Identification and analysis of the signaling pathways, matrix-digestion enzymes, and motility components controlling *Vibrio cholerae* biofilm dispersal

Andrew A. Bridges\(^1,2\), Chenyi Fei\(^1\), Bonnie L. Bassler\(^1,2*\)

\(^1\)Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
\(^2\)The Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA.
*Corresponding author. Email: bbassler@princeton.edu

Abstract (200 words)

Bacteria alternate between being free-swimming and existing as members of sessile multicellular communities called biofilms. The biofilm lifecycle occurs in three stages: cell attachment, biofilm maturation, and biofilm dispersal. *Vibrio cholerae* biofilms are hyper-infectious and biofilm formation and dispersal are considered central to disease transmission. While biofilm formation is well-studied, almost nothing is known about biofilm dispersal. Here, we conduct an imaging screen for *V. cholerae* mutants that fail to disperse, revealing three classes of dispersal components: signal transduction proteins, matrix-degradation enzymes, and motility factors. Signaling proteins dominated the screen and among them, we focused on an uncharacterized two-component sensory system that we name DbfS/DbfR for Dispersal of Biofilm Sensor/Regulator. Phospho-DbfR represses biofilm dispersal. DbfS dephosphorylates and thereby inactivates DbfR, which permits dispersal. Matrix degradation requires two enzymes: LapG, which cleaves adhesins, and RbmB, which digests matrix polysaccharide. Reorientations in swimming direction, mediated by CheY3, are necessary for cells to escape from the porous biofilm matrix. We suggest that these components act sequentially: signaling launches dispersal by terminating matrix production and triggering matrix digestion and, subsequently, cell motility permits escape from biofilms. This study lays the groundwork for interventions that modulate *V. cholerae* biofilm dispersal to ameliorate disease.
Bacteria transition between existing in the biofilm state, in which cells are members of surface-associated multicellular collectives, and living as free-swimming, exploratory individuals. Biofilms consist of cells surrounded by a self-secreted extracellular matrix that protects the resident cells from threats including predation, antimicrobials, and dislocation due to flow.\textsuperscript{1–3} Biofilms are relevant to human health because beneficial microbiome bacteria exist in biofilms, and, during disease, because pathogens in biofilms evade host immune defenses, thwart medical intervention, and exhibit virulence.\textsuperscript{4–7} The biofilm lifecycle consists of three stages: cell attachment, biofilm maturation, and dispersal (Fig. 1A).\textsuperscript{8} Cells liberated during the dispersal step can disseminate and found new biofilms.\textsuperscript{8} The environmental stimuli and the components facilitating biofilm attachment and maturation have been defined for many bacterial species.\textsuperscript{9} In contrast, little is known about the biofilm dispersal stage.

The model pathogen \textit{Vibrio cholerae} forms biofilms in its aquatic habitat, biofilm cells are especially virulent in mouse models of cholera disease, and biofilms are thought to be critical for cholera transmission.\textsuperscript{10–14} Studies of \textit{V. cholerae} biofilms have predominantly focused on matrix overproducing strains that constitutively exist in the biofilm mode and that do not disperse. This research strategy has propelled understanding of \textit{V. cholerae} biofilm attachment and maturation, revealing that the second messenger cyclic diguanylate (c-di-GMP) is a master regulator of biofilm formation, and that expression of vibrio polysaccharide (vps) biosynthetic genes are required.\textsuperscript{15–17} The strategy of characterizing constitutive biofilm formers, while successful for uncovering factors that promote biofilm formation, has necessarily precluded studies of biofilm dispersal. Here, we employed a microscopy assay that allowed us to monitor the full wild-type (WT) \textit{V. cholerae} biofilm lifecycle. We combined this assay with high-content imaging of randomly mutagenized WT \textit{V. cholerae} to identify genes required for biofilm dispersal. Investigation of the proteins encoded by the genes allowed us to characterize the signaling relays, matrix-digestion enzymes, and motility components required for biofilm dispersal, a key stage in the lifecycle of the global pathogen \textit{V. cholerae}.

\section*{Results}

Previously, we developed a brightfield microscopy assay that allows us to monitor the full WT \textit{V. cholerae} biofilm lifecycle in real time.\textsuperscript{18} In our approach, \textit{V. cholerae} cells are inoculated onto glass coverslips at low cell density and brightfield time-lapse microscopy is used to monitor biofilm progression. WT biofilms reach peak biomass after 8-9 h of incubation and subsequently dispersal occurs and is completed by 12-13 h (Fig. 1B, C). To identify genes required for biofilm dispersal, we combined mutagenesis with high-content imaging of the output of this assay. Specifically, WT \textit{V. cholerae} was mutagenized with Tn5 yielding ~7000 mutants that were arrayed in 96-well plates. Following overnight growth, the mutants were diluted to low cell density in minimal medium, a condition that drives initiation of the biofilm lifecycle. Brightfield images of each well were captured 8 h post-inoculation to assess biofilm maturation and at 13 h to evaluate biofilm dispersal. Mutants that showed no defects in biofilm maturation as judged by the 8 h images but displayed significant remaining biofilm biomass at the 13 h timepoint were identified. To verify phenotypes, candidate mutants were individually reevaluated by time-lapse microscopy. Mutants that accumulated at the bottom of wells due to aggregation or that failed to attach to surfaces were excluded from further analysis, eliminating strains harboring insertions in O-antigen and flagellar genes, respectively. The locations of transposon insertions in the 47 mutants that met our criteria were defined and corresponded to 10 loci. The new genes from the screen fell into
three classes: signal transduction (blue), matrix degradation (green), and motility (red) (Fig. 1A, C). In-frame deletions of each gene were constructed, and the biofilm lifecycles of the deletion mutants were imaged to confirm that the genes are required for biofilm dispersal (Table 1, Supplementary Video 1). We also identified insertions in genes encoding proteins with known roles in biofilm dispersal (i.e., RpoS, quorum sensing), which we excluded from further analysis.\textsuperscript{18,19}

**Figure 1**

![Diagram of V. cholerae biofilm lifecycle]

**Fig. 1.** A high-content imaging screen identifies genes required for *V. cholerae* biofilm dispersal. (A) Schematic illustrating the *V. cholerae* biofilm lifecycle. See text for details. (B) Brightfield image series over time of the WT *V. cholerae* biofilm lifecycle. (C) Top panels: Quantitation of biofilm biomass over time as measured by time-lapse microscopy for WT and representative transposon insertion mutants from each of the three functional categories identified in the screen. Note differences in y-axes scales. Data are represented as means normalized to the peak biofilm biomass of the WT strain. $N = 3$ biological and $N = 3$ technical replicates, ± SD (shaded). a.u., arbitrary unit. Bottom panels: Representative brightfield images of biofilms at the final 16 h timepoint for the strains presented in the top panels.
Proteins involved in signal transduction dominated the screen (7 of 10 loci) and included the ribosome-associated GTPase, BipA, multiple cyclic diguanylate (c-di-GMP) signaling proteins, polyamine signaling proteins, and a putative two-component histidine kinase, Vc1639. The signal transduction mutants displayed different severities in their biofilm dispersal phenotypes. The ∆bipA displayed a modest defect: ~19% of its biofilm biomass remained at 16 h, the final timepoint of our data acquisition, while the WT showed ~6% biomass remaining. By contrast, the ∆vc1639 mutant underwent no appreciable dispersal (Table 1). In the category of matrix degradation, two enzymes were identified, LapG a periplasmic peptidase, and RbmB, a putative polysaccharide lyase (Table 1). A single motility mutant was identified with an insertion in the gene encoding the chemotaxis response regulator cheY3 (Table 1). Below, we carry out mechanistic studies on select mutants from each category to define the functions of the components. Other mutants will be characterized in separate reports.

| Gene       | Function                        | Times Hit | Peak Biomass (vs WT) | Peak Time | % Biomass Remaining (16 h) |
|------------|---------------------------------|-----------|---------------------|-----------|---------------------------|
| WT         | -                               | -         | 1.0 ± 0.2           | 8.7 ± 0.4 h | 6 ± 4%                    |
| bipA (vc2744) | ribosome-associated GTPase | 2         | 1.0 ± 0.2           | 9.6 ± 0.3 h | 19 ± 6%                   |
| cdgG (vc0900) | GGDEF domain containing protein | 1         | 1.1 ± 0.3           | 8.4 ± 0.6 h | 34 ± 13%                  |
| cdlI (vc0658) | c-di-GMP phosphodiesterase | 1         | 0.9 ± 0.2           | 8.7 ± 0.4 h | 17 ± 9%                   |
| rocS (vc0653) | c-di-GMP phosphodiesterase | 1         | 1.3 ± 0.3           | 10.4 ± 0.6 h | 59 ± 13%                  |
| mbaA (vc0703) | polyamine sensor, c-di-GMP phosphodiesterase | 2         | 0.9 ± 0.2           | 9.6 ± 0.3 h | 27 ± 10%                  |
| potD1 (vc1424) | polyamine transporter | 6*        | 1.6 ± 0.2           | 11.9 ± 0.9 h | 90 ± 12%                  |
| dbfS (vc1639) | histidine kinase | 8         | 1.8 ± 0.3           | 14.3 ± 0.9 h | 95 ± 8%                   |
| lapG (vca1081) | peptidase | 3         | 0.8 ± 0.2           | 9.4 ± 0.2 h | 55 ± 12%                  |
| rbmB (vc0929) | polysaccharide lyase | 21        | 0.9 ± 0.2           | 10 ± 0.4 h | 69 ± 12%                  |
| cheY3 (vc2065) | chemotaxis response regulator | 2*        | 1.0 ± 0.2           | 9.1 ± 0.6 h | 21 ± 6%                   |

All ± values represent SD
*Value includes transposon insertions in other genes in this operon
A two-component regulatory system controls *V. cholerae* biofilm dispersal

The mutant from our screen that exhibited the most extreme dispersal phenotype had a transposon in a gene encoding an uncharacterized putative histidine kinase (designated HK), Vc1639 (Table 1). A screen for factors required for *V. cholerae* colonization of the suckling mouse intestine repeatedly identified Vc1639, suggesting that this HK is core to the cholera disease. HKs typically contain periplasmic ligand binding domains and internal catalytic domains that switch between kinase and phosphatase activities based on ligand detection. HKs transmit sensory information to cognate response regulators (RR) by altering RR phosphorylation. RRs, in turn, control gene expression and/or behavior depending on their phosphorylation states. Deletion of vc1639 in *V. cholerae* resulted in an 80% increase in peak biofilm biomass relative to WT and nearly all the biofilm biomass remained at 16 h demonstrating that Vc1639 is essential for biofilm dispersal (Fig. 2A, Table 1). Complementation of the \( \Delta vc1639 \) mutant with vc1639 inserted onto the chromosome at an ectopic locus restored WT biofilm dispersal (Extended Data Fig. 1A). Consistent with the extreme dispersal phenotype of the \( \Delta vc1639 \) mutant, vpsL-lux expression was elevated 10-fold throughout the growth curve in the \( \Delta vc1639 \) strain compared to WT *V. cholerae* (Fig. 2B). vpsL is the first gene in the major extracellular matrix biosynthetic operon showing that Vc1639 signaling regulates matrix production. vc1639 is the final gene in a three gene operon that includes genes encoding a hypothetical protein (Vc1637) and an OmpR family RR (Vc1638) (Fig. 2C). We name Vc1639 DbfS for Dispersal of Biofilm Sensor and we name Vc1638 DbfR for Dispersal of Biofilm Regulator. Domain prediction suggests that DbfS contains two transmembrane domains (TM), a periplasmic sensory domain, and a cytoplasmic HAMP domain that likely transmits ligand-binding-induced conformational changes to regulation of the C-terminal kinase/phosphatase activity (Fig. 2C).

To explore the connection between DbfS and DbfR in the control of biofilm dispersal, we deleted dbfR. Typically, cognate HK and RR null mutants have identical phenotypes. To our surprise, the \( \Delta dbfR \) mutant had no biofilm dispersal defect and progressed through the biofilm lifecycle identically to WT (Fig. 2D). We considered the possibility that some other RR is the partner to DbfS. To test this idea, we constructed the \( \Delta dbfS \Delta dbfR \) double mutant. This strain behaved identically to the \( \Delta dbfR \) strain (Fig. 2D), demonstrating that dbfR is epistatic to dbfS and thus, DbfR indeed functions downstream of DbfS. Moreover, because RRs are typically active when phosphorylated, our results suggest that DbfR must be active in the absence of DbfS. Thus, we reason that phosho-DbfR is the species present in the \( \Delta dbfS \) strain. To verify the hypothesis that phosho-DbfR is responsible for the dispersal defect in the \( \Delta dbfS \) strain, we constructed a non-phosphorylatable allele of DbfR (D51V). The *V. cholerae* dbfR\(^{D51V} \) mutant displayed the WT biofilm dispersal phenotype in the presence and the absence of DbfS (Fig. 2E). DbfR-SNAP fusions showed that SNAP did not interfere with WT DbfR function and that DbfR protein abundance was unchanged in the dbfR\(^{D51V} \) strain relative to WT (Extended Data Fig. 1B, C). Thus, phospho-DbfR causes *V. cholerae* cells to remain in the biofilm state in the \( \Delta dbfS \) mutant. It follows that deletion of dbfS causes biofilm dispersal failure due to loss of DbfS phosphatase activity on DbfR. To test this hypothesis, we assessed *in vivo* DbfR phosphorylation in the presence and absence of DbfS. Phos-tag gel analysis enabled separation and visualization of phosphorylated and dephosphorylated DbfR. In the absence of DbfS, DbfR was phosphorylated and induction of DbfS production caused the phosho-DbfR species to disappear (Fig. 2F). Thus, under our experimental conditions, DbfS functions as a DbfR phosphatase. We infer that some other unknown kinase must exist and phosphorylate DbfR (Fig. 2G). We propose that phospho-DbfR is active, and it drives expression of matrix biosynthetic genes, and increased
**Figure 2**

**A** A two-component system that we name DbfS (HK) and DbfR (RR) regulates *V. cholerae* biofilm dispersal. (A) Representative 16 h images and quantitation of biofilm biomass over time measured by time-lapse microscopy for WT *V. cholerae* and the ∆dbfS (i.e., ∆vc1639) mutant. (B) The corresponding *P*<sub>vpsL-lux</sub> output for strains and growth conditions in A over the growth curve. (C) Top panel: operon structure of the genes encoding the DbfS-DbfR two-component system. Bottom panel: Cartoon of the domain organization of DbfS. TM, transmembrane domain. (D) As in A for the ∆dbfR (i.e., ∆vc1638) strain and for the ∆dbfS ∆dbfR double mutant. (E) As in A for the *dbfR<sup>D51V</sup>* and ∆dbfS dbfR<sup>D51V</sup> strains. (F) Representative Phos-tag gel analysis of DbfR-SNAP in the absence (-arabinose) or presence (+arabinose) of DbfS. Fucose was added to repress DbfR production in the uninduced samples. A phosphorylated protein migrates slower than the same unphosphorylated protein. (G) Proposed model for the DbfS-DbfR phosphorylation cascade regulating biofilm dispersal. OM, outer membrane; IM, inner membrane. In all biofilm measurements, *N* = 3 biological and *N* = 3 technical replicates, ± SD (shaded). a.u., arbitrary unit. For *vpsL-lux* measurements, *N* = 3 biological replicates, ± SD (shaded). RLU, relative light units. Phos-tag gel result is representative of *N* = 3 biological replicates.
matrix production prevents biofilm dispersal. It is possible that phospho-DbfR also controls other genes involved in suppressing biofilm dispersal.

BLAST analysis of the DbfS protein sequence against the *Escherichia coli* K-12 genome revealed limited homology to the cation regulated HK, PhoQ, with 32% sequence identity (E value=1e-41), with the lowest region of similarity in the predicted ligand binding domain. We tested whether the ligands that control PhoQ signal transduction also regulate DbfS-DbfR signaling (Extended Data Fig. 2A-D, Supplemental Discussion). They do not. Thus, DbfS and DbfR are not functionally equivalent to PhoQ and its cognate RR, PhoP, respectively. Thus, DbfS responds to a yet-to-be defined stimulus to regulate biofilm dispersal.

Matrix disassembly mediates *V. cholerae* exit from biofilms

The second group of mutants in our screen harbored insertions in the gene encoding the calcium-dependent periplasmic protease LapG that degrades outer-membrane spanning adhesive proteins and in the gene specifying the extracellular polysaccharide lyase RbmB that degrades the VPS component of the biofilm matrix. The \( \Delta \text{lapG} \) strain exhibited slightly lower peak biofilm biomass compared to WT, with a short delay in the onset of dispersal, and ~55% of its biomass remained at 16 h (Fig. 3A, Table 1). The \( \Delta \text{lapG} \) and the WT strains had similar \( \text{vpsL-lux} \) expression patterns (Fig. 3B) consistent with LapG playing no role in repression of matrix production, but rather functioning downstream in matrix degradation. The LapG mechanism is known: When c-di-GMP concentrations are high, the FrhA and CraA adhesins are localized to the outer membrane where they facilitate attachments that are important for biofilm formation (Fig. 3C). Under this condition, LapG is sequestered and inactivated by the inner membrane c-di-GMP sensing protein LapD. When c-di-GMP levels fall, LapD releases LapG, and LapG cleaves FrhA and CraA facilitating cell detachment from biofilms. Our results are consistent with this mechanism; in the absence of LapG, FrhA and CraA remain intact, and *V. cholerae* cells cannot properly exit the biofilm state. To verify that the established c-di-GMP-dependent regulatory mechanism controls LapG activity in our assay, we deleted \( \Delta \text{lapD} \) (Fig. 3C). Indeed, in the \( \Delta \text{lapD} \) strain, biofilm dispersal occurred prematurely indicating that, without LapD, LapG is not sequestered, and unchecked LapG activity promotes premature adhesin degradation, and, as a consequence, early biofilm disassembly (Fig. 3D). The \( \Delta \text{lapD} \Delta \text{lapG} \) double mutant had the same dispersal phenotype as the \( \Delta \text{lapG} \) single mutant confirming that LapG functions downstream of LapD (Fig. 3D). Lastly, in a reciprocal arrangement, overexpression of \( \text{lapG} \) from an ectopic locus caused peak biofilm formation to decrease by ~65% (Extended Data Fig. 3A) suggesting that enhanced LapG-mediated cleavage of adhesins prematurely released cells from the biofilm. Thus, the conserved Lap pathway, which responds to changes in c-di-GMP levels, facilitates biofilm dispersal in *V. cholerae*.

Regarding the RbmB polysaccharide lyase, the \( \Delta \text{rbmB} \) strain formed biofilms to roughly the same peak biomass as WT, however, it exhibited a 2 h delay in dispersal onset and most of its biomass (~70%) remained at 16 h (Fig. 3E, Table 1). The level of \( \text{vpsL-lux} \) expression in the \( \Delta \text{rbmB} \) mutant was similar to the WT, showing that the RbmB dispersal function does not concern production of VPS (Fig. 3F). Complementation with inducible \( \text{rbmB} \) expressed from an ectopic locus in the \( \Delta \text{rbmB} \) strain caused a ~40% reduction in peak biofilm formation, confirming that RbmB negatively regulates biofilm formation, however the complemented strain retained a modest biofilm dispersal defect, suggesting that the timing or level of \( \text{rbmB} \) expression is critical for WT biofilm disassembly (Extended Data Fig. 3B). To verify that the \( \Delta \text{rbmB} \) dispersal defect stems from the lack of vps degradation, we grew \( \Delta \text{rbmB} \) biofilms for 16 h (i.e., post WT biofilm}
Fig. 3. **Matrix-digesting enzymes mediate *V. cholerae* biofilm dispersal.** (A) Representative 16 h images and quantitation of biofilm biomass over time measured by time-lapse microscopy for WT *V. cholerae* and the ΔlapG mutant. (B) The corresponding *P* <sub>vpsL-lux</sub> output for strains and growth conditions in A over the growth curve. (C) Schematic representing the LapG mechanism. (D) As in A for the WT, the ΔlapD single mutant, and the ΔlapD ΔlapG double mutant. (E) As in A for the WT and the ΔrbmB mutant. (F) As in B for WT *V. cholerae* and the ΔrbmB mutant. (G) Representative images and quantitation of WGA-txRed signal in ΔlapG and ΔrbmB biofilms 16 h post-inoculation. To account for differences in biomass, the WGA-txRed signal was divided by the 4', 6-diamidino-2-phenylindole (DAPI) signal in each biofilm. Values were normalized to the mean signal for the ΔlapG strain. >100 individual biofilms were quantified for each strain. An unpaired t-test was performed for statistical analysis, with **** denoting p < 0.0001. (H) Proposed model for the role of RbmB in biofilm dispersal. Gray lines represent the polysaccharide matrix. In all cases, *N* = 3 biological and *N* = 3 technical replicates, ± SD (shaded). a.u., arbitrary unit. For *vpsL-lux* measurements, *N* = 3 biological replicates, ± SD (shaded). RLU, relative light units. OM, outer membrane; IM, inner membrane.
dispersal completion), and subsequently fixed and stained the non-dispersed biofilms with wheat germ agglutinin conjugated to Texas Red (WGA-txRed), which binds to N-acetylglucosamine sugars in the VPS matrix. We used the ΔlapG mutant as our control since its biofilm dispersal phenotype should not involve changes in VPS. On average, the ΔrbmB mutant exhibited ~6x more WGA-txRed signal than the ΔlapG mutant (Fig. 3G). Collectively, our results show that the non-dispersed ΔlapG biofilms contain little VPS, consistent with possession of functional RbmB, while non-dispersed ΔrbmB biofilms contain excess VPS due to the lack of RbmB-mediated polysaccharide digestion. Thus, we suggest that RbmB-directed VPS disassembly is critical for proper biofilm disassembly (Fig. 3H).

Extracellular DNA (eDNA) is a component of the V. cholerae biofilm matrix and two DNAses secreted by V. cholerae, Dns and Xds, digest eDNA. Although we did not identify dns and xds in our screen, we nonetheless investigated whether they contributed to biofilm dispersal. Neither the Δdns and the Δxds single mutants, nor the Δdns Δxds double mutant displayed a biofilm dispersal defect in our assay (Extended Data Fig. 3C), suggesting that eDNA digestion is not required for dispersal. In a similar vein, we did not identify genes encoding the eight V. cholerae extracellular proteases that could degrade matrix proteins. Consistent with this finding, measurement of the phenotypes of mutants deleted for each extracellular protease gene showed that none exhibited a dispersal defect. Thus, no single extracellular protease is required for biofilm dispersal (Extended Data Fig. 3D). It remains possible that proteases contribute to biofilm dispersal by functioning redundantly. Together, our results indicate that two enzymes, LapG and RbmB, are the primary matrix degrading components that enable biofilm dispersal.

Reorientations in swimming direction are required for biofilm dispersal.

The final category of genes identified in our screen are involved in cell motility. As noted above, non-motile mutants were excluded from analysis because they are known to be impaired in surface attachment. Nonetheless, we identified a mutant containing a transposon insertion in cheY3 as defective for biofilm dispersal. cheY3 is one of the five V. cholerae cheY genes specifying chemotaxis RR proteins. Notably, cheY3 is the only V. cholerae cheY homolog required for chemotaxis. The ΔcheY3 mutant exhibited similar peak biofilm timing and biomass as WT V. cholerae, however, ~21% biomass remained at 16 h (Fig. 4A, Table 1). Expression of vpsL-lux in the mutant was identical to the WT indicating that the dispersal phenotype was not due to elevated matrix production (Fig. 4B). The V. cholerae default motor rotation direction is counterclockwise (CCW), which fosters smooth, straight swimming. Transition to clockwise (CW) motor rotation causes reorientations in swimming direction. Phospho-CheY3 binds to the flagellar motor switch complex to mediate the change from CCW to CW rotation. Thus, the ΔcheY3 mutant is non-chemotactic and the cells are locked in the CCW, straight swimming mode (Fig. 4C). We reasoned that the ΔcheY3 mutant dispersal defect could stem from an inability to chemotact or from an inability to reorient swimming direction. To distinguish between these possibilities, we examined biofilm dispersal in a V. cholerae mutant carrying a cheY3 allele, cheY3D16K, Y109W (henceforth, cheY3* that locks the motor into CW rotation and so also disrupts chemotaxis. cheY3* cells undergo frequent reorientations and are unable to swim in smooth straight runs (Fig. 4C). The cheY3* strain had WT biofilm dispersal capability. Thus, being chemotactic is not required for V. cholerae to exit biofilms (Fig. 4A).

We reasoned that analysis of the unique motility characteristics of our strains could reveal
the underlying causes of the $\Delta$cheY3 biofilm dispersal defect. We measured the turning frequencies and swimming velocities of the WT, $\Delta$cheY3, and cheY3* V. cholerae strains. Consistent with previous reports, these three mutants exhibited notable differences: on average, the WT turned once every 3 s, the $\Delta$cheY3 mutant turned less than once every 40 s, and the cheY3* strain turned once every 0.5 s (Fig. 4C and D). The cheY3* strain displayed slightly lower average swimming velocity than the WT and $\Delta$cheY3 strains, due to its high turning frequency as turning necessarily involves a decrease in velocity (Fig. 4E). Together, these results suggest that the low turning frequency of the $\Delta$cheY3 mutant is responsible for the biofilm dispersal defect. We propose that if cells do not frequently change their direction of motion, they become trapped by the biofilm matrix mesh which compromises their ability to escape (Fig. 4F). Indeed, in other bacteria, straight-swimming mutants are deficient in traversing fluid-filled porous media compared to WT organisms that can reorient. To circumvent this problem, we employed phenamil, an inhibitor of the Na$^+$-driven V. cholerae flagellar motor, which, as expected, dramatically reduced planktonic cell motility (Extended Data Fig. 4). To assess the role of swimming motility in biofilm dispersal, we first allowed WT V. cholerae cells to undergo biofilm formation for 5 h, at which point we perfused DMSO or phenamil into the incubation chamber (Fig. 4G). Following phenamil treatment, the WT strain displayed a dispersal defect nearly identical to that of the $\Delta$cheY3 mutant. Additionally, phenamil treatment of the $\Delta$cheY3 mutant did not further impair its biofilm dispersal. Together, these results demonstrate that swimming motility is crucial for V. cholerae biofilm dispersal and an inability to reorient is as detrimental to dispersal as a complete lack of flagellar motility.

**Discussion**

In this study, we developed a high-content imaging screen that allowed us to identify components required for V. cholerae biofilm dispersal. We categorized the identified components into three classes: signal transduction, matrix disassembly, and cell motility. We propose that the three functional categories represent the chronological steps required for the disassembly of a biofilm: First, the stimuli that activate dispersal must accumulate. Subsequently, the gene expression pattern established by detection of these stimuli must repress biofilm matrix production and activate production of enzymes required to digest the biofilm matrix. Finally, cells must escape through the partially digested, porous matrix which requires changes in the direction of movement. Together, these steps ensure that when environmental conditions are appropriate, V. cholerae cells can exit the sessile lifestyle and disseminate to new terrain that is ripe for biofilm formation or, alternatively, during disease, to a new host. One can now imagine targeting the functions identified in this work for small-molecule disruption of the V. cholerae biofilm lifecycle, possibly guiding the development of treatments to reduce the duration of V. cholerae infection or to prevent transmission.
Fig. 4. Reorientations in swimming direction are required for *V. cholerae* biofilm dispersal. (A) Representative 16 h images and quantitation of biofilm biomass over time measured by time-lapse microscopy for WT *V. cholerae*, the ΔcheY3 mutant, and the cheY3^{D16K, Y109W} (cheY3*) mutant. (B) The corresponding *P*_{vpsL-lux} output for WT and the ΔcheY3 strain over the growth curve. (C) Representative, randomly colored, single-cell locomotion trajectories for the strains in A. (D) Turning frequencies of the strains in A. (E) Measured swimming velocities of the strains in A. (F) Proposed model for the role of motility and reorientation in biofilm dispersal. (G) Quantitation of biofilm biomass over time for WT and the ΔcheY3 mutant following treatment with DMSO or the motility inhibitor, phenamil supplied at 5 h post-inoculation. For biofilm biomass assays, *N* = 3 biological and *N* = 3 technical replicates, ± SD (shaded). a.u., arbitrary unit. For *vpsL-lux* measurements, *N* = 3 biological replicates, ± SD (shaded). RLU, relative light units. For motility measurements, 45-125 individual cells of each strain were tracked. In panels D and E, unpaired t-tests were performed for statistical analysis, with *P* values denoted as *P* < 0.05; **P** < 0.01; ***P** < 0.001; ****P** < 0.0001; n.s., *P* > 0.05.
Materials and Methods

Bacterial Strains and Reagents

The *V. cholerae* parent strain used in this study was WT O1 El Tor biotype C6706str2. Antibiotics were used at the following concentrations: polymyxin B, 50 µg/mL; kanamycin, 50 µg/mL; spectinomycin, 200 µg/mL; and chloramphenicol, 1 µg/mL. Strains were propagated in lysogeny broth (LB) supplemented with 1.5% agar or in liquid LB with shaking at 30°C. All strains used in this work are reported in Supplementary Table 1. Unless otherwise stated, exogenous compounds were added from the onset of biofilm initiation. The antimicrobial peptide C18G (VWR) was added at 5 µg/mL. Phenamil (Sigma) was prepared in DMSO and added 5 h post biofilm inoculation to a final concentration of 50 µM. L-arabinose (Sigma) was prepared in water and added at 0.2%.

DNA Manipulation and Strain Construction

To produce linear DNA fragments for natural transformations, splicing overlap extension PCR was performed using iProof polymerase (Bio-Rad, Hercules, CA, USA) to combine DNA pieces. Primers and gene fragments used in this study are reported in Supplementary Table 2. In all cases, ~3 kb of upstream and downstream flanking regions of homology were generated by PCR from *V. cholerae* genomic DNA and were included to ensure high chromosomal integration frequency. DNA fragments that were not native to *V. cholerae* were synthesized as g-blocks (IDT, Coralville, IA, USA).

All *V. cholerae* strains generated in this work were constructed by replacing genomic DNA with DNA introduced by natural transformation as previously described. PCR and Sanger sequencing were used to verify correct integration events. Genomic DNA from recombinant strains was used for future co-transformations and as templates for PCR to generate DNA fragments, when necessary. Deletions were constructed in frame and eliminated the entire coding sequences. The exceptions were *mbaA*, *dbfS*, and *dbfR*, which each overlap with another gene in their operons. In these cases, portions of the genes were deleted ensuring that adjacent genes were not perturbed. For *tagA*, the first 103 base pairs, including the nucleotides specifying the start codon, were deleted. All strains constructed in this study were verified by sequencing at Genewiz.

Microscopy and Mutant Screening

The biofilm lifecycle was measured using time-lapse microscopy as described previously. All plots were generated using ggplot2 in R. To generate the library of *V. cholerae* insertion mutants for the dispersal screen, the WT parent strain was mutagenized with Tn5 as previously described. Mutants were selected by growth overnight on LB plates containing polymyxin B and kanamycin. The next day, mutant colonies were arrayed into 96-well plates containing 200 µL of LB medium supplemented with polymyxin B and kanamycin using an automated colony-picking robot (Molecular Devices). The arrayed cultures were grown in a plate-shaking incubator at 30°C covered with breathe-easy membranes to minimize evaporation. After 16 h of growth, the arrayed cultures were diluted 1:200,000 into 96-well plates containing M9 medium supplemented with glucose and casamino acids. Diluted cultures were incubated statically at 30°C for 8 h (to achieve peak biofilm biomass), at which point, images of each well were captured on a Nikon Ti-E inverted microscope using transmitted-light bright-field illumination,
a 10× Plan Fluor (NA 0.3) objective lens, and an Andor iXon 897 EMCCD camera. Automated image acquisition was performed using NIS-Elements software v5.11.02 and the NIS-Elements Jobs Module to acquire images at four positions within each well to account for heterogeneity within samples. To maintain the focal plane between wells, the Nikon Perfect Focus System was used. After performing microscopy at the 8 h timepoint, 96-well plates were returned to the incubator. To assess biofilm dispersal, a second set of images of the same samples was acquired at 13 h post inoculation. Mutants that displayed biofilm growth at the 8 h timepoint but failed to disperse by the 13 h timepoint were subcultured, grown overnight, and subsequently re-imaged using the time-lapse approach described above to assess their biofilm lifecycles in real-time. Mutants that exhibited biofilm dispersal defects after this reassessment step were analyzed for the locations of transposon insertions using arbitrary PCR.

vpsL-lux Transcription Assay

Three colonies of each strain to be analyzed were individually grown overnight in 200 μL LB with shaking at 30°C in a 96-well plate covered with a breathe-easy membrane. The following morning, the cultures were diluted 1:5,000 into fresh M9 medium supplemented with glucose and casamino acids. The plates were placed in a BioTek Synergy Neo2 Multi-Mode reader (BioTek, Winooski, VT, USA) under static growth conditions at 30°C. Both OD$_{600}$ and bioluminescence from vpsL-lux were simultaneously measured at 15 min time intervals. Results were exported to R, and light values were divided by OD$_{600}$ to produce relative light units (RLUs). Results from replicates were averaged and plotted using ggplot2 in R.

VPS Quantitation

To assess VPS levels in non-dispersed biofilms using WGA-txRED, biofilms were grown for 16 h and subsequently washed 3 times with 1× phosphate buffered saline (PBS), and fixed for 10 min with 3.7% formaldehyde in 1× PBS. After fixation, samples were washed 5 times with 1× PBS and subsequently incubated with a solution containing 1 μg/mL WGA-txRED (ThermoFisher Scientific), 1 μg/mL 4', 6-diamidino-2-phenylindole (DAPI), and 1% bovine serum albumin in 1× PBS for 1 h with shaking at 30°C in the dark. After incubation, samples were washed 5 more times with 1× PBS before imaging. Confocal microscopy was performed on a Leica DMi8 SP-8 point scanning confocal microscope (Leica, Wetzlar, Germany) with the pinhole set to 1.0 airy unit. The light source for DAPI was a 405 laser and the light source used to excite WGA-txRED was a tunable white-light laser (Leica; model #WLL2; excitation window = 470–670 nm) set to 595 nm. Biofilms were imaged using a 10× air objective (Leica, HC PL FLUOTAR; NA: 0.30). Sequential frame scanning was performed to minimize spectral bleed-through in images. Emitted light was detected using GaAsP spectral detectors (Leica, HyD SP), and timed gate detection was employed to minimize the background signal. Image analyses were performed in FIJI software (Version 1.52p). Biofilms were segmented in the DAPI channel using an intensity threshold and the intensities of each channel were measured. The same threshold was applied to all images. WGA-txRED signal was divided by DAPI signal to achieve the normalized WGA signal.

Motility Assay

To prevent biofilm formation during measurements of swimming velocities and turning frequencies for the WT, ΔcheY3, and cheY3* strains, vpsL was deleted. Each strain was grown
for 16 h in LB medium and the following day, cells were diluted to \( \text{OD}_{600} = 0.001 \) in M9 medium supplemented with glucose and casamino acids. Subsequently, diluted cultures were dispensed in 200 µL aliquots into glass-coverslip bottomed 96-well plates (MatTek, Ashland, MA, USA). After a period of 1 h, during which time cells were allowed to adhere to the coverslips, wells were washed 8 times with fresh medium to remove unattached cells. The plates were incubated at 25°C for 3 h, and imaging was performed using the brightfield setup described above for the biofilm dispersal screen. In this case, the frame interval was 50 msec and imaging was conducted at a distance of ~100 µm into the sample. Images were smoothed, background corrected, and imported into the TrackMate (v.5.2.0) plugin in FIJI. Cells were detected with a Laplacian of Gaussian (LoG) detector and were subsequently tracked using the simple Linear Assignment Problem (LAP) approach. To exclude non-motile cells from our analyses in Fig. 4C-E objects with velocities under 40 µm/sec were eliminated. Analyses and plotting of swimming velocities and turning frequencies were performed in MATLAB (The Mathworks, Inc.). Local curvatures for single-cell locomotion trajectories were calculated as described. 

**Phos-tag Gel Analysis**

To monitor DbfR and phospho-DbfR via SDS-PAGE, the endogenous \( \text{dfbR} \) gene was replaced with \( \text{dbfR-SNAP} \) in the \( \Delta \text{dbfS} \) strain, and \( \text{P}_{\text{BAD}}-\text{dbfS} \) was introduced at the ectopic locus, \( \text{vc}1807 \). To assess DbfR-SNAP phosphorylation in the absence and presence of DbfS, overnight cultures of the strain were diluted 1:1000 and subsequently grown for 4 h at 30°C with shaking to an \( \text{OD}_{600} \sim 0.6 \). To each culture, 1 µM SNAP-Cell TMR Star (New England Biolabs) was added to label the SNAP tag, and the culture was subsequently divided into two tubes. To one tube, 0.2% D-fucose was added, and to the other, 0.2% L-arabinose was added to repress and induce DbfS production, respectively. The cultures were returned to 30°C with shaking. After 1 h, the cells were collected by centrifugation for 1 min at 13,000 rpm. Lysis and solubilization were carried out as rapidly as possible. Briefly, cells were chemically lysed by resuspension to \( \text{OD}_{600} = 1.0 \) in 40 µL Bug Buster (Novagen) for 5 min at 25°C with intermittent vortex. The cell lysate was solubilized at 25°C in 1.5× SDS-PAGE buffer for 5 min also with intermittent vortex. Samples were immediately loaded onto a cold 7.5% SuperSep™ Phos-tag™ (50 µM/L) gel (FUJIFILM Wako Pure Chemical, 198-17981). Electrophoresis was carried out at 100 V at 4°C until the loading buffer exited the gel. Gel images were captured on an ImageQuant LAS 4000 imager (GE Healthcare) using a Cy3 filter set.
Extended Data Fig. 1. **Complementation, functional tagging, and mutagenesis of the DbfS-DbfR two-component system.** (A) Quantitation of biofilm biomass over time measured by time-lapse microscopy for the ΔdbfS, P_BAD-dbfs strain following addition of water (Ctrl) or 0.2% arabinose. (B) As in A for SNAP-tagged DbfR in the WT and ΔdbfS strains. (C) Top panel: representative in-gel SDS-PAGE fluorescence following electrophoresis of *V. cholerae* cell lysates containing WT DbfS-SNAP or DbfSD51V-SNAP that had been incubated with SNAP-Cell TMR Star. Bottom panel: Coomassie stained loading control (LC). For all biofilm measurements, N = 3 biological and N = 3 technical replicates, ± SD (shaded). a.u., arbitrary unit.
Extended Data Fig. 2. **DbfS is not functionally equivalent to PhoQ.** (A) Alignment of the sensory domains of PhoQ from *E. coli*, *S. enterica*, and *P. aeruginosa* against that of *V. cholerae* DbfS. Black boxes indicate residues involved in Mg$^{2+}$ binding in PhoQ. (B) Quantitation of biofilm biomass over time measured by time-lapse microscopy in high magnesium (10 mM) and limiting magnesium (10 µM) conditions for WT *V. cholerae* and the ΔdbfR strain. (C) The corresponding P$_{vpsL}$-lux outputs for strains and growth conditions in B over the growth curve. (D) As in B except following the addition of water or 5 µg/mL C18G. In all cases, N = 3 biological and N = 3 technical replicates, ± SD (shaded). a.u., arbitrary unit. For vpsL-lux measurements, N = 3 biological replicates, ± SD (shaded). RLU, relative light units.
Extended Data Fig. 3. **Introduction of lapG and rbmB complements the ΔlapG and ΔrbmB biofilm defects, respectively, and assessment of the roles of extracellular DNAses and secreted proteases in V. cholerae biofilm dispersal.** (A) Quantitation of biofilm biomass over time measured by time-lapse microscopy for the ΔlapG P\textsubscript{BAD}-lapG strain following addition of water (Ctrl) or 0.2% arabinose. (B) As in A, but for the ΔrbmB P\textsubscript{BAD}-rbmB strain. (C) Quantitation of biofilm biomass over time measured by time-lapse microscopy for WT V. cholerae and mutants lacking the designated DNAses. (D) Quantitation of biofilm biomass over time measured by time-lapse microscopy for WT V. cholerae and mutants lacking the designated proteases. In all cases, \( N = 3 \) biological and \( N = 3 \) technical replicates, ± SD (shaded). a.u., arbitrary unit.
Extended Data Fig. 4. **Phenamil inhibits V. cholerae motility.** Mean squared displacement (MSD) of cell trajectories versus lag time for WT *V. cholerae* treated with DMSO solvent or 50 µM phenamil.
Supplemental Discussion

_DbfS is not equivalent to PhoQ_

In _E. coli_, low Mg$^{2+}$ and cationic peptides activate PhoQ kinase activity.\(^{40}\) Sequence alignment of the DbfS sensory domain with that from PhoQ of _E. coli_, _Salmonella enterica_, and _Pseudomonas aeruginosa_ revealed that DbfS lacks all of the key residues involved in Mg$^{2+}$ binding (Extended Data Fig. 2A).\(^{41}\) To test if Mg$^{2+}$ alters DbfS activity, we measured the _V. cholerae_ biofilm lifecycle in response to low Mg$^{2+}$ conditions in WT _V. cholerae_ and in the \(\Delta dbfR\) mutant. If, analogous to PhoQ, DbfS kinase activity is activated by low Mg$^{2+}$, when Mg$^{2+}$ is limiting, WT _V. cholerae_ should exhibit an altered biofilm dispersal phenotype while the \(\Delta dbfR\) mutant would be impervious to Mg$^{2+}$ changes.\(^{40}\) Extended Data Fig. 2B shows that Mg$^{2+}$ limitation does indeed inhibit _V. cholerae_ biofilm dispersal, however, inhibition occurs in both the WT and the \(\Delta dbfR\) strains. Mg$^{2+}$ limitation did not alter _vpsL-lux_ expression in either strain (Extended Data Fig. 2C). Thus, Mg$^{2+}$ does not control DbfS activity. We obtained the same results following exogenous addition of the cationic peptide C18G (Extended Data Fig. 2D). Together, these results demonstrate that DbfS does not respond to the ligands that control PhoQ activity.
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### Supplementary Table 1

Strains used in this study.

| Strain Number | Genotype | Plasmid | Antibiotic Resistance | Parent |
|---------------|----------|---------|-----------------------|--------|
| BB_Vc_0090   | WT O1 El Tor biotype C6706str2 | -       | Sm                    | -      |
| AB_Vc_761    | Δvc1807::Cm\(^{R}\) (Referred to as WT) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_705    | ΔcheY Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_708    | ΔbpA Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_839    | ΔmbaA Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_711    | ΔpotD1 Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_757    | ΔlapG Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_758    | ΔrocS Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_775    | ΔdbfS Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_776    | ΔcdgΔ Δvc1807::Cm\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_485    | ΔrbmB Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| AB_Vc_801    | Δvc1807::Kan\(^{R}\) | PEVS143−P\(_{BAD}\)-lux::Cm\(^{R}\) | Sm, Cm, Kan | AB_Vc_479 |
| AB_Vc_825    | ΔcheY Δvc1807::Kan\(^{R}\) | PEVS143−P\(_{BAD}\)-lux::Cm\(^{R}\) | Sm, Cm, Kan | AB_Vc_705 |
| AB_Vc_829    | ΔlapG Δvc1807::Kan\(^{R}\) | PEVS143−P\(_{BAD}\)-lux::Cm\(^{R}\) | Sm, Cm, Kan | AB_Vc_757 |
| AB_Vc_802    | ΔrbmB Δvc1807::Kan\(^{R}\) | PEVS143−P\(_{BAD}\)-lux::Cm\(^{R}\) | Sm, Cm, Kan | AB_Vc_485 |
| AB_Vc_815    | ΔdbfS Δvc1807::Kan\(^{R}\) | PEVS143−P\(_{BAD}\)-lux::Cm\(^{R}\) | Sm, Cm, Kan | AB_Vc_775 |
| AB_Vc_773    | ΔdbfS Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_701    | ΔdbfS Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_788    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_789    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_863    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_866    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_879    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_881    | dbfS\(^{REV}\) Δvc1807::Kan\(^{R}\) | -       | Sm, Cm               | BB_Vc_0090 |
| AB_Vc_859    | ΔlapG Δvc1807::P\(_{BAD}\)-lapG::Spec\(^{R}\) | -       | Sm, Spec             | AB_Vc_757 |
| AB_Vc_898    | ΔlapD Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| AB_Vc_900    | ΔlapD Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| AB_Vc_862    | ΔrbmB Δvc1807::P\(_{BAD}\)-rbmB::Spec\(^{R}\) | -       | Sm, Spec             | AB_Vc_485 |
| BB_Vc_0252   | Δdns | -       | Sm                    | BB_Vc_0090 |
| BB_Vc_0253   | Δxls | -       | Sm                    | BB_Vc_0090 |
| BB_Vc_0254   | Δdns Δxls | -       | Sm                    | BB_Vc_0090 |
| MJ_552       | ΔhapA Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| MJ_553       | ΔprtV Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| MJ_554       | ΔvesA Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| MJ_555       | ΔvesB Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| MJ_562       | ΔvesC Δvc1807::Kan\(^{R}\) | -       | Sm, Kan              | BB_Vc_0090 |
| Strain   | Description                                                                 | Resistance | Plate     |
|----------|-----------------------------------------------------------------------------|------------|-----------|
| MJ_561   | Δlap ΔlapX lacZ::Ptac-mKO Δvc1807::KanR                                     | -          | Sm, Kan   |
| AB_Vc_792| ΔtagA Δvc1807::CmR                                                           | -          | Sm, Cm    |
| AB_Vc_715| cheY<sup>D16K, Y109W</sup> Δvc1807::KanR                                    | -          | Sm, Kan   |
| AB_Vc_732| ΔvpsL Δvc1807::Ptac-mScarlet::SpecR                                         | -          | Sm, Spec  |
| AB_Vc_735| ΔcheY ΔvpsL Δvc1807::Ptac-mScarlet::SpecR                                    | -          | Sm, Spec  |
| AB_Vc_745| cheY<sup>D16K, Y109W</sup> ΔvpsL Δvc1807::Ptac-mScarlet::SpecR             | -          | Sm, Spec  |

BB_Vc_0090
AB_Vc_705
AB_Vc_715
### Supplementary Table 2
DNA oligonucleotides and gene fragments used in this study.

| Oligo # | Name | Purpose | Direction | 5' to 3' Sequence |
|---------|------|---------|-----------|-------------------|
| 551     | cheY _3000up | Cloning at cheY3 locus | F | CAAGCGTTACAACCTCGACGCTAG |
| 552     | cheY _3000down | Cloning at cheY3 locus | R | CACAACGAGACTCGGCGCTGAC |
| 553     | cheY _100up | Cloning at cheY3 locus | F | GGTGAGGTATCTTGAGTTAGTGATCCTC |
| 554     | cheY _100down | Cloning at cheY3 locus | R | CACTGAAGCGCTCAATCCTGAAG |
| 555     | cheY _B | cheY3 deletion | R | GAGCACCTTTTGCGAGCAAAAGCTGATTTGGAGATCAGTGATATTTAGTCATTCC |
| 556     | cheY _C | cheY3 deletion | F | GGAATGACTAAATATCACTGATCTCAGAGGGTTTTGCTGCGGCAAAAGGTGCTCTATTCACAGCGCAAAAG |
| 557     | cheY _2700up | Cloning at cheY3 locus | F | GATGACGCGTGTCAGTTTGCAATCGAG |
| 558     | cheY _2700down | Cloning at cheY3 locus | R | CTTCCGGGCACTACGTTTGAAGTGAAC |
| 559     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 560     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 561     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 562     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
| 563     | cheY _up_R | Cloning at cheY3 locus | R | CAGCCTCGTGAGCAGACTCTCATGAGGTAGGATCATAC |
| 564     | cheY _down_R | Cloning at cheY3 locus | R | GGCCTTGTGCTGCCGCAAAGGTGCTCTATTC |
| 565     | cheY _2700up | Cloning at cheY3 locus | F | GATGACCGTGTCAGTTTGCAATCGAG |
| 566     | cheY _2700down | Cloning at cheY3 locus | R | CTTCCGGGCACTACGTTTGAAGTGAAC |
| 567     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 568     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 569     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 570     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
| 571     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 572     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 573     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 574     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
| 575     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 576     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 577     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 578     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
| 579     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 580     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 581     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 582     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
| 583     | cheY _up_R | Cloning at cheY3 locus | R | GATTTTGAGATCGATATTTAGCATCAGTCGAG |
| 584     | cheY _down_R | Cloning at cheY3 locus | R | GGATCTTTGCTGCCGCAAAGGTGCTCTATTC |
| 585     | cheY _D16K_Y109 W | Gblock for introduction of cheY3 point mutation | F | GTTAAAGTTCTTTGAGCTCCGAGAAGATCAATATCTCTGAGCTATCTACTGAGCTCACCAGCTGAGTCCTAAATCCGAGTAATTTGGAGATCAGTGATATTTAGTCATTCC |
| 586     | cheY _2700down | Cloning at cheY3 locus | R | CTTCGGTCTAACCAGTATTTGGATAAGTGAAC |
|   | Gene_1 | Cloning at Gene_1 locus |   | Gene_2 | Cloning at Gene_2 locus |
|---|--------|------------------------|---|--------|------------------------|
| 577 | mbaA_100up | Cloning at mbaA locus | F | lapG_3000up | Cloning at lapG locus |
| 578 | mbaA_100down | Cloning at mbaA locus | R | lapG_3000down | Cloning at lapG locus |
| 539 | potD1_3000up | Cloning at potD1 locus | F | lapG_100up | Cloning at lapG locus |
| 540 | potD1_3000down | Cloning at potD1 locus | R | lapG_100down | Cloning at lapG locus |
| 541 | potD1_100up | Cloning at potD1 locus | F | potD1_2700up | Cloning at potD1 locus |
| 542 | potD1_100down | Cloning at potD1 locus | R | potD1_2700down | Cloning at potD1 locus |
| 543 | potD1_B | potD1 deletion | R | potD1_B | potD1 deletion |
| 544 | potD1_C | potD1 deletion | F | potD1_C | potD1 deletion |
| 569 | potD1_2700up | Cloning at potD1 locus | F | lapG_3000up | Cloning at lapG locus |
| 570 | potD1_2700down | Cloning at potD1 locus | R | lapG_3000down | Cloning at lapG locus |
| 602 | lapG_3000up | Cloning at lapG locus | F | lapG_3000up | Cloning at lapG locus |
| 603 | lapG_2700up | Cloning at lapG locus | F | lapG_2700down | Cloning at lapG locus |
| 604 | lapG_100up | Cloning at lapG locus | F | lapG_100down | Cloning at lapG locus |
| 606 | lapG_B | Cloning at lapG locus | R | lapG_B | Cloning at lapG locus |
| 607 | lapG_C | Cloning at lapG locus | F | lapG_C | Cloning at lapG locus |
| 608 | lapG_100down | Cloning at lapG locus | R | lapG_100down | Cloning at lapG locus |
| 609 | lapG_3000down | Cloning at lapG locus | R | lapG_3000down | Cloning at lapG locus |
| 774 | lapD_3000up | Cloning at lapD locus | F | lapD_3000up | Cloning at lapD locus |
| 775 | lapD_2700up | Cloning at lapD locus | F | lapD_2700up | Cloning at lapD locus |
| 776 | lapD_100up | Cloning at lapD locus | F | lapD_100up | Cloning at lapD locus |
| 777 | lapD_B | Cloning at lapD locus | R | lapD_B | Cloning at lapD locus |
| 778 | lapD_C | Cloning at lapD locus | F | lapD_C | Cloning at lapD locus |
| 779 | lapD_100down | Cloning at lapD locus | R | lapD_100down | Cloning at lapD locus |
| 780 | lapD_2700down | Cloning at lapD locus | R | lapD_2700down | Cloning at lapD locus |
| 781 | lapD_3000down | Cloning at lapD locus | R | lapD_3000down | Cloning at lapD locus |
| 784 | lapDG_B | lapDG deletion | R | lapDG_B | lapDG deletion |
| 785 | lapDG_C | lapDG deletion | F | lapDG_C | lapDG deletion |
| 610 | rocS_3000up | Cloning at rocS locus | F | rocS_3000up | Cloning at rocS locus |
| 611 | rocS_2700up | Cloning at rocS locus | F | rocS_2700up | Cloning at rocS locus |
| 612 | rocS_100up | Cloning at rocS locus | F | rocS_100up | Cloning at rocS locus |
| 613 | rocS_B | Cloning at rocS locus | R | rocS_B | Cloning at rocS locus |
| 614 | rocS_C | Cloning at rocS locus | F | rocS_C | Cloning at rocS locus |
| 615 | rocS_100down Cloning at rocS locus | R | GAACCGGATATAAAACGCGATCGGCA |
| 616 | rocS_2700down Cloning at rocS locus | R | GTCACTTGATTAAGCCGGTGATTTTC |
| 617 | rocS_3000down Cloning at rocS locus | R | GCTGTGGTCTTGGCAGTCTCG |
| 533 | vc1639_3000up Cloning at dbfS locus | F | GCTTAGTATGCGAAGCTTGG |
| 534 | vc1639_3000down Cloning at dbfS locus | R | GTGCACTTGATTAAGCCGGTGATTTTC |
| 535 | vc1639_100up Cloning at dbfS locus | F | CAAGATTTTGACCGCGATTCCAATAC |
| 536 | vc1639_100down Cloning at dbfS locus | R | GTAGAATTTTCACACCTATGGAG |
| 626 | vc1639_Real_B dbfS deletion | R | CAACTGAAATCCGTTTTTGCACCGGATTTAATGCGGATCAGCAACTCAGTTGG |
| 627 | vc1639_Real_C dbfS deletion | F | CTTTGGGATACATTGGGTACATGCCCATTAAATAGCGGATTTACATGG |
| 559 | vc1639_2700up Cloning at dbfS locus | F | CAATCGGTGGTGCGCAACTTATCTGAG |
| 560 | vc1639_2700down Cloning at dbfS locus | R | GTAAATGGACTGGAGCAGAATTTAGCTGCG |
| 527 | vc1638_3000up Cloning at dbfR locus | F | GTAGGCTCTTCTGCGGACCTTGTGAG |
| 528 | vc1638_3000down Cloning at dbfR locus | R | GTCCATAACCTTGGCGAAGCTGAT |
| 529 | vc1638_100up Cloning at dbfR locus | F | GACAATCAGTCTTGGTGCAGAATACAC |
| 530 | vc1638_100down Cloning at dbfR locus | R | CTTCCAGAAATTTGAGTGAAGATTTTG |
| 628 | vc1638_Real_B dbfR deletion | R | GAGATTTAATTGGCAGTCAACTGATACCACCCAGGGATCTGCG |
| 629 | vc1638_Real_C dbfR deletion | F | CGTGCAGTAAACAAAAATATACGGGACGACCTTGTAGTACGTTG |
| 557 | vc1638_2700up Cloning at dbfR locus | F | CACCATCGGATTGGTGCAGTACGAT |
| 558 | vc1638_2700down Cloning at dbfR locus | R | GTGGCGCTAGTCCAAAAATCTGTTC |
| 650 | dbfR_D51V_B Generating dbfR D51V | R | CAATTTTCGGATAGGCCGATACGAGTACGACGTC |
| 651 | dbfR_D51V_C Generating dbfR D51V | F | GGACGTCATCGTACTCGTACCCGGTACAGGATTAGGATATGGG |
| 736 | dbfR_SNAP_delta S_Gblock Gblock for generating dbfR SNAP and simultaneously deleting dbfS | F | CCGCGGCTTGGTGATATGGGTAGGGTTCTGCCCATTAGCCAGAAGCGGCTCACGACGCAGTCAAGATTAGGATATGGG |
| 734 | dbfR_R Generating dbfR SNAP and deleting dbfS | R | TTTGGCATCGAAGACTCGCATCCACGACGAGCG |
| 735 | dbfS_down_F Generating dbfR SNAP and deleting dbfS | F | ATGGGCTGACAAAAACGAGGTATTTCAGG |
| 672 | SNAP_UnivR Generating dbfR SNAP | R | TTAACCTAATCTGGTTTACCTAATCAGTGCAGTCTGCAGTCTG |

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| ID     | Description                   | Location          | Action                      | Sequence                                      |
|--------|--------------------------------|-------------------|-----------------------------|-----------------------------------------------|
| 718    | dbfR_SNP_E                      | Generating dbfR-SNP | F                           | GACATAGATTAGTAACCCAGGATTAGTTAGATGTGATCA AAACCTGTCGCCGCCTGTC |
| 634    | cdgl_3000up                      | Cloning at cdgl locus | F                           | CGATGCAAGTAGTGGACACACGAC                        |
| 635    | cdgl_2700up                      | Cloning at cdgl locus | F                           | GAATACATTAGCGCGAGCGCTTGTTT                        |
| 636    | cdgl_100up                       | Cloning at cdgl locus | F                           | GGGACCAACTTACTGTATATTCAAGTATG                        |
| 637    | cdgl_B                           | cdgl deletion      | R                           | GATGCCGATTCATGAGTACTACTATTATTGTTAGTTAAGGCCCCGAC TTCAATTATTACTCTTCG |
| 638    | cdgl_C                           | cdgl deletion      | F                           | GAGATGATAGAAAAAAATAGAATGCGGCGCTTTACAAAAATAGG TAGCTCATGATGCGCATC |
| 639    | cdgl_100down                     | Cloning at cdgl locus | R                           | GGTACAGCACTTTGGCACGACTTTATTG                     |
| 640    | cdgl_2700down                    | Cloning at cdgl locus | R                           | GAGGTGCAACCTGGTGAATGGAATTTTTC                        |
| 641    | cdgl_3000down                    | Cloning at cdgl locus | R                           | CCAGTGGGCTATCATAATGCGCATC                        |
| 642    | cdgG_3000up                      | Cloning at cdgG locus | F                           | GTGTCACTTACGACGACAAGTGGCAATTTTGG                     |
| 643    | cdgG_2700up                      | Cloning at cdgG locus | F                           | GAATAACCCGACACGCGAGTAGAC                          |
| 644    | cdgG_100up                       | Cloning at cdgG locus | F                           | GATAATGCTGCCCCACGTGGCCATAAACCCTGAG            |
| 645    | cdgG_B                           | cdgG deletion      | R                           | GCACAAATTTAATAGTAACTTAATATATCGACTGAGG ATAGTTGAGGATCAATCCTGAGCTCC |
| 646    | cdgG_C                           | cdgG deletion      | F                           | GGTACAGGATTGATCTCAACTACAGTGCAGTTATTAATTTT AAGCTAATATTATATTATTGTG |
| 647    | cdgG_100down                     | Cloning at cdgG locus | R                           | TGTACAGGATCTGACTGAGATGTTGAGATGAGG |
| 648    | cdgG_2700down                    | Cloning at cdgG locus | R                           | CCAGTAAATTCGGTTATGAGGTAAAGGATGAGG |
| 649    | cdgG_3000down                    | Cloning at cdgG locus | R                           | GATCGCCACTTTCGCCGAGTGGATG |
| 105    | BBC1881                          | Cloning at vc1807 locus | F                           | TTTAAGGGGATCGATGACG |
| 106    | BBC1882                          | Cloning at vc1807 locus | R                           | CAATTTGCTTATGACCTCCC |
| 270    | 1807_2700up                      | Cloning at vc1807 locus | F                           | GGCCCGCACCTTTATTGAAAAAT |
| 271    | 1807_2700down                    | Cloning at vc1807 locus | R                           | GTCTTATACGAGCCGCTTAAAGAGG |
| 721    | P_Bac-1807_Univ_B                | Generating P_Bac- dbfIS | R                           | CATTTCACACTTCTGCAGGTAC                        |
| 722    | P_Bac-dbflS-1807_C               | Generating P_Bac- dbfIS | F                           | GTACCTGCAAGAGTTGAATGATGCTGACATGCTGCAATTAAATG |
| 723    | P_Bac-dbflS-1807_D               | Generating P_Bac- dbfIS | R                           | GTGACGAGCTCCGGAATTTAATGGGGATTGTGAGGGGCTTTG |
| 232    | ABD123                           | Generating P_Bac- dbfIS | F                           | ATTTCCGGGGATCCTCGAGCAT |
| 729    | P_Bac-lapG-1807_C                | Generating P_Bac- lapG | R                           | GTTACCTGCAAGAGTTGAATGATGCTGACATGCTGCAATTAAATG |
| 730    | P_Bac-lapG-1807_D                | Generating P_Bac- lapG | F                            | GTGACGAGCTCCGGAATTTAATGGGGATTGTGAGGGGCTTTG |
| 731    | P_Bac-rbmB-1807_C                | Generating P_Bac- rbmB | R                           | GTACCTGCAAGAGTTGAATGATGCTGACATGCTGCAATTAAATG |
| 732    | P_Bac-rbmB-1807_D                | Generating P_Bac- rbmB | F                           | GTGACGAGCTCCGGAATTTAATGGGGATTGTGAGGGGCTTTG |
| 587    | tagA_3000up                      | Cloning at tagA locus | F                           | GGGCTGCAAGAACTGGGATCTGCTAC |
| 588    | tagA_2700up                      | Cloning at tagA locus | F                           | GAGCAAATACCAAGCTCGATCCTCACAGTAGTAAG |
| 662    | tagA_103bpD_B                    | Removes first 103 codons of | R                             | GTCAAATACTCTGTTACCTGAATTGTTGCACTTCTTTAAACAAA AAAATAAAGGACAGGAGGAAAAAGTATTG |
| Tag    | Description                  | Orientation | Sequence                                                                 |
|--------|------------------------------|-------------|--------------------------------------------------------------------------|
| 663    | *tagA* _103bpD_C_ Removing 103 codons of *tagA* including start | F           | CAATACGTTTCCTCCTGTCTTTATTTTTTGTAAAGAATGCAA CATCCAGTAACGACCGTAGTTTGA C |
| 591    | *tagA* _2700down_ Cloning at *tagA* locus | R           | CCACCGAGGATAACCATCCCATCTTGAATAATGT                             |
| 592    | *tagA* _3000down_ Cloning at *tagA* locus | R           | CTCTTGCCATCCATAGACATGTGACCTTTTG                                |
| 593    | *tagA* _100up_ Cloning at *tagA* locus | F           | GTTGACTCATCCATTGACCTTGAATG                                       |
| 594    | *tagA* _100down_ Cloning at *tagA* locus | R           | CCAGTCGAAATTTATTGATCAGCTTTAGC                                    |
| 664    | *tagA* _150down_ Cloning at *tagA* locus | R           | GCAACCATACATCTTCTTCAATTACTACCATAAGAG                              |
References

1. Matz, C. et al. Biofilm formation and phenotypic variation enhance predation-driven persistence of Vibrio cholerae. *PNAS* **102**, 16819–16824 (2005).
2. Yan, J. & Bassler, B. L. Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms. *Cell Host Microbe* **26**, 15–21 (2019).
3. Shaw, T., Winston, M., Rupp, C. J., Klapper, I. & Stoodley, P. Commonality of elastic relaxation times in biofilms. *Phys. Rev. Lett.* **93**, 098102 (2004).
4. Walter, J., Britton, R. A. & Roos, S. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. *PNAS* **108**, 4645–4652 (2011).
5. Roilides, E., Simitsopoulou, M., Katragkou, A. & Walsh, T. J. How biofilms evade host defenses. *Microbiology Spectrum* **3** (2015).
6. Mah, T. F. & O’Toole, G. A. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**, 34–39 (2001).
7. Phillips, P. L. & Schultz, G. S. Molecular mechanisms of biofilm infection: biofilm virulence factors. *Adv Wound Care (New Rochelle)* **1**, 109–114 (2012).
8. Guilhen, C., Forestier, C. & Balestrino, D. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. *Mol. Microbiol.* **105**, 188–210 (2017).
9. Hobley, L., Harkins, C., MacPhee, C. E. & Stanley-Wall, N. R. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev* **39**, 649–669 (2015).
10. Conner, J. G., Teschler, J. K., Jones, C. J. & Yildiz, F. H. Staying alive: Vibrio cholerae’s cycle of environmental survival, transmission, and dissemination. *Microbiol Spectr* **4** (2016).
11. Almagro-Moreno, S., Pruss, K. & Taylor, R. K. Intestinal colonization dynamics of Vibrio cholerae. *PLOS Pathogens* **11**, e1004787 (2015).
12. Silva, A. J. & Benitez, J. A. Vibrio cholerae biofilms and cholera pathogenesis. *PLOS Neglected Tropical Diseases* **10**, e0004330 (2016).
13. Gallego-Hernandez, A. L. et al. Upregulation of virulence genes promotes Vibrio cholerae biofilm hyperinfectivity. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 11010–11017 (2020).
14. Tamayo, R., Patimalla, B. & Camilli, A. Growth in a Biofilm induces a hyperinfectious phenotype in Vibrio cholerae. *Infection and Immunity* **78**, 3560–3569 (2010).
15. Valentini, M. & Filloux, A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: lessons from Pseudomonas aeruginosa and other bacteria. *J Biol Chem* **291**, 12547–12555 (2016).
16. Conner, J. G., Zamorano-Sánchez, D., Park, J. H., Sondermann, H. & Yildiz, F. H. The ins and outs of cyclic di-GMP signaling in Vibrio cholerae. *Curr Opin Microbiol* **36**, 20–29 (2017).
17. Fong, J. C. N., Syed, K. A., Klose, K. E. & Yildiz, F. H. Role of Vibrio polysaccharide (vps) genes in VPS production, biofilm formation and Vibrio cholerae pathogenesis. *Microbiology (Reading, Engl.)* **156**, 2757–2769 (2010).
18. Bridges, A. A. & Bassler, B. L. The intragenus and interspecies quorum-sensing autoinducers exert distinct control over Vibrio cholerae biofilm formation and dispersal. *PLoS Biol.* **17**, e3000429 (2019).
19. Singh, P. K. et al. Vibrio cholerae combines individual and collective sensing to trigger biofilm dispersal. *Curr Biol* **27**, 3359-3366.e7 (2017).
20. Merrell, D. S., Hava, D. L. & Camilli, A. Identification of novel factors involved in colonization and acid tolerance of Vibrio cholerae. *Molecular Microbiology* **43**, 1471–1491 (2002).
21. Gao, R. & Stock, A. M. Biological insights from structures of two-Component proteins. *Annu Rev Microbiol* **63**, 133–154 (2009).
22. Gao, R., Bouillet, S. & Stock, A. M. Structural basis of response regulator function. *Annu. Rev. Microbiol.* **73**, 175–197 (2019).

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23. Boyd, C. D., Chatterjee, D., Sondermann, H. & O'Toole, G. A. LapG, required for modulating biofilm formation by Pseudomonas fluorescens Pf0-1, is a calcium-dependent protease. Journal of Bacteriology 194, 4406–4414 (2012).
24. Fong, J. C. N. & Yildiz, F. H. The rbmBCDEF gene cluster modulates development of rugose colony morphology and biofilm formation in Vibrio cholerae. Journal of Bacteriology 189, 2319–2330 (2007).
25. Kitts, G. et al. A conserved regulatory circuit controls large adhesins in Vibrio cholerae. mBio 10, (2019).
26. Collins, A. J., Smith, T. J., Sondermann, H. & O'Toole, G. A. From input to output: the lap/c-di-GMP biofilm regulatory circuit. Annu. Rev. Microbiol. (2020).
27. Berk, V. et al. Molecular architecture and assembly principles of Vibrio cholerae biofilms. Science 337, 236–239 (2012).
28. Seper, A. et al. Extracellular nucleases and extracellular DNA play important roles in Vibrio cholerae biofilm formation. Mol Microbiol 82, 1015–1037 (2011).
29. Hyakutake, A. et al. Only one of the five cheY homologs in Vibrio cholerae directly switches flagellar rotation. Journal of Bacteriology 187, 8403–8410 (2005).
30. Butler, S. M. & Camilli, A. Going against the grain: chemotaxis and infection in Vibrio cholerae. Nat Rev Microbiol 3, 611–620 (2005).
31. Butler, S. M. & Camilli, A. Both chemotaxis and net motility greatly influence the infectivity of Vibrio cholerae. Proc Natl Acad Sci U S A 101, 5018–5023 (2004).
32. Son, K., Guasto, J. S. & Stocker, R. Bacteria can exploit a flagellar buckling instability to change direction. Nature Physics 9, 494–498 (2013).
33. Licata, N. A., Mohari, B., Fuqua, C. & Setayeshgar, S. Diffusion of bacterial cells in porous media. Biophys J 110, 247–257 (2016).
34. Utada, A. S. et al. Vibrio cholerae use pili and flagella synergistically to effect motility switching and conditional surface attachment. Nature Communications 5, 4913 (2014).
35. Wu, D. C. et al. Reciprocal c-di-GMP signaling: incomplete flagellum biogenesis triggers c-di-GMP signaling pathways that promote biofilm formation. PLOS Genetics 16, e1008703 (2020).
36. Kojima, S., Yamamoto, K., Kawagishi, I. & Homma, M. The polar flagellar motor of vibrio cholerae is driven by an Na+ motive force. J Bacteriol 181, 1927–1930 (1999).
37. Dalia, A. B., McDonough, E. & Camilli, A. Multiplex genome editing by natural transformation. Proc Natl Acad Sci U S A 111, 8937–8942 (2014).
38. Yan, J. et al. Mechanical instability and interfacial energy drive biofilm morphogenesis. eLife 8, e43920 (2019).
39. Qin, B. et al. Cell position fates and collective fountain flow in bacterial biofilms revealed by light-sheet microscopy. Science (2020).
40. Groisman, E. A. The pleiotropic two-component regulatory system PhoP-PhoQ. Journal of Bacteriology 183, 1835–1842 (2001).
41. Prost, L. R., Daley, M. E., Bader, M. W., Klevit, R. E. & Miller, S. I. The PhoQ histidine kinases of Salmonella and Pseudomonas spp. are structurally and functionally different: evidence that pH and antimicrobial peptide sensing contribute to mammalian pathogenesis. Mol Microbiol 69, 503–519 (2008).