THE CONTROL OF PHENOTYPIC DIVERSITY IN *VIBRIO CHOLERAE* DURING THE TRANSITION BETWEEN MOTILITY AND ATTACHMENT

By

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ABSTRACT

THE CONTROL OF PHENOTYPIC DIVERSITY IN *VIBRIO CHOLERAE* DURING THE TRANSITION BETWEEN MOTILITY AND ATTACHMENT

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Vibrio cholerae is a causative agent of human intestinal disease, cholera. It requires both flagellar-based motility and biofilm formation to colonize the small intestine. The secondary messenger molecule, c-di-GMP, plays a central role in controlling transition between motility and biofilm formation. However, the switch between these two lifestyles has been studied on an average population scale, overlooking the heterogeneous phenotypic response that can occur at a single-cell level. *V. cholerae* infections are characterized by the co-occurrence of cells with motile and sessile behaviors, but the determinant of this phenotypic diversity remains poorly understood. We used single-cell tracking to examine the motile behaviors of two *V. cholerae* strains (El Tor C6706 and Classical O395) in response to direct manipulations of c-di-GMP concentration. Both motile and non-motile cells are present in a well-mixed batch culture with distributions of these phenotypes that change depending on the growth phase. We determined that the proportion of motile cells differs between El Tor and Classical strains because they maintain different levels of c-di-GMP. However, even in conditions that promote biofilm formation, *V. cholerae* still generates a sub-population of motile cells. C-di-GMP is known to inversely regulate assembly of mannose-sensitive haemagglutinin (MSHA) pili and flagella. We found that the most cells in clonal populations are flagellated, but MSHA piliation affords the variable opportunity to attach at single-cell level, driving behavioral switching between motile and sessile behaviors. Our results support the hypothesis that c-di-GMP regulates phenotypic diversity in *V. cholerae*, and it does so by simultaneous elaboration of MSHA pili and flagellum.

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KEY TO ABBREVIATIONS

C-di-GMP……..Bis-(3’,5’)-cyclic dimeric guanosine monophosphate

TCP……..Toxin-coregulated pilus

MSHA pilus……...Mannose-sensitive haemagglutinin pilus

DGC……...Diguanylate cyclase

PDE……..Phosphodiesterase

CHAPTER 1

PHENOTYPIC DIVERSITY IN CLONAL BACTERIAL POPULATIONS

1.1 Introduction

A clonal population of bacteria may produce progeny with a wide range of behaviors or characteristics through a process called phenotypic diversity. Phenotypic diversity is defined as non-genetic cell-to-cell variability in the response to identical signals. Stochastic molecular fluctuations in biochemical reactions may contribute to phenotypic diversity, but there are many other biological processes that can trigger non-genetic diversity in bacteria such as asymmetric cell division and local gradients of signals perceived in microenvironments[1,2].

Phenotypic diversity offers opportunities for cells to engage in probabilistic strategies to hedge their bets in anticipation of environmental change[3] and to organize themselves to achieve collective behaviors[4]. The idea that phenotypic diversity confers fitness advantage has long been appreciated and discussed in various contexts such as antibiotic persistence[5], competence for DNA uptake[6], chemotactic response[7], and others. The transition between motility and attachment lifestyles in bacteria plays a key role in establishing colonization in many environmental niches. This process is a potential source of phenotypic diversity, during which single-cell motility phenotypes can be heterogeneous, but a few studies have directly observed and investigated motile behavior of individual bacteria.

1.2 Single-cell tracking method as a tool to study phenotypic diversity

Bacterial behaviors are complex phenomena resulting from multiple sources that contribute to the total diversity of a population[8]. Investigating phenotypic diversity requires measurements of the cell behavior in a large number of individuals on the timescale to collect sufficient information to characterize the proportion of diverse phenotypes. Bacterial motility behavior is a

biologically relevant phenotype for which such measurements are possible. Motility behavior is often implicated in virulence for pathogenic bacteria, but it has also been studied as part of bacterial lifestyles between motile and sessile states, chemotactic strategies, environmental persistence, and its effect on host immune response.

Single-cell tracking is a powerful method to quantitatively characterize individual cell trajectories using the automated multiple-particle tracking and behavioral analysis pipeline previously developed[9]. As many as 1,000 cells can be tracked at once and computationally handled. Recording of cell swimming can be done at high speed (20 frames/s) at low magnification (10X). At this configuration, the microscopy depth of field is large enough so that individual trajectories can be followed for more than a minute. With this capacity, individual motility behavior can be analyzed accurately to characterize the rich diversity of motility phenotypes found in a clonal population.

1.3 *Vibrio cholerae* as a model system of phenotypic diversity

This study focuses on the motility behavior of *Vibrio cholerae* as a model system of phenotypic diversity. *V. cholerae* is an environmental bacterium found in brackish waters and can be an opportunistic human pathogen through consumption of water or food contaminated with this bacterium. It requires both flagellar motility and biofilm formation to colonize the small intestine and to coordinate an exit from the host to resume its environmental life cycle. The spatiotemporal expression of these two phenotypes during infection remain unresolved.

Both flagellar synthesis and biofilm formation are transcriptionally regulated in *V. cholerae* by the second messenger, bis-(3’,5’)-cyclic dimeric guanosine monophosphate (c-di-GMP). Binding

of c-di-GMP to FlrA, the master regulator of flagellar synthesis, inhibits the binding of this transcription factor to the downstream promoter in flagellar synthesis cascade[10]. On the other hand, c-di-GMP binding activates two transcription factors involved in biofilm formation, VpsT[11] and VpsR[12]. Thus, c-di-GMP signaling plays a central role in phenotypic transition between motility and sessile biofilm aggregates. However, this phenotypic transition in response to c-di-GMP has been characterized on average with cell populations.

My preliminary data to characterize motility behavior of *V. cholerae* (C6706str2 strain) using single-cell tracking showed the coexistence of both motile and non-motile cells in a clonal population. The coexistence of motile and sessile phenotypes can be used as a division-of-labor strategy during infection. Given the role of c-di-GMP in transition between motile and sessile phenotypes, this work aims to investigate the regulation of phenotypic diversity by c-di-GMP signaling in *V. cholerae*, a pathogen of public health significance. Studying the molecular mechanism that underlies the control of phenotypic diversity in *V. cholerae* can be a valuable research endeavor, contributing to understanding of the dynamics of *V. cholerae* intestinal colonization and pathogenesis.

1.4 Thesis overview

This thesis work was initiated by defining experimental conditions that affect phenotypic diversity in *V. cholerae* populations. Distributions of diffusion coefficient ($\mu m^2/s$) parameter extracted from individual cell trajectories were used to characterize the proportion of different motility phenotypes in clonal populations. I found that the distribution of phenotypes depends on the growth phase in batch cultures, but the proportion of motile cells differs between two pandemic strains of *V. cholerae*, namely El Tor and Classical biotypes.

In Chapter 2, I will present how c-di-GMP affects phenotypic diversity in El Tor and Classical *V. cholerae*. Here, I will discuss my findings that phenotypic diversity observed in *V. cholerae* populations is not attributable to c-di-GMP-mediated phenotypic switching between flagellated and non-flagellated states. Rather, phenotypic diversity occurs via behavioral switching afforded by pili expression on the cell surface. *V. cholerae* seems to require a certain level of c-di-GMP to activate pili assembly, but phenotypic diversity is driven by the opportunity to attach, not by cell-to-cell variations in flagellation phenotype. Induction of c-di-GMP synthesis increased the proportion of sessile cells in pili-deficient background, suggesting involvement of other c-di-GMP effectors in the regulation of phenotypic diversity. Chapter 3 will summarize significance of this work and propose future studies that can lead to a greater mechanistic understanding on the control of phenotypic diversity.

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CHAPTER 2

THE CONTROL OF PHENOTYPIC DIVERSITY DURING THE TRANSITION BETWEEN MOTILITY AND ATTACHMENT IN TWO PANDEMIC STRAINS OF *V. CHOLERAE*

This chapter is formatted as a manuscript to be submitted for publication:

Lee JS, Kim HS, Hsueh BY, Bedore AM, Waters CM, Dufour YS.

In this chapter, I performed the swim plate assay and single-cell tracking experiments. I prepared the sample for scanning electron microscopy (SEM) and acquired SEM images with the assistance of Center for Advanced Microscopy at MSU. HS Kim produced preliminary results and laid the groundwork for the study. BY Hsueh helped with mass spectrometry trace analysis and constructed *V. cholerae ΔmshA* mutant in El Tor and Classical biotypes. AM Bedore performed motility screen of El Tor genomic cosmid library and constructed the 3DGC knock-out (ΔVCA0956, ΔVCA0960, ΔVCA0965) cosmid library mutant. CM Waters gifted *V. cholerae* strains and provided valuable feedback on the manuscript. YS Dufour and I analyzed the results of single-cell tracking and SEM images of cell flagellation.

2.1 Introduction

Vibrio cholerae is the etiologic agent of cholera that is contracted by consumption of water and food contaminated with bacteria. The pathogenesis of cholera infection has been well established by previous studies, but the dynamics of infection and intestinal colonization are inherently complex, owing to bacterial ability to switch between motile lifestyle and sessile aggregate known as biofilm. Ingested *V. cholerae* transits through the highly acidic environment of the stomach and colonize the small intestine. Intestinal colonization involves penetration through the mucus layer and attachment to epithelial cells lining the base of villi called crypts. Entry into the biofilm state promotes bacterial survival during gastric passage, whereas penetration of the host mucosal layer depends on flagellar motility. Both biofilm formation and motility[1] have been cited as virulence factors, and thus, understanding how these traits are regulated and expressed at different stages of infection is a key to develop new strategies to minimize human infection. *V. cholerae* elaborates cellular appendage called toxin-coregulated pili (TCP) to adhere to the epithelial surface[2], grows to a high cell density in the crypts[3], and secrets cholera toxin (CT), leading to a rapid loss of electrolytes and water to the lumen of the intestine. Water loss triggers bacterial detachment from the host epithelial cells[4] to be shed in the characteristic rice stool containing both sessile aggregates and motile *V. cholerae* cells[5]. This highlights again how establishment and dispersal of infection must be coordinated with sessile and motile lifestyles, respectively. Despite the importance of flagellar motility and biofilm formation as virulence factors, the temporal and spatial expression of these two phenotypes during infection is still unresolved.

Two *Vibrio cholerae* biotype strains are responsible for pandemic outbreaks of cholera: the Classical biotype that is an etiologic agent of past six pandemics (1899-1923) and the El Tor

biotype[6] in the current seventh pandemic that started in 1961[7]. Both biotypes are known to be capable of flagellar motility that has been implicated in virulence contributing to intestinal colonization and disease transmission. However, it is currently unknown what makes El Tor strain more virulent than classical strain and whether the difference in motility behaviors accounts for the dominance of one strain over the other. Past studies that looked into genome-wide comparisons between classical and El Tor strains had identified that classical strain misses 29 genes that are uniquely represented in El Tor strain,[8] constituting two genomic islands known as the Vibrio Seventh Pandemic (VSP) islands (VSP-I and VSP-II). The functional consequences of a few of these genes are only beginning to be appreciated, but their contribution to pathogenicity and environmental persistence remains poorly characterized.

The transition between motility and sessile behaviors has been linked to the second messenger, bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP). The second messenger c-di-GMP is widely conserved in bacteria, and it controls a broad set of phenotypic responses besides flagellar motility including virulence factor expression[9], cell morphology[10], Type II secretion[11], DNA repair[12], and the antioxidant production[13]. The synthesis and degradation of c-di-GMP are regulated by diguanylate cyclases (DGC) and phosphodiesterases (PDE), respectively. In *V. cholerae*, the c-di-GMP regulatory network consists of 40 DGCs with a signature GGDEF domain and 29 PDEs with a signature EAL or HD-GYP motif to control c-di-GMP turnover[14]. Despite the complexity of regulatory network, the sensory domains of most DGCs and PDEs remain largely uncharacterized with their cognate signals yet to be identified. It suggests that c-di-GMP integrates many environmental signals to regulate the transition between motility and biofilm formation.

Only a few sensory inputs or environmental stimuli are known to date that are directly responsible for the transition between motile-to-sessile states in *V. cholerae*. Signals such as bile acid[15] and autoincuder (AI) molecules[16] have been shown to control motility behavior in *V. cholerae* by affecting the level of c-di-GMP. Bile acid promotes biofilm formation by modulating the activity of three DGCs and of one PDE. In contrast, high concentrations of autoinducer (AI) molecules resulting from high cell density promote cell detachment and motility via transcriptional control of 14 genes encoding DGCs and PDEs[16]. These two signals characterize the intestinal milieu during infection, and yet they have conflicting effects on the regulation of *V. cholerae*'s lifestyles.

Previous studies have established that c-di-GMP plays a central role in *V. cholerae*'s decision between motile or sessile lifestyle[17,18]. High intracellular concentration of c-di-GMP promotes biofilm formation, thereby reducing motility, in *V. cholerae*. However, the transition between motility and sessile behavior has been largely studied on an average population scale. Such experimental approach can overlook the potential of individual cells to exhibit heterogeneous behaviors at a single-cell level (referred as phenotypic diversity). Stools from cholera patients are known to contain a heterogeneous mixture of motile cells and biofilm-like aggregates[5]. The coexistence of cells with fundamentally different behaviors in *V. cholerae* infections suggests that phenotypic diversity in single cells contributes to successful colonization of and survival within the host. Despite the importance of phenotypic diversity in understanding the dynamics of *V. cholerae* intestinal colonization and pathogenesis, mechanisms that control and generate phenotypic diversity in clonal populations remain poorly understood. Based on the role of c-di-GMP in regulating motility and biofilm formation, we propose the role of c-di-GMP in controlling phenotypic diversity during the transition between two lifestyles.

Using a single-cell tracking method, we demonstrate that a clonal batch culture of El Tor C6706 *V. cholerae* is phenotypically diverse at a single-cell level, and the proportion of motile cells within a population increases from exponential to stationary phases. We also found that Classical strain of *V. cholerae* exhibits a divergent pattern of phenotypic distributions through the growth cycle. Population phenotypes are uniformly motile, and motile single cells are found in high proportions both during exponential and stationary phases. This biotype-specific behavioral difference is due to the difference in c-di-GMP levels in two pandemic strains, which was not previously detected with the quantification method (two-dimensional thin-layer chromatography) that is not sensitive enough[19]. We also investigated the mechanism by which c-di-GMP produces phenotypic diversity, as c-di-GMP binds to several downstream effectors to mediate a number of different processes, such as flagellar synthesis and pili export onto the cell surface. We found that most *V. cholerae* cells in El Tor and Classical cells are flagellated, and the proportion of flagellated cells does not change between the growth phases. In contrast, the activity of mannose-sensitive hemagglutinin (MSHA) pili appears to dominate behavioral response between the exponential and stationary growth phases in El Tor strain. In Classical strain, that does not naturally express MSHA pili, both the presence and absence of *mshA* gene did not affect single-cell response through the growth cycle. Expression of DGC partially restores non-motile phenotypes in MSHA-deficient background for both El Tor and Classical strains, suggesting that there is yet an unidentified mechanism by which c-di-GMP regulates phenotypic diversity.

2.2 Results

Initial observations of the difference in motile behaviors between the El Tor and Classical strains came from motility plate assay. The Classical strain showed further colony spreading, whereas

the El Tor strain appeared to be more localized (Fig 1A). However, interpreting motility phenotypes on soft agar-based plate assay is complicated by interplay of numerous factors, such as growth, swimming speed, and chemotactic response to the gradient that can be induced by local depletion of nutrients. The use of a multiple-particle tracking method[20] allowed us to characterize single-cell behaviors of these strains in well-mixed batch cultures. We took samples of *V. cholerae* El Tor C6706 and Classical O395 cells from the respective batch cultures during the exponential and stationary phases and tracked the swimming behavior of individual bacteria. The distribution of single-cell behaviors was characterized in terms of diffusion coefficients ($\mu m^2/s$) (Fig 1B). Cells undergoing Brownian motion have diffusion coefficients less than 10 $\mu m^2/s$ and are considered non-motile. Our results showed that both non-motile and motile cells coexist in *V. cholerae* populations in well-mixed conditions. However, the distribution of non-motile and motile phenotypes changed between exponential and stationary phases and as well as between the El Tor and Classical biotypes.

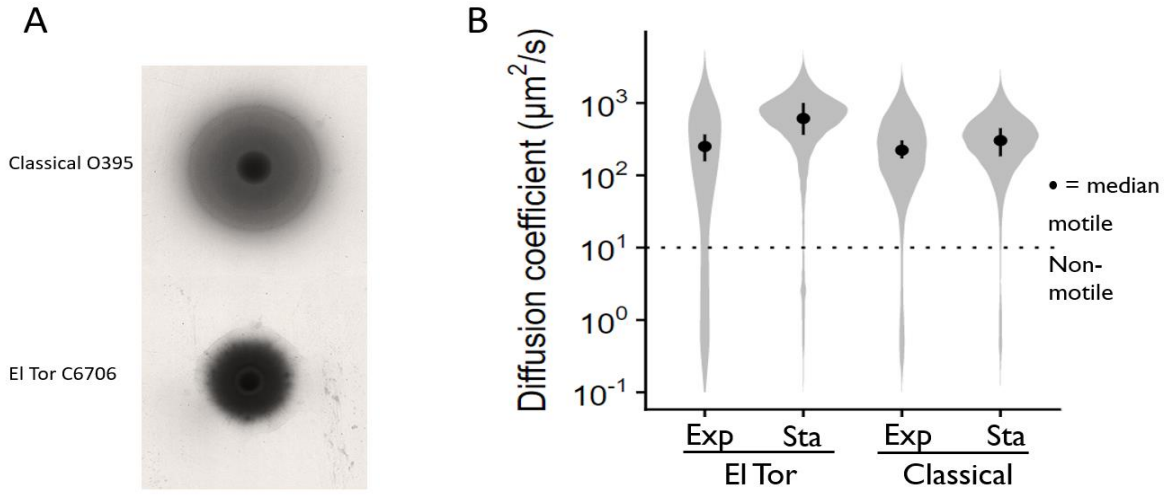

Figure 1. Clonal populations of *V. cholerae* are phenotypically heterogeneous, and proportions of different phenotypes depend on growth phases and biotype background. (A) Representative colonies from the Classical O395 and El Tor C6706 in M9 salts supplemented with pyruvate and 0.3% (wt/vol) agar. (B) Distributions of diffusion coefficient of Classical

O395 and El Tor C6706 from single-cell trajectories during exponential (Exp) and stationary (Sta) growth. (Circles: median of motile population, line: 95% credible interval)

Clonal populations of *V. cholerae* have a wide range of motility behaviors with its diffusion coefficients ranging from 10^{-1} to 10^{3} $\mu m^2/s$. The El Tor populations exhibited a higher degree of phenotypic diversity during the exponential phase and shifted to a more uniformly motile state toward the stationary phase. Interestingly, the classical biotype strain did not undergo such behavioral transition at different growth phases. The populations of classical strain appeared to gain higher diffusion coefficients toward the stationary phase but remained uniformly motile throughout its growth cycle.

El Tor *V. cholerae* motility phenotypes include a dense, localized colony on a swim plate assay (Fig 1A) and the down-regulation of motility during the exponential phase and resultant bimodal population distributions (Fig 1B). In order to identify the genes responsible for altered motility behaviors in El Tor strain, 20-25 kb random genomic fragments of the *V. cholerae* El Tor C6706 were inserted into a low copy number cosmid, pLAFR[21], to generate a genomic library. The resulting genomic library was conjugated into the classical strain and screened for El Tor-like reduced motility on the motility plate assay. Three of those library constructs screened positive for an altered motility phenotype independently hit a region of chromosome that contains three DGC genes. These three DGCs are VCA0956, VCA0960, and VCA0965 located on the second chromosome of El Tor strain. Interestingly, the genes encoding for these three DGCs are also found in the chromosome of Classical strain, suggesting that the difference in motility behaviors between two biotypes is a c-di-GMP-regulated process.

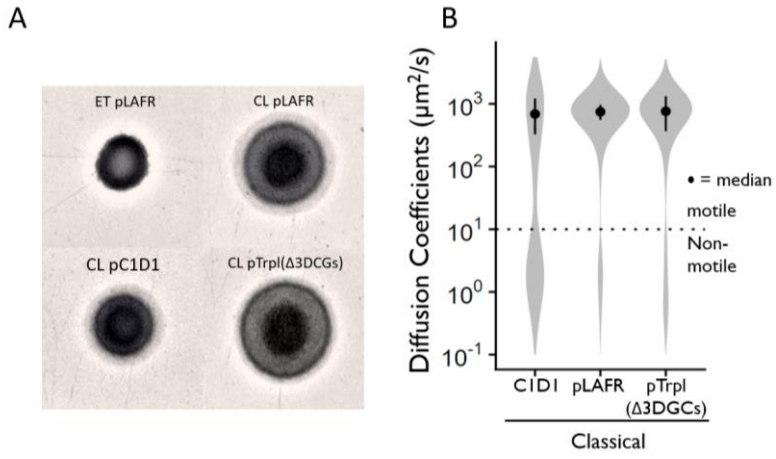

Figure 2. El Tor C6706 DGCs can change swimming phenotypes of Classical O395 *V. cholerae* strain. (A) Basal motility phenotype in El Tor C6706 (ET) or Classical O395 (CL) biotype background harboring the control cosmid (pLAFR) was used as a reference for phenotypic screen of El Tor genomic library (C1D1) and its three DGC-inactivated version (pTrpl) in soft agar. (B) Distribution of diffusion coefficients of Classical O395 containing the control cosmid (pLAFR), El Tor genomic library (C1D1), or C1D1 library with three DGCs functionally removed (pTrpl) from single-cell trajectories in the exponential phase. (Circles: median, line: 95% credible interval)

One of library constructs containing all three DGCs (VCA0956, VCA0960, and VCA0965), called the pC1D1 cosmid, inhibited motility of Classical strain on an agar-based motility plate (Fig 2A). Specific deletion of three DGCs in C1D1 insert, denoted as pTrpl (ΔVCA0956, ΔVCA0960, ΔVCA0965) cosmid, restored wild-type motility in Classical strain (Fig 2A), implicating these three DGCs for El Tor-specific swimming phenotype. Single-cell tracking study revealed that the Classical *V. cholerae* harboring C1D1 cosmid are phenotypically heterogeneous (Fig 2B), reflecting coexistence of non-motile and motile cells in El Tor *V. cholerae* (Fig 1B). The Classical strain expressing either pLAFR control cosmid or DGC-removed cosmid (pTrpl) showed uniformly motile populations (Fig 2B). Overall results of this investigation suggest the role of c-di-GMP in shaping biotype-specific behaviors.

Since the presence of three El Tor DGCs affects phenotypic diversity in different biotype strains of *V. cholerae,* we investigated whether manipulation of c-di-GMP concentration could shift phenotypic distribution to one way or the other. Induction of VC2224 DGC activity, one of strong DGCs to produce a high level of c-di-GMP in *V. cholerae*[22,23], in a flask batch culture did not shift population phenotypes to all non-motile phenotypes. Instead, clonal cultures maintained mixed populations of motile and non-motile cells during the growth cycle of El Tor or Classical strain. We found that DGC induction led to heterogeneous populations comprised of motile and non-motile cells in El Tor *V. cholerae* undergoing stationary growth (Fig 3A) during which phenotypes are predominated by motile cells. Likewise, DGC induction increased the proportion of non-motile cells in Classical *V. cholerae* that maintains uniform swimming motility during its entire growth cycle, leading to bimodal population distributions (Fig 3B). In contrast, induction of VC1086 PDE activity that degrades c-di-GMP, resulted in populations primarily composed of active swimmer cells. It is worth noting that El Tor *V. cholerae* during the exponential phase also shifted to the higher proportion of motile cells with PDE induction, which resembles phenotypic distributions of the stationary phase (Fig 3A). These results suggest that manipulation of c-di-GMP level is sufficient to shift population distribution.

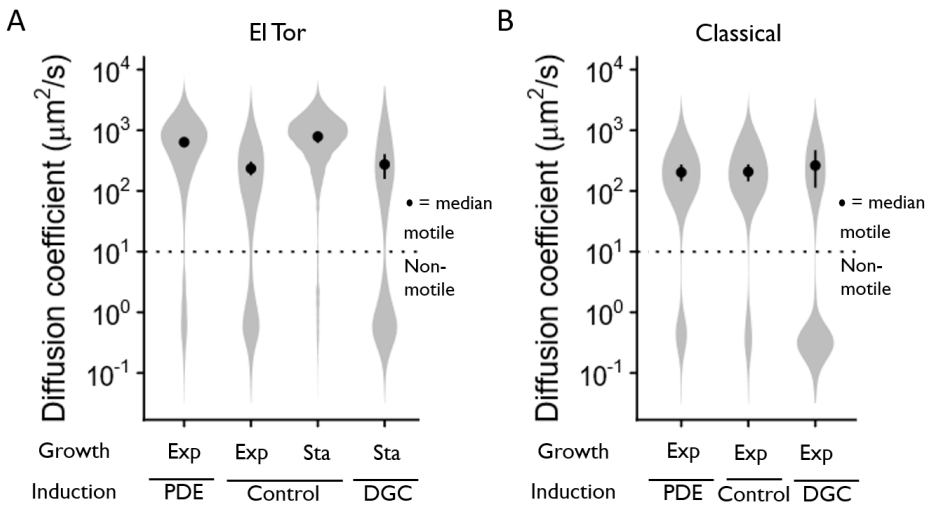

Figure 3. Swimming phenotypes can be controlled by the c-di-GMP turnover enzymes (DGC and PDE) in both El Tor C6706 and Classical O395 *V. cholerae*. (A) Distributions of diffusion coefficients of inducible DGC or PDE system, or control vector strain in El Tor C6706 background from single-cell trajectories in the exponential (Exp) or the stationary (Sta) phase. (B) Distributions of diffusion coefficients of inducible DGC or PDE system or control vector strain in Classical O395 background from single-cell trajectories in the exponential (Exp). (Circles: median, line: 95% credible interval)

The higher concentration of intracellular c-di-GMP has been correlated with motility suppression and biofilm production in *V. cholerae*[24]. In addition, our own cosmid screen to search for El Tor genomic content responsible for altered motility behaviors in El Tor *V. cholerae* showed that the DNA region containing three DGCs, and likely effect thereof, influences biotype-specific behaviors (Fig 2). This led us to a hypothesis that El Tor strain maintains a higher level of c-di-GMP than Classical strain, resulting in divergent motility phenotypes. Using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), we measured the c-di-GMP concentrations of El Tor and Classical strains harvested from either exponential or stationary growth phase. As shown in Figure 4, El Tor strain maintains a higher level of c-di-

GMP than Classical strain. However, the total c-di-GMP concentration showed no measurable change between growth phases.

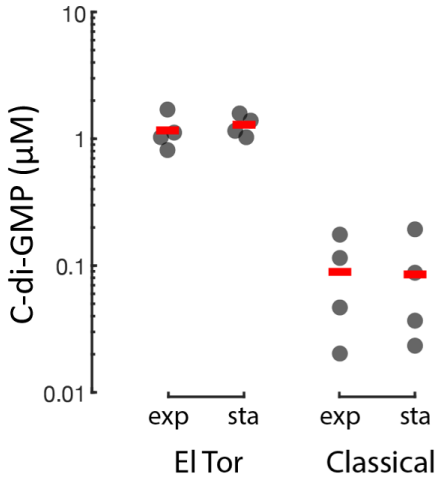

Figure 4. Effects of growth phases and biotype background on c-di-GMP concentrations in *V. cholerae* El Tor C6706 and Classical O395.

These results corroborate our findings that ectopic expression of either DGC or PDE to manipulate c-di-GMP concentrations can mimic biotype-specific behaviors (Fig 3). However, growth phase-dependent transition of phenotypes does not appear to be regulated by c-di-GMP. Recently, it has been shown that *V. cholerae* swims faster at alkaline pH (~pH 8) and tends to attach to the surface at acidic pH (~pH 6)[25]. However, there is no measurable effects of pH on the total c-di-GMP concentrations[25], suggesting there is an environmental cue not relayed through the c-di-GMP signaling.

Despite our study showed that different cytoplasmic levels of c-di-GMP accounts for biotype-specific motility behaviors, the mechanism by which c-di-GMP establishes distinct biotype

behaviors is still unknown. c-di-GMP is known to bind the transcription factor called FlrA that regulates flagellar synthesis in *V. cholerae*[24]. Binding of c-di-GMP to FlrA inhibits the binding of this transcription factor to the downstream promoter in flagellar synthesis cascade. Using scanning electron microscopy (SEM), we investigated flagellation pattern of El Tor and Classical strains during different growth phases (Fig 5A). Despite the higher proportion of non-motile cells in El Tor strain during exponential growth, both biotype strains appeared to have about 90% flagellated cells in their sampled populations regardless of their growth stages (Fig 5B). Single-cell tracking study of El Tor FlrA (R176H) mutant, that constitutively activates flagellar synthesis, showed that the mutant strain still retains its motility response to ectopic expression of DGC or PDE (Fig 5C). This study indicates that single-cell behavioral response to c-di-GMP is not likely to be attributed to flagellar synthesis.

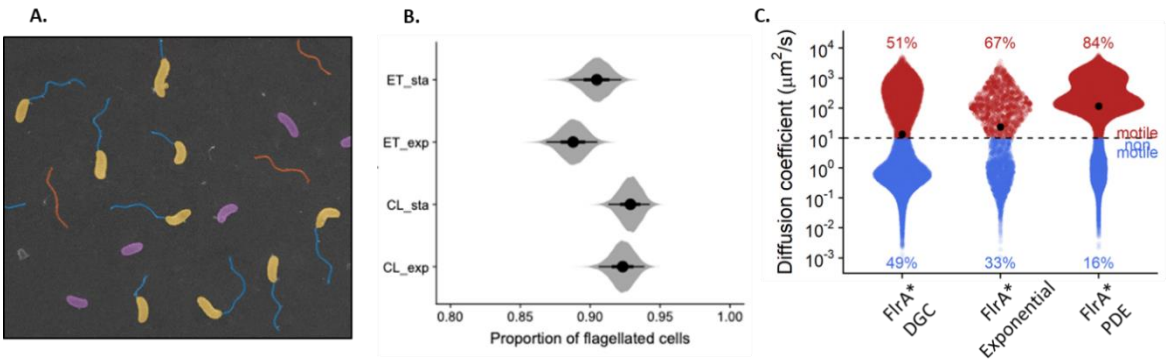

Figure 5. Effects of flagellar synthesis on phenotypic diversity in *V. cholerae* El Tor C6706 and Classical O395. (A) Representative SEM image with the detection of flagellated cell body (yellow), attached flagella (blue), non-flagellated cell body (purple), and broken flagella (red). (B) Distributions of flagellated cells in populations. (C) Distributions of diffusion coefficients of El Tor C6706 c-di-GMP blind mutant of FlrA (FlrA*) with induction of DGC or PDE, or without inducible vector system from single-cell trajectories in the exponential phase. (Circles: median, line: 95% credible interval)

The other way in which c-di-GMP affects motility behavior is through the regulation of MSHA pili synthesis. Binding of c-di-GMP to MshE, an ATPase protein, is required for pilin export and the production of MSHA pili on the cell surface[26]. Elevated c-di-GMP level was shown to enhance MSHA pilus production in El Tor *V. cholerae*, promoting cell attachment and biofilm formation[27]. However, Classical strain *V. cholerae* does not produce MSHA pilus, despite having the gene[28]. Thus, different motility behaviors in two pandemic strains could be attributable to difference in pili elaboration on the cell surface.

MSHA pili was previously thought to play a role in biofilm formation on an abiotic surface, whereas toxin co-regulated pili (TCP) appeared to contribute to bacterial attachment on chitin and the intestinal surface[29]. Single cell study of *tcpA*, the repeating subunit of TCP, showed that the population of *V. cholerae* bifurcates into two sub-populations during the late stage of rabbit ileal loop infection: *tcpA*-expressing and *tcpA*-non-expressing[30]. Bifurcation of *tcpA* expression was also shown to coincide with the entry into the stationary phase, and RpoS is required for heterogeneous expression of *tcpA*. RpoS is also implicated in mucosal escape response, whereby *V. cholerae* detaches from the intestinal surface and enters the luminal space to be shed back into the environment. Thus, coordination of TCP production and motility response plays a crucial role in perpetuating the infectious cycle. The role of MSHA in the infectious cycle, however, has been under appreciated. A recent study showed that MSHA genes are essential for colonization of the environmental predator, *Caenorhabditis elegans*[31]. This suggests that MSHA pili contribute to *V. cholerae*'s survival and persistence in its ecosystem.

With null mutations in *mshA*, the gene encoding the subunit of MSHA pili, El Tor strain exhibited the larger zone of spreading on motility plate (Fig 6A). El Tor *mshA* mutant strain harboring the control vector (pEVS141, See Plasmid Table in Supplementary Section) showed

higher proportion of motile cells (Fig 6B) compared to El Tor wild-type strain harboring the control plasmid (pEVS141). Consistent with its counterpart without the vector system (Fig 2B), El Tor wild-type strain sustained stable phenotypic heterogeneity with mixed motile and non-motile populations (Fig 6B) during its exponential growth. El Tor *mshA* mutant, however, appeared to have lost heterogeneous single-cell response during the exponential phase, exhibiting the population dominated by motile cells. IPTG-induced DGC (VC2224) partially restored phenotypic diversity in El Tor *mshA* population with increased proportion of non-motile cells, but to a lesser degree than that of the wild-type. However, the effect of MshA on swimming behaviors in Classical strain is less clear. Both wild-type and *mshA* mutant Classical strains demonstrated the similar size of zone spreading on motility plate assay (Fig 6A), as well as similar single-cell behaviors (Fig 6B). Production of c-di-GMP via DGC induction still generated non-motile populations in Classical *mshA* mutant strain, suggesting the role of other c-di-GMP effectors in control of phenotypic diversity. There are five proteins belonging to the family containing the PilZ domain (PlzA-E) in *V. cholerae*, and a subset of these proteins were shown to be involved in the regulation of motility[32]. Their involvement in c-di-GMP-mediated phenotypic switching may explain restored single-cell heterogeneity in El Tor and Classical *mshA* mutants by DGC induction.

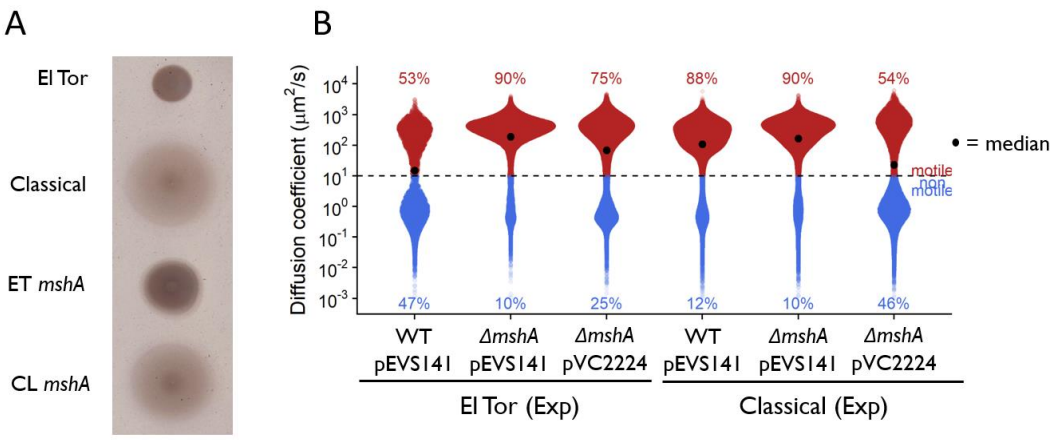

Figure 6. Effects of MSHA pili and c-di-GMP induction on the generation of non-motile cells in *V. cholerae* El Tor C6706 and Classical O395. (A) Representative colonies from El Tor C6706 and Classical O395 *V. cholerae* in wild-type or *mshA* mutant background. (B) Distributions of diffusion coefficients of wild-type (WT) and *ΔmshA* strains in El Tor C6706 or Classical O395 biotype with induction of DGC (pVC2224) or control plasmid (pEVS141) from single-cell trajectories during exponential (Exp) growth. (Circle: median)

2.3 Discussion

Taken together, our work demonstrated that single-cell motile behaviors in *V. cholerae* are phenotypically diverse. The switch between motile and non-motile behaviors is not a binary process but occurs in a gradual fashion with the proportion of phenotypes changing in response to the level of c-di-GMP. Two strains of *V. cholerae*, Classical and El Tor, showed different characteristics of population distribution, owing to different levels of intracellular c-di-GMP. El Tor *V. cholerae* transitions from a phenotypically diverse population with motile and non-motile cells during the exponential phase to a uniformly swimming population in the stationary phase. In contrast, Classical *V. cholerae* maintains lower physiological concentrations of c-di-GMP than El Tor strain and showed a uniformly swimming population through its growth cycle. El Tor *V.*

cholerae has higher physiological concentrations of c-di-GMP that provide bacterial populations with an opportunity to generate phenotypic diversity.

Synthesis and assembly of two motility appendages, flagellum and MSHA pili, are known to be inversely regulated by c-di-GMP. However, there is no measurable difference in the level of flagellation between El Tor and Classical *V. cholerae*. Most cells (roughly 90% or greater) in sampled populations remain flagellated. This may explain why a subpopulation of motile cells persists even under conditions that promote biofilm formation with induction of c-di-GMP synthesis. This finding may not be consistent with results of the consensus in the literature, but we used the minimal media in our experiments and the behavior of *V. cholerae* can be quiet different in rich media, which most other studies had used.

Our result also showed that MSHA pili contributes to generation of non-motile populations and dominates single-cell behavioral response in El Tor *V. cholerae*. Western blot of cell lysates revealed that both El Tor and Classical strains produce MshA protein (results not shown). Induction of DGC activity did not change overall production level of MshA protein in both strains. The difference in biotype-specific cell behaviors could be the result of c-di-GMP-dependent export of MshA onto the cell surface. Physiological concentrations of c-di-GMP in Classical *V. cholerae* may not be high enough to activate MshE ATPase, a protein responsible for MSHA pili extension[33]. We attempted to monitor the surface expression of MSHA pili using primary MshA antibody and secondary antibody conjugated to red fluorescent dye, but it was difficult to implement the experiment due to unspecific binding of primary MshA antibody (results not shown).

It may seem counterintuitive that *V. cholerae* cells do not get rid of flagella while assembling MSHA pili for surface attachment at elevated levels of c-di-GMP. Conflicting effects of flagellar

swimming and MSHA pili-mediated surface attachment may create a tug-of-war situation where cells can either swim away or transiently attach. The range of c-di-GMP concentrations that allows simultaneous elaboration of flagellum and MSHA pili could be a potential source of phenotypic heterogeneity.

We propose a model that c-di-GMP concentrations control phenotypic diversity in *V. cholerae* by cell surface appendages at different stages of its life cycle (Fig 7A). At low c-di-GMP, as in Classical *V. cholerae*, cells in a population uniformly swim with flagellar motility (Fig 7A, Stage 1). At slightly elevated level of c-di-GMP, as in El Tor *V. cholerae*, begins to assemble MSHA pili while retaining its flagellum (Fig 7A, Stage 2). Upon finding an abiotic surface, MSHA pili facilitate initial surface interactions that can be transient, allowing cells an opportunity either to detach or commit to attachment. This intermediary stage where cells can utilize both flagellum and MSHA pili may result in diverse motility phenotypes as in El Tor *V. cholerae*. It remains to be discovered what environmental cues regulate phenotypic switching in El Tor *V. cholerae* as it transitions from the exponential to stationary growth phase. Although the total concentration of c-di-GMP does not change during this growth phase-dependent phenotypic switching, expression of DGCs specifically involved in activation of MSHA pili, potentially three DGCs found in cosmid library screen, could be regulated by environmental cues associated with the growth phase (Fig 7B). Prolonged interactions with the surface was known to increase c-di-GMP levels in *V. cholerae* by an unknown mechanism[33] that promotes microcolony formation (Fig 7A, Stage 3) and biofilm maturation (Fig 7A, Stage 4) by secreting polysaccharide matrix. Flagellar break has been shown to elevate intracellular c-di-GMP levels as part of flagellum-dependent biofilm regulatory (FDBR) response[34]. Three specific DGCs (CdgA, CdgL, and CdgO) were found to be involved in this process[34], suggesting signaling specificity. Although it demonstrates how the

flagellar assembly status can contribute to biofilm development, proportions of flagellated and non-flagellated cells in biofilm remain to be characterized. Under our growth conditions, we were not able to culture *V. cholerae* to produce cell aggregates or mature biofilm in minimal media. Future experiments need to formulate growth conditions to culture the biofilm stage of *V. cholerae* in defined medium so that the regulation of phenotypic diversity can be studied with respect to flagellar motility, MSHA pili-mediated attachment, and biofilm formation (Fig 7C) in a holistic sense.

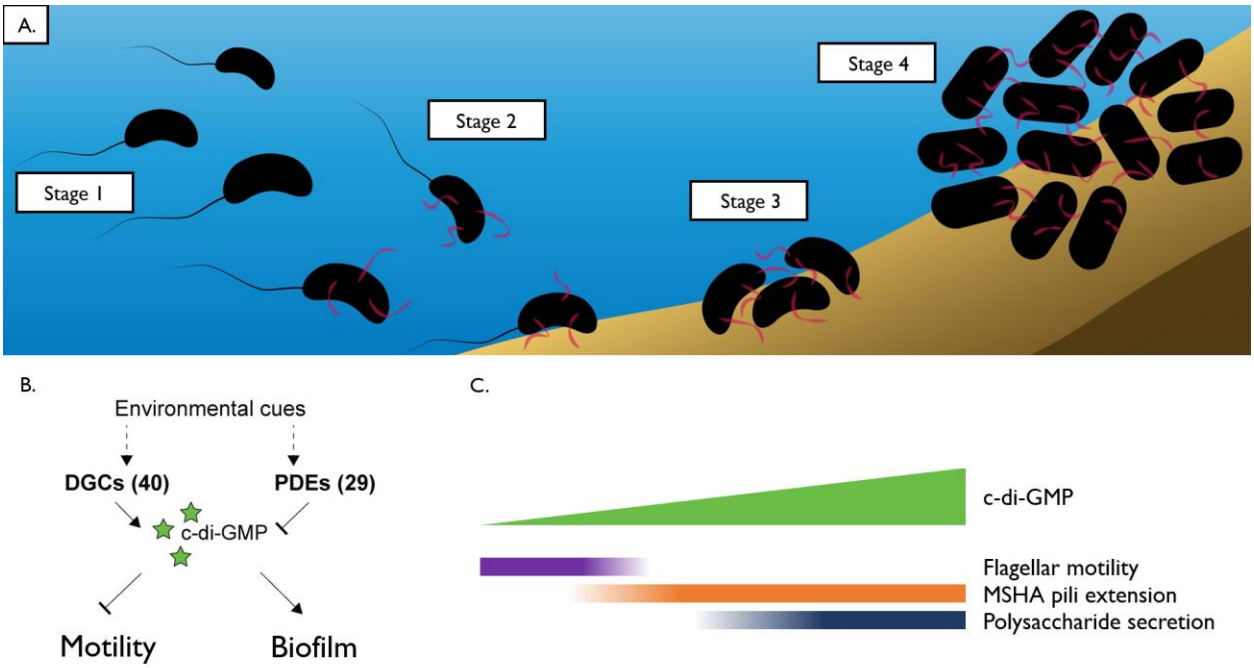

Figure 7. Proposed model of how c-di-GMP controls phenotypic diversity in *V. cholerae*. (A) *V. cholerae*'s developmental shift from a planktonic (swimming) state to a biofilm (sessile) state. (B) Control of DGC and PDE activity by environmental cues regulates the transition between motility and biofilm formation. (C) Expression of different phenotypic parameters, such as flagella and MSHA pili, may overlap in a well-sustained range of c-di-GMP concentrations and contribute to the generation of diverse motility phenotypes. Committed surface attachment

mediated by MSHA pili and continual increase in c-di-GMP levels promote polysaccharide secretion that encases sessile cell aggregates in biofilm matrix.

2.4 Materials and methods

Bacterial strains

V. cholerae strains used in this study were El Tor C6706str2 and Classical O395 biotypes. *V. cholerae* strains with inducible PDE or DGC were obtained by conjugating with *E. coli* S17 strains carrying either PDE (VC1086) or DGC (VC2224) from *V. cholerae* El Tor biotype under promoter-driven expression on a p15a plasmid derivative (gift from Dr. Christopher Waters).

To construct Δ*mshA* mutant strains in Classical and El Tor biotypes, 500 bp upstream and downstream of *mshA* gene (VC0409) were amplified by PCR (NEB). Primers to amplify the flanking regions of *mshA* gene were designed using NEB builder (www.nebuilder.com) (See Primer Table in Supplementary Section). The suicide vector pKAS32[35] was digested by SacI and KpnI and purified with gel extraction (Promega). The three-piece Gibson Assembly[36] was performed using upstream and downstream products and digested pKAS32 backbone. The assembled plasmid (See Plasmid Table in Supplementary Section) was transformed into *E. coli* BW29427 and moved into *V. cholerae* by conjugation. *V. cholerae* with the gene deletion was selected by LB streptomycin (2500 µg/mL) media, that selects for the recombinant clone with allelic exchange.

Growth conditions

M9 minimal salts (47.76 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.69 mM NH_4Cl, 8.55 mM NaCl) were supplemented with 2 mM $MgSO_4$, 100 µM $CaCl_2$, 29.65 µM thiamine hydrochloride, and 18.2 mM sodium pyruvate. *V. cholerae* cultures were grown at 32 °C, shaken at 200 rpm.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the growth medium at 20 µM concentration when indicated to induce DGC or PDE expression. Kanamycin was added to 50 µg/ml when needed. Soft-agar plates were prepared with the same medium with the addition of 0.3% (wt/vol) Bacto agar (BD). Plates were inoculated using an inoculating needle dipped into the bacterial suspension with the optical density value at 600 nm (OD_{600}) normalized to 1.

Intracellular c-di-GMP quantification

$2 * 10^8$ cells were sampled from El Tor C6706 and Classical O395 batch cultures during the exponential and stationary phases. Samples were collected on a polytetrafluoroethylene membrane (PTFE) filter (0.2 µm) disc pre-wet with methanol. Membranes were washed and submerged in extraction buffer (40%, vol/vol, acetonitrile, 40%, vol/vol, methanol, 0.1 N formic acid) for 30 minutes. The extraction solution containing a known amount of N^{15}-labeled c-di-GMP was used to normalize sample loss across samples during extraction. The soluble fractions were collected after centrifuging out cell debris and vacuum-dried overnight. The dried samples were re-suspended in 100 µl distilled water prior to identification and quantification using mass spectrometry (Quattro Premier XE mass spectrometer; Waters Corp.). c-di-GMP and N^{15}-labeled c-di-GMP were detected with electrospray ionization to produce a daughter ion at *m/z* 689.16 → 344.31[22] and *m/z* 699.16 → 349.31, respectively.

Single-cell tracking

V. cholerae cells in liquid medium were tracked using the protocol previously described[20]. *V. cholerae* cells in the mid-exponential and the early stationary phases were diluted to the OD_{600} value of 0.01 (calibrated to be $1.9 * 10^7$ cells/mL) in fresh medium. Polyvinylpyrrolidone (PVP) was added at 0.05% (wt/vol) to the samples to prevent cell attachment on a glass slide. To record

motile behavior, 5 µL of each sample was sealed between the glass slide and a coverslip (22 mm^2 number 1.5) using VALAP (equal weight of petrolatum, lanoline, and paraffin wax). Images of cell motion were recorded using an sCMOS camera (Andor Zyla 4.2; Oxford Instruments) at 20 frames per second with a 10X phase contrast objective (Plan Fluor 10X; Nikon Instruments, Inc.) mounted on an inverted microscope (Eclipse Ti-E; Nikon Instruments, Inc.). Image sequences were analyzed to identify detected cells using custom scripts[20]. Tracked motions of cells from frame to frame were processed using the µ-track package[37] to reconstruct cell trajectories. The cell trajectory statistics were then analyzed and plotted on MATLAB (The Mathworks, Inc.) as previously described[20].

Construction of cosmid library mutants

The cosmid library of El Tor genomic DNA in pLAFR[21] was constructed by the work of Severin et al.[38] The cosmid library was maintained in *E. coli* DH10B strain and transferred to *V. cholerae* Classical O395 strain in a tri-parental mating with *E. coli* S17 strain harboring pRK2013[39] helper plasmid. Cosmids resulting in El Tor-like reduced motility on the soft-agar motility plate were isolated and sequenced using Sanger sequencing. Three of eight sequenced cosmids contain the genomic region encompassing genes for three DGCs (VCA0956, VCA0960, and VCA0965). One such cosmid, pC1D1, (See Plasmid Table in Supplementary Section) was selected for further study.

Genetic replacement of each DGC gene from the cosmid (C1D1) insert was achieved by Lambda Red recombineering method[40]. Briefly, *E. coli* DH10B strain harboring pC1D1 was transformed with a temperature-sensitive plasmid, pKD46, that encodes for Lambda Red recombinase[41]. Transformants were cultured at 30 °C and induced with 1 M L-Arabinose to express the recombinase. Primers were designed with NEBuilder (www.nebuilder.com) to amplify the

chloramphenicol cassette from pKD3 plasmid with target sequences incorporating the 40-bp homologous regions of each DGC gene flanking the cassette (See Primer Table in Supplementary Section). DH10B cosmid strain was made competent to take up the PCR products amplified from pKD3 and allowed to undergo homologous recombination at 35 °C, at which pKD46 is cured from transformants. The pTL17 plasmid[42,43] that encodes flippase gene was transformed into recombinant cells to remove the chloramphenicol cassette. To form triple gene replacement (pTrpl), (See Plasmid Table in Supplementary Section) cells that had already undergone two gene removals were used to repeat the process. The final product, pTrpl, was mobilized into *V. cholerae* O395 with the aid of *E. coli* S17 strain carrying pRK2013 helper plasmid.

Motility screen and single-cell tracking of cosmid strains

V. cholerae strains harboring the cosmid of interest (pLAFR, pC1D1, or pTrpl) were inoculated into M9 medium (47.76 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.69 mM NH_4Cl, 8.55 mM NaCl) supplemented with 2 mM $MgSO_4$, 100 µM $CaCl_2$, 29.65 µM thiamine hydrochloride, 18.2 mM sodium pyruvate, and LB at a 5% concentration of the total volume. The cultures were grown overnight at 35 °C, shaken at 250 rpm. *V. cholerae* cells from overnight cultures were diluted 1:100 in fresh medium (LB supplemented M9 pyruvate) and allowed to grow to mid-exponential phase under the same incubation and shaking conditions. Single-cell tracking, reconstruction of cell trajectories, and analysis were done using the same protocol as previously described.

Scanning electron microscopy (SEM)

$2*10^8$ cells were sampled from El Tor C6706 and Classical O395 batch cultures during the exponential and stationary phases. Bacterial samples were fixed with an equal volume of 4%

glutaraldehyde (prepared in 0.1 M sodium phosphate buffer), diluting cell number to $1*10^8$ cells. Fixation was allowed to proceed for 1.5 hour at 4 °C. One drop of 1% Poly-L-Lysine (Sigma Aldrich P1399) was placed on a plastic petri dish, covered with 12-mm round glass coverslip, and allowed to stand for 5 minutes. After draining the coverslip, one drop of fixed cell sample was placed on the side of the coverslip previously faced down. The sample was allowed to settle for 5 minutes. The coverslip was gently washed with several drops of water and placed in a graded ethanol series (25%, 50%, 75%, 95%) for 5 minutes in each step and three times in 100% ethanol with 5 minutes each. The water in the cell sample was dried in a critical point dryer (EM CPD300, Leica Microsystems) using liquid CO_2 as the transitional fluid. Samples were mounted on aluminum stubs with epoxy adhesive (Quick Cure 5, System Three Resins, Inc.) and dried overnight. Samples were coated with osmium in an osmium coater (NEOC-AT, Meiwafosis Co., Ltd.) and examined in a JEOL scanning electron microscope (JSM-7500F, JEOL Ltd.) at Center for Advanced Microscopy at MSU.

APPENDIX

Strains	Name in this study	Relevant characteristics	Source or reference
E. coli			
BW29427		*RP4-2(TetSkan1360::FRT), thrB1004, lacZ58*(M15), *ΔdapA1341::[erm pir^{+}], rpsL*(strR), *thi-, hsdS-, pro-*	Lab Stock
DH10B		Background for recombineering; donor for conjugation	Lab Stock
S17 λpir		Host of pEVS141, pVC2224, pCMW121, or helper plasmid (pRK2013)	Lab Stock
V. cholerae			
El Tor C6706str2	El Tor *V. cholerae*	Wild-type	Lab stock
Classical O395	Classical V. *cholerae*	Wild-type	Lab stock
El Tor FlrA (R176H)	El Tor FlrA (R176H)	c-di-GMP blind mutant of FlrA	Lab stock
BYH103	Classical *mshA*	O395 Classical Δ*mshA*	This study
BYH174	El Tor *mshA*	O1 El Tor Δ*mshA*	This study

Table 1. List of bacterial strains used in this work.

Plasmids	Name in this study	Relevant characteristics	Source or reference
pEVS141	Control plasmid	pEVS143 without pTac; Km^r	[44]
pVC2224	DGC plasmid	DGC(VC2224)-inducible under pTac promoter (in pEVS143); Km^r	[22]
pCMW121	PDE plasmid	PDE(VC1086)-inducible under pTac promoter (in pEVS143); km^r	[16]
pKAS32	Suicide vector	Suicide vector for mutant construction, Amp^r	[35]
pBYH32		Deletion construct for Δ*mshA* in pKAS32	This study
pLAFR	pLAFR	Cosmid backbone of El Tor genome insert; Tet^r	[21]
pAMB001	pC1D1	pLAFR backbone with C1D1 cosmid insert sequence; Tet^r	This study
pAMB025	pTrpl	pLAFR backbone with C1D1 cosmid insert sequence, ΔVC0956, ΔVC0960, ΔVC0965; Tet^r	This study
pKD46		Lambda Red genes under the araBAD promoter; temperature-sensitive (maintained < 32 °C); Amp^r	[41]
pKD3		Plasmid with chloramphenicol resistance cassette flanked by FRT sites; Cm^r	[41]
pTL17		Flippase gene under pTac promoter; facilitates recombination between FRT sites; Km^r	[42,43]
pRK2013		Helper plasmid for the transfer of cosmid by conjugation; km^r	[39]

Table 2. List of plasmids used in this work.

Name	Primer use	Sequence (5'→3')	Reference
CMW3350	Δ*mshA* up[1] F; BYH103 & BYH174	GTGGAATTCCCGGGAGAGCTTTTGC CCCGTTTTCTTGATG	This study
CMW3351	Δ*mshA* up[1] R; BYH103 & BYH174	CATATTTAAACTCTCTTTCATGTGAA TACGC	This study
CMW3352	Δ*mshA* down[2] F; BYH103 & BYH174	TGAAAGAGAGTTTAAATATGGCTCG TGC	This study
CMW3353	Δ*mshA* down[2] R; BYH103 & BYH174	AGCTATAGTTCTAGAGGTACGTATT GATGCAGACTTGG	This study
GG27 F	VCA0956 upstream homology; pKD3	GCAGCGTTTCTACAGTAAATCCGCT AATCTTAAGGTTATGGTGTAGGCTG GAGCTGCTTC[3]	This study
GG27 R	VCA0956 downstream homology; pKD3	GTGCCGTCGATTTACAACGTGTGGG TACGTATAGCAGAGTCATATGAATA TCCTCCTTA[4]	This study
GG28 F	VCA0960 upstream homology; pKD3	GCAAATTGCAAAATCAAAATTTGCA AAATGCAAAGGCGGTGTAGGCTGGA GCTGCTTC[3]	This study
GG28 R	VCA0960 downstream homology; pKD3	GCGAATGGATCTGTGTGGTTCACTC AGTAATCAACAAACACATATGAATA TCCTCCTTA[4]	This study
GG29 F	VCA0965 upstream homology; pKD3	CGCCTTCAAACAAGCCAATATCATC TAAACTTTAGCTAACGTGTAGGCTG GAGCTGCTTC[3]	This study
GG29 R	VCA0965 downstream homology; pKD3	GGCGCAGTATGTTTATGGCTGATGT ACATGTTTGGATGAGCCATATGAAT ATCCTCCTTA[4]	This study

Table 3. List of oligonucleotides used in this work. [1]Up= Amplifies Upstream Fragment. [2]Down= Amplifies Downstream Fragment. [3]GTGTAGGCTGGAGCTGCTTC = pKD3 chloramphenicol cassette (upstream) + FRT site. [4]CATATGAATATCCTCCTTA = pKD3 chloramphenicol cassette (downstream) + FRT site.

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CHAPTER 3

CONCLUDING REMARKS

3.1 Conclusions and significance of this work

Studies discussed in this thesis shed light on the role of c-di-GMP in regulation of phenotypic diversity in *Vibrio cholerae*. Phenotypic switching between motile and sessile lifestyles in *V. cholerae* batch culture was shown to involve diverse single-cell phenotypes with a wide range of diffusive behaviors. Our results also showed that c-di-GMP controls distribution of different motility phenotypes in a clonal population. This demonstrates that clonal cells do not respond to chemical signaling or environmental cues in a homogenous way. Both flagellar motility and biofilm-like sessile aggregates are implicated in virulence of *V. cholerae*, and the spatial and temporal expression of these two phenotypes during infection remain unresolved. Thus, understanding molecular mechanisms that generate and control phenotypic diversity in clonal populations is expected to facilitate the development of therapeutic strategies to treat cholera.

This thesis began to probe the mechanisms by which c-di-GMP generates and controls phenotypic diversity in *V. cholerae*. I characterized the response to direct manipulation of c-di-GMP concentrations and the contribution of several c-di-GMP-binding proteins to the control of phenotypic diversity. Consistent with population-average studies, elevated c-di-GMP increased proportion of non-motile cells in a population, but do not abolish the generation of motile cells. My results also indicate that the transcription factors regulating biofilm formation, VpsR and VpsT, do not have a direct effect on the generation of motile cells (Fig 8). The c-di-GMP blind mutant of FlrA, which constitutively activates flagellar synthesis, still exhibited down-regulation of motility at elevated c-di-GMP level (Fig 5). These results suggest that the regulation of phenotypic diversity does not depend on transcription factors for biofilm formation and flagellar synthesis.

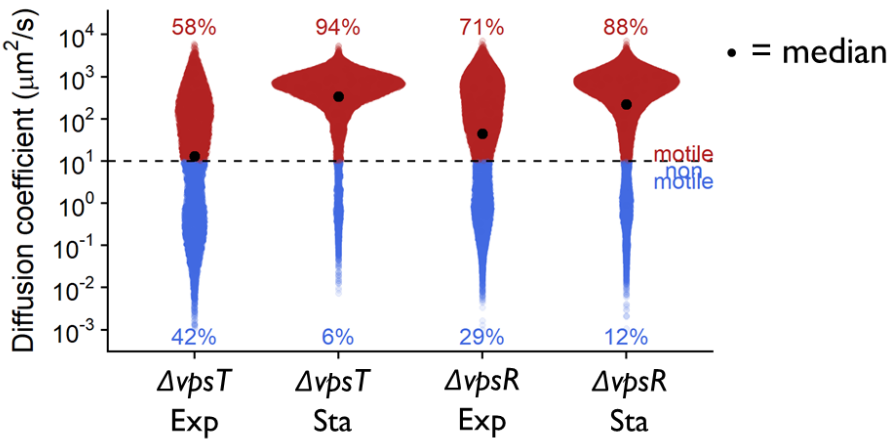

Figure 8. VpsT and VpsR, two transcription factors known to bind c-di-GMP and induce biofilm formation, do not affect the overall control of phenotypic switching in El Tor C6706 strain. Distributions of diffusion coefficients of El Tor *ΔvpsT* strain and El Tor *ΔvpsR* strain from single-cell trajectories in the exponential (Exp) or stationary (Sta) phase. Circles indicate median for the motile populations.

Phenotypic distribution depends on the biotype background and the growth phase in batch cultures. Two strains of *V. cholerae*, namely El Tor and Classical biotypes, show different proportions of motile cells because of different intracellular c-di-GMP levels in these strains. However, the total c-di-GMP concentration does not change between the growth phases, suggesting the presence of alternative signals that primes a shift in phenotypic distribution. Recently published work by Nhu et al. highlights pH as an important factor affecting motility behaviors of *V. cholerae*. Between physiologically relevant pH 6-8, acidic pH promotes surface attachment and alkaline pH promotes swimming without having measurable change in the total c-di-GMP concentration[1]. Thus, pH could be one of environmental cues during the growth cycle not relayed through c-di-GMP signaling that can affect phenotypic diversity. Interestingly, direct manipulation of c-di-GMP was shown to override growth phase-mediated phenotypic

distribution, showing that c-di-GMP is epistatic to growth phase-dependent signal(s) in the control of phenotypic diversity in *V. cholerae*.

I discovered that most cells remain flagellated regardless of the biotype background and the growth phase. This result may account for the fact that *V. cholerae* generates a subpopulation of motile cells at elevated c-di-GMP level that represses motility. On the other hand, the loss of MSHA pili abrogated the generation of non-motile cells in El Tor *V. cholerae* during exponential growth. The effect of MSHA pili was not seen in Classical *V. cholerae,* as this strain has been previously reported not to assemble MSHA pili[2]. Consistent with this report, the work by Nhu et al. also showed that acidic pH promotes surface attachment by activating MSHA pili in El Tor *V. cholerae,* but such attachment behavior was not seen in Classical *V. cholerae* at acidic pH[1].

c-di-GMP regulates MSHA pilus extension and retraction through the regulation of MshE ATPase activity[3]. Activation of MshE by c-di-GMP binding promotes pilus extension. Reduced intracellular c-di-GMP enhances MSHA retraction, the process facilitated by the depolymerization ATPase known as PilT. Western blot study of cell lysates showed that Classical *V. cholerae* strain synthesizes the major pilin subunit protein, MshA (data not shown). However, MSHA extension needs be powered by c-di-GMP-dependent ATPase, MshE. It is possible that Classical *V. cholerae* maintains physiological concentrations of c-di-GMP sufficiently low not to activate MshE function. This could explain why such behavioral difference exists between El Tor and Classical strains. A constitutively active, c-di-GMP non-responsive variant of MshE has been reported to have a simultaneous increase in both MSHA pilus extension and retraction rates. Floyd et al. attributes this phenotype to an altered conformational or functional state of MshE. Both elevated c-di-GMP and acidic pH activate

MSHA pili, and thus, the effect of c-di-GMP-binding and pH on the conformational state of MshE needs to be deciphered to better understand MSHA pili extension and retraction dynamics.

Induction of ectopic DGC expression, however, restored the generation of non-motile cells in *ΔmshA* mutant both in El Tor and Classical biotypes, suggesting other c-di-GMP-binding effectors may play a role in the control of phenotypic diversity. I examined the effect of additional proteins containing c-di-GMP-binding domains from the PilZ family on the distribution of phenotypes. *V. cholerae* encodes five PilZ domain-containing proteins (PlzA – PlzE); among them only PlzC and PlzD have been shown to bind c-di-GMP *in vitro*[4]. El Tor *V. cholerae* quintuple mutant (*ΔplzABCDE,* or *ΔplzA-E* in short) not only failed to transition to a uniformly swimming population in the stationary phase, but also lost its motility response at low c-di-GMP upon PDE induction (Fig 9A). Other notable characteristic of this mutant was much reduced swimming speed (Fig 9B). The function of these Plz proteins remains largely uncharacterized, but this preliminary result shows their possible roles in controlling phenotypic diversity.

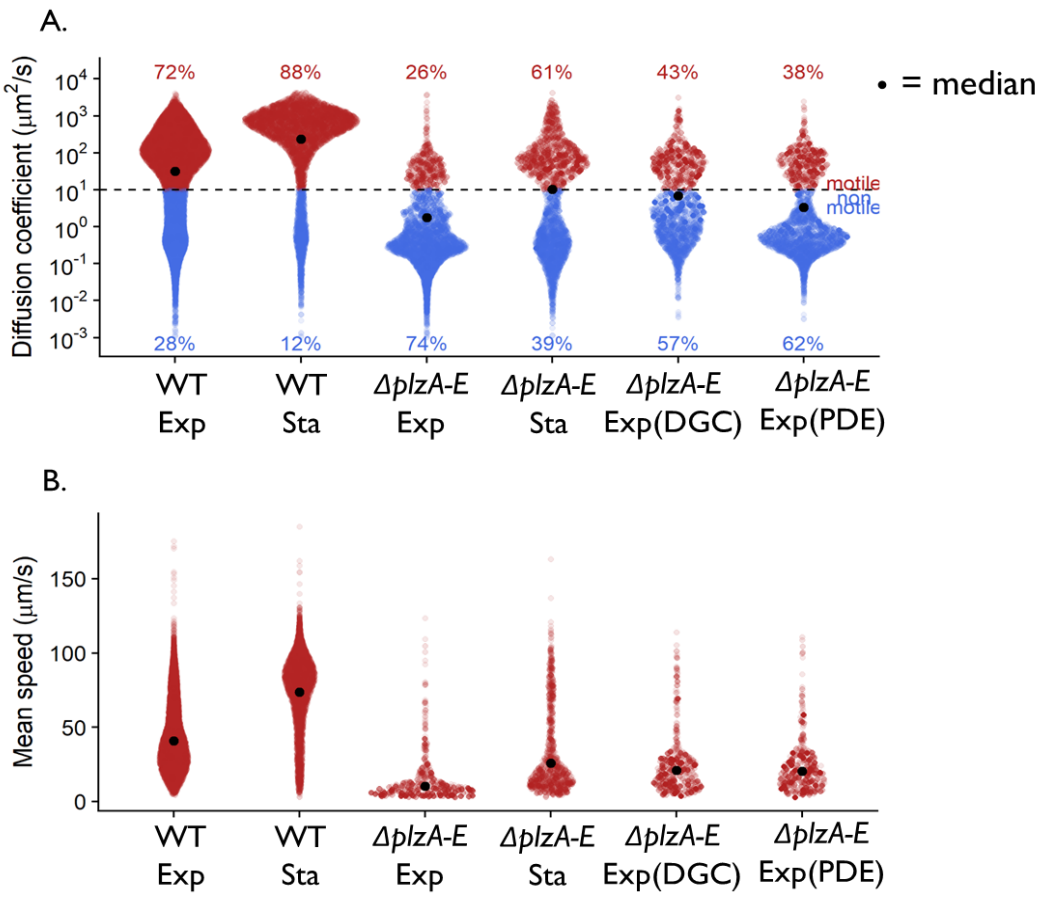

Figure 9. The PilZ family proteins in C6706 El Tor *V. cholerae* regulate swimming speed and phenotypic switching in response to the growth phase and c-di-GMP. (A) Distributions of diffusion coefficients of El Tor wild-type, *ΔplzABCDE*, and inducible DGC or PDE system in *ΔplzABCDE* background from single-cell trajectories in the exponential (Exp) or the stationary (Sta) phase. (B) Distributions of swimming speed from the motile population. Circles indicate median for the motile populations.

To dissect the effect of each Plz protein on phenotypic diversity, I performed the single-cell tracking experiment with single deletion mutant strain for each *plz* gene. I found that the loss of PlzB alone is sufficient to cause reduced swimming speed in El Tor *V. cholerae*, although population of *ΔplzB* mutant is still capable of phenotypic switching in a growth phase-dependent manner (Fig 10A). There seems to be no direct effect of single gene deletion for *plzC* and *plzD*

on phenotypic switching through the growth cycle (Fig 10B). A mutant strain lacking either PlzA or PlzE did not show distinct phenotype (data not shown). The control of phenotypic diversity may involve several branches of the c-di-GMP network that are functionally redundant, and single-gene perturbations in one pathway may not produce measurable effect on phenotypic diversity. Further studies need to be conducted to investigate the combined effect of multiple c-di-GMP effectors and pathways on the regulation of phenotypic diversity.

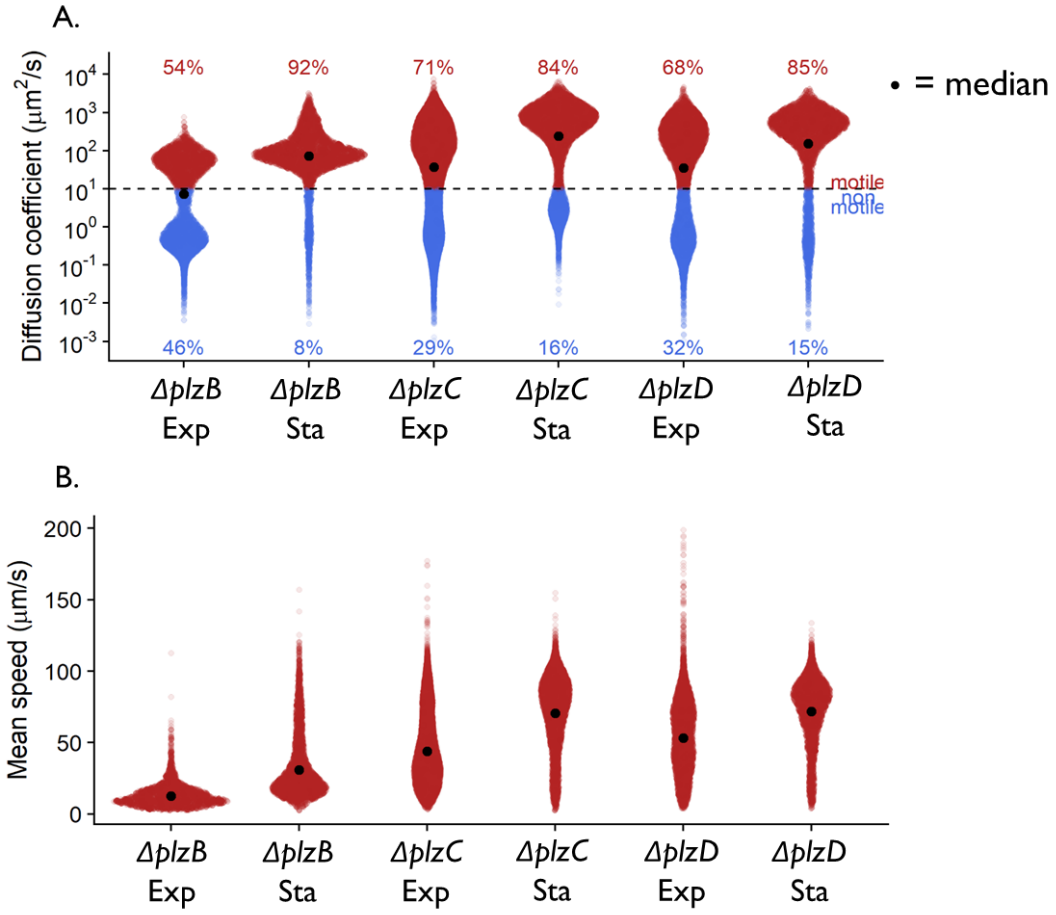

Figure 10. PlzB is required to achieve the full swimming speed in C6706 El Tor *V. cholerae*. The absence of either one of c-di-GMP-binding PilZ proteins (PlzC and PlzD) alone does not affect phenotypic switching primed by growth phase-dependent cues. (A) Distributions of diffusion coefficients of El Tor *ΔplzB*, *ΔplzC*, and *ΔplzD* strains from single-cell trajectories in

the exponential (Exp) or the stationary (Sta) phase. Circles indicate median for the motile populations.

3.2 Future perspectives

The following studies described below are proposed to achieve greater insights into the control of phenotypic diversity in *V. cholerae*.

1. Direct visualization of flagella and MSHA pili using fluorescent labeling

The role of MSHA pili in the control of phenotypic diversity was studied using a reverse genetics approach to characterize single-cell behaviors of *ΔmshA* mutant in El Tor and Classical *V. cholerae*. The flagellated cells in populations were imaged via SEM at 1,600X to sample a large number of cells in a field of view, but this magnification did not allow visualization of MSHA pili on the cell surface. Visualization of MSHA pili can be challenging without specific labeling, because *V. cholerae* is known to have two additional pili, namely TCP and competence pili. I attempted to label MSHA pili with a rabbit anti-MshA antibody to be detected by secondary antibody conjugated to a fluorescent compound, but nonspecific binding of primary antibody caused labeling of whole cell populations (data not shown).

Alternatively, thiol-reactive maleimide dyes have been used to fluorescently label MSHA pili of *V. cholerae* with the pilin subunit, MshA, bearing a single cysteine substitution at amino acid residue 70 (T70C)[3,5]. This method was also successfully applied to visualize fluorescently labeled competence pili[5,6]. I propose to combine this method with NanoOrange staining of flagellar sheath[7]. A polar flagellum of *V. cholerae* is sheathed with what appears to be an

extension of the outer membrane[8]. The membranous sheath wrapping the inner flagellum is rich in hydrophobic contents, which can be stained using lipophilic dyes such as NanoOrange reagent. Unbound reagent is nonfluorescent, but its binding to hydrophobic domains enhances fluorescence[9]. Fluorescent labeling with maleimide dyes or with NanoOrange is a rapid staining method (< 30 minutes) that can be applied to live cells and can be imaged using epifluorescence microscopy. In this case, the maleimide conjugate of choice needs to have a fluorescence label that can be distinguished from NanoOrange using different fluorescence channels available (CFP filter, YFP filter, and mCherry filter).

Simultaneous staining of flagellar sheath and MSHA pili in El Tor and Classical *V. cholerae* will allow proportions of flagellated and piliated cells to be quantified in the same population samples. This experiment can validate my current hypothesis about the source of phenotypic diversity attributable to simultaneous elaboration of flagella and MSHA pili. Or proven otherwise, it can serve as experimental data to guide future experiments.

2. Effect of three putative DGCs (VCA0956, VCA0960, and VCA0965) on Classical *V. cholerae*'s phenotypes

El Tor genomic region containing three putative DGCs (VCA0956, VCA0960, and VCA0965) was independently isolated from multiple cosmid-harboring Classical *V. cholerae* strains with altered motility phenotype resembling that of El Tor *V. cholerae* (Bedore, unpublished data). This chromosomal region is highly conserved between Classical and El Tor biotypes, sharing 99.98% sequence identity (Bedore, unpublished data). The activity of the three El Tor DGCs was shown to raise the c-di-GMP concentration in Classical *V. cholerae* comparable to that of El Tor *V. cholerae* (Bedore, unpublished data). However, *V. cholerae* encodes 40 DGCs that can

contribute to a global pool of c-di-GMP. It is currently unknown whether these three specific DGCs are required for biotype-dependent behavior and distribution of phenotypes. To characterize the role of three DGCs, the native genes for these three DGCs in Classical *V. cholerae* need to be removed and replaced with the DGC genes from El Tor *V. cholerae*, ensuring El Tor DGC genes of interest are present in single copy in the chromosome of Classical *V. cholerae*, rather than on a cosmid. Newly engineered Classical *V. cholerae* strains will allow more accurate characterization of each DGC's contribution to phenotypic diversity.

Previous studies showed that overexpression of VCA0956 DGC results in increased expression of the genes in Msh operon in both El Tor and Classical *V. cholerae* using transcriptomic analysis[10]. Overexpression system of VCA0956 was also used to demonstrate direct correlation between c-di-GMP production and MSHA pilus assembly on the cell surface of El Tor *V. cholerae*[11]. VCA0965 DGC was thought to be an inactive DGC, because it contains a degenerate GGDEF motif (AGDEF). The work by Hunter et al. showed that VCA0965 DGC is capable of c-di-GMP synthesis, and this functionality is linked to its ability to repress motility in *V. cholerae*[12]. Single-cell tracking results showed that single gene replacement of VCA0965 in C1D1 cosmid is sufficient to restore motility in Classical *V. cholerae* (data not shown), suggesting it has the greatest effect on motility inhibition among the three El Tor DGCs.

The concentration of c-di-GMP can be measured in Classical *V. cholerae* strain having its native DGCs (VCA0956, VCA0960, and VCA0965) chromosomally replaced with El Tor DGCs to compare it to the level of c-di-GMP in El Tor *V. cholerae*. It is worth noting that strong induction of VC2224 (DGC overexpression system used throughout this study) impairs the growth of Classical *V. cholerae* in minimal medium, and thus, inducer concentration needs to be adjusted accordingly. This indicates Classical *V. cholerae* strain has the optimal range of c-di-GMP

concentrations that can be physiologically tolerated. The activity of El Tor VCA0956, VCA0960, and VCA0965 DGCs may raise the levels of c-di-GMP within the physiologically tolerated range to activate MSHA pili on the cell surface of Classical *V. cholerae*. Using the fluorescent labeling method described above, proportions of piliated and flagellated cells can be quantified to test this hypothesis. It would be also interesting to know why high concentration of c-di-GMP causes a growth defect of Classical *V. cholerae* in minimal medium.

3. The role of PlzB on the regulation of motility

Previous studies on the regulation of motility by PilZ domain protein primarily focused on YcgR type of PilZ proteins. YcgR type of PilZ proteins refer to two-domain proteins consisting of PilZ-related N-terminal domain (also called YcgR domain) fused to the C-terminal canonical PilZ domain that contains c-di-GMP-binding motifs (RxxR and [D/N]xSxxG). Their regulation of motility in response to c-di-GMP have been shown in *E. coli*[13,14], *S. enterica*[14], *B. subtilis*[15], to name a few. YcgR and its homologs bind to c-di-GMP to inhibit flagellar function by directly interacting with the flagellar motor.

However, structures of PilZ domain proteins are diverse, and as such, they also include atypical PilZ domain structures that share the same canonical PilZ structure with the exception of an ~60 amino acids insertion in the middle that results in two additional α-helices. One of these α-helices promotes stable tetramer formation, and thus, this group of PilZ domain proteins is also known as tetrameric PilZ (tPilZ) family[16]. Some tPilZ proteins retain their c-di-GMP-binding ability, whereas some have lost this ability.

Single-cell tracking data showed that PlzB, a tPilZ protein with non-canonical PilZ domain, is required for *V. cholerae* to achieve the full swimming speed. This phenotype is consistent with the reduction of colony size observed on swim plate assay. Although single-cell phenotypes of *ΔplzB* strain was characterized only in El Tor strain, the loss of PlzB was shown to inhibit swimming in Classical *V. cholerae* as well[4]. However, the study of PlzB has been largely overlooked owing to its inability to bind c-di-GMP and a lack of recognition of tPilZ domains as a subfamily of PilZ. The mechanism by which PlzB regulates motility may be independent of c-di-GMP. Further studies need to be done to explore the effect of PlzB on other phenotypes such as gross cell morphology and flagellar positioning that may affect the swimming speed. El Tor and Classical *V. cholerae* can be engineered to incorporate a vector system with inducible PlzB expression. Single deletion mutant for *plzB* (*ΔplzB*), wild-type, and PlzB-overexpressing strains in El Tor and Classical strains can be characterized with respect to their cell shape and flagellar position and number. Such efforts will lead us to a better understanding of how other cellular phenotypes regulated independently of c-di-GMP may contribute to the generation and control of diverse motility phenotypes.

4. Combined effect of multiple c-di-GMP effectors and pathways on phenotypic diversity

PlzD in *V. cholerae* is a homolog of *E. coli* YcgR, but it showed no direct effect on the distribution of phenotypes under growth conditions to prepare samples for single-cell tracking. It is possible that the wild-type *V. cholerae* do not express PlzD under my experimental conditions, causing the *ΔplzD* strain phenocopy the wild-type. To circumvent this issue, the effect of PlzD also needs to be studied using inducible PlzD expression. Overexpression of PlzD in minimal

media was shown to inhibit motility in *V. alginolyticus* by aggregating at the flagellated cell pole, but the study did not definitively show whether this effect is mediated by c-di-GMP[17].

To investigate the interplay between PlzD and c-di-GMP, El Tor and Classical *V. cholerae* strains can be engineered to have two compatible plasmid systems: one driving inducible expression of *plzD* fused with *gfp* (*plzD-gfp*) and the other driving inducible expression of DGC. PlzD protein can be fused with GFP at the C-terminus using the method described by Kojima et al. to construct C-terminal GFP fusion PlzD construct (PlzD-GFP) in *V. alginolyticus*[17]. The study also showed that PlzD-GFP retains the inhibitory effect on motility when overexpressed in minimal media. The future experiments need to assess whether induced PlzD or PlzD-GFP expression shows a similar motility inhibition in *V. cholerae*. Once the efficacy of PlzD-GFP is validated, then the following experiment can probe the localization of PlzD-GFP in response to c-di-GMP by using inducible DGC system.

The quintuple knock-out mutant for PilZ proteins (*ΔplzABCDE*) was shown to be non-responsive to growth phase-dependent and c-di-GMP-regulated phenotypic transition. Thus, it is important to characterize the combined effects of different PilZ proteins, such as PlzB and PlzD, to the overall control of phenotypic diversity. In addition to PilZ proteins, the transcription factor called TfoY was shown to trigger dispersive motility in *V. cholerae*. Expression of *tfoY* is partially regulated by c-di-GMP-dependent riboswitch Vc2. Upon binding to c-di-GMP, Vc2 functions as an off switch to inhibit TfoY production. The study by Pursley et al. showed that overexpression of TfoY promotes motility in *V. cholerae*, but motility was still induced at low c-di-GMP in *ΔtfoY* knock-out background[18]. This result demonstrates that no single branch of c-di-GMP network will likely account for the generation of diverse motility phenotypes. Future studies

need to be conducted to characterize the combined effect of multiple effectors and pathways on phenotypic diversity including, but not limited to, PilZ proteins and TfoY.

5. Perspective summary

Results discussed in this thesis conclude that both c-di-GMP-dependent and independent effectors can contribute to phenotypic diversity. The difference in levels of c-di-GMP between El Tor and Classical *V. cholerae* contributes to different levels of phenotypic diversity observed in two biotype strains. My experimental data showed that biotype-dependent phenotypic distributions are most likely attributable to differential MSHA pili activation, although it is not clear how El Tor *V. cholerae* transitions from a phenotypically diverse population in the exponential phase to a uniformly swimming population in the stationary phase without showing measurable change in c-di-GMP levels. More studies need to be done to characterize environmental cues during transition between the exponential and stationary growth phases that mediate phenotypic switching.

Three DGCs (VCA0956, VCA0960, and VCA0965) from El Tor *V. cholerae* are sufficient to alter swimming behaviors of Classical *V. cholerae* to mimic El Tor-like phenotypic distributions. Whether these three El Tor DGCs are specifically required for MSHA pili activation remains to be tested. The mechanism of how acidic pH activates MSHA pili and promotes surface attachment behaviors in El Tor *V. cholerae* is still unknown. The total c-di-GMP concentration does not change between pH 6 and 8, where *V. cholerae* exhibits the increased swimming speed from pH 6 to 8[1]. Since MSHA pili assembly is powered by c-di-GMP-dependent ATPase MshE[3,11], its functional or conformational state may contribute to pilus extension and retraction dynamics[3]. The change of pH during the growth cycle and available c-di-GMP that can bind to

MshE may affect the conformational state of this ATPase to either inhibit or promote MSHA pili assembly, a potential interconnection between growth phase-dependent cues and c-di-GMP signaling.

MSHA pili facilitates initial surface attachment. Prolonged interactions with the surface promote c-di-GMP synthesis by an unknown mechanism and lead to biofilm maturation[3]. Although *V. cholerae* cultures under my experimental conditions in minimal media did not form biofilm, other c-di-GMP effectors such as PilZ proteins may be involved in the regulation of motility during later stages of biofilm development. Growth conditions that allows biofilm formation need to be defined in future work to investigate the effect of c-di-GMP effectors on motility inhibition and biofilm formation.

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