient-poor FSLW and incubated overnight statically at room temperature. Expression of each gene was normalized to that of *toxR*, and subsequently the expression of the gene was compared to that of the wild-type N16961S-24. Number one (1) represents the value of expressed gene by N16961S-24. Values above 1 or below 1 represent the positive and negative expression, respectively. Data represent the average results of at least three independent experiments are expressed as means ± standard deviation (SD). P-values are computed by comparing the differential expression of each gene with that of N1961S-24 using one-way ANOVA test. A p-value of <0.005 was considered statistically significant.

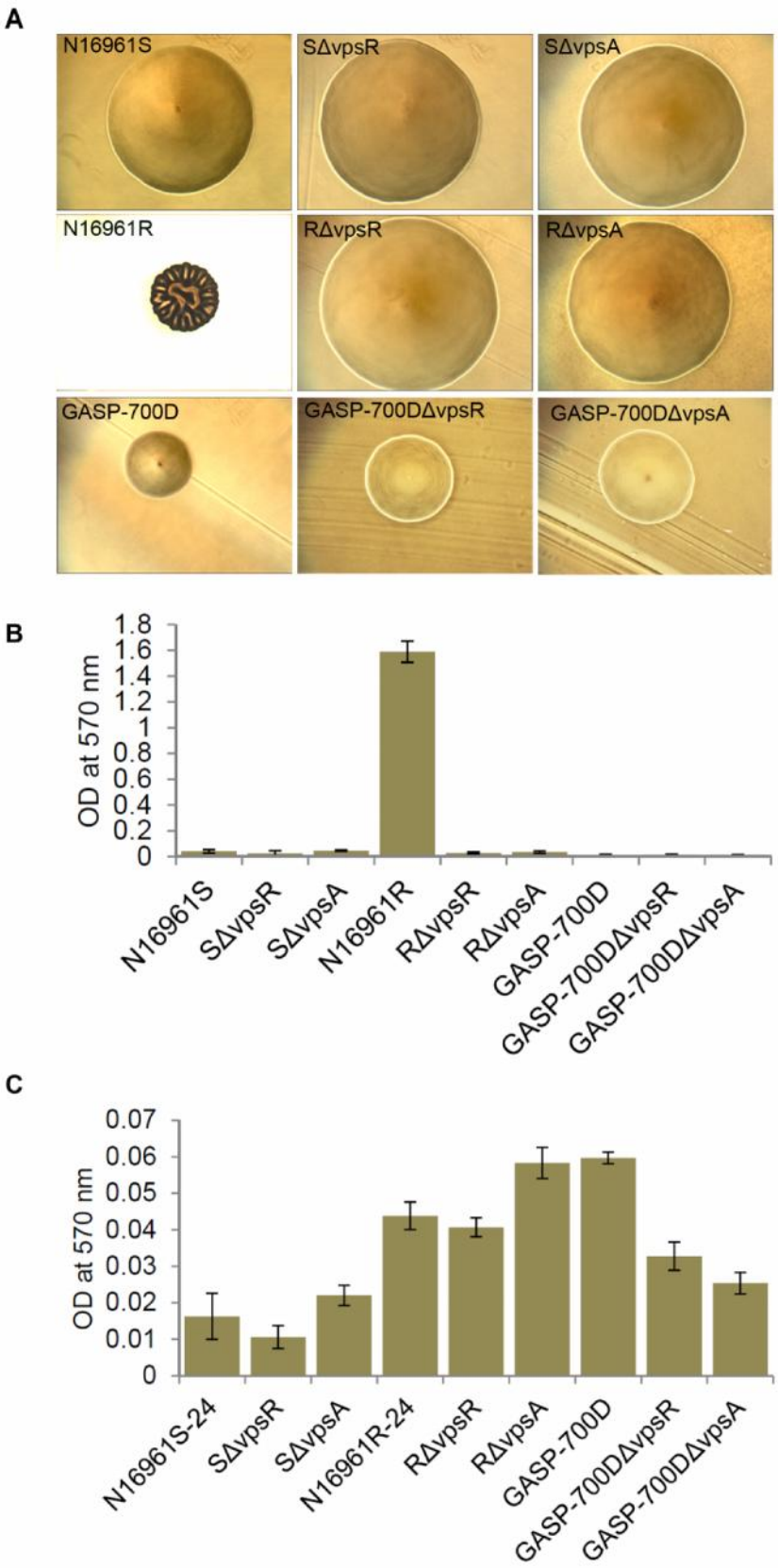

Figure 3-4. Colony morphology and associated biofilms (measured quantitatively) produced by each *V. cholerae* strain. (A) Colony morphology: each *V. cholerae* strain was subcultured on L-agar and incubated overnight at 37^{o}C before images were acquired; (B) Quantitative measurement of biofilm produced by each *V. cholerae* strain in nutrient-rich L-broth; and (C) Quantitative measurement of biofilm produced by each *V. cholerae* strain in nutrient-poor FSLW. All the values are expressed as means ± standard deviation (SD) from at least triplicate experiments. P-values are computed by comparing the biofilm formation of each strain with that of N1961S-24 using one-way ANOVA test. A p-value of < 0.005 was considered statistically significant.

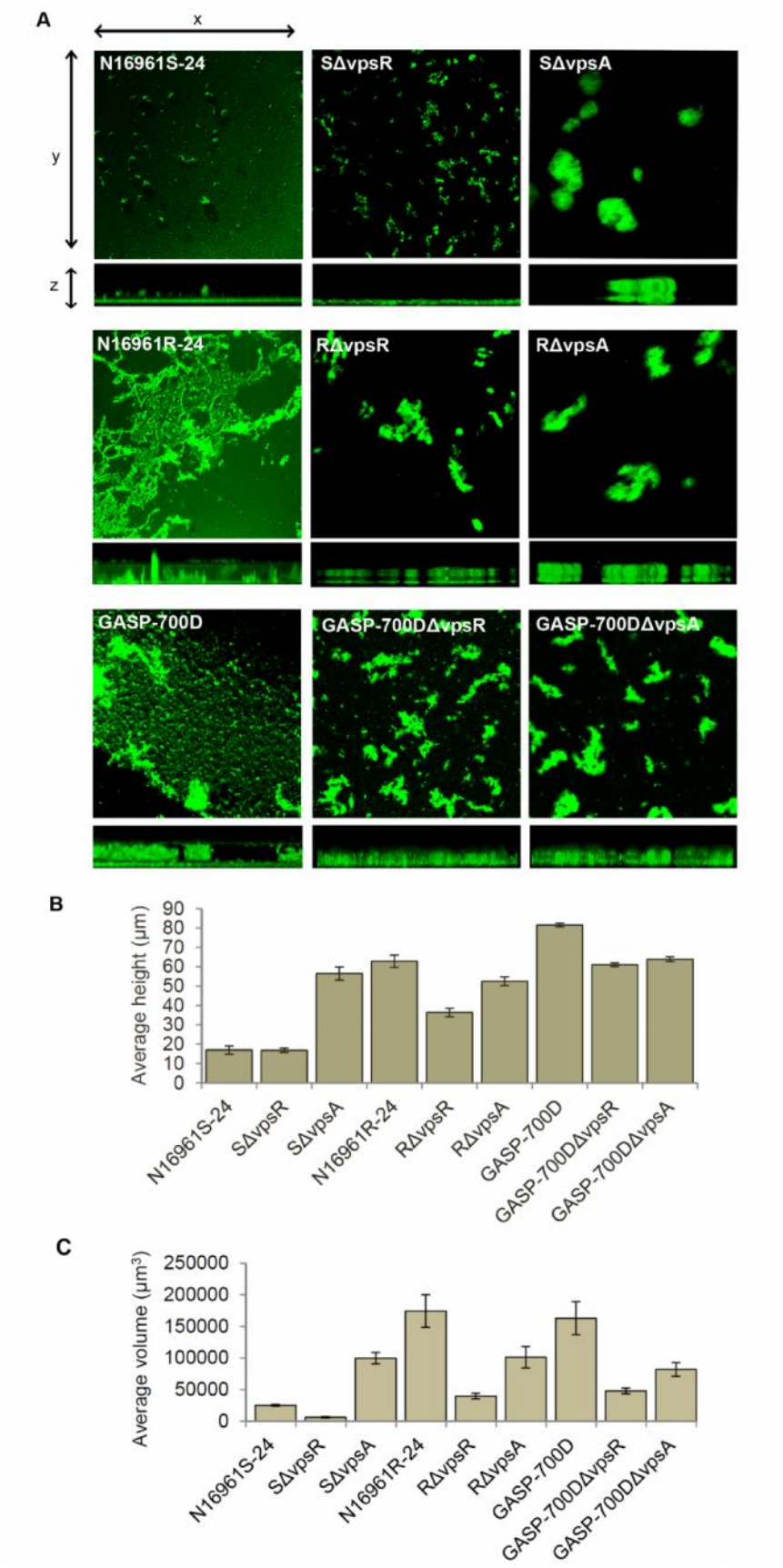

Figure 3-5. Topography and architecture of *V. cholerae* biofilms. Each strain was grown in a 4-well cell culture plate containing 500 µl FSLW. A glass cover slip was dipped into each culture well and incubated overnight statically at room temperature. The glass cover slips were stained with SYTO 9 and the images were obtained using a laser scanning confocal microscopy with an excitation and emission wavelengths of 484 and 500 nm, respectively. (A) Images of x-y sections (top panels) and x-z projections of the same biofilms (bottom panels) were analyzed with DAIME software; magnification, x200. (B) Average biofilm heights (µm) for each strain measured across five random x-z sections. (C) Total volume of biofilm (μm^3) for each strain calculated by x-y and x-z projections. A p-value of <0.005 was considered statistically significant.

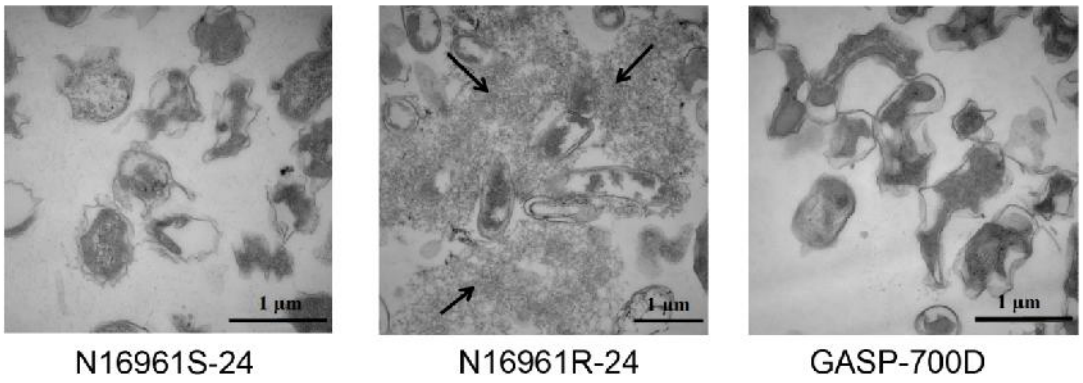

Figure 3-6.Ruthenium red staining of exopolysaccharide produced by *V. cholerae* strains.Each *V. cholerae* strain (ca. 10^8 cfu/ml) was grown in 3 ml FSLW and incubated overnight statically at room temperature. The cultures were stained with ruthenium red stain (as described in Methods section) and images were visualized using transmission electron microscopy (TEM). Exopolysaccharide produced by N16961R-24 is indicated by arrows; N16961S-24 and GASP-700D did not develop any exopolysaccharide. Bars = 1 µm.

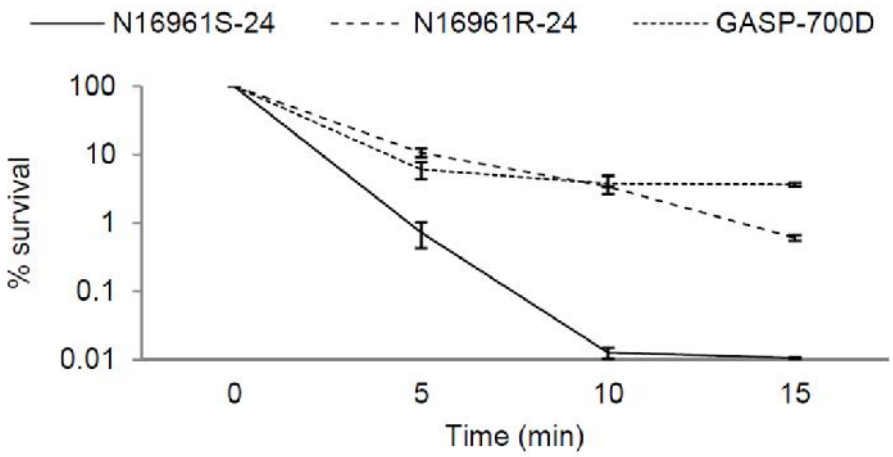

Figure 3-7. Resistance of GASP-700D to oxidative (H_2O_2) stress.*V. cholerae* strains N16961S-24, N16961R-24 and GASP-700D were grown (ca. 10^8 cfu/ml) in FSLW supplemented with 20 mM H_2O_2. The cultures were examined at 5 min interval for 15 min for the presence of culturable bacteria as determined by standard plate count. Error bars indicate means ± standard deviation (SD) from triplicate experiments. The stress resistance of each strain was compared with that of N1961S-24 using one-way ANOVA test. A p-value of <0.005 was considered statistically significant.

CHAPTER 4

Comparative Gene Expression Profile between *V. cholerae* N16961 and Its Growth Advantage Stationary Phase (GASP-700D) phenotype

INTRODUCTION

Cholera is a major public health threat worldwide, particularly in countries where safe drinking water, adequate sanitation and hygiene are suboptimal [1]. Despite decades of studies, the basis of persistence of *V. cholerae* in aquatic reservoirs is not well-understood[37, 42]. Suggestions are made that the microorganism can survive by assuming viable but non-culturable state (VBNC) [32, 51] and bioflim-protected "rugose" phenotype [33]; however, each survival phenotype has its own limitation in regard to disease transmission[30, 33].

To adapt to hostile and stressful survival conditions, bacterial species employ diverse phenotypic changes either by stochastic mechanisms or by adopting a "growth advantage stationary phase" (GASP) phenotype[47, 48, 53]. I have reported that, in response to nutrient starvation in filtered sterilized lake water (FSLW) microcosms, *V. cholerae* N16961 exhibited a novel "persister " phenotype exhibiting long helical cells with bipolar flagella (as early as 24 h of growth in FSLW) that transitioned into very small cells with peritrichous flagella by 700 days of growth [42]. Upon storage of persister cells in glycerol broth at -80°C, the persister cells lost their ability to render into typical cells (a hall mark of persister cells)[92]; however, I found that stored cells become non-motile while promoting biofilm formation specific to FSLW[92]. I termed these cells as growth advantage stationary phase (GASP) of *V. cholerae*[92].

To better understand the GASP phenotype and compare the gene expression profile between GASP-700D (700 day old stored culture) and N16961 in FSLW microcosms, I compared the transcriptome profile of GASP-700D and fresh *V. cholerae* N16961 strains grown for 24 h in FSLW to *V. cholerae* N16961 cells persisting in L-broth for 24-h in otherwise identical growth conditions.

METHODS & MATERIALS

Microarray experiments

An 8X15 custom microarray platform (Agilent Technology, Palo, CA), spotted with oligos (60 nt; 4-replicates) of all *V. cholerae* ORFs and 500 internal controls, was used in this study. Analysis was performed at Interdisciplinary Center for Biotechnology Research (ICBR) Microarray Core in University of Florida. Total RNA samples were extracted using Trizol reagent (Sigma Aldrich, St. Louis, Missouri) from i) *V. cholerae* N16961(N16961S) strain (ca. 10^8 cfu/ml) grown in L-broth overnight statically at room temperature, and ii) *V. cholerae* N16961 strain (ca. 10^8 cfu/ml) grown in L-broth overnight statically at room temperature, washed, starved for 24 h in FSLW statically at room temperature (N16961S-24), and iii) GASP-700D cells (ca. 10^8 cfu/ml) surviving in FSLW statically at room temperature for 24 h. The extracted RNA was digested on column (Qiagen, Valencia, CA) with DNase 1 and purified using an RNeasy mini kit (Qiagen, Valencia, CA). Six biological replicates were used for microarray analysis. RNA quantity was initially determined on a ND-8000 Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Five μg of total RNA from each sample was used to generate

labeled cDNA according to the manufacturer's protocol. Labeled cDNA was generated using the Agilent Two-Color Microarray-Based Prokaryote Analysis (Fairplay III labeling). Briefly, cDNA was synthesized from 5 µg of total RNA with AffinityScript HC using random primers, and modified cDNA was labeled with Cy3. Labeled cDNA was purified following the manufacturer's instructions. A total of 1650 ng of Cy3-labeled cDNA per sample was used for hybridization. Hybridization was performed using a gene expression hybridization kit (Agilent technologies, Santa Clara, CA) for 17.5 h at 65^{o}C. The arrays were washed with gene expression wash buffer 1 (0.005% Triton-102) for 1 min, and dried by Agilent stabilization and dying solution. The arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C, Agilent Technologies, Santa Clara, CA). The data were extracted from scanned images using feature Extraction 10.1.1.1 software (Agilent technologies, Santa Clara, CA).

The data normalization, noise mitigation, statistical analysis was performed by the ICBR[93]. After normalizing the signal intensity values, the Student t-test was performed using a probe-by-probe comparison between each experimental probe group and each control probe group.

qRT-PCR

For qRT-PCR, *V. cholerae* strains, including N16961S, N16961S-24, and GASP-700D were grown in FSLW overnight statically at room temperature. Total RNA was extracted and purified from each culture using the RNeasy kit (Qiagen, Valencia, CA); the contaminating DNA in the preparation was eliminated on-column by DNase digestion. Total RNA (10 ng) was converted to cDNA, and the RT-PCR assay were performed using iScript one-step RT-PCR kit with SYBR green

(Bio-Rad, Hercules, CA) and CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) following manufacturer's instructions[92]. Primers used in this study are listed in Table 4-1. For each sample, the mean cycle threshold of the test transcript was normalized to that of *toxR* (*toxR* was equally expressed both in L-broth and in FSLW) and presented relative to *V. cholerae* N16961S-24 strain that has arbitrarily been taken as 1 (Figure 4-3)[92]. Values above 1 or less than 1 of a selected gene indicate that the transcript was present in higher or lower numbers, respectively, than that of control strain. Data are based on three independent experiments.

RESULTS

Transcriptome profiles analysis between strains N16961S, N16961S-24 and GASP-700D

The comparative transcriptomes between N16961S and N16961S-24, and N16961S and GASP-700D, were highly concordant within each of the various biological groups (Figure 4-1). A p-value and a fold change (FC [2.5]) were computed for each gene locus in the experimental and control groups[93]. The gene expression fold changes were computed based on the normalized log transformed signal intensity data. A total of 3,885 genes are represented by the heat maps in figure 4-1. Each technical replicate clustered into the appropriate biological grouping (Figure 4-1). The p-value measures the minimum statistical false positive rate incurred when setting a threshold for test significance. In addition, false discovery rate (FDR) values were also computed, which are the expected proportion of false positives among all significant hypotheses [94].

Data obtained from this study suggests that, a total of 190 genes were in common and more highly expressed by both GASP-700D and N16961 grown in FSLW (N16961S-24) compared

to N16961 grown in L-broth (N16961S) (Figure 4-2). Furthermore, 387 and 195 genes were expressed by N16961S-24 and GASP-700D as stage specific manner, respectively, when compared to N16961S (Figure 4-2). Similarly, 66 genes in common were repressed by GASP-700D and N16961S-24 compared to N16961S; in addition, 165 and 54 genes were repressed by N16961S-24 and GASP-700D as stage specific manner, respectively, when compared to the N16961S (Figure 4-2). Sixty-one protein synthesis genes were upregulated in GASP-700D compared to 26 of them in N16961S-24 (Table 4-2). Most of these genes were ribosomal protein synthesis genes. As expected, motility and chemotaxis genes were more expressed (32 and 23 genes respectively) in N16961S-24 compared to GASP-700D (zero and 1 gene respectively) (Table 4-2). Interestingly, phosphate transport system genes (5 genes in N16961S-24 and 6 in GASP-700D), and stress adaptation genes (5 genes in N16961S-24 and 3 in GASP-700D) were highly expressed in FSLW, while virulence genes (7 genes in N16961S-24 and 15 in GASP-700D) were repressed by both N16961S-24 and GASP-700D in FSLW (Table 4-2).

To validate my microarray gene expression analysis, I performed RT-PCR assays targeting some known genes expressed or repressed in FSLW compared to *V. cholerae* grown in nutrient-rich L-broth. Consistent with earlier reports [30], we observed that *phoB*, *pstS* and *pstB-2* genes were more highly expressed in N16961S-24 and GASP-700D in FSLW compared to expression of those genes when the N16961S grown in L-broth (Figure 4-3), whereas the virulence genes *tcpA*, *tcpP*, *tcpH* and *ctxA* were repressed (Figure 4-3), validating the results of my RT-PCR analysis.

DISCUSSION

I hypothesize that, during long-term survival (700 days) in stressful stationary culture, *V. cholerae* may have adopted two responses, including: (i) assume "persister" phenotype [42], and (ii) select GASP mutants that successfully adapt to stressful growth conditions [92]. Although I currently do not have anysupporting evidence to conclude that GASP-700D genome has any mutation, I did observe that GASP-700D is defective in productive motility implying that GASP-700D may have possible mutation(s)/alteration in its genome [92]. Indeed, previous reports demonstrated that GASP phenotypes with genetic mutations are common in microorganisms surviving long-term in stressful and stationary growth phase[47, 48].

V. cholerae GASP-700D exhibited significantly differential gene expression profiles relative to N16961S-24 (Table 4-2) as measured by microarray technique. GASP-700D robustly expressed ribosomal proteins and ATP synthesis genes compared to N16961S-24 in FSLW (Table 4-2); however, further investigation is needed to understand whether flagella negatively contribute to ribosomal and ATP synthesis proteins. As expected, motility and chemotaxis genes were more expressed by *V. cholerae* N16961S-24 compared to GASP-700D (Table 4-2). High-affinity phosphate transport system genes, and stress adaptation genes were highly expressed in FSLW, while virulence genes were repressed by both N16961S-24 and GASP-700D in FSLW (Table 4-2).

Previous report using qPCR demonstrated that *V. cholerae* expressed *phoB* and Pst-system genes while repressed *tcp* genes when grown in "filter sterilized" pond water microcosms compared to its growth in nutrient-rich L-broth [30]. Consistent with that previous

study[30], I found that*phoB* and Pst-system genes were highly expressed, while *tcp* genes and *ctxA* were repressed, by N16961S-24 and GASP-700D grown in FSLW relative to N16961S grown in nutrient-rich L-broth (Figure 4-3) validating the my gene expression analysis by microarray technique.

My data indicate that the GASP-700D phenotype have differential physiologic and metabolic adaptive responses compared to its wild-type *V. cholerae* (N16961S-24). I propose that, in addition to persister phenotype, GASP-700D of *V. cholerae* may contribute to environmental persistence and cholera transmission.

Table 4-1. Oligonucleotide primers used in this study

Primer types	Primer name	Direction	Target Gene	Sequence (5' to 3')
Real time PCR				
	aa133	Forward	*phoB*	ATG TCT AGA AGG ATT CTG GT
	aa134	Reverse		GAA TCA TAA TCT TCA GCC TC
	aa135	Forward	*pstB2*	TGA ATC GTA TGA ATG ATC TC
	aa136	Reverse		TCA TAA ATA CTC ATT GGG AA
	aa137	Forward	*pstS*	CAC TTA TAG GAA ATT CGT GA
	aa138	Reverse		ACT GCT GAG ATT GTC TCT TT
	aa139	Forward	*tcpA*	CAA ACT TAT CGT AGT CTT GG
	aa140	Reverse		CAT AGC TGT ACC AGT GAA AG
	aa141	Forward	*tcpP*	GAA TGA ATG CAC TAA TCA AG
	aa142	Reverse		ATT ATT TGA TCA TTT GGA CA
	aa143	Forward	*toxR*	ATA TCG ATG AGT CAT ATT GG
	aa144	Reverse		TAA TCG AAT GAT CTC TTC AC
	aa180	Forward	*tcpH*	GTG TAA CGA TCA TCG CAC TC
	aa181	Reverse		ATA GGT TAC AAA CCG AAT GG
	aa260	Forward	*ctxA*	TCT AGA CCT CCT GAT GAA AT
	aa261	Reverse		AAG GTT GAT ATT CAT TTG AG

Table 4-2. Notable gene expression by *V. cholerae* N16961S-24 and GASP-700D as measured by microarray technique

Genes by function	N16961S-24		GASP-700D	
	Upregulated	Down regulated	Upregulated	Down regulated
Protein synthesis	26	1	61	0
Outer membrane protein	5	1	4	1
Motility	32	1	0	0
Chemotaxis	23	2	1	2
Virulence	0	7	0	15
Phosphate transport system	5	2	6	0
Stress adaptation	5	1	3	2
Polysaccharide production	0	2	4	0
ATP synthesis	2	2	9	0
Hypothetical or conserved hypothetical protein synthesis	280	57	154	47

Figure 4-1. Heat maps of *V. cholerae* genes (3,885) expressed differentially: (A) comparative expression between V. cholerae N16961S grown in L-broth and in FSLW (N16961-24); (B) relative expression between N16961S grown in L-broth and in FSLW (GASP-700D). Red and green represent expressed (max=14-fold) and repressed (max=11-fold) genes, respectively. Cut-off value was considered as 2.5.

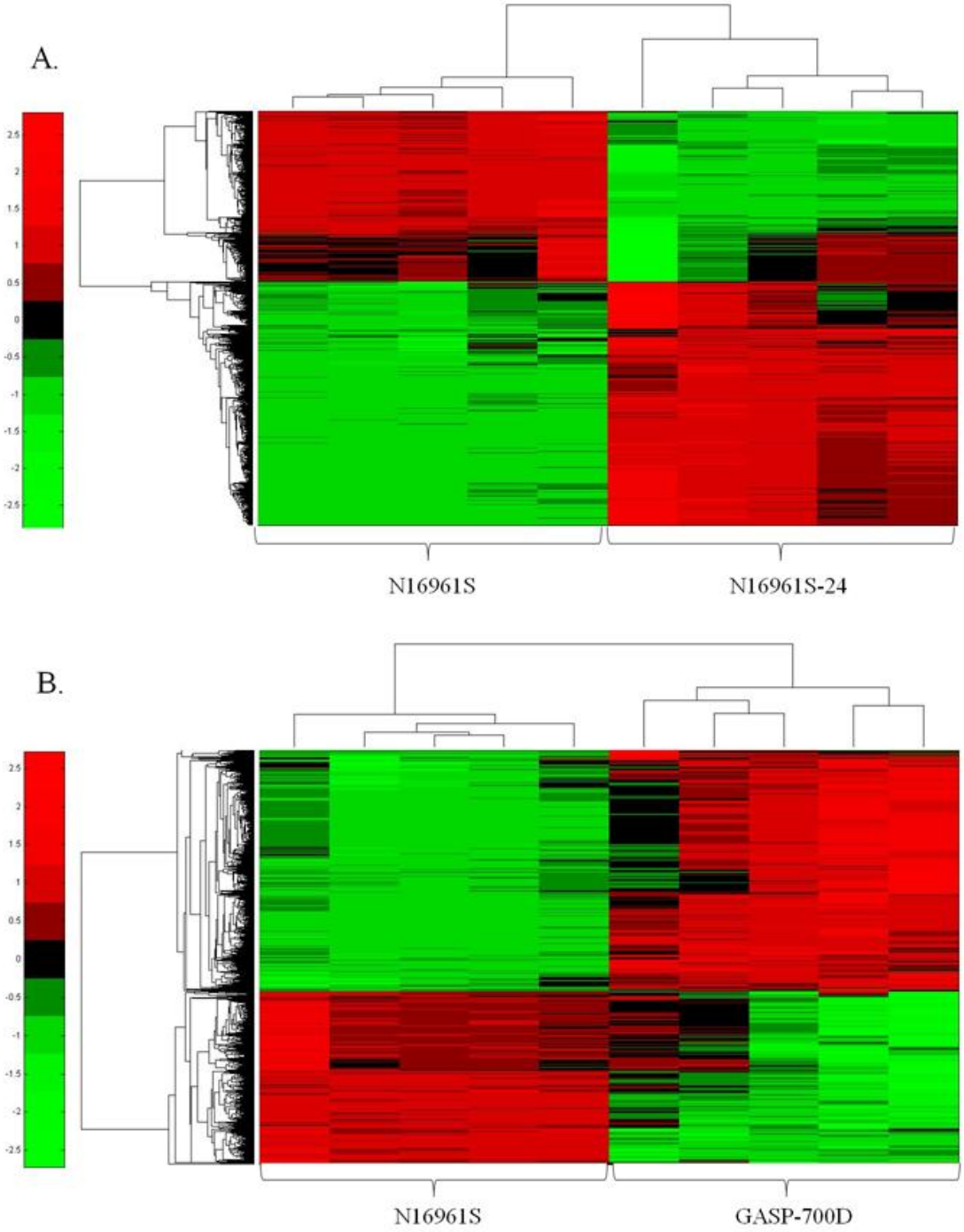

Figure 4-2. Comparative gene expression profiles between N16961S-24 and GASP-700D grown in FSLW and their isogenic wild-type *V. cholerae* grown in L-broth. E, expressed; R, repressed.

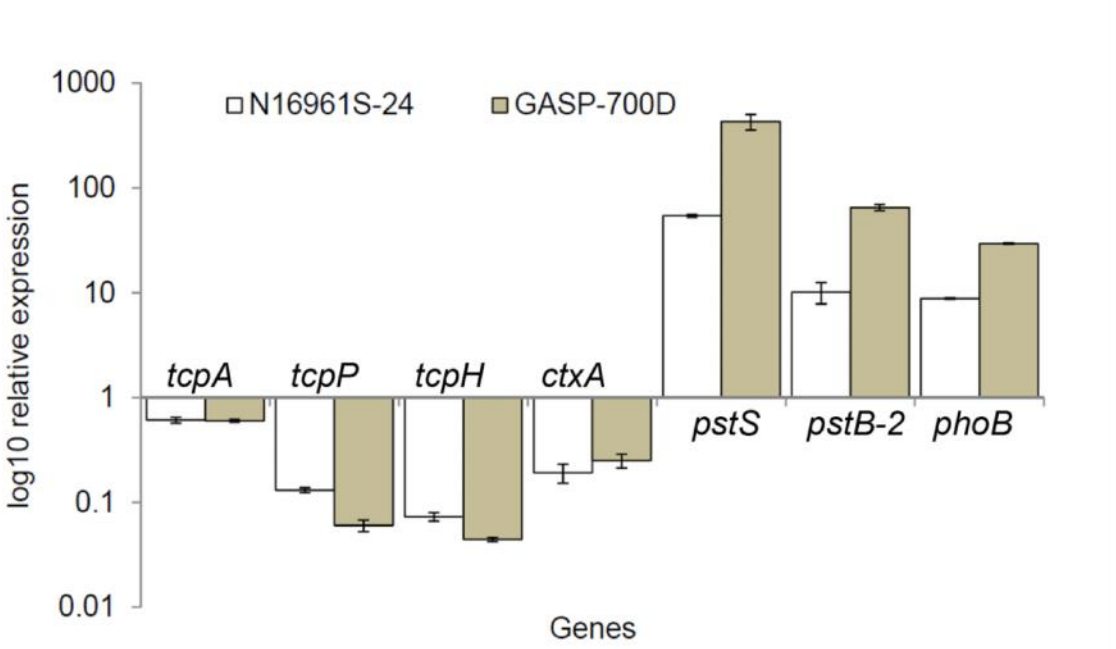

Figure 4-3. Comparative analysis of the differential gene expression among *V. cholerae* strains N16961S and GASP-700D using qRT-PCR. N16961S was grown both in nutrient-rich L-broth and in nutrient-poor FSLW (N16961S-24) (ca. 108 cfu/ml), and the cultures were incubated overnight statically at room temperature. GASP-700D was grown (ca. 108 cfu/ml) in FSLW only. Expression of each gene was normalized to that of toxR, and subsequently compared to that of the wild-type N16961S grown in L-broth. Data represent the average results of three independent experiments and error bars indicate as means ± standard deviation (SD).

CHAPTER 5

CONCLUSIONS

It is well established that *V. cholerae* is a natural component of aquatic environments and humans acquire the bacterium upon consumption of food and water contaminated with the bacterium [1, 2]. In spite of decades of research, the genetic and physiologic basis of persistence of *V. cholerae* in nutrient-poor/limited and stressful conditions is largely unknown [42]. In the present work, I, for the first, demonstrated that a subset of *V. cholerae* can assume a novel persister cells exhibiting differential cellular morphologies, including cells with no flagella, bipolar and peritrichous flagella, along with cells displaying short rod to very long helical cells and coccoid cells [42]. Although, the roles for diverse cell morphologies to environmental persistence are yet to be determined, I reasoned that, in response to nutrient starvation, *V. cholerae* promotes cellular diversity as seen in other bacterial species [95-99] to diversify a cellular portfolio to ensure that, a component of *V. cholerae* cellular portfolio will evade even increased stressors in the event that other components of the cellular portfolio fell victim to more stringent stressors. Alternatively, while coccoid cells may immediately turn into robust persister cells, the long helical cells may scavenge scarce nutrients using their high surface to volume ratio as is reported with other bacterial species before being turned into more homogeneous small rod -shaped cells with cellular aggregation in biofilm-like consortium [42].

While I do not have direct evidence, a subset of persister cells may also turn further into what we termed as growth advantage stationary phase (GASP)[92] to better adapt to more

stringent nutrient-poor conditions. Evidence supporting this notion came from my findings that, in contrast to 180-days old cells, 700-day old cells seemingly sustained a positive selective phenotype/genotype change inhibiting motility[92]. It's entirely possible that this non-motile GASP phenotype out-competed other persister cells to withstand and persist in nutrient-poor conditions. Indeed I found that GASP phenotype produced elevated polysaccharide and biofilm compared to its wild type phenotype[92], and that GASP-linked biofilm was specific to FSLW but not in L-broth[92], suggesting my idea that GASP is a better persister compared to its parent strain (N16961). Given that *V. cholerae* genome is very plastic, the role of GASP phenotype can be attributed to at least in the evolution of new toxigenic strain in the aquatic reservoirs.

To further explore the roles for *V. cholerae* persister cells and GASP phenotype in environmental persistence and disease transmission more work is needed, including single cell genomic analysis, toxin-antitoxin model testing, RNAseq analysis, and animal studies. In this context, I performed gene expression studies (Chapter 4); with that study, I identified five toxin antitoxin genetic elements, and a few other genes highly expressed in persister and GASP phenotypes, indicating their roles in environmental persistence. I suggest that future work should focus on to employ appropriate genetic manipulations to determine if these gene products are indeed control the persister and GASP phenotypes.

LIST OF REFERENCES

1. Morris, J.G., Jr., *Cholera - Modern pandemic disease of ancient lineage.* Emerg. Infect. Dis., 2011. **17**: p. 2099-2104.

2. Kaper, J.B., J.G. Morris Jr., and M.M. Levine, *Cholera.* Clinical Microbiology Reviews, 1995. **8**: p. 48-86.

3. Ali M, L.A., You YA, Kim YE, Sah B, Maskery B, Clemens J, *The global burden of cholera.* Bulletin of the World Health Organization, 2012. **90**: p. 209-218.

4. WHO, *Weekly epidemiological record.* World Health Organization, 2014. **89**(31): p. 345-56.

5. Banerjee, R., et al., *Dynamics in genome evolution of* Vibrio cholerae*.* Infect Genet Evol, 2014. **23**: p. 32-41.

6. Basak, S., et al., *Influence of domain architecture and codon usage pattern on the evolution of virulence factors of* Vibrio cholerae*.* Biochem Biophys Res Commun, 2009. **379**(4): p. 803-5.

7. Stine, O.C. and J.G. Morris, Jr., *Circulation and transmission of clones of* Vibrio cholerae *during cholera outbreaks.* Curr Top Microbiol Immunol, 2014. **379**: p. 181-93.

8. Safa, A., et al., Vibrio cholerae *O1 hybrid El Tor strains, Asia and Africa*: Emerg Infect Dis. 2008 Jun;14(6):987-8. doi: 10.3201/eid1406.080129.

9. Sack, D.A., et al., *Cholera.* Lancet, 2004. **363**(9404): p. 223-33.

10. Mukhopadhyay, A.K., Y. Takeda, and G. Balakrish Nair, *Cholera outbreaks in the El Tor biotype era and the impact of the new El Tor variants.* Curr Top Microbiol Immunol, 2014. **379**: p. 17-47.

11. Gardner, A.D. and K.V. Venkatraman, *The antigens of the cholera group of vibrios.* Journal of Hygiene, Cambridge, 1935. **35**: p. 262-282.

12. Morris Jr., J.G., *Non-O1 group 1* Vibrio cholerae *strains not associated with epidemic disease*, in Vibrio cholerae *and Cholera: Molecular to Global Perspectives*, I.K. Wachsmuth, P.A. Blake, and Ø. Ølsvik, Editors. 1994, American Society for Microbiology: Washington. p. 103-115.

13. Saha, P.K., et al., *Nontoxigenic* Vibrio cholerae *01 serotype Inaba biotype El Tor associated with a cluster of cases of cholera in southern India.* J Clin Microbiol, 1996. **34**(5): p. 1114-7.

14. Kim, E.J., et al., *CTX prophages in* Vibrio cholerae *O1 strains.* J Microbiol Biotechnol, 2014. **24**(6): p. 725-31.

15. Pollitzer, R., *Cholera.* Monograph Series 43. Geneva: World Health Organization, 1959.

16. Nair, G.B., et al., *New Variants of* Vibrio cholerae *O1 Biotype El Tor with Attributes of the Classical Biotype from Hospitalized Patients with Acute Diarrhea in Bangladesh.* Journal of Clinical Microbiology, 2002. **40**(9): p. 3296-3299.

17. Nair, G.B., et al., *Cholera due to altered El Tor strains of* Vibrio cholerae *O1 in Bangladesh.* J Clin Microbiol, 2006. **44**(11): p. 4211-3.

18. Colwell, R.R., *Global climate and infectious disease: the cholera paradigm.* Science, 1996. **274**(5295): p. 2025-31.

19. Islam, M.S., B.S. Drasar, and R.B. Sack, *The aquatic flora and fauna as reservoirs of* Vibrio cholerae*: a review.* J Diarrhoeal Dis Res, 1994. **12**(2): p. 87-96.

20. Lutz, C., et al., *Environmental reservoirs and mechanisms of persistence of* Vibrio cholerae*.* Front Microbiol, 2013. **4**(375): p. 00375.

21. Faruque, S.M., M.J. Albert, and J.J. Mekalanos, *Epidemiology, genetics, and ecology of toxigenic* Vibrio cholerae*.* Microbiology and Molecular Biology Reviews, 1998. **62**: p. 1301-1314.

22. Huq, A., et al., *Detection of* Vibrio cholerae *O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods.* Applied and Environmental Microbiology, 1990. **56**: p. 2370-2373.

23. Epstein, P.R., *Algal blooms in the spread and persistence of cholera.* Biosystems, 1993. **31**(2-3): p. 209-21.

24. Costerton, J.W., et al., *Microbial biofilms.* Annual Review of Microbiology, 1995. **49**: p. 711-745.

25. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases.* Nat Rev Microbiol, 2004. **2**(2): p. 95-108.

26. Nelson, E.J., et al., *Cholera transmission: the host, pathogen and bacteriophage dynamic.* Nat Rev Microbiol, 2009. **7**(10): p. 693-702.

27. Faruque, S.M. and J.J. Mekalanos, *Phage-bacterial interactions in the evolution of toxigenic* Vibrio cholerae*.* Virulence, 2012. **3**(7): p. 556-65.

28. Colwell, R.R., *Viable but nonculturable bacteria: a survival strategy.* J Infect Chemother, 2000. **6**(2): p. 121-5.

29. Thomas, K.U., et al., *Salinity-induced survival strategy of* Vibrio cholerae *associated with copepods in Cochin backwaters.* Mar Pollut Bull, 2006. **52**(11): p. 1425-30.

30. Nelson, E.J., et al., *Transmission of* Vibrio cholerae *is antagonized by lytic phage and entry into aquatic environment.* PLoS pathogens, 2008. **4**: p. 1-15.

31. Reidl, J. and K.E. Klose, Vibrio cholerae *and cholera: out of the water and into the host.* FEMS Microbiol. Rev., 2002. **26**: p. 125-129.

32. Colwell, R.R. and A. Huq, *Vibrios in the environment: viable but nonculturable Vibrio cholerae*, in Vibrio cholerae *and cholera: molecular to global perspectives*, I.K. Wachsmuth, P.A. Blake, and Ø. Olsvik, Editors. 1994, American Society for Microbiology: Washington.

33. Ali, A., M.H. Rashid, and D.K.R. Karaolis, *High-Frequency Rugose Exopolysaccharide Production by* Vibrio cholerae*.* Appl. Environ. Microbiol., 2002. **68**(11): p. 5773-5778.

34. Yildiz, F.H. and G.K. Schoolnik, Vibrio cholerae *O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation.* Proc. Natl. Acad. Sci. USA, 1999. **96**: p. 4028-4033.

35. Wai, S.N., et al., Vibrio cholerae *O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation.* Applied and Environmental Microbiology, 1998. **64**: p. 3648-3655.

36. Rahman, M., et al., *High-frequency rugose exopolysaccharide production by* Vibrio cholerae *strains isolated in Haiti.* PloS ONE, 2014. **9**(11).

37. Lewis, K., *Persister cells.* Annu. Rev. Microbiol., 2010. **64**: p. 357-372.

38. Balaban, N.Q., *Persistence:mechanisms for triggering and enhancing phenotype variability.* Current Opinion in Genetics and Development, 2011. **21**: p. 768-775.

39. Balaban, N.Q., et al., *Bacterial persistence as a phenotypic switch.* Science, 2004. **305**: p. 1622-1625.

40. Lewis, K., *Riddle of biofilm resistence.* Antimicrob. Agents Chemother., 2001. **45**: p. 999-1007.

41. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. **284**: p. 1318-1322.

42. Jubair, M., G.J.J. Morris, and A. Ali, *Survival of* Vibrio cholerae *in nutrient-poor environments is associated with a novel "persister" phenotype.* PloS ONE, 2012. **7**: p. e45187.

43. Schuster, C.F. and R. Bertram, *Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate.* FEMS Microbiol Lett, 2013. **340**(2): p. 73-85.

44. Wagner, E.G. and C. Unoson, *The toxin-antitoxin system tisB-istR1: Expression, regulation, and biological role in persister phenotypes.* RNA Biol, 2012. **9**(12): p. 1513-9.

45. Gibson, R.L., J.L. Burns, and B.W. Ramsey, *Pathophysiology and management of pulmonary infections in cystic fibrosis.* Am J Respir Crit Care Med, 2003. **168**(8): p. 918-51.

46. Lafleur, M.D., Q. Qi, and K. Lewis, *Patients with long-term oral carriage harbor high-persister mutants of* Candida albicans*.* Antimicrob Agents Chemother, 2010. **54**(1): p. 39-44.

47. Finkel, S.E., *Long-term survival during stationary phase: evolution and the GASP phenotype.* Nature reviews. Microbiology, 2006. **4**(2): p. 113-20.

48. Finkel, S.E. and R. Kolter, *Evolution of microbial diversity during prolonged starvation.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(7): p. 4023-7.

49. Zambrano, M.M. and R. Kolter, *GASPing for life in stationary phase.* Cell, 1996. **86**(2): p. 181-4.

50. Zambrano, M.M., et al., *Microbial competition:* Escherichia coli *mutants that take over stationary phase cultures.* Science, 1993. **259**(5102): p. 1757-60.

51. Colwell, R.R., et al., *Viable, but non-culturable* Vibrio cholerae *and related pathogens in the environment: implications for release of genetically engineered microorganisms.* Bio/Technology, 1985. **3**: p. 817-820.

52. Huq, A., et al., *Critical Factors Influencing the Occurrence of* Vibrio cholerae *in the Environment of Bangladesh.* Appl. Environ. Microbiol., 2005. **71**(8): p. 4645-4654.

53. Zinser, E.R. and R. Kolter, Escherichia coli *evolution during stationary phase.* Research in microbiology, 2004. **155**(5): p. 328-36.

54. White, P.B., *The rugose variant of vibrios.* Journal Pathol. Bacteriol., 1938. **46**: p. 1-6.

55. Rice, E.W., et al., *Chlorine and survival of "rugose"* Vibrio cholerae*.* Lancet, 1992. **340**: p. 740.

56. Morris Jr., J.G., et al., *Vibrio cholerae O1 can assume a chlorine-resistant rugose survival form that is virulent for humans.* J. Infect. Dis., 1996. **174**: p. 1364-1368.

57. Singh, P.K., et al., *Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms.* Nature, 2000. **407**: p. 762-764.

58. Moker, N., C.R. Dean, and J. Tao, Pseudomonas aeruginosa *increases formation of multidrug-tolerant persister cells in response to quorum-sensing signalling molecules.* J. Bacteriol., 2010. **192**: p. 1946.

59. Miller, M.B., et al., *Parallel quorum sensing systems converge to regulate virulence in* Vibrio cholerae*.* Cell, 2002. **110**: p. 303-314.

60. Kulp, A. and M.J. Kuehn, *Biological functions and biogenesis of secreted bacterial outer membrane vesicles.* Annu. Rev. Microbiol., 2010. **64**: p. 163-184.

61. Alam, M., et al., *Viable but nonculturable* Vibrio cholerae *O1 in biofilms in the aquatic environment and their role in cholera transmission.* Proceedings of the National Academy of Sciences, 2007. **104**(45): p. 17801-17806.

62. Hansen, S., K. Lewis, and M. Vulic, *Role of global regulators and nuclotide metabolism in antibiotic tolerance in* Escherichia coli*.* Antimicrob. Agents. Chemother., 2008. **52**: p. 2718-2726.

63. Kayama, S., et al., *The role of rpoS gene and quroum-sensing system in ofloxacin tolerance in* Pseudomonas aeruginosa*.* FEMS Microbiol. Lett, 2009. **298**: p. 184-192.

64. Franco, A.A., et al., *Cholera in Lima, Peru, Correlates with Prior Isolation of* Vibrio cholerae *from the Environment.* Am. J. Epidemiol., 1997. **146**(12): p. 1067-1075.

65. Meibom, K.L., et al., *The* Vibrio cholerae *chitin utilization program.* PNAS, 2004: p. 0308707101.

66. Carrel, M., et al., *Spatio-temporal clustering of cholera: The impact of flood control in Matlab, Bangladesh, 1983-2003.* Health & Place, 2009. **15**: p. 771-782.

67. Bompangue, D., et al., *Cholera epidemics, war and disasters around Goma and Lake Kivu: An eight-year survey.* PLoS Neglected Tropical Dis., 2009. **3**: p. 1-8.

68. Watnick, P.I. and R. Kolter, *Steps in the development of a* Vibrio cholerae *El Tor biofilm.* Mol. Microbiol., 1999. **34**: p. 586-595.

69. Klose, K.E. and J.J. Mekalanos, *Differential regulation of multiple flagellins in* Vibrio cholerae*.* J Bacteriol, 1998. **180**(2): p. 303-16.

70. Ali, A., et al., *Sequence analysis of TnphoA insertion sites in* Vibrio cholerae *mutants defective in rugose polysaccharide production.* Infect. Immun., 2000. **68**: p. 6857-6864.

71. Fong, J.C., et al., *Role of Vibrio polysaccharide (vps) genes in VPS production, biofilm formation and* Vibrio cholerae *pathogenesis.* Microbiology, 2010. **156**(Pt 9): p. 2757-69.

72. Wang, R.F. and S.R. Kushner, *Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in* Escherichia coli*.* Gene, 1999. **100**: p. 195-199.

73. Ali, A., et al., *Mutations in the extracellular protein secretion pathway genes (eps) interfere with rugose polysaccharide production in and motility of* Vibrio cholerae*.* Infect. Immun., 2000. **68**: p. 1967-1974.

74. Donnenberg, M.S., et al., *Role of the* eaeA *gene in experimental enteropathogenic* Escherichia coli *infection.* Journal of Clinical Investigation, 1993. **92**: p. 1412-1417.

75. Daims, H., S. Lucker, and M. Wagner, *daime, a novel image analysis program for microbial ecology and biofilm research.* Environ Microbiol, 2006. **8**(2): p. 200-13.

76. Beyenal, H., et al., *Three-dimensional biofilm structure quantification.* Journal of microbiological methods, 2004. **59**(3): p. 395-413.

77. Casper-Lindley, C. and F.H. Yildiz, *VpsTIs a Transcriptional Regulator Required for Expression of* vps *Biosynthesis Genes and the Development of Rugose Colonial Morphology in* Vibrio cholerae *O1 El Tor.* J. Bacteriol., 2004. **186**(5): p. 1574-1578.

78. Watnick, P.I., et al., *The absence of a flagellum leads to altered colony morphology, biofilmdevelopment and virulence in* Vibrio cholerae *O139.* Molecular Microbiology, 2001. **39**: p. 223-235.

79. Yildiz, F.H., N.A. Dolganov, and G.K. Schoolnik, *VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of* vps *biosynthesis genes and EPS^{ETr}-associated phenotypes in* Vibrio cholerae *O1 El Tor.* Journal of Bacteriology, 2001. **183**: p. 1716-1726.

80. Harrison, J.J., R.J. Turner, and H. Ceri, *Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic* Pseudomonas aeruginosa*.* Environ. Microbiol., 2005. **7**: p. 981-994.

81. Banning, N., S. Toze, and B.J. Mee, *Persistence of biofilm-associated* Escherichia coli *and* Pseudomonas aeruginosa *in groundwater and treated effluent in a laboratory model system.* Microbiology, 2003. **149**(1): p. 47-55.

82. Prouty, M.G., N.E. Correa, and K.E. Klose, *The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of* Vibrio cholerae*.* Mol Microbiol, 2001. **39**(6): p. 1595-609.

83. Mann, E.E. and D.J. Wozniak, *Pseudomonas biofilm matrix composition and niche biology.* FEMS Microbiol Rev, 2012. **36**(4): p. 893-916.

84. Correa, N.E., F. Peng, and K.E. Klose, *Roles of the regulatory proteins FlhF and FlhG in the* Vibrio cholerae *flagellar transcription hierarchy.* J Bacteriol, 2005. **187**(18): p. 6324-32.

85. Yildiz, F.H., et al., *Molecular analysis of rugosity in a* Vibrio cholerae *O1 Eltor phase variant.* Molecular Microbiology, 2004. **53**: p. 497-515.

86. Pruzzo, C., et al., *Persistence of adhesive properties in* Vibrio cholerae *after long-term exposure to sea water.* Environ. Microbiol., 2003. **5**: p. 850-855.

87. Ali, A., J.G. Morris, Jr., and J.A. Johnson, *Sugars Inhibit Expression of the Rugose Phenotype of* Vibrio cholerae*.* J. Clin. Microbiol., 2005. **43**(3): p. 1426-1429.

88. Boles, B.R. and A.R. Horswill, *Agr-mediated dispersal of* Staphylococcus aureus *biofilms.* PLoS Pathog, 2008. **4**(4): p. 1000052.

89. O'Neill, E., et al., *A novel* Staphylococcus aureus *biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB.* J Bacteriol, 2008. **190**(11): p. 3835-50.

90. Simon, R., U. Priefer, and A. Puhler, *A broad host range mobilization system for in vivo genetic engineering:transposon mutagenesis in Gram negative bacteria.* Biotechnology, 1983. **1**: p. 784-791.

91. Donnenberg, M.S. and J.B. Kaper, *Construction of an* eae *deletion mutant of enteropathogenic* Escherichia coli *by using a positive-selection suicide vector.* Infect. Immun., 1991. **59**: p. 4310-4317.

92. Jubair, M., et al., Vibrio cholerae *persisted in microcosm for 700 days inhibits motility but promotes biofilm formation in nutrient-poor lake water microcosms.* PloS ONE, 2014. **9**(3): e92883.

93. Quackenbush, J., *Microarray data normalization and transformation.* Nat Genet, 2002. **32**: p. 496-501.

94. Storey, J.D. and R. Tibshirani, *Statistical methods for identifying differentially expressed genes in DNA microarrays.* Methods Mol Biol, 2003. **224**: p. 149-57.

95. Ausmees, N., J.R. Kuhn, and C. Jacobs-Wagner, *The bacterial cytoskeleton: an intermediate filament-like function in cell shape.* Cell, 2003. **115**(6): p. 705-13.

96. Motaleb, M.A., et al., Borrelia burgdorferi *periplasmic flagella have both skeletal and motility functions.* Proc Natl Acad Sci U S A, 2000. **97**(20): p. 10899-904.

97. Beveridge, T.J., *The bacterial surface: general considerations towards design and function.* Can J Microbiol, 1988. **34**(4): p. 363-72.

98. Koch, A.L., *What size should a bacterium be? A question of scale.* Annu Rev Microbiol, 1996. **50**: p. 317-48.

99. Schulz, H.N. and B.B. Jorgensen, *Big bacteria.* Annu Rev Microbiol, 2001. **55**: p. 105-37.

BIOGRAPHICAL SKETCH

Mohammad Jubair grew up in Dhaka, Bangladesh where he developed a passion for science at an early age. After graduating high school in 1999, he attended University of Dhaka in Dhaka, Bangladesh where he earned a Bachelor of Science (Honors) in Microbiology from Department of Microbiology, University of Dhaka. Jubair continued study and earned Master of Science (MS) in Microbiology from Department of Microbiology, University of Dhaka in 2007. After finishing Masters, Jubair started his career in Incepta pharmaceuticals in Dhaka, Bangladesh as a Quality Control Microbiologist for a short time of 6 months in 2008. Then he moved to his research career joining in International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B) in Dhaka, Bangladesh as a Microbiologist. He gained experiences on viruses working with different respiratory viruses (mainly influenza) in Virology laboratory in ICDDR,B. In 2010, he took admission as a PhD student in Department of Microbiology of University of Dhaka in a collaboration PhD program between University of Dhaka, Bangladesh and Emerging Pathogens Institute, University of Florida at Gainesville, FL, USA. In December 2010, he moved to Gainesville, Florida, USA continuing his graduate studies as a Research Scholar in the Emerging Pathogens Institute of University of Florida. Jubair did his doctoral thesis studying genetic mechanisms of culturable *Vibrio cholerae* in the aquatic environment and completed his PhD dissertation in January 2015. After graduation, Jubair plans to continue working with cholera as a post-doc in Emerging Pathogens Institute at University of Florida.