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Living in the matrix: assembly and control of *Vibrio cholerae* biofilms

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Preface

Nearly all bacteria form biofilms as a strategy for survival and persistence. Biofilms are associated with biotic and abiotic surfaces and are composed of aggregates of cells that are encased by a self-produced or acquired extracellular matrix. *Vibrio cholerae* has been studied as a model organism for understanding biofilm formation in environmental pathogens, as it spends much of its life cycle outside of the human host in the aquatic environment. Given the important role of biofilm formation in the *V. cholerae* life cycle, the molecular mechanisms underlying this process and the signals that trigger biofilm assembly or dispersal have been areas of intense investigation over the past 20 years. In this Review, we discuss *V. cholerae* surface attachment, various matrix components and the regulatory networks controlling biofilm formation.

Filippo Pacini first isolated and described the Gram-negative bacterium *Vibrio cholerae* in 1854 the same year that John Snow's 'ghost maps' revealed that a tainted water supply was the source of a deadly cholera outbreak. Pathogenic strains of *V. cholerae* cause the acute diarrheal disease cholera, which can result in hypotonic shock and death within 12 hours of the first symptoms[1]. Approximately 3–5 million people are infected with *V. cholerae* annually and 100,000–120,000 cases are fatal[1].

V. cholerae forms biofilms during aquatic and intestinal phases of its life cycle[2-4]. Both toxigenic and nontoxigenic *V. cholerae* strains live in the aquatic environment year-round, either in a planktonic state or in a biofilm. When toxigenic strains of *V. cholerae* enter the human host, typically through the ingestion of contaminated water or food, they colonize the small intestine. Once in the small intestine, *V. cholerae* multiplies and produces the cholera toxin, which causes severe illness in the host. *V. cholerae* is then shed in the stool, where it can reenter the aquatic environment or infect a new host[1]. The role of biofilms in *V. cholerae* environmental persistence, dissemination and transmission has been well established (Fig.

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1). This growth mode provides protection from a number of environmental stresses, including nutrient limitation, predation by unicellular eukaryotes (known as protozoa) and attack by bacterial viruses (known as bacteriophages)[5,6]. While *V. cholerae* can form biofilms on many biotic and abiotic surfaces, several field studies showed that *V. cholerae* preferentially forms biofilms on phytoplankton, zooplankton and oceanic chitin rain[7,8]. The exoskeletons of zooplankton contain chitin, which *V. cholerae* can utilize as its sole carbon source[9,10]. Growth on chitin also induces natural competence and enables cells to acquire new genetic material[11]. As physical carriers and primary sources of nutrients for *V. cholerae*, zooplankton serve as reservoirs and disease vectors of cholera[12].

Although *V. cholerae* is found year-round in the coastal and estuarine environments where cholera is endemic, outbreaks are seasonal and correlate with changes in environmental conditions[12]. Plankton blooms, which are influenced by water temperature, hours of sunlight, sea surface height, rainfall and salinity, are thought to be the major environmental factor affecting seasonal outbreaks[13]. Simple filtration practices that remove particles larger than 20 μm were shown to significantly reduce cholera cases, which suggests that the removal of biofilm-associated and plankton-associated *V. cholerae* from the environment can reduce transmission[14].

Between epidemics, metabolically quiescent *V. cholerae* cells have been observed in both the planktonic state and in biofilms. These cells seem to contribute to *V. cholerae* persistance[3,4]. These quiescent cells may lose their typical curved rod shape, becoming coccoid, and cannot be cultured under standard laboratory conditions. They can return to an active state in response to signals produced by active cells present in the environment or from passage through a host, though the mechanism of host-mediated activation is unknown[3,15,16]. Biofilms containing metabolically quiescent *V. cholerae* may have important biological relevance, as their reduced metabolic needs and slowed growth may enable them to survive harsh environmental conditions until circumstances improve. Once activated, they may act as seed cells for *V. cholerae* growth in the water supply and contribute to an outbreak[15].

V. cholerae biofilms contain both higher doses of bacteria and hyperinfective cells and therefore have a key role in transmission[3,17]. The hyperinfective state refers to a decrease in the number of cells required to cause disease. In other words, the infectious dose required for infection is decreased and the risk of disease transmission is increased. However, the role of *V. cholerae* biofilm formation inside the host is poorly understood. Both single cells and dense clumps of *V. cholerae* were observed in a rabbit ileal loop infection model, supporting an earlier finding that biofilms may form *in vivo* and subsequently be excreted in stool[3,18]. Biofilms are composed of aggregates of cells encased by a self-produced or acquired extracellular matrix and thus may have increased resistance to host defenses. Though the role of biofilms in host resistance has not been well explored, several studies suggest that a key component of the biofilm, *Vibrio* polysaccharide (VPS), is produced during host infection[19,20]. Additionally, deletion of genes involved in the production of VPS and the extracellular matrix protein RbmA led to a defect in intestinal colonization in a mouse model[21]. Collectively, these findings imply that biofilms play a part during *V. cholerae* infection, but further studies are needed to elucidate the underlying mechanisms and

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functions of *in vivo* biofilms. Currently, much of what we know about *V. cholerae* biofilm structure, function and regulation is based on *in vitro* findings.

In this Review we discuss recent advances in our understanding of initial surface attachment, provide an overview of the matrix components and discuss dispersal. Next, we review the regulatory network that governs *V. cholerae* biofilm formation, including transcriptional regulators of key genes involved in biofilm formation as well as the roles of small nucleotides and small RNAs. Finally, we discuss the impact of aquatic and host environmental inputs on biofilm formation and highlight new discoveries in small-molecule therapeutics that have the potential to control and inhibit *V. cholerae* biofilms.

Surface attachment

V. cholerae biofilm formation is a multistep process: bacteria mechanically 'scan' the surface using 'roaming' or 'orbiting' movements, attach to the surface and subsequently form microcolonies, which lead to the generation of organized, three-dimensional structures[22] (Fig. 2).

Orbiting and roaming motility

Bacteria swimming in close proximity to surfaces experience hydrodynamic forces that both attract them towards the surface and cause them to move in circular trajectories[23]. *V. cholerae* is equipped with a single polar flagellum driven by a Na^+ motor[24]. Viscous drag forces act on the flagellum as it sweeps past the surface, which induces torque on the cell body; this surface-induced torque deflects the directional movement of the cells into curved clockwise paths[25].

By using high-speed tracking of *V. cholerae* grown in flow cell chambers, two types of trajectories have been identified: orbiting involves tight, repetitive, near-circular orbits with high curvatures (Radius of gyration (R_{gyr}) < 8 μm), whereas roaming involves long directional persistence and small curvatures (R_{gyr} > 8 μm)[22]. In both motility modes cells move in an oblique direction that strongly deviates from the cell axis and have strong nutations along the trajectory. Moreover, the direction of motion seems to be exclusively in the clockwise direction for both motility modes[22]. These motility modes are ablated in strains lacking mannose sensitive haemagglutinin pili (MSHA) type IV pili (TFP) or the flagellum, which suggests that both appendages are necessary for these characteristic behaviors[22].

Theoretical modeling was used to elucidate the origins of orbiting and roaming motility behavior[22]. In free-swimming cells, flagellar rotation causes the cell body to counter rotate. For surface-skimming *V. cholerae*, this body rotation associated with swimming causes MSHA appendages to have periodic mechanical contact with the surface, enabling surface skimming cells to continuously assay the surface mechanically via friction. Orbiting enables *V. cholerae* to loiter over surface regions that interact more strongly with MSHA pili, whereas roaming *V. cholerae* pass over surface regions that interact more weakly with MSHA pili[22] (Fig. 2a).

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Orbiting *V. cholerae* cells exhibit intermittent pauses of different durations before eventually attaching to the surface. Both the frequency and duration of these pauses significantly decreased when cells were incubated with a non-metabolizable mannose derivative to saturate MSHA pili binding[22], which suggests that MSHA pili-surface interactions are mechano-chemical in nature. Moreover, strains lacking MSHA are defective in initial surface attachment[22,26]. Taken together, these observations suggest that MSHA pili-surface binding is crucial to arrest cell motion near the surface and to transition to surface attachment and microcolony formation.

It is important to note that the initial surface attachment behavior of *V. cholerae* is unlike the case for *Pseudomonas aeruginosa*, which reversibly attach to surfaces in a vertical orientation and move along random trajectories with TFP-driven 'walking motility' after initial attachment[27,28]. These cells transition to an irreversibly attached state in which the cell axis is oriented parallel to the surface; these cells move along the surface by TFP-driven 'twitching', guided by a network of secreted polysaccharides and extracellular DNA (eDNA)[29,30], which ultimately results in the formation of microcolonies. By contrast, as discussed above, *V. cholerae* use their polar flagellum and MSHA pili synergistically to scan a surface mechanically before surface attachment. The sites of surface attachment strongly correlate with the positions of microcolonies, which indicates that TFP-driven motility has a minor role in determining positions of *V. cholerae* microcolonies[22].

After surface attachment, it is unknown whether the *V. cholerae* flagellum is functional, whether it is lost and degraded, or if it acts as a structural component in the biofilm. However, mutations in a flagellar structural gene, *flaA*, resulted in increased exopolysaccharide production, which suggests that the lack of a flagellum serves as a signal for biofilm formation[31,32]. Surprisingly, mutations in the genes encoding the flagellar motor genes *motB* and *motY* rescue this phenotype[31,32], which suggests that the Na^+ driven flagellar motor may act as a mechanosensor, enabling *V. cholerae* to recognize when it encounters a surface and subsequently induce the appropriate attachment response[31,32].

Macro-colony formation and the matrix

Following the initial stages of cell attachment cells produce the extracellular matrix, which is essential to achieve mature biofilms with a three-dimensional structure. Distinct morphological and phenotypic differences can be observed depending on the quantity of biofilm matrix components being produced (Box 1). Compositional analysis of an intact *V. cholerae* biofilm matrix by solid state nuclear magnetic resonance (NMR) using ^{15}N profiling and spectroscopic analysis of the extracellular matrix carbon pools showed that the extracellular matrix is primarily composed of polysaccharides, phospholipids, proteins and small amounts of nucleic acids[33]. Additionally, the *V. cholerae* biofilm matrix seems to be sugar-rich, especially when compared to the protein-rich biofilm matrix of *Escherichia coli*[33]. Defining and quantifying the major building blocks of the *V. cholerae* biofilm not only furthers our understanding of how individual components interact to support the formation of a complete biofilm matrix, but also highlights differences between species that may inform how biofilm components better facilitate pathogen survival and transmission.

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VPS

VPS makes up 50% of the biofilm matrix mass and is essential for the development of three dimensional biofilm structures[21,34,35]. It has an essential role in *V. cholerae* biofilm formation and is secreted from cell surfaces shortly after initial attachment, and VPS extrusion from cells is observed throughout biofilm development[36](Fig. 2b). VPS is composed of a polysaccharide conjugated to an, as of yet, unidentified component and its chemical structure was only recently revealed[35]. Two types of VPS are produced during biofilm formation: the repeating unit of the major variant of the polysaccharide portion of VPS is [→4)-α-L-Gul*p*NAcAGly3OAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→]$_n$, whereas the minor variant partially replaces α-D-Glc with α-D-GlcNAc[35]. It is still unclear whether VPS remains tethered to the cell or if it is cleaved after secretion; the identification of its unknown component may reveal how VPS is retained in the biofilm.

Genes involved in VPS production are organized into two *vps* clusters — 12 are found in *vps-1* and 6 are found in *vps-2*[21,34]. These genes are divided into six classes with different predicted functions: class I encodes for the nucleotide sugar precursors, VpsA and VpsB; class II encodes glycosyltrasferases, VpsD, VpsI, VpsK, and VpsL; class III encodes VPS polymerization and export proteins, VpsE, VpsH, VpsN, and VpsO; class IV encodes acetyltransferases, VpsC and VpsG; class V encodes the phosphotyrosine-protein phosphatase VpsU; and class VI encodes the hypothetical proteins, VpsF, VpsJ, VpsM, VpsP and VpsQ[21]. Deletion of *vpsF, vpsJ* or *vpsM* results in the complete loss of colony corrugation, an inability to form pellicles and a reduction in biofilm and VPS production, which implies that these hypothetical proteins may have an important role in biofilm formation[21]. In-frame deletion of 15 of the 18 *vps* genes resulted in strains with reduced colony corrugation phenotypes compared with wild-type[21].

The structure of VPS is in agreement with many of the proteins encoded by the *vps* genes, as their predicted functions, described above, match potential steps in the VPS biosynthesis pathway[21,35]. The two *vps* clusters are separated by an 8.3 kb *rbm* cluster containing 6 genes, some of which encode matrix proteins (see below)[34,37,38]. The *vps-1, rbm* and *vps-2* clusters comprise a functional genetic module, here referred to as the *V. cholerae* biofilm-matrix cluster (VcBMC), that encodes many genes involved in the generation of VPS and the major biofilm proteins RbmA, Bap1, and RbmC, described in more detail below. Two additional genes involved in UDP-glucose and UDP-galactose synthesis, *galU* and *galE*, respectively, are also necessary for biofilm production, which suggests that these substrates may be essential for VPS biosynthesis[39].

Matrix proteins

Three matrix proteins, RbmA, RbmC and Bap1, are produced and secreted from *V. cholerae* at various times during biofilm formation and play different parts within the biofilm. *rbmA* (*r*ugosity and *b*iofilm structure *m*odulator A) is the thirteenth gene of the VcBMC, encoded within the *rbm* cluster, and encodes a protein involved in cell-cell and cell-biofilm adhesion[36,37,40]. Analysis of the crystal structure revealed that RbmA contains two fibronectin type III (FnIII) folds, commonly found in cell surface receptors and cell adhesion proteins. The FnIII folds of two RbmA monomers are connected by a linker segment and

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form a bilobal structure with unique surface properties[41]. The dimer interface forms a wide groove, capable of accommodating large, filamentous substrates, such as VPS, and a tight groove, capable of binding negatively charged carbohydrates found on cell surfaces. Saturation transfer difference (STD) experiments indicate that these two binding sites preferentially bind monosaccharides from VPS and lipopolysaccharide (LPS), which implies that RmbA possibly acts as a biofilm scaffolding protein[42]. RbmA accumulates on the cell surface after initial attachment and VPS production[36] (Fig. 2b). RbmA was also shown to contribute to early elasticity and corrugation in pellicle biofilms, further corroborating its role in the development of biofilm architecture and stability[43].

Two other major biofilm matrix proteins, Bap1 and its homolog RbmC, share 47% sequence similarity, but have non-redundant roles in biofilm formation[37]. Bap1 contains four overlapping *Vibrio-Colwellia-Bradyrhizobium-Shewanella* repeat (VCBS) domains, which may be involved in cell adhesion, and four FG-GAP domains, which are thought to be important for recognition and binding of an, as of yet, unidentified ligand[36,37,44]. During biofilm formation, Bap1 is secreted at the cell-surface interface and gradually radially accumulates on nearby surfaces, although the concentration of Bap1 remains the highest near the founder cell (also known as parent cell). These findings support a role for Bap1 in surface adhesion and suggest that the founder cell and its earliest descendants are primarily responsible for the production of Bap1[36] (Fig. 2b). In rugose pellicles, Bap1 was found to be uniquely required for maintaining pellicle strength over time, and scanning electron microscopy revealed that a Bap1 mutant exhibited a distinctly different pellicle microstructure. Bap1 was also shown to considerably contribute to pellicle hydrophobicity, enabling it to spread and remain at an air-water interface[43].

V. cholerae's RbmC protein also has four VCBS domains but only contains two FG-GAP domains. RbmC is larger than Bap1 and has two carboxy-terminal β-prism domain, while Bap1 only has one, and two amino-terminal domains of unknown function[40]. The β-prism domain has lectin- and carbohydrate-binding activity in other bacterial proteins, but the significance of its binding properties in RbmC is still being explored[45]. As biofilms develop and more cell division occurs, RbmC is secreted at discrete sites on the cell surface, and RbmC and Bap1 form flexible envelopes surrounding the cell that can grow as cells divide[36] (Fig. 2b). During biofilm formation on a solid-water interface, RbmA, RbmC and Bap1 were unable to accumulate on the surface of cells that did not produce VPS, and RmbC was shown to be critical for incorporating VPS throughout the biofilm. Thus, the mature biofilm is a composite of organized clusters composed of cells, VPS, RbmA, Bap1 and RbmC[36] (Fig. 2b).

A recent study demonstrated that the type II secretion system (T2SS), a multiprotein system that exports proteins from the cell by translocating proteins from the periplasm through the outer membrane, is responsible for the secretion of RbmA, RbmC and Bap1[46]. T2SS mutants were unable to secrete RbmA, RbmC, and Bap1 into culture and exhibited diminished biofilm formation, although VPS excretion from the cell remained unaffected. Additionally, deletion of the T2SS in a rugose strain abolished colony corrugation and pellicle formation, further supporting the crucial role of the T2SS in biofilm formation and morphology[46].

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V. cholerae biofilm proteins have also been associated with outer membrane vesicles (OMVs), which act as secretory vehicles. In *V. cholerae*, 90 proteins are associated with OMVs, including RbmA, RbmC and Bap1[47]. It is unknown whether the association of biofilm proteins with OMVs is a regulated cellular programme or whether it is the result of the random inclusion of proteins that pass through the periplasm. However, in the presence of antimicrobial peptides, Bap1 was shown to bind to the surface of OMVs via its association with the outer membrane protein OmpT. Evidence suggests that OMV-associated Bap1 then binds to antimicrobial peptides and attenuates their impact on *V. cholerae*, thus increasing bacterial resistance[44]. Future studies are required to determine the contribution of other OMV-associated and free matrix proteins to biofilm structure and function.

Dispersal

The last stage in biofilm development is dispersal. Although dispersal is an important step in the biofilm cycle, enabling exiting cells to seek out and colonize new resources, little is known about this process in *V. cholerae*. Two extracellular nucleases, Dns and Xds, have been implicated in biofilm development and dispersal through their regulation of eDNA, which plays a role in nutrient delivery and biofilm structure[48]. eDNA released by cell lysis or active secretion may be taken up by competent cells during growth on chitin, where it can act as a source of organic nutrients or become incorporated in the genome. It may alternatively remain in the biofilm matrix, where it seems to act as an important structural component[48]. Deletion of Dns and Xds promoted biofilm formation independently of *vps* production, altered biofilm structure and impaired detachment from biofilms[48]. Evidence indicates that degradation of eDNA by these nucleases reduce biofilm formation and might facilitate dispersal. Impaired *in vivo* colonization was also observed, which suggests that dispersal may be necessary for colonization of the host[48].

Additionally, *rbmB*, a gene encoded in the *rbm* cluster of the VcBMC, encodes a putative polysaccharide lyase that has been proposed to have a role in VPS degradation and cell detachment. Strains lacking RbmB exhibit enhanced biofilm formation compared with strains that encode the protein, although the enzymatic activity of RbmB has not been experimentally demonstrated[37]. The downregulation of biofilm components, discussed in more detail below, is likely to play a role in dispersal; however, genes involved in the degradation of biofilm proteins remain to be identified. Extracellular signals, such as the bile salt taurocholate (Box 2), may also act as a signal for biofilm dispersal[49]. The identification of the proteins that are crucial for dispersal is essential and would further our understanding of how and when *V. cholerae* disperses from a biofilm.

V. cholerae biofilm regulation

V. cholerae biofilm formation is controlled by an integrated regulatory network of transcriptional activators — VpsR, VpsT and AphA — transcriptional repressors — HapR and H-NS — alternative sigma factors — RpoN, RpoS and RpoE — small regulatory RNAs and signaling molecules (Fig. 3). The regulation of biofilm matrix production is controlled by a highly connected regulatory network that integrates at least three different nucleotide second messengers and the quorum-sensing (QS) response (Fig. 3b). Biofilm formation is an

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energetically costly process; commitment to the biofilm lifestyle has major biological consequences and must therefore be both tightly regulated and plastic, enabling biofilm bacteria to be responsive to the various environmental cues that they experience during their life cycle (Box 2).

Positive regulation

VpsR, the master regulator of biofilm formation in *V. cholerae*, is a member of the two-component signal transduction system (TCS) response regulator family. It harbors an N-terminal response regulator receiver domain (REC), an ATPases associated with a wide variety of cellular activities (AAA+) domain and a C-terminal helix-turn-helix (HTH) DNA-binding domain[50]. VpsR is required for biofilm formation, as disruption of *vpsR* prevents expression of the Vps and matrix proteins and abolishes the formation of biofilms. VpsR binds to the *vps* promoter regions to directly control their expression[51] (Fig. 3a). VpsR also upregulates *eps* genes that form part of the type II secretion system, matrix protein genes and *aphA*, which is a major virulence regulator, demonstrating that it may also have a role in pathogenesis[50,52,53]. VpsR contains a conserved aspartate residue, Asp59, which seems to be critical for its function. Conversion of this aspartate to alanine renders VpsR inactive, whereas conversion to glutamate creates constitutively active VpsR, supporting the premise that phosphorylation controls DNA binding of VpsR[32]. It has been shown that VpsR can bind the second messenger cyclic dimeric guanosine monophosphate (c-di-GMP), however c-di-GMP does not alter its DNA-binding ability[54]. Sensor histidine kinase or kinases that have a role in activating VpsR and positively regulating *vps* gene expression and biofilm formation are not known. The expression of VpsR is positively regulated by VpsT and negatively regulated by HapR, although other factors are likely to be involved and further study is needed to fully characterize its regulation[52].

A second positive regulator of biofilm formation, VpsT, is also a response regulator. VpsT consists of an N-terminal REC domain and a C-terminal HTH domain. Unlike other REC domains, the canonical (α/β) 5-fold in VpsT is extended by an additional helix (α6) at its C terminus[55]. Disruption of *vpsT* reduces the expression of *vps* and matrix protein genes and reduces biofilm forming capacity. Similarly to VpsR, VpsT binds to the *vps* promoter region to directly control the expression of *vps* genes[51,55] (Fig. 3a). VpsT binding to c-di-GMP is required for DNA association and transcriptional regulation[55], with a dimer of c-di-GMP binding to a VpsT dimer with an affinity of 3.2 μM. The VpsT c-di-GMP-binding motif is W[F/L/M][T/S]R[55]. Mutations in the putative phosphorylation site intended to produce a constitutively inactive or active state do not alter the efficiency of VpsT, which indicates that its role in gene expression regulation is independent of its phosphorylation status[55]. *vpsT* is positively regulated by VpsR, AphA and the alternative sigma factor RpoS; and negatively controlled by HapR[52,56-58].

The VpsR and VpsT regulons extensively overlap and, although both proteins positively regulate the transcription of *vps* and biofilm-related genes (Fig. 3a), the magnitude of gene regulation varies. The fact that VpsR and VpsT can modulate each other's expression could, in part, be responsible for the overlap in their regulons[52,53]. A recent study identified the VpsR and VpsT recognition sequences in the regulatory region of the first gene in the *vps-2*

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operon, *vpsL*[51]. Moreover, *in silico* analysis to determine promoter recognition sites revealed that both VpsR and VpsT could bind to the regulatory region of the first gene in the *vps-1* cluster, *vpsU*, as well as to the regulatory regions of *rbmA* and *vpsT*. This study also showed that the promoter of another gene in the *vps-1* cluster, *vpsA*, harbors only the VpsT recognition sequence, whereas *rbmC* and *bapI* promoters harbor only the VpsR recognition sequence. These findings support the premise that these two regulators act in concert by directly targeting all the regulatory regions in the VcBMC[53,55,57] (Fig. 3a).

Negative regulation

HapR is the main negative regulator of biofilm formation in *V. cholerae*, as disruption of *hapR* enhances biofilm formation[59-61]. HapR directly binds to the regulatory regions of *vpsL*, the first gene in the *vps-2* operon, and *vpsT*[62]. HapR has homology to TetR regulators; the N-terminus contains a HTH domain and the C-terminus contains a dimerization domain, which is predicted to have a binding pocket for an unidentified amphipathic ligand that contains anionic moieties[63].

The timing of *hapR* repression and activation is controlled by QS and modulates the formation of mature biofilm structures and dispersal from the biofilm, respectively[60,64]. QS-deficient mutants form thicker biofilms and do not detach as easily from the biofilm structure when compared to wild-type[64]. HapR production is negatively controlled through the QS pathway (reviewed in [65]). Briefly, membrane-bound sensor histidine kinases, LuxQ and CqsS, recognize the signaling molecules autoinducer 2 (AI-2) and cholerae autoinducer 1 (CAI-1), respectively, and initiate a phosphorelay event that culminates at the histidine phosphotransfer protein, LuxU, and the response regulator, LuxO[65] (Fig. 3b). At low cell density, when the concentrations of AI-2 and CAI-1 are low, LuxO-P, in concert with the alternative sigma factor RpoN, activates transcription of the quorum-regulated small RNAs (sRNAs), Qrr1–4, which work in conjunction with the sRNA chaperone Hfq to prevent the translation of *hapR*. This ultimately results in the upregulation of biofilm formation[65] (Fig. 3b). In contrast, at high cell densities, when AI-2 and CAI-1 levels are high, LuxO is dephosphorylated via the autoinducer receptors, CqsS and LuxPQ, and the Qrr1-4 sRNAs are repressed. This activates HapR expression and results in the downregulation of biofilm formation[65].

Several additional regulators have been shown to be involved in QS-mediated regulation of HapR and thereby affect biofilm formation (Fig. 3b). The two-component system, VarS-VarA, upregulates *hapR* expression post-transcriptionally via a pathway that involves the regulatory sRNAs CsrB, CsrC, and CsrD[66,67]. These sRNAs bind to and titrate the RNA-binding protein CsrA, interfering with the LuxO activation of Qrr1-4, thus leading to decreased levels of Qrr1-4 and enhanced HapR production[67]. By contrast, the small protein Fis is a direct positive regulator of the QS-responsive sRNAs, Qrr 1-4[68], thereby promoting HapR repression. VpsS, a hybrid histidine kinase, also increases biofilm formation through the QS pathway by donating phosphate groups to the phosphotransfer protein LuxU[69]. The global regulator, cAMP receptor protein (CRP), has been shown to upregulate HapR production through its positive regulation of the CAI-I autoinducer synthase and its negative regulation of Fis, suggesting that CRP functions at two regulatory junctions in the QS

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pathway[70]. Finally, the *hapR* gene is also regulated independently of the QS pathway: the transcriptional regulator VqmA can directly activate *hapR* expression; and the sigma factor RpoS promotes expression of *hapR*[53,71].

The H-NS protein is a histone-like protein that has an important role in modulating nucleoid topology and also functions as a transcriptional regulator. It has low sequence-specificity and shows preference for AT-rich regions with high curvature[72]. In *V. cholerae*, H-NS negatively controls the expression of biofilm and virulence genes[72,73]. A strain lacking *hns* has a significantly enhanced ability to form biofilms; it has been shown that H-NS acts as a direct negative regulator of *vpsL, vpsA* and *vpsT* both *in vitro* and *in vivo*, although little is known about the role of H-NS in controlling other biofilm genes[72] (Fig. 3a). A recent study revealed that when VpsT is bound to the *vpsL* regulatory region, it prevents H-NS-mediated silencing; however, in the same study it was shown that VpsT also regulates biofilm formation independently of H-NS[51].

Small nucleotide signaling

A key signaling molecule controlling *V. cholerae* motility and biofilm matrix production, and thus the planktonic-to-biofilm transition, is the nucleotide-based, second messenger c-di-GMP[74] (Fig. 3b). C-di-GMP is synthesized by diguanylate cyclases (DGCs), which contain a GGDEF domain, and it is degraded by phosphodiesterases (PDEs), which contain an EAL or HD-GYP domain[75]. The *V. cholerae* genome encodes 31 proteins with a GGDEF domain, 12 proteins with an EAL domain, and 9 proteins with a HD-GYP domain[76]. Although an additional 10 genes encode proteins with both GGDEF and EAL domains, this does not necessarily suggest that the protein exhibits both DGC and PDE activity, as it is common for one domain to be degenerate. In *V. cholerae*, c-di-GMP is sensed by receptor proteins, including PilZ and VpsT, or c-di-GMP riboswitches[55,77,78].

At present, little is known about the precise molecular mechanisms by which c-di-GMP affects motility and planktonic-to-biofilm transition in *V. cholerae*. Systematic phenotypic characterization of isogenic *V. cholerae* mutants with in-frame deletions in the genes encoding predicted DGCs and EAL domain-containing PDEs revealed that four DGCs (CdgH, CdgK, CdgL and CdgD) inhibit motility and two PDEs (CdgJ and RocS) promote motility[79], and regulation of the abundance or activity of these proteins is predicted to be critical for the motile-to-sessile transition. Increases in cellular c-di-GMP can repress the transcription of flagellar genes, or act post-transcriptionally to regulate swimming velocity and alter flagellar rotational switching, possibly by interacting with a yet-to-be-identified c-di-GMP receptor or with flagellar motor proteins[75]. Transcriptional profiling experiments revealed that high concentrations of c-di-GMP promote transcription of *msh*, the operon encoding the MSHA pilus, *vps* and other biofilm genes, and repress transcription of flagellar genes[80]. Flagellar regulatory protein A (FlrA) represses flagellar genes when it is in the c-di-GMP-bound state; however, the molecular details of c-di-GMP-mediated repression of motility are not completely understood[55,81].

High cellular levels of c-di-GMP promote enhanced transcription of genes involved in biofilm formation[80]. For example, when bound to c-di-GMP, VpsT induces the expression of biofilm genes[55]. Analysis of *vpsL* expression and biofilm formation in strains containing in-

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frame deletions of genes encoding proteins with GGDEF, EAL, or GGDEF and EAL domains revealed that strains lacking the DGCs CdgA, CdgH, CdgK, CdgL and CdgM show a decrease in *vpsL* expression and biofilm formation[76,82]. Furthermore, whereas c-di-GMP levels decreased to 54–86% of wild-type levels in each single DGC deletion strain, in the 5DGC strain (containing deletions in the genes encoding all five of these DGCs), c-di-GMP levels decreased to 17% of wild-type levels[82]. These results show that multiple DGCs are involved in maintaining cellular c-di-GMP levels and additively contribute to biofilm formation and *vps* expression, likely due to increased binding of c-di-GMP to VpsT to enable *vps* expression. Conversely, mutants lacking PDEs (CdgJ, CdgC, RocS, MbaA, VieA) exhibited enhanced biofilm formation compared with wild-type[74,79,82,83].

Cellular c-di-GMP levels could be maintained by transcriptional or post-transcriptional regulation of proteins involved in c-di-GMP signaling and we are only beginning to understand how the vast repertoire of *V. cholerae* DGCs and PDEs is regulated. VpsR, VpsT and HapR all seem to play a part in the regulation of these genes. Transcriptome studies indicate that VpsR and VpsT influence the expression of ten genes involved in the regulation of c-di-GMP levels, specifically upregulating key DGCs that enhance biofilm formation while repressing key PDEs that decrease biofilm formation[52,53]. The promoter regions of genes encoding proteins predicted to be involved in c-di-GMP signaling, including *cdgA, cdgC cdgD*, and *VCA0165*, have predicted VpsR-binding domains, which indicates that they may be directly regulated by VpsR[53,84]. HapR was shown to influence the expression of 14 DGCs and PDEs and was demonstrated to directly bind to the promoter region of *cdgA, cdgG, VCA0080, VC2370*, and *VC1851* and *VC1086* genes. HapR was shown to specifically upregulate PDEs that promote a decrease in biofilm formation and downregulate DGCs that have been shown to increase biofilm formation[52,62]. In addition, some DGCs and PDEs seem to be regulated by the QS pathway independently of HapR, through LuxO and Qrr1-4[85]. Environmental signals, such as polyamines and bile components, have also been shown to modulate abundance and activity of c-di-GMP signaling enzymes[52,62,86,87].

The second messenger cyclic adenosine-monophosphate (cAMP) is involved various cellular responses and acts as a repressor of *V. cholerae* biofilm formation[88] (Fig. 3b). When glucose is limited, cAMP is synthesized by the adenylyl cyclase, CyaA, and binds CRP to initiate the carbon catabolite repression response. The cAMP-CRP complex downregulates expression of *rbmA, rbmC, bap1, vpsR* and other *vps* genes[89]. A number of DGC and PDE genes controlling c-di-GMP levels are also regulated by cAMP-CRP; for example, *rocS, cdgA, cdgH*, and *cdg*I were shown to be downregulated by cAMP-CRP[89]. Interestingly, all of these genes contain the GGDEF domain required for DGC activity, but RocS and CdgI also contain EAL domains and are thought to act as PDEs rather than DGCs. This further highlights the complexity of the regulatory network governing c-di-GMP synthesis and degradation and its influence on biofilm formation. As mentioned above, cAMP-CRP also upregulates HapR and the biosynthesis of the autoinducer CAI-I[70,89]. Thus, cAMP-CRP links the nutritional status and biofilm formation.

The *V. cholerae* stringent response is triggered by nutritional stress and results in the synthesis of the two small molecules guanosine 3’-diphosphate 5’-triphosphate and guanosine 3’,5’-bis(diphosphate), collectively called (p)ppGpp, by RelA, SpoT and

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RelV[58,90,91]. Mutants deficient in the stringent response were shown to have decreased, although not completely deficient, biofilm formation[58]. All three (p)ppGpp synthases — RelA, SpoT, and RelV — are necessary for *vpsR* transcription, but only RelA is necessary for *vpsT* transcription. Whereas the regulation of *vpsT* expression through the stringent response is strongly dependent on RpoS, the regulation of *vpsR* probably involves additional factors that remain to be identified[58] (Fig. 3b).

sRNA regulation of V. cholerae biofilms

The importance of sRNAs in the regulation of cellular processes is becoming increasingly recognized (Fig. 3b). Besides sRNAs controlling HapR levels, two additional sRNAs were shown to regulate biofilm formation in *V. cholerae*. VrrA, the expression of which is controlled by the sigma factor RpoE, negatively regulates the expression of the biofilm matrix protein RbmC by directly pairing with the 5' end of its mRNA transcript, thereby inhibiting the translation of *rbmC* mRNA and downregulating biofilm formation[92,93]. This is the first link between biofilm formation and RpoE and the first example of a sRNA bypassing the master regulators of biofilm formation to directly regulate a biofilm matrix component[92]. Furthermore, the sRNA RyhB, which is negatively regulated by iron and Fur, is involved in biofilm formation. A *ryhB* mutant was shown to exhibit a defect in biofilm formation when grown in low-iron medium, but this defect can be rescued by the addition of excess iron or succinate; however the molecular basis by which RyhB controls biofilm formation remains to be determined[94]. Although the role of sRNAs in biofilm formation is unexplored, these examples add another level of regulation to the elaborate regulatory network that controls biofilm development.

Conclusions and future directions

Over the past 20 years, *V. cholerae* biofilms have been extensively studied and great strides have been made in understanding the molecular mechanisms of biofilm formation and the role of biofilms in environmental persistence of the pathogen and transmission to the human host.

Mature biofilms depend on the production of extracellular matrix components — polysaccharides (VPS) and matrix proteins — to establish cell-cell interactions and attach biofilms to environmental and host surfaces. Key structural components of the biofilm matrix, including VPS, RbmA, Bap1 and RbmC, have been identified and characterized. The genes within the *V. cholerae* polysaccharide gene clusters (*vps*) that are required for biofilm formation and VPS synthesis, as well as the structure of the repeating unit of the VPS polysaccharide, have been identified. Three matrix proteins, RbmA, RbmC and Bap1, were shown to influence biofilm stability and architecture through their distinct locations in the matrix and their interactions with each other.

The major biofilm regulators VpsR, VpsT, HapR and H-NS directly control the expression of structural and regulatory genes. The nucleotide second messengers c-di-GMP, cAMP and (p)ppGpp are instrumental in controlling the expression and activity of these regulators and, in turn, their regulatory targets. The role of sRNAs in QS-dependent and -independent regulation of biofilm is being explored and integrated into our understanding of the major

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regulatory pathways. What is known about the molecular underpinnings of biofilm regulation provides a platform for further study and discovery to gain a complete understanding of this important process.

While recent advances have bolstered our understanding of how, why and when *V. cholerae* biofilms are formed, much remains to be discovered about the functions of each biofilm matrix component and biofilm regulation in response to signals experienced by *V. cholerae* during its intestinal and aquatic life stages. Our understanding of the impact of biological factors on biofilm formation, such as growth in mixed species biofilms, predation by protozoans and infection by phages, is limited and should be explored further. Similarly, more work is required to understand the mechanisms and regulation of biofilm dispersal as well as the role of biofilms *in vivo*.

However, as our understanding of *V. cholerae* biofilm physiology has evolved, a number of new key players have emerged that are not only crucial for biofilm development, but are also suitable candidates for targeting with small-molecule therapeutics. These developments, coupled with continued improvements in biofilm screening technologies, are now providing medicinal chemists with a toolbox of screening strategies for the discovery of small-molecule inhibitors of biofilm formation (Box 3). Known biofilm inhibitory compounds fall into three main classes: quorum sensing inhibitors; disruptors of c-di-GMP signaling; and compounds with unknown targets that were discovered through unbiased biofilm imaging methods. Thus far, several compounds have shown promise for biofilm inhibition and treatment. Further exploration of the use of small molecules to target and inhibit biofilm formation may led to the discovery of new therapeutics and better equip us to prevent and treat deadly cholera outbreaks.

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Glossary

Plankton	A variety of microscopic drifting organisms that inhabit the water column.
Flagellum	A motility structure composed of a cytoplasmic basal body that functions as a motor, a rod that extends from the cytoplasm through the membrane and a long filamentous polymer projecting from the cell.
Flow cell	Used for *in vitro* culture and examination of bacterial biofilms under hydrodynamic flow conditions.
Radius of gyration (R_{gyr})	The R_{gyr} is used here as a statistical measure of the spatial extent of a bacterial track. It is defined as the root mean square distance between each point of a track and the center of mass of that track. For a perfect circle of radius (r), $R_{gyr} = r$.
Pili	Proteinaceous filaments that are found on the surface of many bacteria and are often involved in adhesion or motility.
Chitin	A (1→4)-β-linked homopolymer of *N*-acetyl-D-glucosamine found in the exoskeleton of zooplankton and other crustaceans. It is an abundant source of carbon, nitrogen and energy for many microorganisms.
Orbiting	A motility mode of surface-skimming *V. cholerae* cells that is associated with tightly-curved, circular trajectories that hover over small areas of the surface.
Roaming	A motility mode of surface skimming *V. cholerae* cells associated with meandering, gently-curved trajectories that range over large areas of the surface.
Serogroups	A classification of bacterial strains based on the structure of their surface O-antigen group.
Rugose	A corrugated colony phenotype associated with higher biofilm matrix production.
Pellicle	A biofilm formed at an air-liquid interface.
***V. cholerae* biofilm-matrix cluster (VcBMC)**	a genetic module composed of the *vps-1, rbm*, and *vps-2* genes that encode many of the proteins that generate VPS and major biofilm proteins.
Cyclic diguanylate (c-di-GMP)	A key signaling molecule that controls the motile-to-biofilm transition and biofilm formation by inhibiting motility and stimulating the synthesis of cell-surface adhesins and/or exopolysaccharides.
TetR regulators	A family of proteins involved in the transcriptional control of a number of cell processes, including biofilm formation, pathogenesis, catabolic pathways, antibiotic resistance, and differentiation processes. TetR members harbor a helix-turn-helix (HTH) motif that is highly similar to

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	the DNA binding motif of TetR, which controls the expression of *tet* genes required to confer tetracycline resistance.
Riboswitch	A regulatory RNA sensor composed of structured non-coding RNA that binds to specific small molecules and regulates gene expression
cAMP	A second messenger signaling molecule that is involved in the regulation of a number of cell processes, including cell division, catabolite repression, motility and biofilm formation.
(p)ppGpp	Refers to two alarmones, pppGpp and ppGpp, that are synthesized in response to nutrient limitation and other stress conditions to induce the stringent response and subsequent changes in cell physiology.
Stringent response	a stress response triggered by nutritional stress that results in the synthesis of (p)ppGpp, which in turn controls anabolic and catabolic processes and thereby regulates growth rate.

Biographies

Jennifer Kateri Teschler received her B.Sc. from the University of California, San Diego and is currently working towards her Ph.D. in microbiology at the University of California, Santa Cruz under the advisement of Fitnat Yildiz. She studies the regulation of *Vibrio cholerae* biofilms and is currently focused on the role of two components systems in biofilm formation.

David Zamorano-Sánchez received his B.Sc and Ph.D. from Universidad Nacional Autónoma de México, where he started his studies in bacterial genetics. He currently holds a position as a Postdoctoral fellow at Fitnat Yildiz's research group. He is interested in signal transduction and signal integration within bacterial communities such as biofilms.

Andrew Shinichi Utada graduated from Harvard University, USA, with a Ph.D. in Applied Physics. During his Ph.D. training, he worked in the area of soft-matter physics focusing on complex fluids and droplet formation in microfluidic devices. As a postdoctoral fellow, his research interests focus on bacterial surface motility and biofilm formation.

Christopher Warner obtained his Ph.D. from the University of Sheffield, UK in October 2012. In January 2013, he joined the laboratory of associate professor Roger Linington where he is involved in discovering marine derived natural products as small molecule disruptors of *V. cholerae* biofilm formation.

Gerard Wong received his B.Sc. at Caltech in physics and Ph.D. at Berkeley in physics. Wong's current research interests include bacterial biofilm communities and antibiotic design. He has been the recipient of a Beckman Young Investigator Award, an Alfred P Sloan Fellowship, and is a Fellow of the American Physical Society.

Roger G. Linington is an Associate Professor of Chemistry and Biochemistry at the University of California Santa Cruz. His research focuses on the discovery and development

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of marine natural products for a range of applications including antibiotic development, control of biofilm formation and persistence, and development of antiparasitics against global health targets.

Fitnat Yildiz received her B.Sc. from Hacettepe University, Turkey, her Ph.D. from Indiana University, Bloomington, and completed postdoctoral studies at Carnegie Institution of Washington, Department of Plant Biology and Stanford University Medical School. She was a recipient of the Ellison Medical foundation New Scholar Award in Global Infectious Disease and is a Fellow of the American Academy of Microbiology. Her current research focuses on understanding molecular mechanisms of biofilm formation, c-di-GMP signaling, and environmental stress response. http://yildizlab.sites.ucsc.edu/

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Box 1

Analysis of *Vibrio cholerae* biofilm formation

More than 200 serogroups of *Vibrio cholerae* have been identified, of which only the O1 and O139 serotypes are capable of causing pandemic cholera outbreaks[95]. Serotype O1 is further classified into classic and El Tor biotypes on the basis of their biochemical properties and phage resistance[95]. Most of our understanding of *V. cholerae* biofilms has developed from studying O1 El Tor and O139 strains, which include the commonly studied A1552, C6706, N16961 and MO10 strains. *V. cholerae* can generate phenotypic variants, that is smooth and rugose, that vary in their ability to form biofilms. The ability of *V. cholerae* to switch between a smooth and wrinkled colony morphology was first noted by Ioan Balteanu in 1926[96]. The wrinkled variant was termed rugose by Bruce White in 1938[97], who observed "that the rugose growth habit in vibrios results from abnormally active secretion of mucinous material". Due to their high production of VPS and formation of robust biofilms, rugose variants have been used extensively to characterize biofilm matrix components and regulation[34]. In fact, formation of corrugated colonies (a, Scale is 0.5mm), also termed colony biofilms, is dependent on the production of biofilm matrix materials.

Various experimental methods are used to study and characterize *V. cholerae* biofilms. Crystal violet staining or high-throughput imaging microscopy can be used to visualize and quantify biofilms formed in multi-well plates[26,98]. Biofilms that are formed by *V. cholerae* under static or flow conditions (typically using strains engineered to constitutively produce a fluorescent protein) can be visualized by laser scanning confocal microscopy (LSCM) to analyse biofilm structure (b shows the top down view of the biofilm as the central box, and the side view of the biofilm in the adjacent rectangles. Scale is 40μm)[26,34]. Biofilm parameters such as biomass, surface colonization, biofilm thickness and heterogeneity can be quantified using the COMSTAT biofilm analysis program[99]. Architectural details of the biofilm structure can be evaluated by electron microscopy and high resolution microscopy.

Pellicle biofilms (c, Scale is 2mm) have also been used to assess the role of biofilm components in biofilm integrity, strength of attachment and thickness[34,38]. Recently, interfacial rheology was used to study the mechanical properties of pellicles and analyse pellicle strength and morphology of biofilms containing cells lacking matrix protein[43].

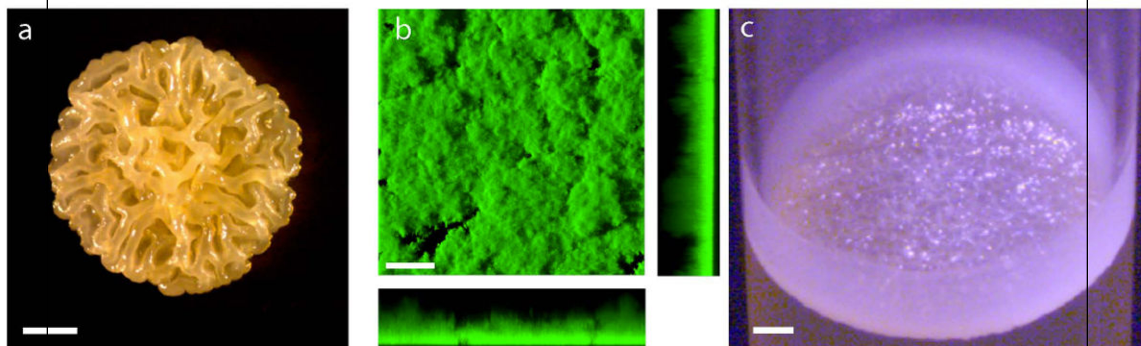

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Figure.

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Box 2

Environmental signals controlling *Vibrio cholerae* biofilm formation

Vibrio cholerae encounters a number of fluctuating environmental signals during its life cycle. Regulation of biofilm formation in response to these external signals is an important factor in survival and persistence. The identification of signals and determining the molecular mechanism of signal integration into the biofilm regulatory network is crucial to increase our understanding of the regulation of biofilm formation during intestinal and aquatic survival of *V. cholerae*.

The bacterial phosphoenolpyruvate phosphotransferase system (PTS) is a highly conserved system that controls transport of select sugars into the cell. Sugars transported by the PTS into the cell, such as mannose and glucose, increase biofilm formation in *V. cholerae*, which suggests a role for the PTS in determining environmental suitability for biofilm growth[100]. The PTS contains the general components enzyme I (EI) and histidine protein (HPr), which function upstream of the carbohydrate-specific enzymes II (EIIA and EIIB/C)[100]. The phosphorylation state of PTS components reflect nutrient availability — PTS enzymes will become phosphorylated when no sugars are available for import, whereas components will quickly become dephosphorylated when sugar transport is active[100]. Four independent PTS pathways have been identified in activation or repression of *V. cholerae* biofilm formation, thus providing another link between the nutrition status and biofilm formation[101,102].

In addition to nutrient availability, a number of other environmental signals are thought to have a role in *V. cholerae* biofilm formation. Salinity and osmolarity fluctuations in the aquatic environment can affect biofilm formation and *vps* expression[103-105]. Two transcriptional regulators, OscR and CosR, regulate biofilm in response to osmolarity and ionic strength, respectively. At low salinities, transcription of *oscR* is increased and OscR inhibits *vps* production and upregulates motility[104]. As ionic strength increases, CosR activates biofilm formation and represses motility[105]. The mechanism by which OscR and CosR sense a shift in osmolarity and ionic strength remain to be determined.

Phosphate limitation in the aquatic environment has been implicated in the negative regulation of *V. cholerae* biofilm formation[106,107]. PhoBR, a regulatory system that responds to phosphate limitation, upregulates motility and downregulates biofilm formation, possibly by repressing *vpsR* and regulating the expression levels of genes encoding DGCs and PDEs[108,109]. PhoB regulation of biofilm formation and the stress response was shown to be independent of the transcriptional repressor HapR and the alternative sigma factor RpoS and may play a role in dispersal from environmental biofilms or host intestines[108,110]. Small organic cations (polyamines), such as norspermidine and spermidine, have been shown to induce or represses biofilm formation, respectively, in response to environmental signals[87].

Calcium (Ca^{+2}) levels vary in the aquatic environment, and extracellular Ca^{+2} has been shown to decrease *vps* transcription and lead to the dissolution of biofilms[111,112] by regulating the expression of the two-component regulatory system CarRS[111]. Indole, which is produced by bacteria found in the human gut, is thought to act as an

extracellular signaling molecule that activates *vps* genes via a signaling cascade[113]. The role of bile in *V. cholerae* biofilm formation is not completely clear. Bile has been shown to induce biofilm formation in a VpsR-dependent manner[114]; it was also demonstrated that bile acids increase intracellular c-di-GMP levels and biofilm formation[86]. However, a recent study demonstrated that exposure to a component of bile, taurocholate, can lead to abiotic degradation of the biofilm matrix and therefore may lead to *in vivo* biofilm dispersal and inhibit biofilm formation[49].

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Box 3

Small-molecule therapeutics that target *Vibrio cholerae*

As our understanding of the physiology of the *V. cholerae* biofilm has evolved, new targets have emerged for the disruption of biofilm formation with small-molecule therapeutics. These compounds fall into three classes: QS inhibitors; disruptors of c-di-GMP signaling; and compounds with unknown molecular targets (see the figure).

The *V. cholerae* quorum sensing (QS) mechanism is unlike that of many other pathogenic bacteria because both suppression of virulence factors and dispersal of biofilms are observed in the presence of high concentrations of QS signaling molecules[65]. Therefore, both virulence factors and biofilm formation and dispersal can theoretically be controlled using a single QS molecule mimic. A number of reports have identified compounds capable of targeting the response regulator LuxO and the transcriptional repressor HapR, two key regulators in the quorum sensing pathway, as well as processes involved in the production of the two known QS signaling molecules produced by *V. cholerae*, CAI-1 and AI-2[115-117]. The recent discovery of Ea-CA1-1 (see the figure, part a), a biosynthetic precursor of CAI-1 that targets the CqsS receptor, has enabled the development of the more potent pyrrole analogue of this precursor. This analogue is capable of repressing transcription of the toxin-coregulated pilus, TcpA, and activating the production of HapR to the same levels as CAI-1 at up to ten-fold higher dilution[118,119].

The c-di-GMP signaling system is also an attractive target for therapeutic intervention (see the figure, part b). In *V. cholerae* a number of compounds have been identified that can target DGCs. In particular, two compounds (denoted as DGC inhibitor 1 and 2 in the figure) were identified as DGC inhibitors from an initial screen of 66,000 synthetic compounds. These compounds were shown to inhibit biofilm formation under both static and flow cell culture conditions[120]. One compound (DGC inhibitor 1 in the figure) was also shown to be effective against other biofilm-forming pathogens, including *P. aeruginosa* and *S. aureus*, and to inhibit the formation of biofilms on the surface of silicone catheters. A second set of seven structurally related poly-aromatic inhibitors discovered from the same screening campaign were also shown to inhibit DGCs. Interestingly, only two of these compounds (denoted DGC inhibitor 3 and 4 in the figure) showed a direct relationship between a decrease in c-di-GMP concentration and a reduction in biofilm coverage, as measured by crystal violet staining[121]. The remaining five compounds reduced biofilm formation, but not global c-di-GMP levels, which suggests that their mechanism of action may involve inhibiting specific DGCs that affect biofilm formation, but not overall c-di-GMP concentration.

Whole-cell phenotypic imaging is well suited to biofilm screening because biofilm structures are of a suitable size for segmentation and quantification using standard imaging tools[98,122]. Coupling this technique with cellular viability measurements permits the differentiation of bactericidal agents and compounds that selectively disrupt biofilm formation without affecting cell survival. Using this approach, two novel scaffolds have been reported: the natural product oxazine and a quinoline-based molecule (unnamed)[123,124]. In both cases, the authors have developed strategies for the synthesis of

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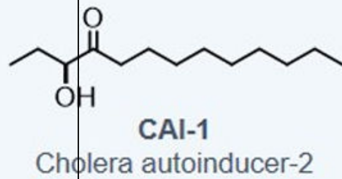
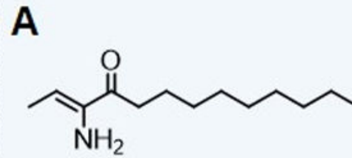
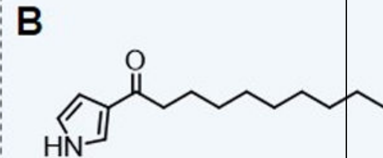
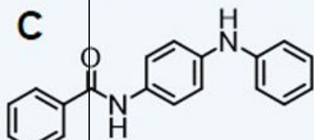
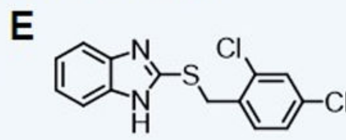
libraries of analogues of these compounds to determine the structural features required for biofilm inhibition and to develop synthetic analogues with improved potencies compared with the original lead compounds[123,125].

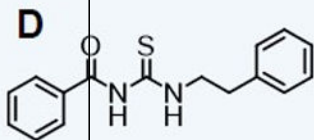


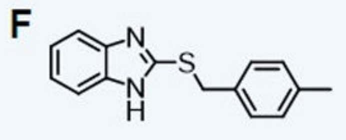


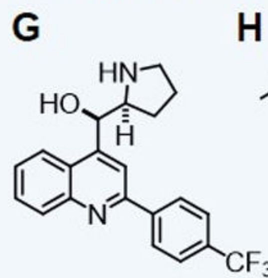












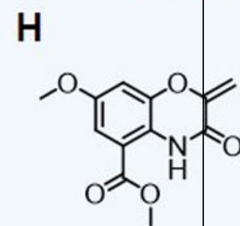


Figure.

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Key points

- *Vibrio cholerae* biofilms play an important role during the aquatic and intestinal phases of its lifestyle, conferring higher resistance to environmental stresses and increasing infectivity.
- *V. cholerae* biofilm formation is a multistep process that begins with initial attachment via its mannose sensitive haemagglutinin pili (MSHA). The key components of a *V. cholerae* biofilm are secreted by the cell at various times during biofilm formation and include *Vibrio* polysaccharide (VPS), the biofilm proteins RbmA, Bap1, RbmC, and extracellular DNA (eDNA), which are critical for the formation of mature biofilms.
- *V. cholerae* biofilm formation is controlled by an integrated regulatory network of transcriptional activators. The major transcriptional activators include VpsR, VpsT and AphA and major transcriptional repressors include HapR and H-NS; alternative sigma factors, small regulatory RNAs, and signaling molecules also function as regulators of this complex process.
- Nucleotide-based signals play an important role in controlling biofilm formation and include cyclic dimeric guanosine monophosphate (c-di-GMP), which positively regulates biofilm formation and negatively regulates motility to influence the planktonic-to-biofilm transition. Additionally, cyclic adenosine-monophosphate (cAMP) represses biofilm formation and guanosine 3'-diphosphate 5'-triphosphate and guanosine 3',5'-bis(diphosphate), collectively called (p)ppGpp, enhances biofilm formation.
- *V. cholerae* biofilm formation is influenced by a number of fluctuating environmental factors, including nutritional status, shifts in salinity and osmolarity, phosphate limitation, the presence of polyamines, variations in calcium levels, and exposure to indole and bile. The ability of *V. cholerae* to activate or repress biofilm formation in response to external signals likely contributes to its environmental survival and persistence and demonstrates the complexity of plasticity of its biofilm regulation program.
- New small-molecule therapeutics have emerged that target and disrupt *V. cholerae* biofilm formation and include: quorum sensing (QS) inhibitors; disruptors of c-di-GMP signaling; and compounds with unknown molecular targets. Whole-cell phenotypic imaging coupled with cellular viability measurements have been used to differentiate bactericidal agents and compounds that selectively disrupt biofilm formation without affecting cell survival and have led to the discovery of several compounds that show promise for biofilm inhibition and treatment.

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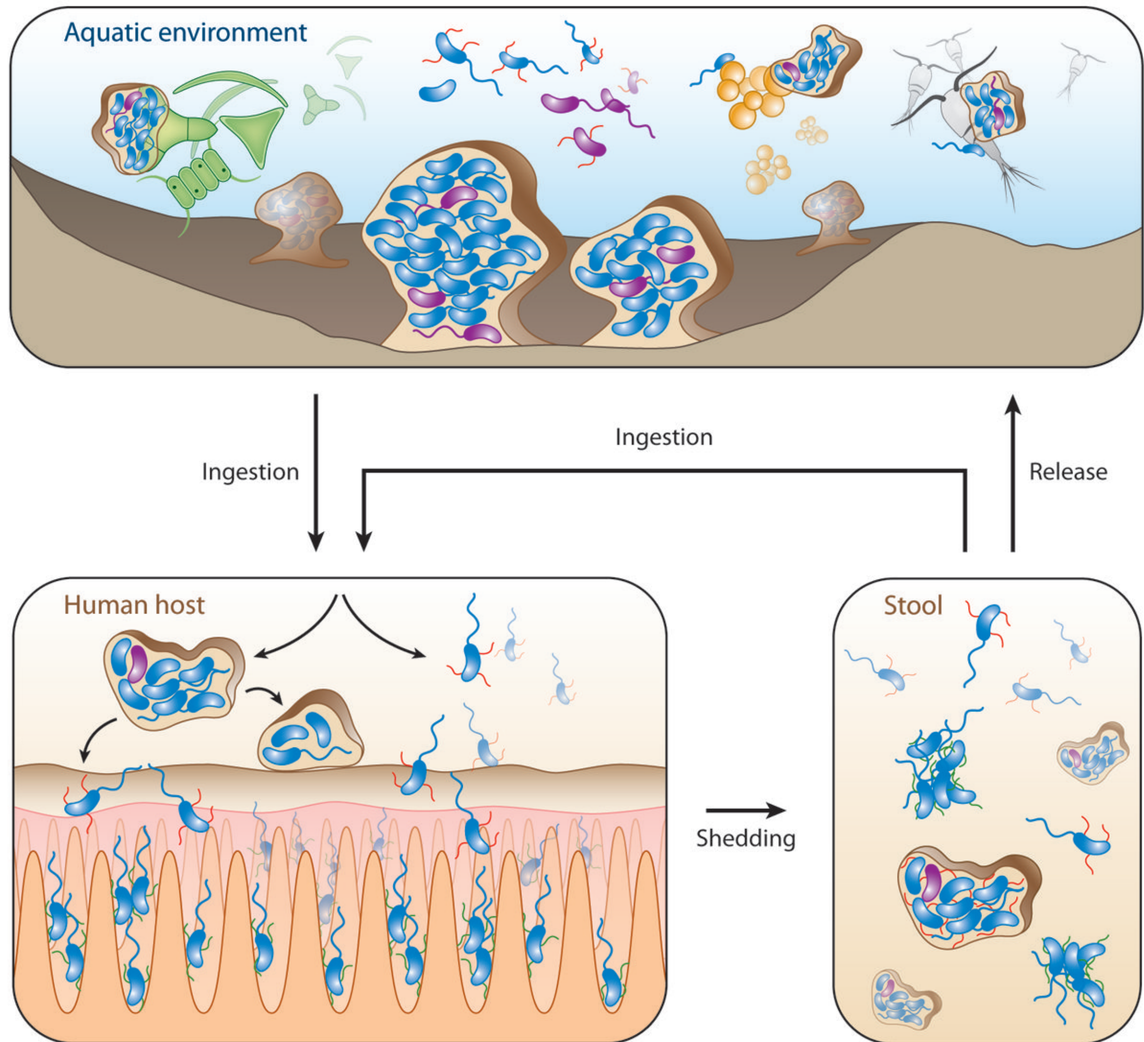

Figure 1. Biofilms in *V. cholerae* life cycle

In the aquatic environment *V. cholerae* is found in its highly mobile planktonic form as well as in biofilms formed on zooplankton, phytoplankton, detritus, and other surfaces, such as sediments. Following the initial stages of attachment to abiotic and biotic surfaces, which involves the type IV pili mannose-sensitive haemagglutinin (MSHA) pili, cells produce the extracellular matrix, which is essential to achieve mature biofilms with a three-dimensional structure. Because it is unknown whether the flagellum is lost during biofilm formation, cells are depicted with or without the flagellum in biofilms. *V. cholerae* can be ingested by humans from environmental sources causing seasonal outbreaks. During intestinal colonization, *V. cholerae* produce toxin co-regulated pili (TCP). Both planktonic cells and biofilm aggregates are found in patient stool, and these cells can re-infect a new host or return to the aquatic environment.

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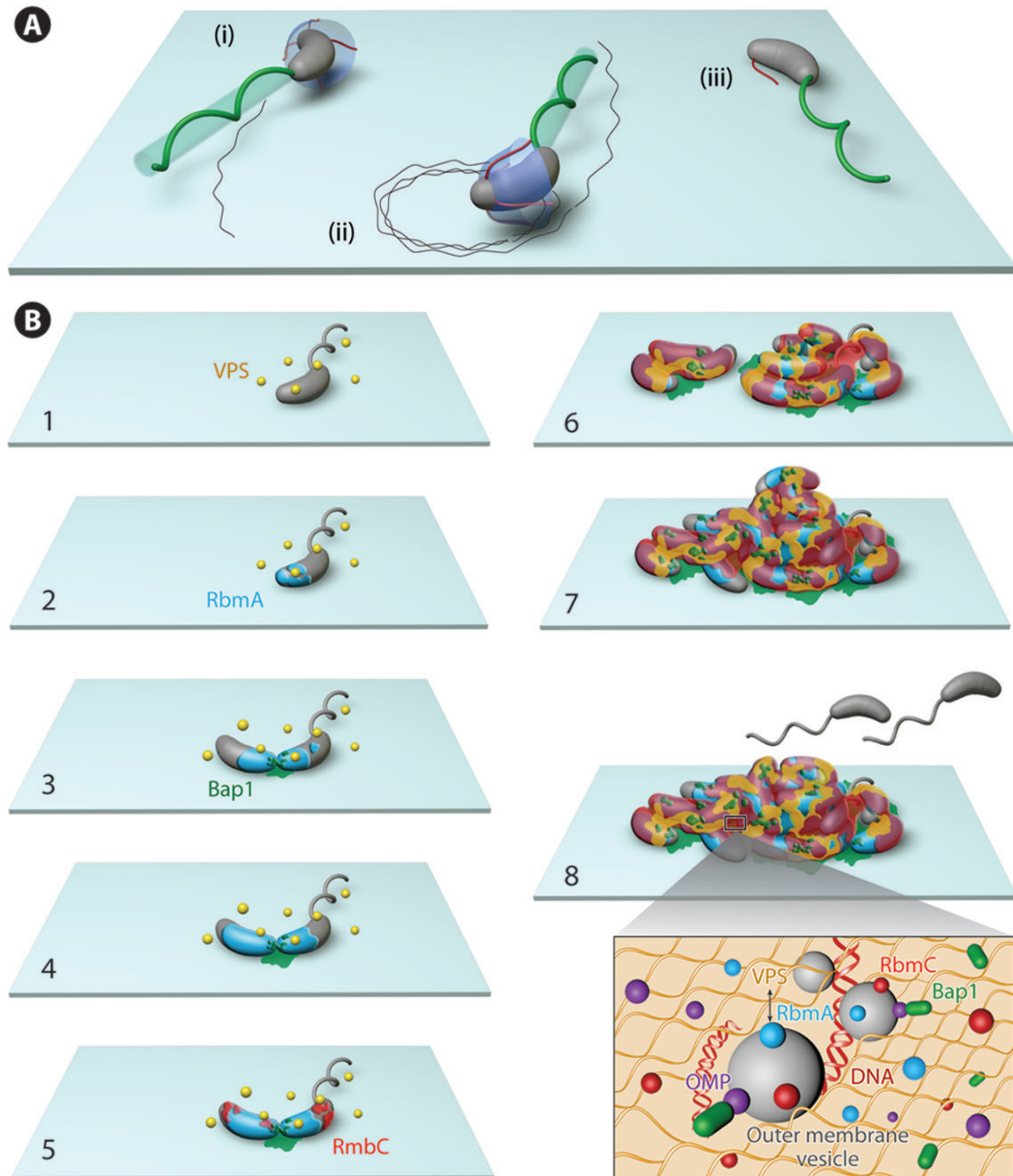

Figure 2. Building a *V. cholerae* biofilm

A) Surface motility and initial attachment: Surface-skimming cells use flagella to move and mechanically 'scan' the surface via mannose-sensitive haemagglutinin (MSHA) pili appendages. Weak interactions between surfaces and pili lead to 'roaming' behavior (tight, repetitive, near-circular orbits with high curvatures), whereas strong surface-pili interactions lead to 'orbiting' behavior (long directional persistence and small curvatures), which allow cells to loiter over these regions and eventually attach and initiate microcolony formation. Motility trajectories are depicted by dashed lines on the surface and correspond to roaming and orbiting behavior.

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B) Microcolony formation and matrix production. Soon after initial attachment, *Vibrio* polysaccharide (VPS) is excreted from cell surfaces (B1), and VPS extrusion is observed throughout biofilm formation. Next, the biofilm matrix protein RbmA accumulates on the cell surface (B2). During cell division, the daughter cell remains attached to the founder cell (also known as parental cell), confirming the role of RbmA in cell-cell adhesion, and the biofilm matrix protein Bap1 is excreted between the two cells and on the substrate near the two cells (B3). Bap1 gradually radially accumulates on nearby surfaces, although the concentration of Bap1 remains the highest near the founder cell. Subsequently, the biofilm matrix protein RbmC is excreted and found on discrete sites on the cell surface (B4). As biofilms develop, VPS, RbmC and Bap1 form envelopes that can grow as cells divide (B5). The mature biofilm is a composite of organized clusters composed of cells, VPS, RbmA, Bap1 and RbmC, in addition to other matrix components, such as outer membrane vesicles (OMVs) and extracellular DNA (eDNA) (inset). Outer membrane proteins (OMPs) associate with Bap1 in OMVs and bind to antimicrobial peptides thereby increasing *V. cholerae* resistance. The last stages in biofilm development are dispersal, whereby exiting *V. cholerae* cells seek out and colonize new resources (B6); however, the underlying mechanism remains to be determined.

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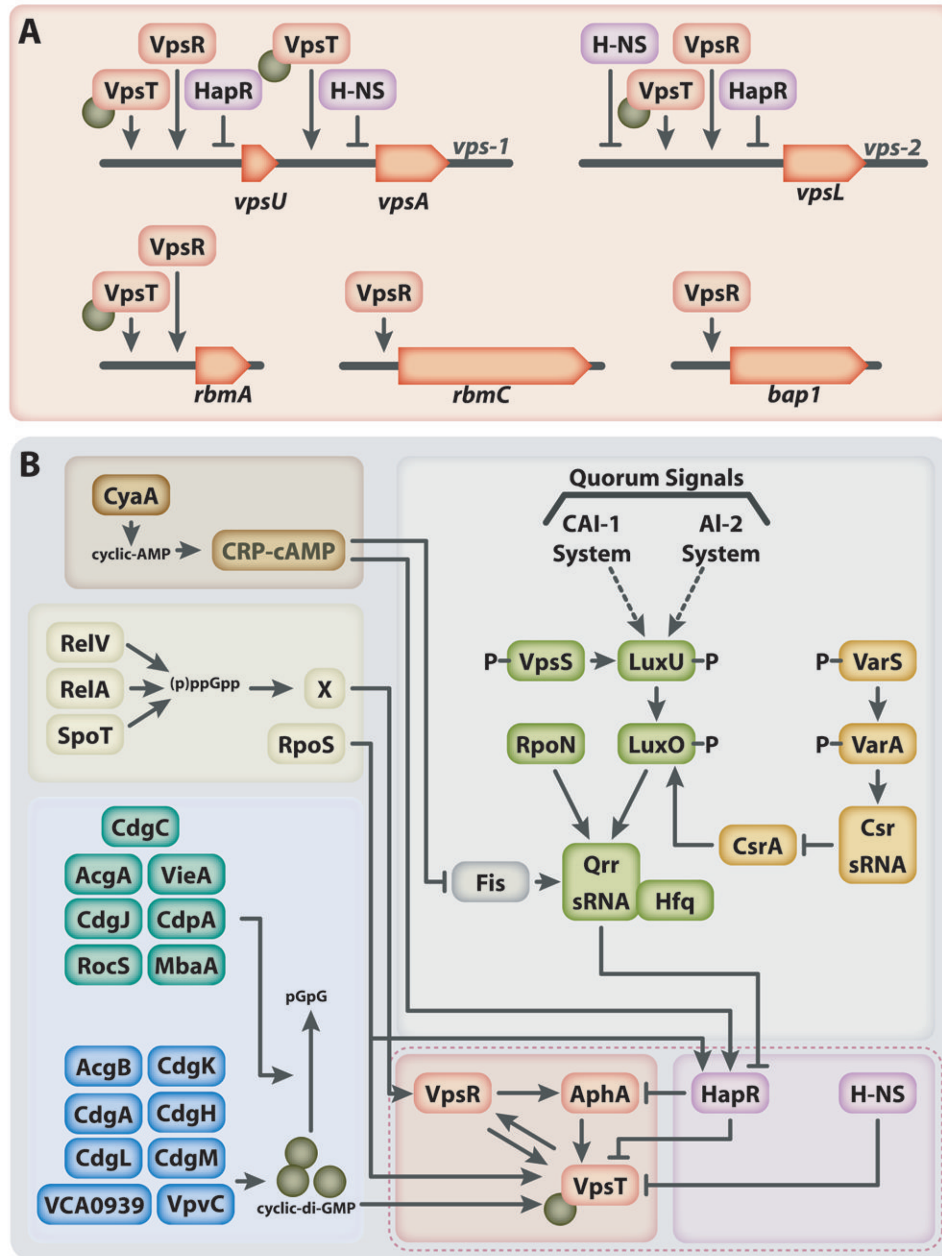

Figure 3. *V. cholerae* biofilm regulatory network

A) The transcriptional activators VpsR, VpsT and transcriptional repressors HapR and H-NS directly and indirectly regulate several genes that have key roles in biofilm formation. Positive regulators of biofilm are shown in orange, while negative regulators are shown in purple. These include the *vps* (*vibrio* polysaccharide) cluster and the *rbm* (rugosity and biofilm structure modulator) cluster, which contain genes that encode proteins involved in VPS production and matrix proteins. The *vps* and *rbm* clusters comprise a functional genetic module, the *V. cholerae* biofilm-matrix cluster (VcBMC). In addition, the *bap1* (biofilm-associated extracellular matrix protein) gene has also been shown to be regulated by these core regulators. The recognition sequences for VpsR, VpsT, HapR and H-NS have been

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identified in the regulatory region of *vps-1* and *vps-2* clusters and the genes encoding the extracellular matrix proteins RbmA and RbmC. Binding of VpsT to promoter regions requires its interaction with c-di-GMP. As shown, the VpsR and VpsT targets extensively overlap, though some biofilm genes appear to be only directly regulated by one. Additionally, the negative regulators directly downregulate many of the genes encoding proteins involved in VPS production and matrix proteins, as well as the genes that encode the positive transcriptional regulators of those genes (shown in part b).

B) An extensive regulatory network governs *V. cholerae* biofilm formation. VpsR, VpsT and AphA are the main activators of biofilm formation, and HapR and H-NS are the main repressors (shown in the core the dashed box). VpsR, VpsT, HapR and H-NS directly regulate genes involved in biofilm formation (see part a).

These core regulators directly and indirectly regulate each other and are modulated by a complex regulatory network in response to a number of environmental and host signals. The quorum sensing (QS) pathway, which responds to cell density via bacterial signaling, has a key role in the regulation of HapR and, thus, the other major biofilm regulators. The signaling molecules autoinducer 2 (AI-2) and cholerae autoinducer 1 (CAI-1) regulate a phosphorelay event that culminates at the histidine phosphotransfer protein, LuxU, and the response regulator, LuxO. Together with the alternative sigma factor RpoN, LuxO activates transcription of the quorum-regulated small RNAs (sRNAs), Qrr1–4, which work in conjunction with the sRNA chaperone Hfq to prevent the translation of *hapR*.

HapR production is repressed at low cell density, when CAI-1 and AI-2 production is not high, shown by dashed arrows, leading to LuxO phosphorylation by the QS signal transduction pathway. The VarS-VarA system responds to an unknown environmental cue and represses biofilm production by post-transcriptionally upregulating HapR. This process involves the regulatory sRNAs CsrB, CsrC, and CsrD, which bind to and titrate the RNA-binding protein CsrA, thereby interfering with LuxO-mediated activation of Qrr1-4. This leads to decreased levels of Qrr1-4 and enhanced HapR production. By contrast, the small protein Fis is a direct positive regulator of the QS-responsive sRNAs, Qrr1-4 thereby promoting HapR repression. The histidine kinase VpsS, donates phosphate groups to LuxU, thus promoting HapR repression. The integration of many regulatory pathways enables the induction or repression of *V. cholerae* biofilm formation in response to a number of extracellular and intracellular signals.

Small-nucleotide molecules, including cyclic-AMP (cAMP), (p)ppGpp, and cyclic-di-GMP regulate the induction and repression of major regulators, including HapR, VpsT and VpsR. The sigma factor RpoS promotes expression of *hapR*. Of note, RpoS is depicted with (p)ppGpp because the stringent response regulation of *vpsT* and *vpsR* has been shown to partially occur through RpoS. A key signaling molecule controlling *V. cholerae* motility and biofilm matrix production is the second messenger c-di-GMP. High cellular levels of c-di-GMP promote enhanced transcription of genes involved in biofilm formation, possibly by promoting VpsT-mediated transcriptional expression of *vps* genes. Several diguanylate cyclases (DGCs), which cumulatively contribute to c-di-GMP levels, and phosphodiesterases (PDEs), known to degrade cellular c-di-GMP to pGpG or GMP, are shown.

The second messenger cyclic adenosine-monophosphate (cAMP) is involved various cellular responses and acts as a repressor of *V. cholerae* biofilm formation. cAMP in complex with cAMP receptor protein (CRP), has been shown to upregulate HapR production through its

positive regulation of the CAI-I autoinducer synthase and its negative regulation of Fis. Finally, the sigma factor RpoS promotes expression of *hapR*.

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