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Mechanisms underlying *Vibrio cholerae* biofilm formation and dispersion

Authors:

Jennifer K. Teschler[1], Carey D. Nadell[2], Knut Drescher[3], Fitnat H. Yildiz[1#]

[1]Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz, 95064, California, USA

[2]Department of Biological Sciences, Dartmouth College, Hanover 03755, New Hampshire, USA

[3]Biozentrum, University of Basel, 4056 Basel, Switzerland

[#]Correspondence: fyildiz@ucsc.edu

1. ABSTRACT

Biofilms are a widely observed growth mode in which microbial communities are spatially structured and embedded in a polymeric extracellular matrix. Here we focus on the model bacterium *Vibrio cholerae* and summarize the current understanding of biofilm formation including initial attachment, matrix components, community dynamics, social interactions, molecular regulation, and dispersal. The regulatory network that orchestrates the decision to form and disperse from biofilms coordinates various environmental inputs. These cues are integrated by several transcription factors, regulatory RNAs and second messenger molecules, including bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP). Through complex mechanisms, *V. cholerae* weighs the energetic cost of forming biofilms against the benefits of protection and social interaction that biofilms provide.

Keywords: *Vibrio cholerae,* biofilm, surface attachment, extracellular matrix, extracellular polymeric substances (EPS), dispersal, biofilm regulation, emergent properties

2. INTRODUCTION

Biofilms are surface associated multicellular microbial communities surrounded by a protective extracellular matrix; they are evolutionarily ancient and the dominant mode of growth among bacteria and archaea (12, 59). Biofilm formation and dispersal are dynamic processes that begin with initial attachment, followed by microcolony formation and matrix production (174). Biofilms break up through different mechanisms including detachment, desorption, and dispersal (142). Committing to biofilm formation is inherently risky: producing extracellular matrix, for example, can incur > 20% reduction in maximal growth rate compared to free-living planktonic cells (75). The regulatory circuits governing biofilm development must therefore be complex and include sensitivity to physical, chemical, and ecological stimuli. However, the cost of biofilm formation can be counteracted by cooperative benefits that include protection from environmental stresses (58, 174).

Study of *Vibrio cholerae* has greatly increased our understanding of biofilm formation, structure, regulation, dispersal, and, more recently, the emergent architectural properties of biofilms (58, 75, 174). *V. cholerae* is the causative agent of the diarrheal disease cholera. As a facultative human pathogen, *V. cholerae* spends much of its life cycle in the aquatic environment and is transmitted to a human host through the ingestion of contaminated food or water (34). Biofilms are critical to the environmental survival, transmission, and pathogenesis of *V. cholerae*, making it an ideal model system for studying biofilms (174). Various cell-surface and cell-cell interactions are essential for *V. cholerae* biofilm attachment and stability (13). A complex network of signal transduction pathways modulate biofilm formation in response to extracellular and intracellular signals. In addition, chemical gradients generate heterogeneity within the biofilm as cells respond to local environmental conditions (94, 169). Here we discuss mechanisms that regulate different stages of *V. cholerae* biofilm formation, highlighting the diverse signaling mechanisms and cellular responses that underpin this process. We explore the socio-ecological factors that trigger microbes to cooperate in biofilm production or signal for them to defend against competing strains and species in the vicinity. Finally, we discuss mechanisms by which *V. cholerae* biofilms disband.

3. SURFACE ATTACHMENT

3.1. Pili

The transition of *V. cholerae* from motile-to-biofilm involves moving from swimming, to landing on a surface, attaching to the surface, then transitioning to a sessile state (Figure 1). *V. cholerae* motility swims using a polar flagellum and surface attachment is initiated by type IV mannose-sensitive hemagglutinin (MSHA) pili (105). The viscous drag force on the flagellum as it sweeps past a surface results in torque on the cell body, causing cells to move in clockwise, curved paths (105). Flagellar rotation also causes the *V. cholerae* cell body to counter rotate, which allows MSHA pili to periodically contact the surface. MSHA pili are typically between 0.4 μm and 1.2 μm in length, are evenly distributed across the cell body, and can act as a brake and anchor during attachment to various surfaces (210). High-speed cell tracking of *V. cholerae* during initial surface attachment revealed two types of near-surface swimming trajectories, termed orbiting and roaming (174, 182). Roaming motility is associated with weaker MSHA-surface interactions and results in near-surface motility that has less curvature and longer directional persistence. Orbiting motility is associated with stronger MSHA interactions with the surface, resulting in tight, repetitive, highly circular near-surface motility that ultimately leads to surface attachment and microcolony formation. Both motility modes were ablated in the absence of the MSHA pili, indicating these structures are essential for initial surface attachment (182, 189).

The ubiquitous signaling molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) promotes the transition of *V. cholerae* from a motile to a sessile biofilm state by repressing motility and upregulating surface attachment and biofilm matrix production (Figure 1) (174). C-di-GMP is synthesized by diguanylate cyclase enzymes (DGCs) and degraded by phosphodiesterase enzymes (PDEs) and can interact with various receptor proteins or RNA to modulate cellular processes. One such c-di-GMP receptor protein is MshE, which is required for the polymerization of the MshA subunits that make up the MSHA pilus (92, 148, 186). MshE regulates MSHA dynamics and extension, while retraction is mediated by the retraction ATPase PilT (Figure 1B) (31, 60, 92, 188, 189). MshE is a AAA+ ATPase and binds c-di-GMP with a K_d of 1.9 ± 0.4 μM, (186). The crystal structure of the N-terminal domain of MshE bound to c-di-GMP identified a c-di-GMP binding motif (RLGxx(*L/V/I*)(*L/V/I*)*xxG*(*L/V/I*)(*L/V/I*)xxxxLxxxLxxQ) (186). Amino acid substitution of residues involved in c-di-GMP binding (G11, R9, and L10) reduced c-di-GMP binding to MshE, resulting in decreased MSHA pilus secretion and biofilm formation. In short, c-di-GMP-mediated synthesis of the MSHA pilus is a critical early step in biofilm formation (186). When c-di-GMP levels are high, MshE binds c-di-GMP to promote pilus extension, while low c-di-GMP levels enhance MSHA pilus retraction (60). C-di-GMP binding also regulates the optimal conformational state of MshE to control appropriate extension and retraction dynamics. Indeed, a “constitutively active” MshE results in enhanced MSHA extension and increased rates of retraction (60).

Regulation of *V. cholerae* MSHA pilus extension and retraction not only contributes to initial attachment, but also long-term colonization and robust biofilm formation. The retraction ATPase, PilT, is required for MSHA retraction and *pilT* deletion results in a significant decrease in MSHA pili on the cell surface. Genetic analysis reveals that PilT-mediated MSHA pilus retraction also impacts motility and surface attachment through

modulating the average speed and spatial spread of bacterial trajectories during near-surface swimming (60). C-di-GMP levels and pilus retraction also affect the detachment of cells after initial attachment. Prolonged MSHA pilus retraction results in enhanced detachment from the surface, correlating with lower levels of intracellular c-di-GMP (Figure 1B). *pilT* deletion decreases initial surface attachment overall. However, once *pilT* mutant cells do attach, the rates of detachment are decreased relative to the wild-type strain (60). *V. cholerae* carries a second retraction ATPase, PilU. However, PilU is unable to mediate type IV pilus retraction in the absence of *pilT* (3, 92, 153) and PilU is not sufficient for MSHA pilus retraction. However, PilU has a PilT-dependent role, as it functions in the presence of a catalytically inactive PilT but not when *pilT* is deleted (3, 32, 60).

V. cholerae can produce two additional type IV pili implicated in attachment to specific surfaces: the toxin co-regulated pilus (TCP) and the chitin-regulated pilus (ChiRP), also referred to as the competence pilus or DNA-uptake pilus. TCP is a virulence factor contributing to intestinal adherence, bacterial microcolony formation, and auto-aggregation in the gut via pilus-pilus contact (101, 173). Intriguingly, TCP is upregulated in biofilm compared to planktonic cells and this upregulation is known to contribute to the biofilm hyperinfectivity phenotype (65). TCP also impacts colonization and bacterial interactions on chitinous surfaces (147). While MSHA contributes to chitin colonization (31) and is important for landing and attaching in viscoelastic environments, such as the mucus layer of the intestine (210), constitutive expression of MSHA is deleterious to intestinal colonization and leads to a failed immune escape response (81). Two recently identified positive regulators of MSHA, VC1371 and VcRfaH, also positively contribute to biofilm formation and intestinal colonization (156). Thus, coordinated regulation of TCP and MSHA during infection is important for intestinal colonization and subsequent adherence to environmental surfaces once *V. cholerae* is shed in the stool (150). The ChiRP pilus contributes to chitin colonization, kin selection, and other emergent properties and is discussed further below (3, 19, 123, 124).

3.2. Flagellum

A single Na^+-driven flagellum, located on one of the cell poles, is responsible for swimming motility of *V. cholerae*. After initial surface attachment, inhibition of flagellar rotation stabilizes cell surface attachment and prevents detachment during early stages of biofilm formation. Motility and biofilm formation in *V. cholerae* are inversely regulated by c-di-GMP (37).

Genetic disruption of the *V. cholerae* flagellin, FlaA, or flagellum biogenesis elevates cellular c-di-GMP levels compared to wild type and enhances biofilm formation (106, 128, 193). FlaA deletion also results in the flagellum-dependent biofilm regulatory (FDBR) response, which upregulates c-di-GMP levels and biofilm formation (Figure 1B) (193). The FDBR response is partially dependent on the flagellar stator and Na^+ flux. Deletion of the flagellar stator T ring component, MotX, and mutations that alter either Na^+ motive force or the Na^+ binding ability of the stator in the *flaA* deletion background result in a reversal or reduction of the FDBR response, respectively (193). Irrespective of the presence of the flagellar filament, the stator was essential for maintaining both c-di-GMP levels and MSHA production during surface colonization (193). Three DGCs, CdgA, CdgL, and CdgO, contribute to the FDBR response. While these DGCs do not localize to

the flagellar poles, they are essential for increased c-di-GMP production and signaling cascades that lead to FDBR-mediated enhanced biofilm formation (193). It has been proposed that an unknown signal is transduced by the flagellar stators to enhance c-di-GMP production and biofilm formation (193). Analysis of c-di-GMP production in single cells during initial surface attachment showed an increase in cellular c-di-GMP levels which may underlie a decrease in flagellar function and production (193). While it is unclear if cells maintain flagellum production during biofilm formation, at the transcriptional level, c-di-GMP represses transcription of flagellar genes by binding and inhibiting FlrA, the master regulator of flagellar biogenesis, with a K_d of 2.4 μM (Figure 5) (166).

3.3. Large adhesion proteins

The family of repeats-in-toxin (RTX) adhesins have been linked to surface attachment, cell-cell, and cell-matrix interactions of several microorganisms (72). *V. cholerae* possesses two such adhesins, FrhA and CraA, which are predicted to localize to the outer membrane via the type I secretion system (102). Cell surface retention of these adhesins depends on a well-characterized, conserved, c-di-GMP-dependent post-translational proteolytic mechanism, discussed below in the section on biofilm dispersal (Figure 1B). FrhA and CraA contribute to initial attachment and biofilm formation in a strain- and substrate- dependent manner. Loss of FrhA and CraA in the *V. cholerae* O1 classical strain decreases initial attachment and biofilm formation. In a *V. cholerae* El Tor strain, the contribution of this system to biofilm formation is more evident in biofilms grown on chitin, a nutritious substrate found in aquatic environments (102). Though *V. cholerae* O1 classical strains express the MSHA pilin subunit, they do not assemble surface associated MSHA pili. This adhesion pathway may, therefore, play a greater role in MSHA pilus null strains. The N-acetylglucosamine-containing protein, GbpA, which is upregulated by a c-di-GMP riboswitch, also contributes to *V. cholerae* adhesion to chitin and mucin surfaces (95), further highlighting the different adhesion proteins and strategies linking attachment to specific surfaces (17, 100, 167).

4. MATRIX PRODUCTION

Biofilm formation relies on the production of extracellular matrix components—polysaccharides, proteins, nucleic acids, and other biomolecules that keep cells attached to one another and to surfaces (Figure 2) (96). Recent studies revealed how the biofilm matrix is built and how biofilms attain their structural integrity.

4.1. Vibrio polysaccharide

A major component of the *V. cholerae* biofilm matrix is an exopolysaccharide termed *Vibrio* polysaccharide (VPS). *V. cholerae* produces two different types of VPS. The repeating unit of the major variant (80% of total VPS) consists of [-4)-α-GulNAcAGly3OAc-(1-4)-β-D-Glc-(1-4)-α-Glc-(1-4)-α-D-Gal-(1-] (204). In the minor variant (20% of total VPS), α-D-Glc is replaced with α-D-GlcNAc (204). The functions of specific polysaccharides in biofilm matrix assembly are unknown. However, VPS production is essential for native cell-packing in biofilms and for developing 3-D biofilm structures. VPS is secreted upon surface attachment and is present throughout mature biofilms.

The *V. cholerae* biofilm matrix cluster (VcBMC) contains the 18 *vps* genes organized into two operons on the large chromosome of *V. cholerae* O1 El Tor [*vpsU*, *vpsA-K*, (*vps*-I operon); *vpsL-Q*, (*vps*-II operon)] (62, 207). While the molecular mechanisms underlying VPS production are largely unknown, recent studies indicate that a tyrosine phosphoregulatory system controls VPS biosynthesis (152). VpsO is a tyrosine kinase and VpsU is a low molecular weight protein tyrosine phosphatase. VpsO autophosphorylates multiple tyrosine residues in the C-terminal tail, which VpsU dephosphorylates. VpsO autophosphorylation inhibits its kinase activity; unphosphorylated, high oligomeric state VpsO promotes VPS production. Conversely, autophosphorylation of the C-terminal tail of VpsO inhibits VPS production by disrupting oligomerization. VpsO C-terminal tyrosine-cluster phosphorylation states, and the cycling between high and low levels, fine-tune VPS production. Additionally, the phosphorylation state of VpsO regulates its proteolytic degradation; the catalytically inactive VpsO is rapidly degraded and the absence of tyrosine phosphatase leads to an increase in VpsO abundance (152). Regulation of VPS biosynthesis at the transcriptional and post-transcriptional levels illustrates that biofilm formation is tightly regulated, likely because of the large energetic investment of cells into matrix production during biofilm formation.

4.2. Matrix proteins

Several proteins are present in the matrix, and, of these, RbmA, RbmC, and Bap1 have been extensively studied (Figure 2). Production and accumulation of matrix proteins is temporally controlled, and matrix proteins have complementary architectural functions (1, 14, 63). RbmA, RbmC, and Bap1 are secreted into the matrix via the type II secretion system (T2SS); loss of the T2SS reduces biofilm formation (91).

RbmA is one of the first proteins secreted into biofilm matrix following attachment of a founder cell to surfaces, and it is present throughout the mature biofilm. RbmA facilitates the initial stages of microcolony and cluster formation by controlling cell orientation relative to a substratum and by promoting the attachment of daughter cells to founder cells by cell-to-cell adhesion (14). In the absence of *rbmA*, the lack of cell-to-cell interaction leads to less dense and looser biofilms that are more susceptible to invasion and predation (49, 133, 195). Thus, RbmA is critical for the architecture and structural integrity of the biofilm. The crystal structure of RbmA shows that it contains two Fibronectin type III (FnIII) domains, FnIII (1) and FnIII (2), which are connected by a well-resolved linker segment (69). The two FnIII folds run antiparallel to each other within a monomer, but do not interact extensively. In contrast, the N-terminal FnIII domain of one monomer interacts tightly with the C-terminal FnIII domain of the second monomer in the asymmetric unit. As a result, RbmA forms a dimer consisting of two hemi-complexes with domain contributions from each monomer (69).

RbmA directly binds VPS, through a patch on the exposed outer surface of the FnIII-2 domain (61). VPS-binding leads to the formation of higher order RbmA-VPS structures. The presence of two VPS binding motifs on each RbmA dimer suggests multivalent binding to VPS. In addition, regulation of the RbmA FnIII-1:FnIII-2 dimer interface via a structural switch within its first fibronectin type III (FnIII-1) domain is critical for RbmA function (69). RbmA can assume distinct structures at the dimer interface: FnIII-1 can from a disordered-loop (D-loop) or ordered-loop (O-loop) structure. Both the RbmA O-loop and D-loop states are present at FnIII-1:FnIII-2 dimer interfaces and form the basis of a dynamic binary switch. Locking RbmA in the O-loop dimer state markedly changes

the organization of biofilm matrix components and the biofilm's architectural properties (61). Therefore, the structural dynamics of RbmA appear to be an important mechanism regulating biofilm matrix assembly and biofilm structural properties. RbmA also binds to *V. cholerae* lipopolysaccharide glycans, though the residues involved in these interactions, and the influence of RbmA structural dynamics on these sites, remain unclear (113).

In both the planktonic and biofilm-grown states, RbmA is proteolytically processed (14, 160). Three proteases, HapA, PrtV and IvaP, cleave RbmA and the main proteolytic product is the C-terminal peptide, RbmA*, which harbors the FnIII-2 domain (160). Expression of either RbmA* or FnIII-2 domain can partially complement the biofilm defect phenotype of strains lacking RbmA, suggesting that they can bind and cross-link VPS polymers in biofilms (160). Proteolytic regulation of RbmA may be involved in the recruitment of *V. cholerae* cells to biofilms, as full-length RbmA mainly interacts with VPS-producing cells, while RbmA* interacts with both VPS-producing and non-producing cells (61, 160).

RbmC and Bap1, which share 47% similarity and 35% identity, are the two other biofilm matrix proteins critical for biofilm formation. During the initial stages of biofilm formation and in mature biofilms, Bap1 is concentrated at the interface between the founder cell-surface and the substrate surface, whereas RbmC is absent from the interface (14). However, in mature biofilms, both RbmC and Bap1 appear to encapsulate cell clusters. Bap1 and RbmC have similar, but non-redundant, functions in biofilm matrix assembly. They facilitate sustained attachment of cells and growing biofilms to surfaces (1, 14, 63). While Δ*rbmC* and Δ*bap1* single mutants form biofilm structures that are minimally altered, a strain lacking both proteins (Δ*rbmC*Δ*bap1)* forms a thin biofilm which readily detaches (63). Both RbmC and Bap1 are multi-domain proteins. Recently, the crystal structure of Bap1 (lacking the 57 amino acids from the Bap1 beta-prism domain) was solved (97). Bap1 has an eight-bladed beta-propeller structural core, with an accessory beta-prism domain inserted within blade 6, via a flexible linker (97). The crystal structures of RbmC's two beta-prism domains have also been solved. Beta-prism domains can bind glycans, and, in RbmC, these domains do indeed interact with the mannotriose core, a complex N-glycan. Beta-prism domains can bind to (GlcNAc-Man)2Man-GlcNAc-GlcNAc and residues critical for these interactions have been identified (43).

4.3. Other matrix components

Imaging and proteomic analyses demonstrate that MSHA pili are a highly abundant component of the biofilm matrix (Figure 2) (1, 159). In addition, MSHA pili may bind to other matrix components (159). The flagellum contributes to biofilm structure in other species, though this role has yet to be tested for *V. cholerae* (79).

Extracellular DNA (eDNA) and outer membrane vesicles (OMVs) are also found in biofilms, though the regulation and contribution of these components to the biofilm is an active area of exploration (Figure 2). eDNA contributes to the biofilm structure (154), interacts with VPS (93), and represents a potential source of nutrients and genetic material through horizontal gene transfer (22, 71). Two extracellular nucleases, Dns and Xds modulate eDNA levels; deletion of these genes enhance biofilm formation and impair detachment from the biofilm (154). Dns is an endonuclease and Xds an exonuclease. Both factors degrade eDNA to form nucleoside-monophosphates, allowing *V. cholerae* to

use eDNA as its sole phosphate source (120, 154). Three NupC family proteins are required for nucleoside uptake (71). Deletion of all three nucleoside transporters increases biofilm formation to levels similar to a *dns* and *xds* null mutant background, suggesting that impaired uptake of eDNA degradation products may trigger an inhibitory feedback loop, where nucleoside accumulation in the matrix inhibits extracellular nucleases and enhances biofilm production (71). Mechanisms of eDNA release in the biofilm are not well understood, though cell lysis, autolysis, active secretion, and transport via OMVs are all suggested avenues (85).

OMVs, composed of an outer leaflet of lipopolysaccharide (LPS), an inner leaflet of phospholipids, and outer membrane proteins (OMPs), harbor the biofilm matrix proteins RbmA, RbmC, and Bap1 (4). Although it has been shown that OmpT, an abundant OMP, can interact with Bap1 (52), the role of OMVs and OMV-associated biofilm matrix proteins in *V. cholerae* biofilm formation remains unclear.

5. BIOFILM ECOLOGY AT THE CELLULAR SCALE

Biofilm-linked phenotypes, like matrix production and the secretion of other extracellular compounds, can often be interpreted as classical social behaviors that likely evolved in part due to their positive or negative fitness impact on other cells in the vicinity, including clonemates and cells of other strains or species (132, 190). Whether or not such behaviors are ultimately favored by natural selection depends on which cells express them, the costs of expressing these phenotypes, and positive or negative fitness impact on other cells that are affected by them (68, 73, 74). Because many biofilm-associated behaviors are secreted factors that affect neighbors in a distance-dependent manner, the spatial arrangement of different genetic lineages, strains, and species within biofilms is a key factor influencing evolution in biofilms (132, 202).

5.1. Lineage structuring during surface engagement and matrix secretion

Recent work indicates that *V. cholerae* cells can self-assort according to lineage or strain background during the early stages of surface engagement. *V. cholerae*'s ChiRP is produced specifically in response to chitin colonization and is important for chitin-induced natural transformation (124). This pilus can interact with other copies of itself on nearby cells based on strain-specific variations in *pilA*, the primary subunit that makes up ChiRP (3). Via this mechanism *V. cholerae* can bias the spatial distribution of patches of colonizing cells on chitin such that individuals with the same *pilA* sequence become preferentially clustered. The relative assortment or mixing of different *V. cholerae* strains during surface attachment and subsequent matrix secretion can exert a strong impact on the relative success of downstream phenotypes associated with protection from external threats, exploitation of surface-embedded nutrient sources, and competition with other strains or species in the vicinity.

Controlled lineage spatial structuring continues to manifest in *V. cholerae* biofilms as they advance from initial surface occupation to matrix secretion and growth into groups of ten or more cell layers in depth (Figure 2C). Four of the primary *V. cholerae* biofilm matrix components have been closely studied: the polysaccharide VPS, and the three proteins RbmA, RbmC, and Bap1. RbmC and Bap1 are freely diffusible, can move some distance from secreting cells, and reach neighbors that do not produce these proteins (1, 14, 133). VPS and RbmA, in contrast, remain close to the secreting cells, with little cross-complementation of co-cultured cells. The net result is that clonal lineages of cells established during initial surface attachment remain tightly associated with each other

with boundaries demarcated by localized retention of VPS and RbmA (Figure 2C) (133). This structure is matrix-dependent and also varies with the interaction of population density, surface adhesion and fluid flow during surface attachment (114). Imaging of differentially labelled, isogenic *V. cholerae* strains show that clonal structuring occurs in biofilms on the intestinal epithelium of an infant mouse model (127), and on chitin particles in artificial sea water (3, 50).

Once formed, the tightly bound clonal cell groups of *V. cholerae* are resistant to attachment and invasion of other cells into their interior or to the underlying substratum (133). Recent work has also shown that the *V. cholerae* matrix establishes a difference in osmotic pressure with the external environment, which contributes both to colony expansion, the ability to outcompete matrix non-secreting cells, and the ability to resist invasion by incoming planktonic bacteria (127, 133, 201). The cell-packing conferred by *V. cholerae* matrix structure can protect from spatial invasion by potential competitors, and from T6SS attack on the cluster exterior (177). Finally, the matrix counteracts grazing by protozoa (118) and predatory microbes, including phages and the bacterium *Bdellovibrio bacteriovorus* (21, 51, 195).

While clonal clustering is a major organizational principle in *V. cholerae* biofilm during VPS-dependent growth, other cell group formations have been documented. In the planktonic phase, *V. cholerae* can aggregate in a VPS-independent manner, translating into limited VPS-independent growth on surfaces under flow. This growth mode was recently found to be quorum sensing-dependent (88, 89, 99), and it can involve aggregation and mixing of multiple cell lineages, contrasting with VPS-dependent biofilm production (89).

In addition, some strains of *V. cholerae* can produce filamentous cells. These cells are up to 20-fold longer than typical cell length (2-3 μm) and can colonize chitin particles under flow. Once surface-bound, they produce groups of entangled filaments that can accumulate substantial biofilm biomass in the absence of normal *V. cholerae* extracellular matrix components (194). Though they can colonize surfaces rapidly, in the absence of normal matrix secretion and the architectural cell-packing that it confers, these filamentous biofilms are susceptible in long-term competition to *V. cholerae* variants of normal cell length producing matrix-replete biofilm clusters. Other cell shape alterations can impact biofilm formation. Recent work demonstrates that increased c-di-GMP levels alter *V. cholerae* morphology, shifting cells from a curved to straight shape that enhances surface adherence and biofilm formation (56).

5.2. Matrix secretion and biofilm population dynamics

Cells in biofilms compete more strongly for limited space, which in turn can control access to growth substrates and exposure to exogenous threats (38, 137, 151, 198). Increasing evidence suggests that biofilm formation is often upregulated in response to ecological competition (151), and the extracellular matrix can be a key factor influencing spatial competition within other cells (131, 200, 201). Evidence contradicts the initial expectation that cells which fail to produce extracellular matrix can exploit matrix produced by other cells (131, 151, 200, 201). *V. cholerae* strains that constitutively produce matrix dramatically outcompete those that do not, despite incurring costs as high as a 20% reduction in maximum growth rate in mixed liquid culture. The matrix is critical for the retention and anchoring of layered, multicellular groups to solid substrata and at least two *V. cholerae* matrix components–VPS and RbmA–are not shared with

neighboring expanding clonal cell groups. This is consistent with data from other model organisms, though the experimental environment and the diffusion of key matrix components impacts the extent to which matrix components are shared between clonal groups in different microbial biofilms (48, 86, 138).

The urgency of local competitive ability contrasts with the need to disperse to new locations. Within biofilms, the balance between these two different avenues to ecological success is, in large part, mediated by the timing and magnitude of extracellular matrix production and degradation in response to dispersal cues. While conferring a strong local competitive advantage, constitutive investment into matrix production also reduces dispersal ability (131). High rates of environmental fluctuation favor investment in matrix production and tuned, intermediate biofilm production strategies versus dispersal back into the planktonic phase (200, 203).

By controlling cell-cell association and lineage structure within biofilms, the secreted matrix can also impact the population dynamics of other biofilm-associated phenotypes that target neighboring cells to provide a benefit or inflict harm. When growing on particles of chitin in oceanic or estuarine conditions, for example, *V. cholerae* secrete chitinases (39, 77, 103, 124), which liberate N-Acetylglucosamine (GlcNAC) that can be directly imported into the cells as a sole source of carbon and nitrogen. Chitinase secretion is a cooperative behavior, with the substantial resources invested by one cell benefiting the local community via the diffusible GlcNAC released by chitinase. Mutant strains that do not produce chitinase can out-grow chitinase-secreting cells in well-mixed conditions, and within biofilms in static culture, wild type chitinase activity remains exploitable by non-producing mutants. In biofilms under flow, however, matrix-secreting strains of *V. cholerae* form thick cell groups that sequester all of the released GlcNAC, preventing exploitation by cheating strains (50).

V. cholerae has also recently allowed direct study of antagonistic behavior in structured populations. T6SS activity, for example, increases spatial assortment of clonal lineages via killing activity within and along the borders of T6SS-active cell groups (122). The accumulation of dead cells along strain group borders limits the extent of contact-mediated cell killing and, in the absence of conditions that continue to spatially mix the population, tends to produce coexistence of T6SS-active and target cells into spatially separated cell groups (162, 168). This clonal spatial separation, in turn, makes conditions more favorable for cooperative extracellular secretion behaviors to remain evolutionarily stable (122, 132). Understanding the balance between cooperative and antagonistic interaction in *V. cholerae* in both marine and host environments is an important area for future work (9, 57); clarifying the dynamics of extracellular polymer digestion, in particular among more realistic multispecies biofilm communities on chitin, also remains an active area of research in this domain (41, 53–55).

6. REGULATORY CIRCUITRIES GOVERNING BIOFILM FORMATION AND DISPERSAL

Optimizing biofilm gene expression can provide a fitness advantage to community members at a single cell or population level, under steady-state and fluctuating environmental conditions. In the following section, we first discuss the primary transcriptional regulatory circuitry that controls the biofilm matrix synthesis genes (Figure 3). We then discuss how secondary circuitries integrate into the primary network to

activate or repress biofilm gene expression under different environmental conditions. Finally, we explore the regulatory pathways that inform biofilm dispersal.

6.1. Primary regulatory circuitry

VpsR is the central positive regulator of biofilm genes and additionally upregulates the T2SS, the secretion system responsible for translocating key biofilm proteins into the matrix (205, 206). Disruption of *vpsR* eliminates expression of biofilm genes and abolishes the formation of typical 3D biofilm structure (205). VpsR shares homology with the enhancer-binding NtrC family of response regulators, which act in concert with alternative sigma factor RpoN (sigma-54). Such regulators typically have conserved domains: an N-terminal receiver (REC) domain, a central AAA+ (adenosine triphosphatase (ATPase) associated with diverse cellular activities) activator domain involved in ATP hydrolysis, a domain binding to sigma-54, and a C-terminal DNA-binding domain (66). However, VpsR lacks the residues necessary for interactions with sigma-54 (the GAFTA motif) and ATP hydrolysis, and its target promoters lack sigma-54 binding sequences. Altogether, these observations show that VpsR is an atypical enhancer-binding protein. VpsR contains the conserved aspartate residue, D59. Conversion of this aspartate to alanine renders VpsR inactive, while conversion to glutamate creates constitutively active VpsR, arguing that VpsR phosphorylation controls DNA binding (66). However, the cognate histidine kinase for VpsR phosphorylation has not been identified (106, 157). Interestingly VpsR can bind c-di-GMP, likely through the AAA+ domain, with a K_d of 1.6 µM (165). *In vitro* DNA binding studies using the *vpsL* promoter region showed that VpsR does not require c-di-GMP to bind to DNA or to dimerize. The affinity of VpsR for DNA and the protein–DNA contacts made by VpsR at the *vpsL* promoter are also unaffected by c-di-GMP (83, 209). However, an *in vitro* transcription assay using the *vpsL* promoter region (start of *vps-II* operon) as a VpsR target showed that c-di-GMP is needed to observe distinct protein–DNA contacts within the activated transcription complex containing sigma-70, VpsR, and c-di-GMP (82).

VpsR binds to two different sites, a proximal site and a distal site relative to the *vpsL* transcriptional start site (209). The proximal binding site of the VpsR is immediately upstream of the -35 region of the *vpsL* promoter, indicating that VpsR functions as a class-II transcriptional activator. The distal VpsR binding site is critical for anti-histone-like nucleoid structuring (H-NS) repressor activity. Thus, VpsR acts as both an anti-repressor and direct activator at the *vpsL* promoter (209). VpsR regulon analysis, *in vitro* DNA binding studies, and *in silico* analysis have uncovered VpsR binding motifs in the upstream regulatory sequences of multiple biofilm genes: *vpsU*, *rbmA*, *rbmB*, *rbmC*, *bap1*, and *vpsL* (Figure 3) (15, 206, 209). *In vitro* studies using a subset of these genes revealed that VpsR functions as class-II activator at the *vpsU* promoter region (start of *vps-I* operon) and as a class-I activator at the *rbmA* and *rbmF* promoter regions; and that the affinity of VpsR for biofilm gene promoters and dependency of such interactions to c-di-GMP varies considerably (83). Therefore, VpsR levels, phosphorylation state, and cellular c-di-GMP levels modulate the activity of VpsR on different biofilm genes.

Disruption of *vpsT* also reduces biofilm gene expression and biofilm-forming capacity (15, 28, 104). The VpsT structure contains an N-terminal receiver (REC) and a C-terminal helix-turn-helix (HTH) domain. Constitutively active and inactivating mutations in the VpsT putative phosphorylation site indicate that biofilm gene expression is not impacted by VpsT phosphorylation. VpsT binds to c-di-GMP with a K_d of 3 µM with a 1:1

stoichiometry, consistent with a dimer of c-di-GMP binding to a dimer of VpsT, and this interaction is necessary for binding to the *vpsL* promoter (104). The VpsT binding motif is present at the *vpsL*, *vpsA*, and *rbmA* promoters (Figure 3) (7, 209). A recent ChiP-seq study showed that VpsT binds to 23 loci involved in motility, biofilm formation, and c-di-GMP metabolism (70). In addition to responding to the c-di-GMP signal, VpsT maintains c-di-GMP homeostasis via its activation of the *vpvABC* operon, which encodes a VpvC, a DGC (70).

H-NS is a key negative regulator of biofilm gene expression and *V. cholerae hns* mutants have enhanced biofilm-forming ability (6, 7, 185, 209). H-NS regulates nucleoid topology and binds to AT-rich promoter sequences thereby repressing transcription (46, 47). VpsT disrupts H-NS-DNA complexes formed at *vpsA* and *vpsL* promoters in a dose-dependent and c-di-GMP dependent manner, thereby activating biofilm gene transcription (185, 209). Increasing c-di-GMP levels promotes displacement of H-NS from *vpsA* and *vpsL* promoters by VpsT binding; conversely, in the absence of c-di-GMP, H-NS replaces VpsT at these promoters (8). Overlapping VpsT and H-NS binding motifs are also present in the upstream regulatory sequences of multiple biofilm genes: *vpsU*, *rbmA*, and *rbmF* (8). A H-NS binding site is also present at the *vpsT* promoter (Figure 3) (185). VpsR also positively regulates *vpsT* expression and the lack of *vpsR* increases H-NS occupancy at the *vpsT* promoter (8, 165). Furthermore, decreased H-NS occupancy at the *vpsT* promoter in response to high c-di-GMP levels requires VpsR (8). Thus, coordinated VpsR and VpsT action and increased c-di-GMP levels are needed to relieve the HN-S mediated repression of biofilm matrix production.

H-NS also interacts with the master virulence regulator, ToxR, which can antagonize H-NS at shared binding sites upstream of both virulence and biofilm related genes. Deletion of *toxRS* results in reduced biofilm formation that is rescued when *hns* is also deleted, suggesting that ToxR's primary regulatory role is to antagonize H-NS activity at key promoter locations (98). Other studies indicate that the iron-sensing regulatory protein Fur, which is discussed in further detail below, also directly binds to the regulatory region of *vps* genes and potentially antagonizes other transcriptional regulators of biofilm genes, as its binding sites overlap with VpsT and H-NS (67).

HapR, a TetR family transcriptional regulator, is another major negative regulator of biofilm gene expression; disruption of *hapR* enhances biofilm formation. HapR has an all-helical structure with an N-terminal helix-turn-helix (HTH) DNA-binding domain and a large C-terminal dimerization domain. The dimerization interface is predicted to have a ligand-binding pocket for an unidentified small molecule ligand which may impact its DNA binding ability (44). HapR represses expression of *vps*L, *vpsT* and key c-di-GMP metabolizing enzymes and the HapR binding motif is present at these promoters (Figure 3) (180, 187, 206).

AphA and HapR are defined as low cell density and high cell density master quorum sensing (QS) regulators, respectively. While HapR negatively regulates biofilm formation, AphA, a key regulator of virulence gene expression, also positively regulates biofilm gene expression, through direct regulation of *vpsT* expression (Figure 3). Furthermore, AphA expression is directly activated by VpsR and induced by high cyclic di-GMP levels (111).

6.2. Small RNAs in the biofilm regulatory circuitries

Non-coding small RNAs (sRNAs) in the range of 50 – 250 nucleotides are widespread among bacteria. sRNAs can modulate the translation or stability of complementary, target mRNAs. The RNA chaperone Hfq facilitate sRNA base-pairing interactions. In *V. cholerae*, sRNAs affect biofilm formation by controlling production of key transcriptional regulators of biofilm matrix genes and by directly controlling biofilm matrix production (Figure 4).

One key group of biofilm-regulating sRNAs are termed the quorum regulatory RNAs (Qrr1-4). Qrr1-4 act *in trans* and are Hfq-dependent. QS, which involves the production, release, and recognition of extracellular signaling molecules termed autoinducers (AIs). QS allows microorganisms to monitor cell-population density and coordinate group behaviors, including biofilm formation. Bacterial QS has been recently reviewed in greater detail and is briefly described in Figure 4 (130).

The Qrr sRNAs negatively regulate the *luxO* mRNA and thus establish a feedback loop to control Qrr production (171). The Qrr sRNAs also positively regulate translation of VCA0939, encoding a DGC, by direct interaction of the Qrr sRNAs with 5 ′ UTR of VCA0939, which is enhanced by Hfq. While the ΔVCA0939 mutant does not show a biofilm defect, increased translation of VCA0939 via the Qrr sRNAs results in increased c-di-GMP production, biofilm gene expression, and biofilm formation, thus impacting a *V. cholerae* QS phenotype, independently of HapR.

Production of DPO (5-dimethylpyrazin-2-ol), a recently identified AI, relies on L-threonine degradation by threonine dehydrogenase (Tdh). DPO is recognized by VqmA (VCA1078, *Vibrio* QS modulator A) (140), which positively regulates the *vqmR* sRNA. VqmR is Hfq-dependent and represses *vpsT* translation, presumably by inhibiting ribosome binding (Figure 4) (140). In strains lacking *vqmR* or *vqmA*, *vpsT* mRNA and protein levels are increased compared to wild-type *V. cholerae*. Furthermore, overexpression of VqmR or DPO supplementation inhibits biofilm formation (140).

QS is further regulated by carbon storage regulator (*csr*) sRNAs. The carbon storage regulator protein, CsrA, is a global, post-transcriptional regulator that activates central carbon metabolism pathways and growth-phase dependent phenotypes, including biofilm formation (26, 176). CsrA activity is inhibited through sequestration by CsrBCD (176). These sRNAs are positively regulated by the VarSA two-component system and VarA is, in turn, positively regulated by CsrA in an autoregulatory feedback loop (Figure 4) (27). CsrA impacts biofilm formation in *V. cholerae* by repressing QS, which represents a complex signal transduction cascade (87, 108). Negative QS regulation by *V. cholerae* CsrA increases biofilm formation, while HapR, the key QS regulator, strongly represses biofilm formation (87, 108, 181). RNA-seq and RNA-CsrA coimmunoprecipitation analysis identified key regulatory and structural biofilm genes as potential CsrA targets, including the *aphA* and *rbmA* transcripts (26). Mutation of *csrA* decreased expression of a number of biofilm regulators, including *vpsT*, but appeared to regulate structural genes in the *vps* and *rbm* clusters in a growth-dependent manner: positively regulating them during exponential phase and negatively regulating them during stationary phase (26).

VadR (VxrB activated small RNA) was initially identified as an Hfq-dependent sRNA regulating cell curvature in *V. cholerae* (141). VadR is a direct repressor of the *crvA* mRNA. CrvA levels are increased in a *vadR* mutant and such mutants display increased cell curvature without impacting cell length or volume (141). VadR is expressed during

the initial stages of biofilm formation and this expression promotes straighter cells (141). Intriguingly, c-di-GMP has also been shown to drive the conversion of *V. cholerae* from curved to straight cell morphology to promote biofilm formation, although VadR represses biofilm formation (56, 141). Subsequent studies showed that the VxrAB two-component system activates VadR expression and that VadR negatively regulates the production of genes located in the VcBMC; VadR directly base pairs with *vpsU*, *vpsL*, *rbmA* and post-transcriptionally represses biofilm formation in *V. cholerae* (Figure 4). *vadR* mutants show a slight increase in biofilm biomass and VadR overexpression decreases biofilm formation (141). The VxrAB system promotes biofilm formation in *V. cholerae*, yet the exact molecular mechanism of such activation remains to be determined. VxrB and VadR form a mixed regulatory network involving transcriptional regulators as well as noncoding RNAs. Given that VxrB and VadR inversely regulate biofilm gene expression, VxrB, VadR, and *vpsL* may form a type 1 incoherent feed-forward loop (I1-FFL), a regulatory network motif in which a regulator activates both a gene and a repressor of the gene (141).

The envelope stress induced sRNA VrrA (*Vibrio* regulatory RNA of *ompA*) impacts biofilm formation by targeting the biofilm matrix protein RbmC (Figure 4). The expression of VrrA is dependent on the alternative sigma factor RpoE. VrrA represses *ompA* translation by binding to the 5′ untranslated region of the *ompA* mRNA (164). *vrrA* mutants overproduce the OmpA porin while OmpA is repressed in a VrrA-overexpressing strain. VrrA promotes OMV production through repressing OmpA synthesis (164). The VrrA sRNA also represses *ompT* translation by base-pairing with the 5′ region of the mRNA in a Hfq-dependent manner (164). In addition to regulating OmpA and OmpT production, the VrrA sRNA represses *rbmC* translation by binding to the 5 ′ untranslated region of the *rbmC* mRNA, in an Hfq-independent process (164). Overproduction of VrrA does not impact the initial stages of biofilm formation, but significantly reduces mature biofilm formation (164). As expression of VrrA is controlled by RpoE, VrrA serves as a molecular link between the RpoE and biofilm formation in *V. cholerae* (164).

6.3 Biofilm dispersal

The transition from the planktonic lifestyle to the biofilm lifestyle is well studied in *V. cholerae*, while the reversal of this process is relatively nascent. The metabolically costly commitment to biofilm formation requires the repression of major cellular functions and production of large amounts of extracellular matrix. Release from this adherent matrix is, by design, intricate. Degradation or alteration of cell-to-surface and cell-to-cell attachment factors, such as pili, adhesion proteins, and polysaccharides, can lead to dispersal during various stages of biofilm formation. Dispersal can involve individual cells or collective release that results in a major loss of biofilm biomass (149). Cellular dispersal from biofilms can either be actively regulated or a passive process such as shearing by fluid flow (142, 149, 191). Active biofilm dispersion requires a regulatory response, in which *V. cholerae* senses and responds to changing biofilm microenvironments or external stimuli. The characterization of dispersal, and the regulatory response in *V. cholerae* is far from complete, but several parallel regulatory processes that contribute to biofilm dispersion have recently been identified and are summarized below (Figure 5).

In other species, active dispersal is generally concurrent with pili retraction, the downregulation of biofilm matrix production, upregulation of matrix-degrading enzymes, and upregulation of motility, which are process that are often driven by intracellular levels

of c-di-GMP levels (90, 94). The abundance of c-di-GMP in *V. cholerae* can be reduced through diverse mechanisms i.e., transcriptional regulation and posttranslational activation of c-di-GMP metabolizing enzymes. One such example is the modulation of MSHA pili interactions with a surface by cellular c-di-GMP levels. Low c-di-GMP levels induce MSHA pilus retraction, and enhanced retraction leads to detachment of *V. cholerae* from surfaces during the early stages of biofilm formation (60, 182). Microaerophilic conditions stimulate MSHA pili retraction and detachment from biofilms in a CdpA-dependent manner. CdpA is a NO-responsive PDE, and it was suggested that under these low oxygen conditions, *V. cholerae* produces NO to promote biofilm dispersal (84). Nitric oxide (NO) is a near ubiquitous free radical that acts as a signaling molecule. NO functions as a biofilm dispersal agent in several species (10, 121, 149), though its impact on *V. cholerae* biofilms is still being elucidated. *V. cholerae* biofilms grown in liquid shaking conditions disperse upon exposure to NO (11), a result not seen in *V. cholerae* biofilms grown in flow chambers (192). In addition to c-di-GMP mediated regulation of pili motor activity, the biofilm dispersal regulator, VbdR, induces MSHA pili retraction through transcriptional regulation, discussed further below (159).

C-di-GMP also influences a conserved surface attachment/detachment c-di-GMP signaling module known as LapDG (Figure 1B). The c-di-GMP receptor, LapD, and its cognate periplasmic protease, LapG, enables c-di-GMP-dependent adhesion and degradation of the large surface adhesins FrhA and CraA in *V. cholerae* (33, 36, 102). *V. cholerae*'s FrhA and CraA contain LapG consensus sites that follow an N-terminal periplasmic retention domain (102, 134, 135, 161). When the cytoplasmic domain of *V. cholerae* LapD binds to c-di-GMP, its periplasmic domain sequesters and thus inactivates LapG (102, 134), which is analogous to the function of the LapDG system in *P. fluorescens*. Conversely, when no c-di-GMP is bound to LapD, LapG is liberated in the periplasm and can act as a protease that cleaves the large adhesins, FrhA and CraA, to facilitate dispersal (102). Lack of a functional LapG leads to incomplete biofilm dispersion in a static culture dispersal assay and continuous overexpression of LapG causes a reduced peak biofilm biomass in a static culture dispersal assay (25). In addition to post-translational regulation by LapDG, both FrhA and CraA are transcriptional regulated by c-di-GMP-dependent transcriptional activators FlrA, VpsT, and VpsR, adding another node of c-di-GMP input to this system (16, 102).

Changes in cellular c-di-GMP levels through activation and suppression of c-di-GMP metabolizing enzymes also impact biofilm matrix production, flagellar motility, and, consequently, biofilm dispersion, through alterations of the active state of core biofilm regulators. One well-studied signal c-di-GMP signaling module is the NspS/MbaA system, which responds to polyamines (24, 35, 42). NspS is a periplasmic polyamine binding protein that binds and inhibits the transmembrane protein MbaA (35). MbaA localizes to the inner membrane and contains SGDEF and EVL domains, which are predicted to synthesize and degrade c-di-GMP. When spermidine levels are high, NspS binds spermidine and does not bind to MbaA, which functions as a PDE in its unbound state to reduce c-di-GMP levels (20, 24, 163). The presence of extracellular spermidine results in premature *V. cholerae* biofilm dispersion in static cultures and inhibits biofilm formation (129). However, it is unclear whether spermidine exposure is sufficient to disperse pre-grown biofilms. It is worth noting that norspermidine, which differs from spermidine by one methylene group, can inhibit dispersal through the same pathway. Norspermidine also

674 binds NspS but, in contrast to spermidine, promotes NspS binding to MbaA, thus inhibiting
675 its PDE activity and promoting biofilm formation (24). It appears that MbaA is also able to
676 act as a DGC through its SGDEF domain in the presence of norspermidine (24).
677 Additional environmental cues, including oxygen, temperature, and host signals, that
678 modulate c-di-GMP levels through the activation or reduction of one or more of *V.*
679 *cholerae*'s PDE/DCGs are also likely to impact biofilm dispersion (37).
680 Reduced production of matrix components can promote biofilm dispersal, to
681 enable cellular departure from biofilms, but cells also must detach from and/or degrade
682 the matrix. Several additional enzymes that are involved in *V. cholerae* biofilm matrix
683 processing have been identified. The proteases IvaP, PrtV, and HapA can process the
684 major matrix protein RbmA (76, 160), which also undergoes autoproteolysis (112).
685 Directly adjacent to *rbmA* on the chromosome, is the *rbmB* locus, which encodes a
686 putative polysaccharide lyase that potentially hydrolyses VPS (63). The presence of
687 RbmB reduces the accumulation of VPS in biofilms (63). Overexpression of RbmB in pre-
688 grown biofilms reduces biofilm biomass (45), and deletion of RbmB causes a defect in
689 spontaneous dispersal (25), indicating that RbmB modulates VPS production. The
690 nucleases Xds and Dns can process extracellular DNA; deletion of these nucleases
691 impacted biofilm dispersion in one assay (146, 155), but not in another assay (25),
692 indicating that these nucleases may have a condition-dependent impact on biofilm
693 dispersal.

694 QS signaling circuitry also contributes to the regulation of biofilm dispersal, as HapR
695 responds to cell density cues to repress matrix production and promote motility. High
696 levels of the QS autoinducers CAI-1, AI-2, and DPO enhanced HapR production and the
697 transcriptional regulator/regulatory RNA pair VqmA/VqmR, which repress VpsT
698 transcription and translation, respectively, to effectively inhibit matrix production (Figure
699 5) (139, 140). It remains unclear if exposure of pre-grown biofilms to autoinducers alone
700 is sufficient to induce *V. cholerae* biofilm dispersion or if synthetic pro-QS molecules can
701 trigger dispersion (136). However, the presence of high levels of QS autoinducers is
702 known to contribute to increased biofilm dispersion following carbon source removal in
703 flow chambers (158) and result in earlier biofilm dispersion in static cultures (23).
704 Dispersal following flow cessation or carbon starvation in flow-chamber-grown biofilms
705 depends on HapR and the alternative sigma factor, RpoS (Figure 5) (158). RpoS
706 regulation of biofilm dispersal may be conditional, as the stringent response partially
707 positively regulates key biofilm regulators VpsR and VpsT through RpoS (158, 159) and
708 given that VpsT represses RpoS in a c-di-GMP dependent manner (5, 175). However,
709 RpoS also promotes biofilm detachment via HapR and motility gene regulation (158, 206).
710 Wurm et al. propose a model in which RpoS initiates early stages of biofilm formation
711 before being repressed by c-di-GMP and VpsT (196). As the biofilm matures, RpoS is
712 activated spatially and temporally in response to nutrient limitation, enhancing motility and
713 detachment (23).
714 HapR and VqmA/VqmR also upregulate the expression of the biofilm dispersal
715 regulator VbdR (159), which was identified using a genetic screen to identify factors
716 whose overexpression could cause dispersion of pre-grown biofilms. Subsequent studies
717 showed that *vbdR* expression is induced by carbon starvation (159). *vbdR* overexpression
718 liberates cells from the matrix of pre-grown biofilm through PilTU-mediated MSHA pilus
719 retraction and simultaneous upregulation of the serine protease IvaP (Figure 5) (159). As

discussed previously, the matrix protein RbmA is processed by IvaP, HapA, and PrtV (76, 160), but VbdR only significantly controls IvaP during dispersion. Interestingly, VbdR-controlled cellular dispersal from biofilms leaves behind empty shells of extracellular matrix, indicating that the biofilm matrix does not need to be completely degraded for cellular dispersal–instead, cellular detachment from the matrix is a key process during biofilm dispersion (159).

Biofilm dispersion can also occur spontaneously in growth systems without flow or fluid agitation after a certain cultivation period (24, 25, 154). Multiple two-component systems negatively regulate the expression of biofilm matrix genes, including CarRS (18), NtrBC (30), PhoBR (145), VieSA (115), and DbfSR (25). DbfSR (VC1639/VC1638) is a recently characterized regulatory module that contributes to biofilm dispersion in a static biofilm culture assay (25). DbfS can act as a phosphatase on DbfR, while phosphorylated DbfR enhances matrix production. The absence of DbfS leads to a defect in the downregulation of matrix production that is generally required for biofilm dispersion (25). A genetic screen showed that DbfS is involved in polymyxin B resistance (116, 117). In addition, CpxR, a response regulator for envelope stress, regulates DbfS (2, 116). However, the signal that binds to the periplasmic sensory domain of DbfS remains unknown.

It is likely that additional proteases, polysaccharide lyases, or surfactants are involved in matrix degradation during biofilm dispersal, and that further signals and regulatory modules at the unicellular and multicellular levels control biofilm dispersion in *V. cholerae*.

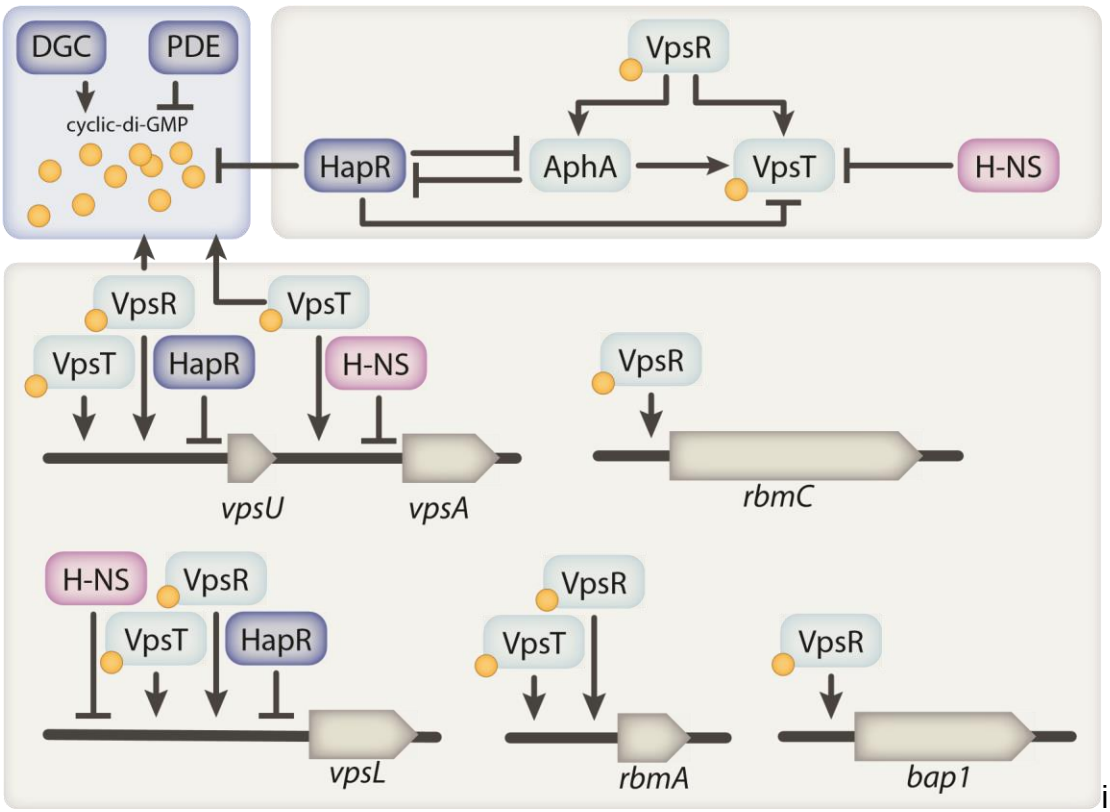
8. ENVIRONMENTAL SIGNAL INPUT INTO BIOFILM REGULATORY CIRCUITRIES

The transition from free-swimming cells to a sessile biofilm, and vice versa, is informed by numerous sensory inputs controlled by complex signal transduction networks that integrate extracellular and intracellular signals. Nitrogen, calcium, salinity, and bile are environmental signals known to influence biofilm formation and dispersal, reviewed in the following citations (94, 174). This section focuses on select environmental inputs that impact biofilm formation and dispersal whose mechanism of action has been recently characterized, including carbon, phosphate, iron, and temperature.

8.1 Carbon availability

As discussed, carbon availability is a major main nutritional cue that informs the metabolically costly decision to form or disperse biofilms. The phosphoenolpyruvate (PEP) phosphotransferase system (PTS), which transports and phosphorylates carbohydrates during the process, regulates biofilm formation in many bacterial species. At least four independent PTS pathways have been identified that influence biofilm formation in *V. cholerae* (78, 80, 183, 199, 208).

PTS systems are comprised of two general proteins, enzyme I (EI) and the histidine phosphocarrier protein (HPr), as well as several substrate-specific proteins known as the enzyme II (EII) proteins. The phosphorylation states of PTS components reflect the nutritional status of the cell. The glucose specific PTS plays a complex role in regulating *V. cholerae* biofilm formation, as some members of the system activate biofilm formation while others repress it (80, 143). $EIIA^{Glc}$, part of the glucose-specific PTS system, has different targets when phosphorylated compared to the unmodified state. $EIIA^{Glc}$



interaction partners also influence its effect on biofilm formation. Removal of an EIIA^{Glc} membrane-associated domain revealed membrane interaction partners repress biofilm formation. In the absence of this domain constitutive association with cytoplasmic partners promotes biofilm formation. Full length EIIA^{Glc} may therefore integrate internal and external cues to illicit an appropriate regulatory response (183). Phosphorylated EIIA^{Glc} was also recently shown to interact with the PDE, PdeS, under glucose limited conditions to repress biofilm formation (78). Additionally, a screen designed to identify EIIA^{Glc} binding partners revealed that unphosphorylated EIIA^{Glc} interacts with MshH, which is homologous to *E. coli* CsrD protein. In *E. coli*, unphosphorylated EIIA^{Glc} promotes degradation of the inhibitory sRNAs CsrB and CsrC through binding to the CsrD protein (107). While CsrD contains domains which are often responsible for the synthesis of c-di-GMP, however, CsrD does not display any c-di-GMP activity. Instead it appears to be required for unphosphorylated EIIA^{Glc}-mediated decay of CsrB and CsrC (107). Whether *V. cholerae* MshH has a similar function to the CsrD homolog remains unclear. However, given its interaction with unphosphorylated EIIA^{Glc}, it may represent a connection between carbon source availability and sRNA CsrA regulation, both of which are known to regulate biofilm formation (143).

Carbon source availability also modulates production of cyclic adenosine-monophosphate (cAMP), an important intracellular signal transduction molecule that modulates biofilm formation. Phosphorylated EIIA^{Glc} activates cAMP synthesis by binding to adenylate cyclase (CyaA). cAMP then binds the regulator cAMP receptor protein (CRP) to initiate carbon catabolite repression (CCR). cAMP-CRP represses biofilm formation in *V. cholerae* (64, 109, 110) and upregulates the key biofilm repressor, HapR (see above) (109). Intriguingly, in a mutant lacking *hapR*, loss of the genes encoding for CyaA and

CRP decreases biofilm formation. In this background, cAMP-CRP positively regulates the master biofilm regulator, VpsR. However, in the wild-type background this regulation appears to be superseded by cAMP-CRP-mediated upregulation of HapR, indicating that multiple signal cascades may feed into CCR regulation of *V. cholerae* biofilm formation (110). Carbon metabolism can also impact c-di-GMP synthesis and degradation and thus biofilm formation and dispersal. cAMP-CRP represses biofilm in *V. cholerae* via repression of the DGC CdgA (64). CRP also positively regulates genes involved in nucleoside uptake, which reduce biofilm formation (71).

8.2 Phosphate availability

Other forms of nutrient limitation have been shown to repress biofilm formation in *V. cholerae*. Phosphate is an essential nutrient, contributing to nucleic acid synthesis and signal transduction. However, it is often limited in both the aquatic environment and human host. Like many bacteria, *V. cholerae* encodes multiple phosphate transport systems that can take up inorganic phosphate from the surrounding environment, including the phosphate specific transport systems, *pst* and *pst2*, and a low affinity phosphate transport system homolog, *pitA* (VC2442) (119). While the role of VC2442 in phosphate uptake remains characterized, both *pst* and *pst2* are part of the *V. cholerae* PhoBR TCS regulatory pathway (119, 145, 184). When extracellular phosphate is abundant a component of the Pst, PstS, binds phosphate and represses autophosphorylation of the histidine kinase PhoR. Under phosphate conditions, PhoR interacts with the Pst system to autophosphorylate and activate the response regulator PhoB via phosphotransfer (29). Activated PhoB binds upstream of Pho regulon genes, upregulating *pst2* expression while repressing virulence and biofilm formation via repression of *tcpPH*, *vpsR*, and c-di-GMP production (40, 144, 145, 170).

Precise and appropriate regulation of the PhoBR system is important to the *V. cholerae* life cycle, playing a vital role in infection, transmission, environmental survival, and biofilm formation. The deletion of the *pst* system results in constitutive induction of the Pho regulon, while deletion of *phoB* results in inactivation of the Pho regulon (144). Proper regulation of the Pho regulon seems to be an important within biofilms as well. Though PhoB downregulates biofilm genes, both *phoB* and the *pst2* system are enriched in biofilms, suggesting that cells may be responding to phosphate starvation within biofilms. In addition, *pst2* upregulation within biofilms contributes to the hyperinfectivity of biofilm-grown cells in the host (129). It has been suggested that too much or too little phosphate may lead to biofilm dispersal, further demonstrating the intricacies and interplays of the biofilm regulatory complexes. *V. cholerae* can utilize extracellular DNA, a structural component of the biofilm matrix, as a phosphate source and uptake of degraded eDNA contributes to environmental fitness following transition from the host (71, 119, 154). Phosphate starvation may be a signal for extracellular DNA degradation and contribute to biofilm dispersal, in tandem with PhoB-mediated downregulation of biofilm genes.

8.3 Iron Availability

V. cholerae experiences varying iron availability in the human host and in the aquatic niches it occupies. Although iron is an essential micronutrient, excess cellular iron is toxic since Fenton chemistry damages macromolecules. *V. cholerae* has multiple iron acquisition systems that are primarily regulated by Fur, an iron-sensing regulatory protein

(126, 197). Under iron-replete conditions, Fur is present in an iron-bound state and directly represses specific iron acquisition gene through direct binding to the Fur box consensus sequence (126, 197). In addition, Fur represses expression of the small regulatory RNA, RyhB, which reduces the transcript abundance of a set of Fur and iron-regulated genes (42, 125). RyhB interacts with the RNA-binding protein Hfq to promote the degradation of target mRNAs. Under iron depleted conditions, Fur is present in the iron-free state, allowing expression of iron acquisition genes and sRNA-RyhB. Iron availability, Fur, and RyhB regulate biofilm formation by targeting the expression and abundance of biofilm matrix genes and their regulators (42, 67, 125). Fur represses biofilm formation by inhibiting *vps* gene expression through Fur boxes present in the regulatory regions of *vpsU* and *vpsA,* the first gene in the *vps*-I operon (67). In addition, Fur regulates cellular c-di-GMP levels independently of iron availability by repressing expression of a DGC (*cdgD*) and activating expression of a PDE (*vieA*). The net result is a decrease in cellular c-di-GMP levels (67). Fur-mediated repression by direct binding to *vps* genes is likely to be further amplified as *vps* expression is positively regulated by increased cellular c-di-GMP levels in a VpsT and VpsR dependent manner (67). It should be noted earlier gene expression profiling studies and ChIP-seq studies did not identify biofilm matrix and c-di-GMP signaling genes as targets of Fur or RyhB. The authors suggested that Fur may antagonize other transcriptional activators of biofilm genes and work with other transcriptional repressors of biofilm genes, noting that the Fur regulatory regions for *cdgD*, *vpsA-K*, and *vpsU* overlapped with both VpsT and H-NS binding sites (67).

Biofilm formation is markedly reduced in response to iron depletion by the addition of iron chelator, suggesting that biofilm formation is inhibited under iron-limited conditions. RhyB is required for biofilm formation and iron or succinate supplementation rescues the biofilm formation defect associated with the *ryhB* mutant. These findings indicate that iron and *ryhB* regulate biofilm formation and suggest that RyhB may target a negative regulator of biofilm formation. The RyhB regulon includes the c-di-GMP dependent transcriptional regulator VpsT, whose abundance is decreased by two-fold in the *ryhB* mutant (42). However, RyhB overexpression does not alter biofilm formation and the mechanism underlying VpsT regulation remains unknown (125). Furthermore, under iron limitation, *ryhB* mutant cells had decreased motility compared to the wild-type strain.

8.4 Temperature

Temperature changes during seasonal shifts or as the microbe moves from an external environment into a host can alter biofilm formation. Biofilm formation is enhanced when *V. cholerae* experiences temperature shifts from host temperatures of 37°C to lower temperatures of 25°C and 15°C, via the activation of 6 DGCs that enhance c-di-GMP production (178, 179). However, warmer water temperatures can also enhance *V. cholerae* attachment to chitin by upregulating the MshA and GpbA adhesins (167). In addition, a conserved ribosome-associated GTPase, BipA (VC2744), negatively regulates rugosity at low temperatures in *V. cholerae* strains that naturally lack the QS master regulator HapR (172). BipA deletion alters the abundance of over 300 proteins when *V. cholerae* is grown at 22°C, including almost all known biofilm related proteins. Though the exact function of BipA is unknown, one study indicated that it is important for assembly of the 50S ribosomal subunit, arguing that BipA modulation of 50S ribosomal

subunit abundance impacts mRNA translation of biofilm-related genes (172). The authors propose a model in which BipA provides an additional layer of biofilm repression at low temperatures that are masked in a HapR+ background due to the dominant exertion of HapR on biofilm repression (172).

9. CONCLUSIONS AND PERSPECTIVES

The multicellular communities known as biofilms represent a unique, yet dominant, mode of bacterial growth in the environment. Understanding the mechanisms and consequences of biofilm formation is important to both basic research and practical applications in medicine and bioremediation. Building and breaking *V. cholerae* biofilms is a complex, sequential, and highly regulated process, as reviewed here. Recent studies have revealed new mechanistic insights into initial attachment and adhesion, biofilm matrix interactions, regulatory pathways, including the growing sRNA regulatory networks, emergent properties, and mechanisms and signals of biofilm dispersal. The interplay between the small nucleotide signaling molecules, environmental signals, and signal transduction pathways highlights the complex regulatory network responsible for determining if bacteria stay or disperse from biofilms in response to fluctuations in their surroundings. Building on over 25 years of study, recent *V. cholerae* biofilm research has resulted in the development of new tools for study that can be applied across bacterial species, as well as in the burgeoning field of bacterial social interactions.

While an incredible amount of new information has been uncovered in the field of *V. cholerae* biofilms, there is still so much that is unknown. The fate of the flagellum after initial attachment remains a mystery. Interactions between both cells and various matrix components within the biofilm are still being explored. Despite prodigious work untangling the complex regulatory network governing biofilm formation and dispersal, many mechanistic details remain unknown. There is growing study of the relatively unexplored realm of multispecies biofilms in the human host and natural environment (9, 57), as well the complex community interactions within and around biofilms. Variations in biofilm forming abilities of the pandemic *V. cholerae* strains further complicate our understanding of biofilm formation and highlight the necessity of integrating disparate genomic and phenotypic results into the depth of available biological information.

Figures

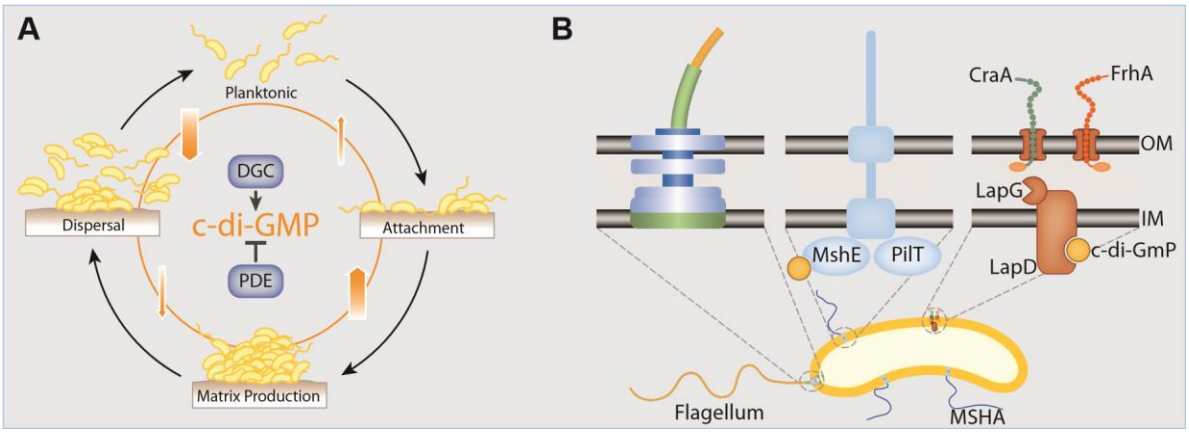


Figure 1. Cycle of biofilm formation and dispersal. A) c-di-GMP promotes the transition from a motile, planktonic state to the sessile biofilm lifestyle in *V. cholerae* by repressing motility and upregulating surface attachment and biofilm matrix production. C-di-GMP is synthesized by diguanylate cyclase enzymes (DGCs) and degraded by phosphodiesterase enzymes (PDEs). As c-di-GMP concentrations increase, *V. cholerae* attachment to a surface and production of biofilm matrix components is enhanced, while decreasing levels of c-di-GMP leads to dispersal from the biofilm. B) The flagellum and flagellar motility are necessary for initial attachment. Loss of the flagellum results in the flagellum-dependent biofilm regulatory (FDBR) response, which positively regulates c-di-GMP levels and biofilm formation. Higher levels of c-di-GMP results in efficient production of MSHA pili, which are required for initial attachment. MshE is a c-di-GMP receptor protein; when bound to c-di-GMP, MshE is responsible for polymerizing the MshA subunits that makeup the MSHA pilus. The retraction ATPase, PilT, mediates MSHA pilus retraction, which facilitates near-surface motility and surface attachment. C-di-GMP also regulates a conserved surface attachment/detachment c-di-GMP signaling module known as LapDG. The *V. cholerae* protease LapG targets two adhesion proteins, FrhA and CraA. When LapD binds c-di-GMP in its cytoplasmic domain, its periplasmic domain has a higher binding affinity for LapG and will sequester and inactivate LapG. Conversely, when no c-di-GMP is bound to LapD, LapG is liberated in the periplasm and can act as a protease that cleaves the large adhesins, FrhA and CraA, to facilitate dispersal.

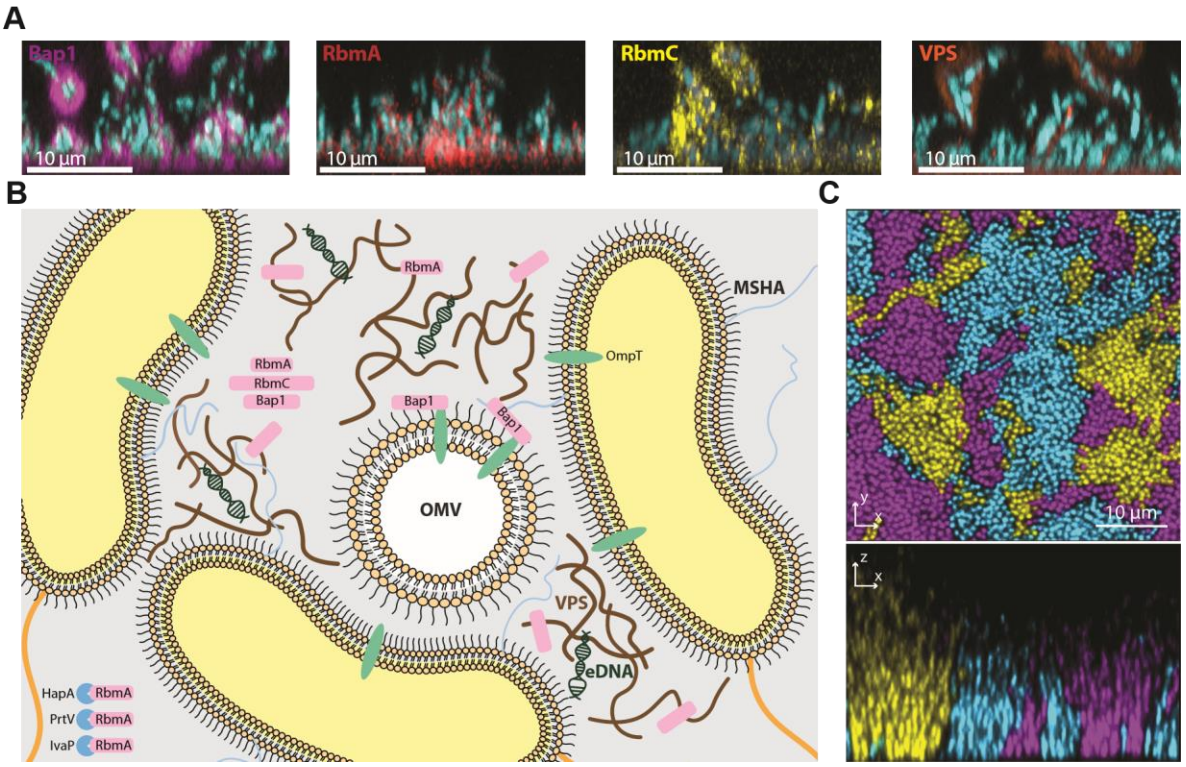


Figure 2. *Vibrio cholerae* biofilm matrix components. A) Localization of the major biofilm matrix components RbmA, Bap1, RbmC and VPS in mature biofilms. B) Biofilm formation relies on the production of extracellular matrix components—polysaccharides, proteins, nucleic acids, and other biomolecules to keep cells attached to each other and to surfaces. A major component of the *V. cholerae* biofilm matrix is VPS, which is present throughout mature biofilms. VPS can interact with other matrix components, including extracellular DNA (eDNA) and the biofilm matrix protein RbmA. RbmA is secreted following attachment of a founder cell to surfaces and it is present throughout the mature biofilm. RbmA facilitates the initial stages of microcolony and cluster formation by controlling cell orientation relative to a substratum and by promoting the attachment of daughter cells to founder cells by cell-to-cell adhesion. RbmA can be processed by the proteases IvaP, PrtV, and HapA. In addition to RbmA, RbmC and Bap1 have similar, but non-redundant, functions in biofilm matrix assembly facilitating sustained attachment of cells and growing biofilms to surfaces. Bap1 has been found in association with outer membrane vesicles (OMVs). Though OMVs and eDNA are known components of the biofilm matrix, their contribution to biofilm architecture and structure is still an area of open exploration. MSHA is also a component of the biofilm matrix and may bind to other matrix components. C) Biofilm formed with three isogenic hyper-matrix producing strains expressing different fluorescent proteins, illustrating that the clonal structuring of strains that occurs during biofilm formation. Cell lineages that are established during initial attachment remain closely associated to one another during biofilm development, resulting in little to no mixing between neighboring cell groups.

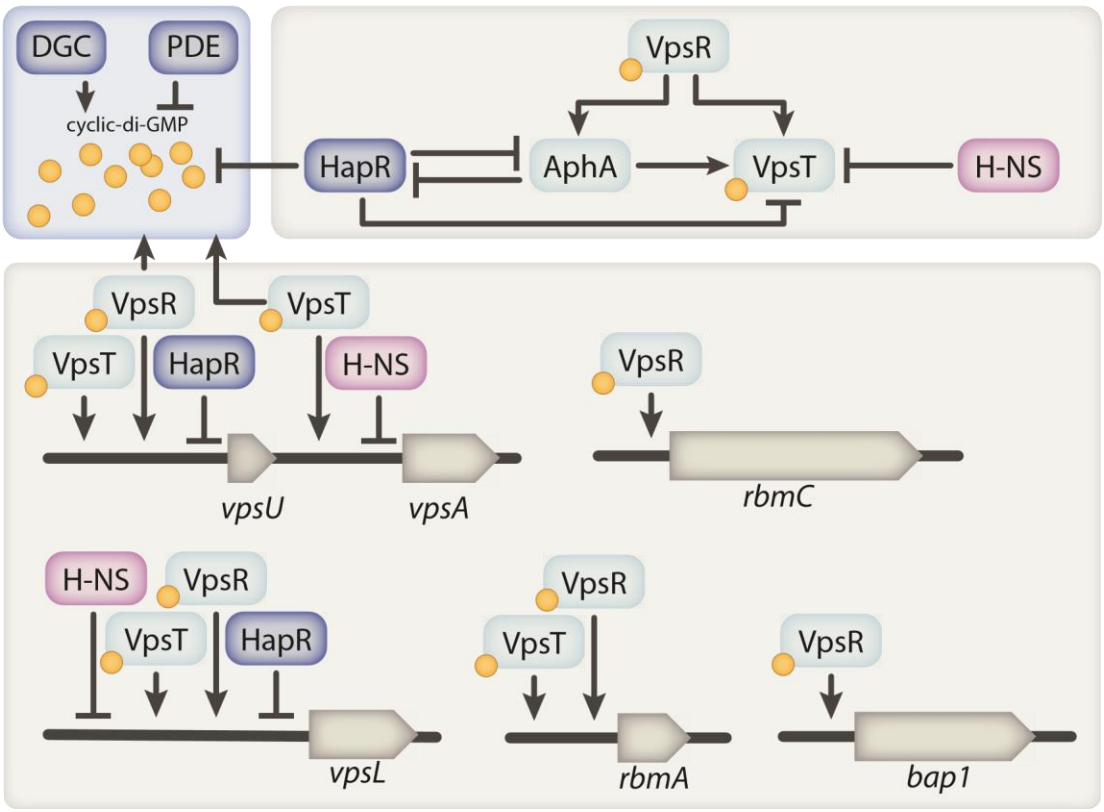


Figure 3. Core regulators of *Vibrio cholerae* biofilm. Regulation of biofilm formation is governed by an interconnected core regulatory network that includes the positive regulators VpsR, VpsT, and AphA, and the negative regulators, HapR and H-NS. C-di-GMP, which is synthesized by diguanylate cyclase enzymes (DGCs) and degraded by phosphodiesterase enzymes (PDEs), feeds into this regulatory network and has been shown to bind to both VpsR and VpsT. The master biofilm regulator VpsR positively regulates both AphA and VpsT, in addition to genes encoding for essential biofilm matrix components. AphA positively regulates VpsT and negative regulates HapR, while HapR negatively regulates AphA, VpsT, and the production of c-di-GMP. H-NS represses VpsT, in addition to repressing genes encoding for *Vibrio* polysaccharide. VpsR, VpsT, HapR, and H-NS have overlapping binding sites in the promoter regions of genes encoding for VPS. VpsR and VpsT also promote biofilm formation through direct binding to the promoter of the gene encoding the key biofilm matrix protein, *rbmA*. VpsR additionally binds to the promoters of the genes encoding the other two biofilm matrix proteins, *rbmC* and *bap1*, to positively regulate their transcription.

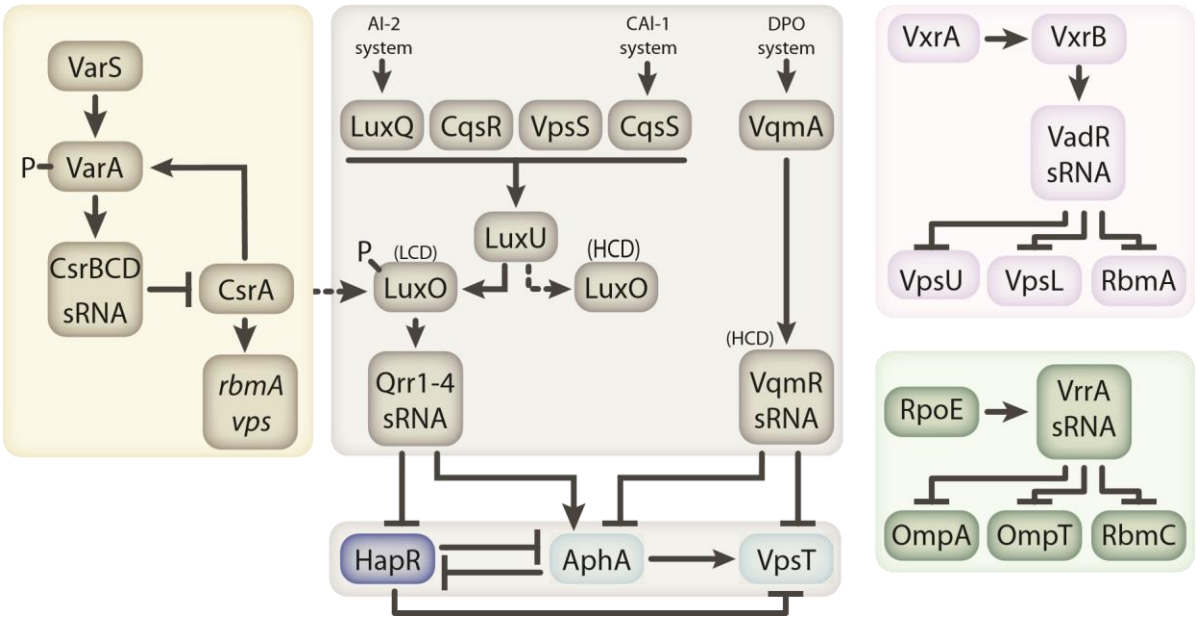


Figure 4. small RNA regulation of biofilm formation. Non-coding small RNAs (sRNAs) regulate biofilm formation by controlling production of key transcriptional regulators of biofilm matrix genes and by directly controlling biofilm matrix production. Here, we highlight several sRNA pathways that feed into *V. cholerae* biofilm regulation, including the CrsBCD sRNAs, the Qrr1-4 sRNAs, VqmR, VadR, and VrrA. CsrBCD are sRNAs that negatively impact biofilm formation by targeting and sequestering CsrA to inhibit its activity. CsrBCD are positively regulated by the VarSA two-component system and VarA is positively regulated by CsrA in an autoregulatory feedback loop. CsrA positively influences biofilm formation through positive regulation of biofilm genes and negative regulation of quorum sensing (QS) through LuxO. The quorum regulatory RNAs (Qrr1-4) play an important role in regulating biofilm formation. QS involves the production, release, and recognition of extracellular signaling molecules termed autoinducers (AIs). *V. cholerae* produces three AIs: CAI-1 ((S)-3-hydroxytridecan-4-one), AI-2 ((2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate), and DPO (5-dimethylpyrazin-2-ol). CAI-1, produced by CqsA, and AI-2, produced by LuxS, are detected by different two-component system regulatory circuits that function through a phosphorelay cascade. Sensor histidine kinase CqsS senses CAI-1, while sensor histidine kinase LuxQ, together with a periplasmic protein LuxP, senses AI-2. At low cell density (LCD), CqsS and LuxQ phosphorylate the response regulator LuxO through the phosphorelay protein LuxU. Phosphorylated LuxO works with the alternative sigma factor sigma-54 to activate Qrr1-4 expression. At low cell density Qrr1-4 promote translation of *aphA* and destabilize *hapR* mRNA, thus repressing HapR translation. Conversely, at high cell density, when autoinducers are abundant CqsS and LuxQ function as phosphatases so that LuxO is dephosphorylated and inactivated. In this case, the Qrr1-4s are not transcribed, which relieves *hapR* repression. HapR then negatively regulates VpsT and other biofilm related genes. High levels of DPO enhances production of the regulatory RNA pair VqmA/VqmR, which represses VpsT translation. VadR is a sRNA that negatively regulates biofilm by

binding to and inhibiting the translation of *vpsU*, *vpsL*, and *rbmA*. VadR form a mixed regulatory network with the VxrAB two-component system, which is a positive regulator of VadR and biofilm genes. The VrrA sRNA is induced by envelope stress and represses *ompT* and *ompA* translation to promote outer membrane vesicle (OMVs) production. VrrA additionally targets and represses *rbmC* translation and overexpression of VrrA results in a reduction of mature biofilm formation. RpoE is required for VrrA expression, providing a link between RpoE and biofilm formation.

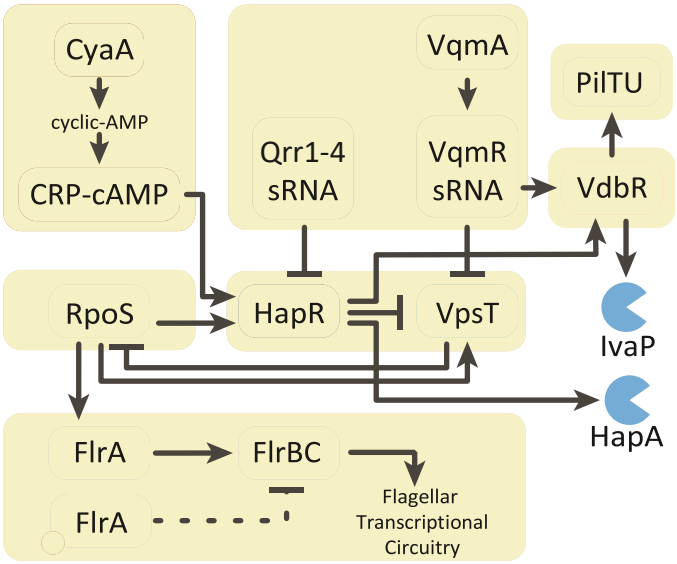


Figure 5. Regulation of biofilm dispersal.
Active dispersal coincides with pili retraction, the downregulation of biofilm matrix production and upregulation of motility. HapR is the main negative regulator of biofilm formation, directly repressing major biofilm regulators and biofilm genes. Quorum sensing sRNAs repress HapR, while RpoS and CRP-cAMP activate HapR. HapR activates flagellar gene expression and expression of the protease HapA, leading to increased flagellum production and motility. RpoS also contributes to the expression of motility genes and is negatively regulated by VpsT, in a c-di-GMP dependent manner. The sRNA VqmR represses the biofilm regulator VpsT and activates VdbR, the biofilm dispersal regulator. VdbR induces PilTU-dependent MSHA retraction and production of the protease IvaP. The dual action of enhanced retraction and RbmA processing via IvaP leads to detachment. VdbR is also activated by HapR. Additional signals and regulatory networks that feed into dispersal and are discussed in greater detail in the text.

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