Published in final edited form as:

Environ Microbiol. 2015 November ; 17(11): 4290–4305. doi:10.1111/1462-2920.12799.

Temperature affects c-di-GMP signaling and biofilm formation in *Vibrio cholerae*

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SUMMARY

Biofilm formation is crucial to the environmental survival and transmission of *Vibrio cholerae*, the facultative human pathogen responsible for the disease cholera. During its infectious cycle *V. cholerae* experiences fluctuations in temperature within the aquatic environment and during the transition between human host and aquatic reservoirs. In this study, we report that biofilm formation is induced at low temperatures through increased levels of the signaling molecule, cyclic diguanylate (c-di-GMP). Strains harboring in-frame deletions of all *V. cholerae* genes that are predicted to encode diguanylate cyclases (DGCs) or phosphodiesterases (PDEs) were screened for their involvement in low-temperature-induced biofilm formation and *Vibrio* polysaccharide (VPS) gene expression. Of the 52 mutants tested, deletions of six DGCs and three PDEs were found to affect these phenotypes at low temperatures. Unlike wild type, a strain lacking all six DGCs did not exhibit a low-temperature-dependent increase in c-di-GMP, indicating that these DGCs are required for temperature modulation of c-di-GMP levels. We also show that temperature modulates c-di-GMP levels in a similar fashion in the Gram-negative pathogen *Pseudomonas aeruginosa* but not in the Gram-positive pathogen *Listeria monocytogenes*. This study uncovers the role of temperature in environmental regulation of biofilm formation and c-di-GMP signaling.

INTRODUCTION

Vibrio cholerae, the causative agent of the diarrheal disease cholera, is a natural inhabitant of aquatic environments (Faruque, 1998; Alam, 2006). The ability of this pathogen to form biofilms, matrix-enclosed surface-associated communities, is important for enhancing environmental survival and increasing infectivity and transmission (Alam, 2006; Charles, 2011; Faruque, 1998; 2006; Cowell, 2003; Harris, 2012; Huq, 1996; Nelson, 2009; Tamayo, 2010). In *V. cholerae*, biofilm formation is controlled by a complex regulatory network and is dependent on the production of *Vibrio* exopolysaccharides (VPS) and biofilm matrix proteins that facilitate cell-cell and cell-surface interactions (Berk, 2012; Fong, 2006; 2007; 2010; Yildiz, 1999; 2009). Although key regulators of biofilm formation and their genetic interactions have been studied, little is known regarding the mechanisms by which environmental signals are integrated into the biofilm regulatory network.

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c-di-GMP is an intracellular signaling molecule that controls diverse cellular processes including biofilm formation, motility, and virulence (Römling, 2013). c-di-GMP is synthesized by diguanylate cyclases (DGCs), which contain GGDEF domains, and degraded by phosphodiesterases (PDEs), which contain EAL or HD-GYP domains (Ryan, 2006; Ryjenkov, 2005; Schmidt, 2005). Many proteins with GGDEF, EAL or HD-GYP domains also harbor additional domains including REC, PAS and GAF domains, which strongly suggests that the activity of c-di-GMP metabolizing enzymes can be modulated by sensing domains. Examples of such proteins are as follows. The AxPDEA1 protein in *Acetobacter xylinum* contains a heme-binding PAS domain, which increases its PDE activity when bound to oxygen (Chang, 2001). The globin-coupled DGCs BpeGReg, DosD, and YddV in *Bordetella pertussis*, *Shewanella putrefaciens*, and *Escherichia coli* (respectively) promote c-di-GMP synthesis upon oxygen binding (Wan, 2009; Wu, 2013; Kitanishi, 2010). And finally, in *E. coli*, the YcgF protein contains a BLUF domain at the N-terminus and an EAL domain at the C-terminus that relieves repression of biofilm-associated genes at low temperatures (Tschowri, 2009). Levels of c-di-GMP can also be affected by sensor proteins that interact with DGCs or PDEs. For instance, in *Rhodospseudomonas palustris*, the BLUF protein PapB enhances PDE activity of PapA in response to blue light conditions (Kanazawa, 2010). Moreover, proteins with H-NOX (heme-nitric oxide/oxygen-binding) domains have been shown to modulate DGC activity and biofilm formation in response to nitric oxide (NO) (Plate, 2012). Once produced, c-di-GMP is sensed by different types of receptors that interact with a multitude of target proteins and regulate diverse cellular processes (Sondermann, 2012).

The genome of *V. cholerae* A1552 encodes 31 proteins with predicted GGDEF domains, 12 with EAL domains, 10 with both GGDEF and EAL domains, and 9 with HD-GYP domains (Galperin, 2001). *V. cholerae* GGDEF/EAL domain proteins often include sensory input domains (Galperin, 2001) that are likely responsible for modulating enzymatic activities of DGCs and PDEs. At present, few studies have identified environmental signals that affect c-di-GMP signaling in *V. cholerae*. The activity of the *V. cholerae* DGC Vc Bhr-DGC is controlled by the redox state of the non-heme di-iron within the Bhr (bacterial hemerythrin) domain. Specifically, the DGC activity has been shown to be 10-times higher when the iron is in its diferrous state, which occurs under anaerobic conditions (Schaller, 2012). Polyamine norspermidine levels affect biofilm formation through the regulatory pathway involving the phosphodiesterase MbaA (Karatan, 2005; Cockerell, 2014). Furthermore, it was recently demonstrated that growth medium and growth conditions significantly impact c-di-GMP levels in *V. cholerae*, through modulation of DGC and PDE cellular activity (Koestler, 2013), however the specific signal(s) responsible for this phenotype was not determined. Additionally bile and bicarbonate, signals encountered by *V. cholerae* during infection, were also shown to impact c-di-GMP levels (Koestler, 2014). Taken together, these findings support the notion that diverse environmental conditions modulate c-di-GMP signaling in *V. cholerae*. Receptors of c-di-GMP identified in *V. cholerae* thus far include 2 riboswitches, 5 PilZ domain proteins, biofilm regulators VpsR and VpsT, degenerate GGDEF domain containing protein CdgG, and flagellar biosynthesis regulator FlrA (Pratt, 2007; Krasteva, 2010; Srivastava 2011; Sudarsan, 2008; Beyhan, 2008; Srivastava 2013). While components

of c-di-GMP signaling networks are being identified, molecular mechanisms by which c-di-GMP signaling operates remain unclear.

V. cholerae is a facultative pathogen that is able to grow in both the human host and aquatic reservoirs during its life cycle. Thus, *V. cholerae* must endure significant temperature shifts during the transition between human hosts and the aquatic environment. Furthermore, *V. cholerae* experiences seasonal and inter-annual changes in temperature within aquatic reservoirs; and it has been demonstrated that cholera outbreaks are highly correlated with sea surface temperature and seasonal temperature fluctuation (Gil, 2004; Huq, 2005; Turner, 2013). However, despite its critical influence on the life cycle of *V. cholerae*, little is known about the effects that temperature has on the physiology of *V. cholerae*.

In this study, we show that a decrease in growth temperature enhances biofilm formation in *V. cholerae* through increased c-di-GMP levels. We determined that six DGCs, *cdgA* (VCA0074), *cdgH* (VC1067), *cdgK* (VC1104), *cdgL* (VC2285), *cdgM* (VC1376), and *vpvC* (VC2454) are required for a low-temperature increase in c-di-GMP levels and biofilm formation. These results collectively show that c-di-GMP signaling is critical for integration of environmental signals with biofilm formation.

RESULTS

Growth at low temperatures increases biofilm formation

V. cholerae experiences temperature changes during its lifecycle and biofilms are crucial to the environmental persistence and transmission of this organism. We therefore asked whether biofilm formation would be affected by temperature. We analyzed the ability of *V. cholerae* to form biofilms at 15°C, 25°C, and 37°C as the pathogen experiences this temperature range during its life cycle (Gil, 2004; Huq, 2005) and *V. cholerae* have been isolated from aquatic environments at these temperatures (Gil, 2004; Turner, 2013). To determine if temperature affects biofilm formation, biofilms were formed under static conditions using wild-type *V. cholerae*. We found that biofilms formed at low temperatures (15°C and 25°C) exhibited increased biomass, thickness, and were more structured compared to those formed at 37°C (Fig. 1). COMSTAT analysis (Table 1) revealed that biofilm biomass is 19.8-fold and 6.8-fold higher at 15°C and 25°C, respectively, when compared to those formed at 37°C. We note that it was previously demonstrated using crystal violet staining assay that biofilm formation in a *V. cholerae* O1 strain and several non-O1 strains was slightly increased at 30°C when compared to 37°C (Hořtacká, 2010), corroborating our results that biofilm forming ability of *V. cholerae* is enhanced at lower temperatures.

Growth at low temperatures increases cellular c-di-GMP levels

Since biofilm formation was impacted by temperature and enhanced c-di-GMP levels induce biofilm formation, we reasoned that cellular c-di-GMP levels may change with growth temperature. Cellular c-di-GMP quantification in wild-type *V. cholerae* grown for 24 hours at different temperatures revealed that c-di-GMP levels were approximately 1.5-fold higher at 25°C when compared to 37°C ($P < 0.05$), and approximately 5-fold higher at 15°C when

compared to 37°C ($P < 0.005$) (Fig. 2A). Because *V. cholerae* must endure shifts in temperature during its transition between human host and the environment, we analyzed if c-di-GMP synthesis could be induced by a shift from high to low temperature. To this end, *V. cholerae* was grown at 37°C until mid-exponential phase then subjected to a low-temperature shift (25°C and 15°C) for 1 hour. Cellular c-di-GMP levels in these cultures were quantified and compared to control cultures that were kept at 37°C. We observed that c-di-GMP levels were over 2-fold higher after the shift from 37°C to 15°C ($P < 0.0005$), however there was no significant change after the shift to 25°C at this time point (Fig. 2B). Cultures maintained at 37°C showed an almost 2-fold decrease in c-di-GMP after 1 hour ($P < 0.005$) (Fig. 2B). In *V. cholerae*, cellular c-di-GMP levels decrease as cell density increases (Koestler, 2013); thus, a decrease in cellular c-di-GMP levels is expected. These results show that c-di-GMP levels are increased at lower temperatures.

Having determined that biofilm formation and cellular c-di-GMP levels are increased in cells grown at low temperatures, we next evaluated the impact of the overexpression of a DGC (CdgA) and a PDE (CdgC) on biofilm formation at 15°C. We determined that overexpression of CdgA or CdgC from an arabinose inducible promoter resulted in an increase and a decrease in biofilm formation, respectively (Fig. 2C, Table 2).

Identification of DGCs that control *vpsL* expression at low temperatures

We next wanted to determine if there are specific DGCs and/or PDEs responsible for modulating c-di-GMP metabolism in response to low temperatures. We previously generated 52 isogenic mutants with in-frame deletions of almost all of the predicted c-di-GMP-related proteins of *V. cholerae* (Beyhan, 2008; Liu, 2010; Shikuma, 2012). To identify specific DGCs that contribute to low-temperature-induced c-di-GMP production, we measured promoter activity of the *vps* genes using a luciferase transcriptional reporter *vpsLp-lux* in wild-type *V. cholerae* and 31 strains containing in-frame deletions of each gene encoding a protein with a predicted GGDEF domain. In *V. cholerae*, *vpsL* is the first gene in the *vps*-II cluster, which together with the *vps*-I cluster genes, encode proteins that are required for VPS production and biofilm formation. Because *vpsL* transcription is positively regulated by c-di-GMP, we used the promoter activity of *vpsL* as an indicator of cellular c-di-GMP levels. To measure *vpsL* promoter activity, each strain was grown at 37°C until it reached an OD₆₀₀ of 0.4, at which time they were shifted to 15°C for one hour and luminescence was measured (Supplementary Fig. 1). A total of six mutants, *cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, and *vpvC*, were found to exhibit an over 2-fold decrease in *vpsL* promoter activity compared to wild type ($P < 0.05$) (Fig. 3A). We also measured *vpsL* promoter activity in these strains after 24 hours of growth at 15°C (Fig. 3B). *cdgH*, *cdgL*, and *cdgM* still exhibited an over 2-fold decrease in *vpsL* promoter activity, whereas *cdgA*, *cdgK*, and *vpvC* exhibited an approximately 1.5-fold decrease when compared to wild type ($P < 0.05$) (Fig. 3B). These results show that multiple DGCs are involved in *vpsL* regulation and likely c-di-GMP homeostasis at low temperatures.

We hypothesized that each of these DGCs could contribute additively to low temperature-mediated increase in *vpsL* expression. Therefore, we created a strain lacking all six DGCs, designated as $\Delta 6$ DGC. We then analyzed *vpsL* gene promoter activity in $\Delta 6$ DGC strain

following a temperature shift from 37 °C to 15 °C, and also in cells grown at 15°C for 24 hours (Fig. 3A and B). The Δ 6 DGC mutant exhibited an approximately 10-fold and 25-fold lower *vpsL* promoter activity when compared to wild type following a low-temperature shift and constant incubation at 15°C ($P < 0.005$) (Fig. 3A and B), supporting the hypothesis that multiple DGC are contributing to cellular c-di-GMP levels at low temperatures.

To further understand how these DGCs could contribute to low temperature sensing, we checked if these proteins are predicted to harbor sensory input domains (Fig. 3C). CdgH contains a tandem bacterial extracellular solute-binding domain 3 (SBP bac 3). Periplasmic solute-binding proteins of Gram-negative bacteria can be classified into eight families (Tam, 1993). Family 3 proteins bind to polar amino acids and opines. CdgM has a Cyclases/Histidine kinases Associated Sensory Extracellular domain (CHASE). This domain is predicted to bind diverse low molecular weight ligands, such as the cytokinin-like adenine derivatives or peptides. CdgH and CdgM activity was recently shown to be enhanced by bile acids, an environmental signal critical for sensing host environment (Koestler, 2014). CdgL harbors a CHASE 4 domain. While the ligand-binding specificities of CHASE 4 domain are yet to be determined, it is reported that in bacteria this domain is found exclusively with diguanylate cyclases (Mougel, 2001). VpvC has a Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases domain (HAMP). HAMP domains are involved in transmembrane signaling by relaying stimulus-induced conformational changes to cytoplasmic signaling domains (Parkinson, 2010). It is yet to be determined how these domains could be involved in sensing or responding low-temperature mediated changes to cell physiology. In contrast to the four DGCs discussed above, CdgA and CdgK periplasmic sensing domains have not been formally described.

Identification of DGCs that regulate biofilm formation at low temperatures

Since our objective was to identify c-di-GMP signaling proteins contributing to both c-di-GMP levels and biofilm formation at low temperatures, we further characterized mutants discussed above for their ability to form biofilms at 15°C. We observed that *cdgH*, *cdgK*, *cdgL*, and *cdgM* exhibited less biofilm formation than wild type (Fig. 4 and Table 3), consistent with the *vpsL* expression data (Fig. 3A). *vpvC* and *cdgA* exhibited a small but reproducible decrease in biofilm formation when compared to wild type (Fig 4 and Table 3). These data show that each DGC affects biofilm formation at low temperatures to a different degree.

To further confirm the contribution of these DGCs to biofilm formation at low temperatures, we generated complementation plasmids where expression of DGC genes were placed under the control of an arabinose-inducible promoter of the pBAD plasmid. Each plasmid was then introduced to their respective deletion strain and biofilm formation was analyzed and quantified. We determined that each DGC gene was able to complement the biofilm defect of its corresponding deletion strain (Table 4).

Next, biofilm formation of the Δ 6 DGC mutant was analyzed after incubation at 15°C for 24 hours. The Δ 6 DGC mutant exhibited a severe defect in biofilm formation at 15°C with an 18-fold decrease in biomass and a 16-fold and 3-fold decrease in average and maximum thickness, respectively, when compared to biofilms formed by the wild-type strain (Fig. 4A

and Table 2). We then evaluated the ability of each complementation plasmid to restore biofilm formation at 15°C in the Δ 6 DGC mutant (Table 4). Thus, we introduced *pcdgA*, *pcdgH*, *pcdgK*, *pcdgL*, *pcdgM*, or *vpvC* into the Δ 6 DGC strain. We then analyzed and quantified biofilm formation by measuring total biomass, average and maximum thickness, and the roughness coefficient. The Δ 6 DGC strain harboring *pcdgA* was able to complement the Δ 6 DGC strain to wild-type levels. Partial complementation was observed with *pcdgH* and *pcdgL*, and *vpvC* (Table 3). On the other hand, the Δ 6 DGC strain harboring *pcdgM* and *pcdgK* exhibited a low but reproducible increase in biofilm properties compared to the Δ 6 DGC harboring the vector alone. These results suggest that these DGCs may differ in their ability to produce c-di-GMP, thus contributing differently to low temperature biofilm formation.

To better evaluate the ability of each DGC to produce c-di-GMP at 15°C, we analyzed cellular c-di-GMP levels in the Δ 6 DGC strain harboring individual complementation plasmids. The expression of each gene resulted in an increase in cellular c-di-GMP levels of the Δ 6 DGC strain (Fig. 5A). However, each DGC differed in its ability to produce c-di-GMP. Overall, biofilm formation and cellular c-di-GMP levels resulting from complementation were corroboratory.

Analysis of motility phenotypes of DGC mutants

c-di-GMP inversely regulates motility and biofilm formation in *V. cholerae*. To understand the effect of the six DGCs on cell motility at low temperatures, we analyzed the motility phenotype of each mutant at 15°C on LB motility plates. While *cdgH*, *cdgK*, *cdgL* and *vpvC* mutants exhibited an increase in migration (Fig. 6A), *cdgA* and *cdgM*, and did not show a major difference in migration compared to wild type. In previous studies performed at 30°C, *cdgH*, *cdgK* and *cdgL* exhibited increased motility, however a *vpvC* mutant did not show an altered motility phenotype when compared with wild type (Liu, 2010), indicating that the increase in motility in the *vpvC* mutant is low-temperature specific. All strains harboring their respective complementation plasmids exhibited decreased motility compared to the same strains carrying vector only ($P < 0.05$) (Fig. 6A). This data further supports that all the above-mentioned DGCs were active at 15°C and able to produce c-di-GMP. We also analyzed the motility phenotype of wild type harboring a plasmid where expression of *cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, or *vpvC* was controlled from an arabinose-inducible promoter at 37°C and 15°C. At 37°C, wild type harboring *pcdgA*, *pcdgH*, *pcdgK*, and *cdgL* exhibited a decrease in motility compared to vector only ($P < 0.005$), while *pcdgM* and *vpvC* did not (Fig. 6B). At 15°C, expression of each gene decreased motility when compared to vector only control ($P < 0.05$) (Fig. 6B and C). These results suggest that *cdgM* and *vpvC* affect motility specifically at low temperatures. Together these data indicate that this set of DGC's inversely control motility and biofilm in *V. cholerae* at low temperatures.

Identification of PDEs that control *vps* expression and biofilm formation at low temperatures

Having identified DGCs that contribute to *vps* gene expression and biofilm formation, we also entertained the possibility that at low temperatures a decrease in PDE activity or abundance could also modulate cellular c-di-GMP levels, and in turn *vps* expression and

biofilm formation. To identify specific PDEs that regulate low-temperature-induced biofilm formation, we measured *vpsL* promoter activity in the 22 strains containing in-frame deletions of each gene encoding a protein with a predicted EAL, or GGDEF and EAL domain (Supplementary Fig. 1). A total of three mutants, *cdgC* (VCA0785), *cdgJ* (VC0137), and *rocS* (VC0653) were found to exhibit an over 2-fold increase in *vpsL* expression compared to wild type ($P < 0.05$) (Fig. 8A). We also observed that a set of mutants VC0398, VC0658, VC1934, VC2750, and VCA0080 exhibited over 2-fold decrease in *vpsL* promoter activity. However, for this study, we focused only on the strains that exhibited an increase in *vpsL* promoter activity. We also generated a 3 PDE mutant with deletions in *cdgC*, *cdgJ*, and *rocS*. The 3 PDE mutant exhibited 13-fold higher *vpsL* expression when compared to wild type ($P < 0.05$) (Fig. 8A).

Typically, strains with enhanced biofilm matrix production are able to form pellicle biofilms at the air-liquid interface and corrugated colonies on agar plates. We analyzed pellicle formation and colony corrugation in *cdgC*, *cdgJ*, *rocS*, and the 3 PDE mutant. We determined that single deletion mutants did not form pellicle biofilms or corrugated colonies, whereas the 3 PDE mutant did form both pellicle biofilms and corrugated colonies (Fig. 8B), which is indicative of increased production of VPS and biofilm matrix proteins (Yildiz, 1999; Fong, 2007).

When we analyzed biofilm formation under static conditions using CSLM, *cdgC*, *cdgJ*, and *rocS* mutants exhibited an increase in biofilm formation relative to wild type at 15°C (Fig. 8C and Table 3). The 3 PDE mutant exhibited a defect in biofilm formation at 15°C with a 5-fold decrease in biomass, a 4-fold decrease in average thickness, and a 1.3-fold increase in maximum thickness when compared to biofilms formed by the wild-type (Fig. 8C and Table 3). As discussed above the 3 PDE mutant has enhanced ability to form pellicles. When biofilms are formed under static conditions, cells that are distributed between the air-liquid and solid-liquid interfaces; therefore biofilms imaged by CSLM at the solid-liquid interface may not be a true representation of the biofilm forming ability of the 3 PDE mutant. These results collectively show that these 6 DGCs and 3 PDEs are additively affecting *vps* gene expression and biofilm formation at low temperatures.

Analysis of the contribution of DGCs and PDEs for enhancement of c-di-GMP levels at low temperatures

To examine the contribution of the DGCs to the low-temperature induction of c-di-GMP, we first analyzed c-di-GMP levels in each of the DGC mutants grown at 15°C for 24 hours (Fig. 8A). We determined that c-di-GMP levels in *cdgA*, *cdgH*, *cdgK*, *cdgL*, and *cdgM* single deletion strains were approximately 1.5-fold lower than wild type ($P < 0.05$) when grown at 15°C for 24 hours; however, there was no decrease in c-di-GMP in the *vpvC* mutant. In a 2 DGC strain lacking *cdgL* and *cdgM*, the strains with lowest *vpsL* expression at 15°C, we observed a 1.7-fold decrease ($P < 0.05$) and in a 6 DGC strain lacking all 6 DGCs a 2.1-fold decrease ($P < 0.005$) in c-di-GMP relative to that of wild type. c-di-GMP levels were not completely abolished in the 6 DGC strain, suggesting that under the conditions utilized in this study other DGCs could also contribute to c-di-GMP levels.

To determine if the DGCs discussed above are involved in the induction of c-di-GMP levels observed after shifting cells from 37°C to 15°C, we determined the amount of c-di-GMP produced by the 6 DGC strains under these conditions and compared them to wild type. Results showed that c-di-GMP levels in 6 DGC grown at 37°C were not significantly different than that of wild type ($P > 0.05$) (Fig. 8B). This suggests that these DGCs are not required to maintain the basal level of the c-di-GMP pool at 37°C. However, when the 6 DGC strain was shifted to 15°C c-di-GMP levels were not induced as observed in wild type, but instead reduced 1.5-fold ($P < 0.05$) (Fig. 8B). These results strongly suggest that these DGCs are required for increasing c-di-GMP in response to low temperatures. It is also possible that reduced phosphodiesterase activity might also contribute to the observed increase in c-di-GMP levels at low temperature. To explore this possibility we determined the c-di-GMP levels in the 3 PDE strain and found that they were 1.5-fold higher than wild type even before the shift (37°C) ($P < 0.05$), and 2-fold higher than wild type after the shift to 15°C ($P < 0.05$) (Fig. 7A). This indicates that these PDEs are not responsible for low temperature-mediated increase in c-di-GMP levels.

Analysis of DGCs expression

The increase in c-di-GMP levels observed at 15°C could be caused by an increase in the abundance or activity of these six DGCs. To determine if expression of these genes is altered by growth at low temperatures or by temperature shift, we determined the transcript abundance for each gene using Real-Time PCR. First, we found that mRNA transcript levels of *cdgA*, *cdgH*, *cdgK*, *cdgL* and *cdgM* are increased 15.3, 6.1, 3.8, 3.4, and 7.2-fold ($P < 0.05$), respectively, after 24 hours of growth at 15°C compared to 37°C (Fig. 9A). However, levels of *vpvC* were not significantly different between these conditions (Fig. 9A). Next, we found that one hour after a shift from 37°C to 15°C, expression of *cdgA* and *cdgM* was slightly increased 1.7 and 1.2-fold respectively ($P < 0.05$); however, transcript levels of *cdgH*, *cdgK*, *cdgL*, and *vpvC* were not increased (Fig. 9B) despite the increase of c-di-GMP levels at this time point. This suggests that post-transcriptional regulation is required for modulating c-di-GMP levels in response to temperature shift.

Effects of temperature on c-di-GMP signaling in other facultative pathogens

Because c-di-GMP is a ubiquitous signaling molecule in bacteria, and temperature changes are encountered by a wide variety of bacteria, we hypothesized that temperature might also affect c-di-GMP signaling in other facultative pathogens. We selected *P. aeruginosa* PAO1, a Gram-negative opportunistic pathogen that is ubiquitous in the environment and *L. monocytogenes*, a Gram-positive pathogen that is capable of growing at low temperatures. To test if c-di-GMP levels were affected by temperature in these two pathogens, cells were grown at 15°C, 25°C, and 37°C for 24 hours and c-di-GMP levels were quantified. Our results revealed that there was a significant increase in c-di-GMP levels at low temperatures in *P. aeruginosa* with 11.50 pmol/mg protein at 15°C, 2.07 pmol/mg protein at 25°C, and 3.15 pmol/mg protein at 37°C (Fig. 10A). Yet growth temperature did not have the same drastic effect on c-di-GMP levels in *L. monocytogenes* with 14.05 pmol/mg protein at 15°C, 16.63 pmol/mg protein at 25°C, and 13.78 pmol/mg protein at 37°C (Fig. 10B). These results indicate that c-di-GMP levels can be affected by temperature in other environmental pathogens but may vary between species.

DISCUSSION

The objective of this study was to determine how temperature affects biofilm formation in *V. cholerae*. We determined that low temperature increases biofilm formation in part through increased cellular c-di-GMP levels. We found that six DGCs (*cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, and *vpvC*) are required for a low-temperature increase in c-di-GMP levels and biofilm formation. Previous studies by our group have identified these DGCs to be important for c-di-GMP signaling and biofilm formation at 30°C in LB (Lim, 2006; Beyhan, 2006; Beyhan, 2007; Beyhan, 2008; Fong, 2008; Liu, 2010; Shikuma, 2012). Shikuma *et al* demonstrated that 5 of these 6 DGCs (*cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*) affect c-di-GMP levels when cultures are grown at 30°C (Shikuma, 2012) and contribute to *vps* gene expression by providing c-di-GMP pool necessary for activation of the c-di-GMP sensor VpsT. In this study, we showed that these 6 DGCs are critical for environmental regulation of c-di-GMP signaling and biofilm formation.

Cellular c-di-GMP levels could be controlled by changes in abundance of c-di-GMP metabolizing enzymes. Studies have shown that either transcription or translation of genes encoding c-di-GMP signaling enzymes could be modulated in response to environmental signals. In *E. coli*, transcription of genes encoding GGDEF/EAL domain proteins is increased at 28°C when compared to 37°C (Sommerfeldt, 2009). It has been previously documented that translation of the DGC VCA0939 is activated through the quorum sensing Qrr sRNA (Hammer, 2007; Zhao, 2013), demonstrating that DGC levels could be controlled post-transcriptionally under some conditions. Here we show that expression of 5 DGCs (*cdgA*, *cdgH*, *cdgK*, *cdgL* and *cdgM*) is increased in cells grown at 15°C for 24 hours. In contrast, short cold-shifts did not lead to a significant increase in transcription of four of these DGC genes. Surprisingly, mRNA message abundance of *cdgH* and *cdgK* was lower one hour after cold-shift. It is yet to be determined if translation of any of these genes or protein stability are impacted by growth temperature.

Cellular c-di-GMP levels could also be controlled by changes in the activities of DGCs upon environmental signal sensing. We observed that these DGCs differ in their ability to complement the biofilm phenotype of a 6 DGC strain at 15°C. This suggests that these DGCs may not be equally active at low temperatures, which was corroborated by our c-di-GMP measurements in the 6 DGC strain harboring over-expression plasmids with each DGC. Alternatively, DGCs might contribute to an increase in the c-di-GMP pools locally or may not on their own surpass the threshold level of c-di-GMP necessary to rescue the biofilm defect in 6 DGC strain. The mechanisms responsible for the differences in activities of these DGCs are yet to be determined.

As mentioned above, four of these DGCs harbor a sensory input domain. These domains could potentially allow these DGCs to sense environmental signals like low temperature and modulate their activity accordingly, but another possibility is that these proteins sense temperature via their transmembrane domains. All six of the DGCs in this study contain transmembrane domains and are predicted to localize in the membrane. Studies of other bacteria have shown that membrane fluidity is affected by temperature (Cronan, 1975; Garwin, 1980; Aguilar, 2001; Zhu, 2005), which could be a means of integrating

temperature sensing into the c-di-GMP network. Specialized sensing domains or changes to protein structure due to membrane fluidity could be important for relaying temperature signals to these DGCs. We are currently investigating how the activity of these DGCs is impacted by environmental signals.

Temperature affects biofilm formation in other bacteria such as *Staphylococcus epidermidis*, *E. coli*, *Legionella pneumophila*, and *Burkholderia pseudomallei* (Fitzpatrick, 2005; White-Ziegler, 2008; Piao, 2006; Ramli, 2012). The mechanism for temperature-mediated biofilm formation and the involvement of c-di-GMP in these microbes, however, has yet to be elucidated. In this study we revealed that c-di-GMP levels are also regulated by temperature in an opportunistic human pathogen, *P. aeruginosa*, indicating that integration of environmental signal perception to c-di-GMP signaling is a common phenomenon of bacteria. c-di-GMP levels were not as dramatically affected by growth temperature in the Gram-positive pathogen *L. monocytogenes*. *L. monocytogenes* is able to grow at a wider temperature range than *V. cholerae*, therefore it is possible that the temperatures used in this study may not be broad enough to see an effect in this organism.

Our study demonstrates that temperature is a crucial environmental signal that modulates biofilm formation and c-di-GMP signaling in *V. cholerae*. Multiple DGCs modulate cellular c-di-GMP levels in response to low-temperature, which in turn increases biofilm formation and represses motility. The identity of effectors responsible for converting c-di-GMP levels to phenotypic outputs at low temperatures is yet to be determined.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

V. cholerae O1 El Tor A1552 was used as our wild-type strain. *E. coli* CC118 λ pir and S17-1 λ pir were used for cloning and conjugation, respectively. *P. aeruginosa* PAO1 and *L. monocytogenes* 10403S were used for experiments designed to determine temperature effect on c-di-GMP levels. Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.2M NaCl; pH 7.5) was used to grow all strains. Temperature-shift experiments were performed by inoculating overnight-grown cultures of *V. cholerae* in a 1:200 dilution in LB and incubating at 37°C until exponential phase (OD₆₀₀=0.4); cultures were then shifted to 15°C and 25°C for the time indicated. Constant temperature experiments were performed by inoculating overnight-grown cultures in a 1:200 dilution in LB followed by incubation at 15°C, 25°C, or 37°C for 24 hours. When needed, chloramphenicol was used at 5 µg/ml, and ampicillin and rifampicin were used at 100 µg/ml, except for biofilm complementation analysis where ampicillin was used at 25 µg/ml. L-arabinose was added to growth medium at a final concentration of 0.05%, 0.1%, or 0.3% (wt/vol) for complementation and DGC activity experiments.

Plasmid Construction

Overexpression plasmids were constructed by cloning the open reading frame of each gene of interest into pBAD/Myc-His B (Invitrogen) using ligation-independent cloning (LIC).

Each insert was sequenced and clones without mutations were used for subsequent experiments.

Generation of in-frame deletion mutants

Deletion mutants were generated using previously published protocols (Fong, 2006). Briefly, approximately 500 bp up and downstream of the gene of interest was amplified by PCR using two sets of deletion primers. These two PCR products were then joined via splicing by overlap extension PCR (Fong, 2006) and cloned into a pGP704-*sacB28* suicide plasmid. The deletion plasmids were maintained in *E. coli* CC118 (λ *pir*). Biparental matings were carried out with *V. cholerae* A1552 and *E. coli* S17-1 (λ *pir*) harboring the deletion plasmid. Ampicillin- and rifampin-resistant transconjugants, resulting from single homologous recombinations, were selected and subjected to sucrose-based selection. Ampicillin-sensitive and sucrose-resistant *V. cholerae* deletion strains, which had undergone double homologous recombination, were selected and verified by PCR.

Generation of GFP-tagged strains

V. cholerae strains were tagged with green fluorescent protein (GFP) according to the procedure previously described (Fong, 2006). Briefly, triparental matings were carried out with donor *E. coli* S17-1 (λ *pir*) carrying pMCM11, helper *E. coli* S17-1 (λ *pir*) harboring pUX-BF13, and various *V. cholerae* strains. Transconjugants were selected on thiosulfate-citrate-bile salts-sucrose (Difco) agar medium containing gentamicin at 30°C. GFP-tagged *V. cholerae* strains were verified by PCR.

Biofilm Imaging

V. cholerae from overnight-grown cultures were diluted into LB broth to an OD₆₀₀ of about 0.02, then 3 ml were inoculated into glass chambers (Lab-Tek) and incubated statically at 15°C, 25°C, or 37°C. After 24 hours, planktonic bacteria were removed by gently inverting the chambers and washed gently twice with LB. Biofilm formation was visualized using confocal laser-scanning microscopy (CLSM) with an LSM 5 Pascal laser-scanning microscope (Zeiss). Three-dimensional images were reconstructed using Imaris 7.6 and analyzed using COMSTAT (Heydorn, 2000). Each experiment included three independent biological replicates and three images were taken for each replicate.

Determination of intracellular c-di-GMP levels

c-di-GMP extraction was performed as described previously (Liu, 2010). Briefly, 40 ml of culture was centrifuged at 3220 g for 30 min. Cell pellets were allowed to dry briefly then re-suspended in 1 ml extraction solution (40% acetonitrile, 40% methanol, 0.1% formic acid, 19.9% water), and incubated on ice for 5 min. Samples were then centrifuged at 16,100 g for 5 min and 800 μ l of supernatant was dried under vacuum and then lyophilized. Samples were re-suspended in 50 μ l of 184 mM NaCl and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Thermo-Electron Finnigan LTQ mass spectrometer coupled to a surveyor HPLC. The Synergin Hydro 4u Fusion-RP 80A column (150 mm $\times$ 2.00 mm diameter; 4- μ m particle size) (Phenomenex, Torrance, CA) was used for reverse-phase liquid chromatography. Solvent A was 0.1% acetic acid in

10 mM ammonium acetate, solvent B was 0.1% formic acid in methanol. The gradient used was as follows: time (t) = 0–4 min, 98% solvent A, 2% solvent B; t = 10–15 minutes, 5% solvent A, 95% solvent B. The injection volume was 20 μ l and the flow rate for chromatography was 200 μ l/min.

The amount of c-di-GMP in samples was calculated with a standard curve generated from pure c-di-GMP suspended in 184 mM NaCl (Biolog Life Science Institute, Bremen, Germany). Concentrations used for standard curve generation were 50 nM, 100 nM, 500 nM, 2 μ M, 3.5 μ M, 5 μ M, 7.5 μ M, and 10 μ M. The assay is linear from 50 nM to 10 μ M with an R² of 0.999. c-di-GMP levels were normalized to total protein per ml of culture.

To determine protein concentration, 4 ml from each culture was pelleted, the supernatant was removed, and cells were lysed in 1 ml of 2% sodium dodecyl sulfate. Total protein in the samples was estimated with BCA assay (Thermo Fisher) using bovine serum albumin (BSA) as standards. Each c-di-GMP quantification experiment was performed with four biological replicates. Levels of c-di-GMP were compared between temperatures in *V. cholerae* and *P. aeruginosa* using a two-tailed student's *t* test. Levels of c-di-GMP in *L. monocytogenes* were compared between temperatures using the Mann Whitney U test.

Luminescence Assays

V. cholerae wild type, and DGD/PDE mutants harboring the *pvpsLp-lux* plasmid were grown overnight (15–17 hours) aerobically in LB supplemented with chloramphenicol. Cells were diluted 1:200 in LB + chloramphenicol and grown at 37°C until OD₆₀₀ = 0.4, at which time cultures were shifted to 15°C. One hour after shift, luminescence was measured using a Victor3 Multi-label Counter (PerkinElmer) and is reported as counts min⁻¹ ml⁻¹/ OD₆₀₀. Assays were repeated with at least two biological replicates for all 52 strains tested, and three biological replicates for the six DGC, three PDE, 6DGC, and 3PDE strains. Four technical replicates were measured for all assays. Statistical analysis was performed using two-tailed student's *t* test. All six DGC and three PDE strains exhibited a *P* value of < 0.05 when compared to wild type.

Motility Assays

LB soft-agar (0.3% agar) motility plates were used to determine motility phenotypes of each strain. Single colonies were stabbed into LB motility plates with and without 0.1% arabinose. Plates were incubated at 37°C for 8 hours or 15°C for 3 days at which time the diameter of the migration zone was measured. Assays were repeated with at least three biological replicates. Statistical analysis was performed using two-tailed student's *t* test.

Expression analysis - Real Time PCR

Total RNA was isolated from *V. cholerae* cells that were grown in LB either at 15°C or 37°C for 24 hours or from *V. cholerae* cells that were exposed to cold-shift. For this experiment, overnight-grown cultures of *V. cholerae* were diluted 1:200 in LB and incubated at 37°C until exponential phase (OD₆₀₀=0.4); cultures were then shifted to 15°C or maintained at 37°C for one hour. 2 ml aliquots were collected by centrifugation, immediately resuspended in 1 ml of Trizol reagent (Invitrogen) and 0.2 ml of chloroform

was added into each tube. Tubes were shaken, incubated at room temperature for 5 minutes and then centrifuged for 20 minutes at 12,000 g, 4°C. Aqueous layer was collected into a new tube. Isopropanol (250 µl) and 250 µl high salt solution (0.8 M Na-citrate, 1.2 M NaCl) was added and the suspension was incubated for 10 minutes at room temperature to precipitate the RNA. Isopropanol was removed by centrifugation for 30 minutes at 12,000 g, 4°C. Pellets were washed with 1 ml of 75% ethanol, and ethanol was removed by centrifugation for 5 minutes at 7,500 g, 4°C. Pellets were dried at room temperature for 10 minutes. Dried pellets were then resuspended in nuclease-free water. To remove contaminating DNA, total RNA was incubated with TURBO DNase (Ambion), and the RNeasy Mini kit (QIAGEN) was used to clean up the RNA after DNase digestion.

cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) from 1 µg of total RNA. Real-time PCR was performed using a Bio-Rad CFX1000 thermal cycler and Bio-Rad CFX96 real-time imager with specific primer pairs (designed within the coding region of the target genes) and SsoAdvanced SYBR green supermix (Bio-Rad). Results are from two independent experiments performed in triplicate. All samples were normalized to the expression of the housekeeping gene 16S using the Pfaffl method (Pfaffl, 2004). Relative expression was calculated by normalizing expression at 15°C by that at 37°C. Statistical analysis was performed using two-tailed student's *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a NIH grant AI102584 to F.H.Y. L. T. was supported in part by the National Human Genome Research Institute (NHGRI) funded Research Mentoring Institute (RMI) at UC Santa Cruz and the Eugene Cota-Robles (ECR) Fellowship. c-di-GMP quantification was performed at the UCSC Mass Spectrometry Facility, which is funded by NIH grant S10-RR20939 (MS equipment grant). We kindly thank Q. Zhang for help with the HPLC/MS-MS experiments and analysis. B. Abrams at the UCSC Microscopy center for help with confocal microscopy. V. Auerbuch for the *Listeria monocytogenes* 10403S strain. We also thank M. Gomelsy, K. Ottemann, M. Camps, J.N.C. Fong, D. Zamorano-Sanchez, and other members of the Yildiz group for helpful discussions and reading of this manuscript.

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Fig. 1. *V. cholerae* biofilm formation at various temperatures

Three-dimensional biofilm structures of wild-type *V. cholerae* formed at 15°C, 25°C, and 37°C after 24 hours incubation in static biofilm chambers. Images of horizontal (*xy*) and vertical (*xz*) projections of biofilms are shown. The results shown are from one representative experiment of three independent experiments. Scale bars = 40 μ m.

Fig. 2. c-di-GMP levels in *V. cholerae* grown at various temperatures

c-di-GMP was extracted from whole cells and quantified using HPLC-MS/MS. (A) c-di-GMP levels in *V. cholerae* cells grown at 37°C, 25°C, or 15°C for 24 hours. (B) c-di-GMP levels in cells grown at 37°C then subjected to low-temperatures. *V. cholerae* cells grown at 37°C to OD₆₀₀ of 0.4 then shifted to 25°C, 15°C or kept at 37°C, for one hour; and c-di-GMP levels were quantified. Error bars indicate standard deviations of four biological replicates. * $P < 0.05$, ** $P < 0.005$, n.s., $P > 0.05$. Biofilms formed after incubation at 15°C for 24 hours by wild-type *V. cholerae* harboring either *cdgA* or *cdgC* from an overexpression

plasmid in LB with 0%, 0.05%, or 0.2% arabinose. The results shown are from one representative experiment of three independent experiments. Scale bars represent 40 μm .

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Fig. 3. Analysis of *vpsL* promoter activity in DGC mutants

vpsL promoter activity in wild-type *V. cholerae* and strains that harbor in-frame deletions of genes encoding proteins with GGDEF domains. *V. cholerae* cells were grown at (A) 37°C to OD₆₀₀ of 0.4 then shifted to 15°C for one hour or (B) at 15°C for 24 hours with no shift. Expression is reported in luminescence counts min⁻¹ ml⁻¹/OD₆₀₀. Error bars indicate standard deviations of three biological replicates. All strains exhibited a statistically significant difference when compared with wild type ($P < 0.05$). (C) Schematic representation of predicted domains for each DGC, black boxes represent predicted transmembrane domains within each protein.

Fig. 4. Biofilm formation in strains lacking DGCs

Biofilms formed by wild-type *V. cholerae* and strains containing in-frame deletions of genes encoding DGCs (*cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, *vpvC* and a $\Delta 6$ mutant) after incubation at 15°C for 24 hours. The results shown are from one representative experiment of three independent experiments. Scale bars represent 40 μ m.

Fig. 5. c-di-GMP levels in complementation strains

A) c-di-GMP levels in wild type *V. cholerae* cells harboring pBAD and the $\Delta 6$ DGC strain harboring pBAD only or pBAD expressing *cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, or *vpvC* genes grown in LB with 0.1% arabinose at 15°C for 24 hours. Error bars indicate standard deviations of four biological replicates. C-di-GMP levels in the $\Delta 6$ DGC strain harboring each complementation plasmid was compared to strains harboring pBAD. * $P < 0.05$, ** $P < 0.005$.

Fig. 6. Motility in strains lacking DGCs

(A) Diameters of migration zones of DGC deletion strains harboring pBAD or their respective complementation plasmid. Migration of *cdgH*, *cdgK*, *cdgL*, and *vpvC* were increased relative to wild type ($P < 0.05$) while *cdgA* and *cdgM* did not differ significantly. (B and C) Diameters of migration zones of wild-type *V. cholerae* harboring a pBAD plasmid where expression of *cdgA*, *cdgH*, *cdgK*, *cdgL*, *cdgM*, or *vpvC* is controlled from an arabinose-inducible promoter. Strains were grown on motility plates with 0.1% arabinose. The diameter of migration zones were measured after (B) 8 hours incubation at 37°C and (C) three days incubation at 15°C. The results represent an average of three biological replicates; error bars indicate standard deviations. * $P < 0.05$, ** $P < 0.005$, n.s., $P > 0.05$.

Fig. 7. Phenotypic analysis of PDEs mutants

(A) *vpsL* promoter activity (B) pellicle and colony morphology and (C) biofilm formation of wild-type *V. cholerae* and strains containing single in-frame deletions of genes encoding PDEs (*cdgC*, *cdgJ*, *rocS*) and a 3 PDE strain. Expression of *vpsL* and biofilm formation was analyzed in cells that were grown at 15°C for 24 hours. Colony morphology was evaluated after 48 h of growth on LB agar at room temperature. Scale bars in colony morphology and confocal images represent 1 mm and 40 μm respectively. (D) Schematic representation predicted domains for each PDE, black boxes represent predicted transmembrane domains within each protein.

Fig. 8. Analysis of c-di-GMP levels in DGC and PDE mutants of *V. cholerae*

(A) c-di-GMP quantification of wild-type and strains containing in-frame deletions of DGCs grown at 15°C for 24 hours. (B) c-di-GMP quantification of *V. cholerae* wild-type and Δ3 PDE and Δ6 DGC strains grown at 37°C to an OD₆₀₀ of 0.4 then shifted to 15°C for one hour. Error bars indicate standard deviations of four biological replicates. * $P < 0.05$, ** $P < 0.005$, n.s., $P > 0.05$.

Fig. 9. Comparison of DGC gene expression in *V. cholerae* grown at 37°C and 15°C
 Message levels of DGCs were determined by Real-Time PCR using total RNA isolated from wild-type *V. cholerae* grown in LB incubated at (A) 37°C and 15°C for 24 hours. Impact of low temperature shift on expression was analyzed using total RNA isolated from wild-type *V. cholerae* grown at (B) 37°C to 0.4 OD₆₀₀ then shifted to 15 °C or maintained at 37 °C for one hour. The Pfaffl method was used to compare expression levels of each gene-of-interest to that of 16s rRNA, and relative expression was calculated by normalizing expression at 15°C by that at 37°C. * $P < 0.05$, ** $P < 0.005$, n.s., $P > 0.05$.

Fig. 10. Analysis of c-di-GMP levels in *P. aeruginosa* and *L. monocytogenes* grown at different temperatures

(A) *P. aeruginosa* and (B) *L. monocytogenes* grown at 15°C, 25°C, and 37°C for 24 hours, c-di-GMP was extracted from whole cells and quantified using HPLC-MS/MS. Error bars indicate standard deviations of four biological replicates. * $P < 0.05$, ** $P < 0.005$, n.s., $P > 0.05$.

Table 1

COMSTAT analysis of biofilms formed by wild-type *V. cholerae* grown at 15°C, 25°C, and 37°C in static chambers in LB for 24 hours.

Temperature (°C)	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)		Roughness coefficient
		Average	Maximum	
15	6.15 (0.46)	6.40 (0.28)	17.01 (5.47)	0.37 (0.03)
25	2.12 (0.76)	2.05 (0.66)	9.53 (0.87)	0.85 (0.35)
37	0.31 (0.32)	0.73 (0.92)	6.60 (1.21)	1.67 (0.34)

Table 2

COMSTAT analysis of biofilms formed by wild-type *V. cholerae* overexpressing either a DGC or PDE at 15°C, in static chambers in LB + 0%, 0.05%, or 0.2% arabinose for 24 hours.

Plasmid	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)		Roughness coefficient
		Average	Maximum	
pBAD-0%	6.15 (1.11)	6.88 (0.98)	15.54 (2.57)	0.26 (0.03)
pBAD-0.05%	5.65 (0.92)	5.20 (0.42)	17.89 (2.33)	0.38 (0.08)
pBAD-0.2%	6.37 (0.90)	5.92 (1.06)	16.13 (1.09)	0.35 (0.06)
pcdgA-0%	8.20 (1.37)	8.75 (0.46)	20.53 (0.62)	0.28 (0.02)
pcdgA-0.05%	9.47 (1.12)	9.70 (1.16)	28.18 (0.45)	0.30 (0.09)
pcdgA-0.2%	10.52 (0.88)	10.88 (2.11)	28.77 (0.66)	0.24 (0.07)
pcdgC-0%	5.10 (0.35)	4.84 (0.45)	10.73 (0.40)	0.31 (0.05)
pcdgC-0.05%	4.91 (0.18)	4.055 (0.33)	10.44 (0.62)	0.20 (0.08)
pcdgC-0.2%	4.22 (0.12)	3.59 (0.28)	9.72 (0.80)	0.26 (0.11)

Table 3

COMSTAT analysis of biofilms formed by wild-type *V. cholerae* and DGC/PDE mutants grown at 15°C, in static chambers in LB for 24 hours.

Strain	Gene Name	Domain	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)		Roughness coefficient
				Average	Maximum	
Wild type	N/A	N/A	7.18 (2.38)	7.65 (2.28)	20.97 (3.54)	0.42 (0.08)
VC0137	<i>cdgJ</i>	EAL	8.80 (0.91)	9.22 (0.42)	24.79 (1.45)	0.36 (0.02)
VC0653	<i>rocS</i>	GGDEF-EAL	10.05 (1.24)	10.80 (1.05)	29.48 (2.70)	0.34 (0.06)
VC1067	<i>cdgH</i>	GGDEF	1.81 (0.49)	1.58 (0.35)	6.75 (0.41)	0.96 (0.26)
VC1104	<i>cdgK</i>	GGDEF	4.04 (0.17)	4.14 (0.20)	16.28 (0.21)	0.69 (0.04)
VC1376	<i>cdgM</i>	GGDEF	3.91 (0.83)	3.68 (1.48)	14.52 (0.62)	0.51 (0.23)
VC2285	<i>cdgL</i>	GGDEF	2.67 (0.35)	2.41 (0.42)	8.36 (0.21)	0.90 (0.15)
VC2454	<i>vpvC</i>	GGDEF	7.67 (0.90)	7.96 (0.51)	22.88 (0.83)	0.35 (0.02)
VCA0074	<i>cdgA</i>	GGDEF	6.00 (2.36)	6.41 (2.38)	23.32 (0.62)	1.32 (0.40)
VCA0785	<i>cdgC</i>	GGDEF-EAL	8.82 (0.14)	9.48 (3.30)	24.95 (3.30)	0.35 (0.06)
6 DGC		GGDEF	0.39 (0.06)	0.47 (0.05)	6.01 (0.62)	1.72 (0.05)
3 PDE		EAL	1.33 (0.15)	1.86 (0.45)	27.28 (4.98)	1.11 (0.39)

COMSTAT analysis of biofilms formed by wild type *V. cholerae* and DGC mutants harboring complementation plasmids. Biofilms were grown at 15°C, in static chambers in LB for 24 hours.

Table 4

Strain	Gene Name	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)		Roughness coefficient
			Average	Maximum	
Wild type	vector	7.23 (0.75)	7.61 (1.12)	20.77 (1.33)	0.34 (0.05)
<i>cdgA</i>	vector	4.60 (0.40)	4.84 (0.69)	15.55 (2.59)	0.37 (0.05)
	<i>pcdgA</i>	6.69 (1.55)	7.47 (2.35)	22.35 (6.54)	0.37 (0.04)
<i>cdgH</i>	vector	3.83 (0.37)	3.96 (0.11)	9.97 (1.02)	0.30 (0.02)
	<i>pcdgH</i>	6.10 (0.61)	6.90 (1.32)	14.67 (0.51)	0.33 (0.05)
<i>cdgK</i>	vector	5.07 (1.23)	4.93 (1.52)	14.08 (3.77)	0.40 (0.15)
	<i>pcdgK</i>	7.50 (3.25)	7.74 (3.84)	17.6 (4.62)	0.34 (0.08)
<i>cdgL</i>	vector	1.94 (0.43)	2.42 (0.61)	12.03 (1.64)	0.85 (0.14)
	<i>pcdgL</i>	5.61 (0.92)	5.59 (1.34)	12.04 (1.81)	0.32 (0.01)
<i>cdgM</i>	vector	4.69 (0.92)	5.17 (1.90)	14.08 (4.16)	0.35 (0.05)
	<i>pcdgM</i>	6.13 (1.79)	7.39 (3.08)	19.21 (6.50)	0.40 (0.03)
<i>cdgN</i>	vector	4.47 (0.62)	4.51 (0.91)	12.47 (2.69)	0.36 (0.04)
	<i>pydN</i>	5.74 (1.09)	5.98 (1.51)	15.25 (4.61)	0.32 (0.02)
9 DGC	vector	1.14 (0.05)	1.14 (0.05)	4.69 (0.51)	1.27 (0.03)
	<i>pcdgA</i>	6.57 (0.42)	6.46 (0.23)	21.71 (1.02)	0.36 (0.02)
	<i>pcdgH</i>	1.98 (0.18)	2.35 (0.23)	13.79 (1.02)	0.86 (0.01)
	<i>pcdgK</i>	1.24 (0.13)	1.16 (0.34)	5.28 (0.88)	1.11 (0.03)
	<i>pcdgL</i>	1.89 (0.26)	2.33 (0.27)	10.85 (1.06)	0.86 (0.01)
	<i>pcdgM</i>	1.29 (0.12)	1.92 (0.30)	4.40 (0.88)	1.34 (0.03)
	<i>pydN</i>	2.33 (0.40)	2.22 (0.34)	7.04 (0.88)	0.69 (0.18)