Review

Vibrio biofilms: so much the same yet so different

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Vibrios are natural inhabitants of aquatic environments and form symbiotic or pathogenic relationships with eukaryotic hosts. Recent studies reveal that the ability of vibrios to form biofilms (i.e. matrix-enclosed, surface-associated communities) depends upon specific structural genes (flagella, pili and exopolysaccharide biosynthesis) and regulatory processes (two-component regulators, quorum sensing and c-di-GMP signaling). Here, we compare and contrast mechanisms and regulation of biofilm formation by *Vibrio* species, with a focus on *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fischeri*. Although many aspects are the same, others differ dramatically. Crucial questions that remain to be answered regarding the molecular underpinnings of *Vibrio* biofilm formation are also discussed.

Vibrios and biofilms

Vibrio species are ubiquitous in aquatic ecosystems. Although many *Vibrio* species are free living, a small group can form pathogenic or symbiotic interactions with eukaryotic hosts. Indeed, these *Vibrio* species alternate between growth within their hosts and prolonged survival in aquatic habitats. *Vibrio cholerae*, for example, causes periodic occurrences of the severe diarrheal disease cholera. These epidemics typically result from consumption of drinking water contaminated with the pathogen. In between epidemics, *V. cholerae* survives within brackish water [1].

Like *V. cholerae*, the pathogens *Vibrio parahaemolyticus* and *Vibrio vulnificus* are most often delivered to human hosts through the consumption of contaminated water or food, particularly raw seafood. *V. parahaemolyticus* is responsible for the most common *Vibrio*-associated, seafood-borne gastroenteritis [2]. *V. vulnificus* causes gastroenteritis, severe wound infections and septicemia in susceptible hosts [3]. The symbiont *Vibrio fischeri* similarly alternates between free-living and host-associated lifestyles [4].

Adaptation of *Vibrio* species to changing parameters of the aquatic ecosystem, in addition to those of their respective hosts, is crucial to their survival and colonization success. One key factor for environmental survival and transmission is the ability to form biofilms (i.e. matrix-enclosed, surface-associated communities). The biofilm mode of growth is the preferred lifestyle in the microbial world as it enhances growth and survival by providing access to nutrients and protection from predators and antimicrobial compounds (reviewed in Ref. [5]).

The biofilm forming capacity of *V. cholerae* is well documented, both in natural habitats and under laboratory conditions (Figure 1a) [1,6,7]. Stool samples from cholera patients, for example, contain not only planktonic *V. cholerae* but also biofilm-like aggregates that are more infectious [8]. Furthermore, removal from water of particles >20 μm in diameter can reduce cholera incidence by 48% [9]. Taken together, these studies highlight the importance of the biofilm growth mode in both the intestinal and aquatic phases of *V. cholerae*'s life cycle. Biofilm formation also has a key role in host colonization by *V. fischeri* (Figure 1b) [10,11]. It is likely to also be important for the ecology, transmission and/or virulence of *V. vulnificus* and *V. parahaemolyticus*, which are found on surfaces of plankton and colonize shellfish [2,3], however, this area of research remains underdeveloped.

In recent years, numerous studies have investigated biofilm formation in *Vibrio* species. Many of these studies rely on colony morphology as an indicator of biofilm formation, including translucent (TR), opaque (OP) and rugose or wrinkled colonies; these and other methods for investigating *Vibrio* biofilms are described in Box 1. These studies have identified many key proteins, including those involved in the biosynthesis of flagella, pili and polysaccharides, and in the regulators that control their expression, predominantly two-component and quorum sensing regulators and the small signaling molecule c-di-GMP. Here, we compare and contrast mechanisms and regulation of biofilm formation in *Vibrio* species, focusing on *V. cholerae*, *V. parahaemolyticus, V. vulnificus* and *V. fischeri* as they are the species most intensively studied for biofilm formation.

Flagella are involved in initial stages of biofilm formation by *Vibrio* spp.

Biofilm formation begins when a bacterium reaches and attaches to a surface. After the initial attachment, subsequent formation of microcolonies and 3D structures is mediated by movement and growth of attached bacteria. In many bacteria, flagella-mediated motility promotes the initial stages of biofilm formation, usually by enhancing movement towards and along the surface [12]. In vibrios, the impact of motility seems to extend beyond attachment.

In *V. cholerae*, loss of flagellar genes usually results in decreased attachment, although the contribution of the flagellum varies between strains [6,13]. In *V. cholerae*

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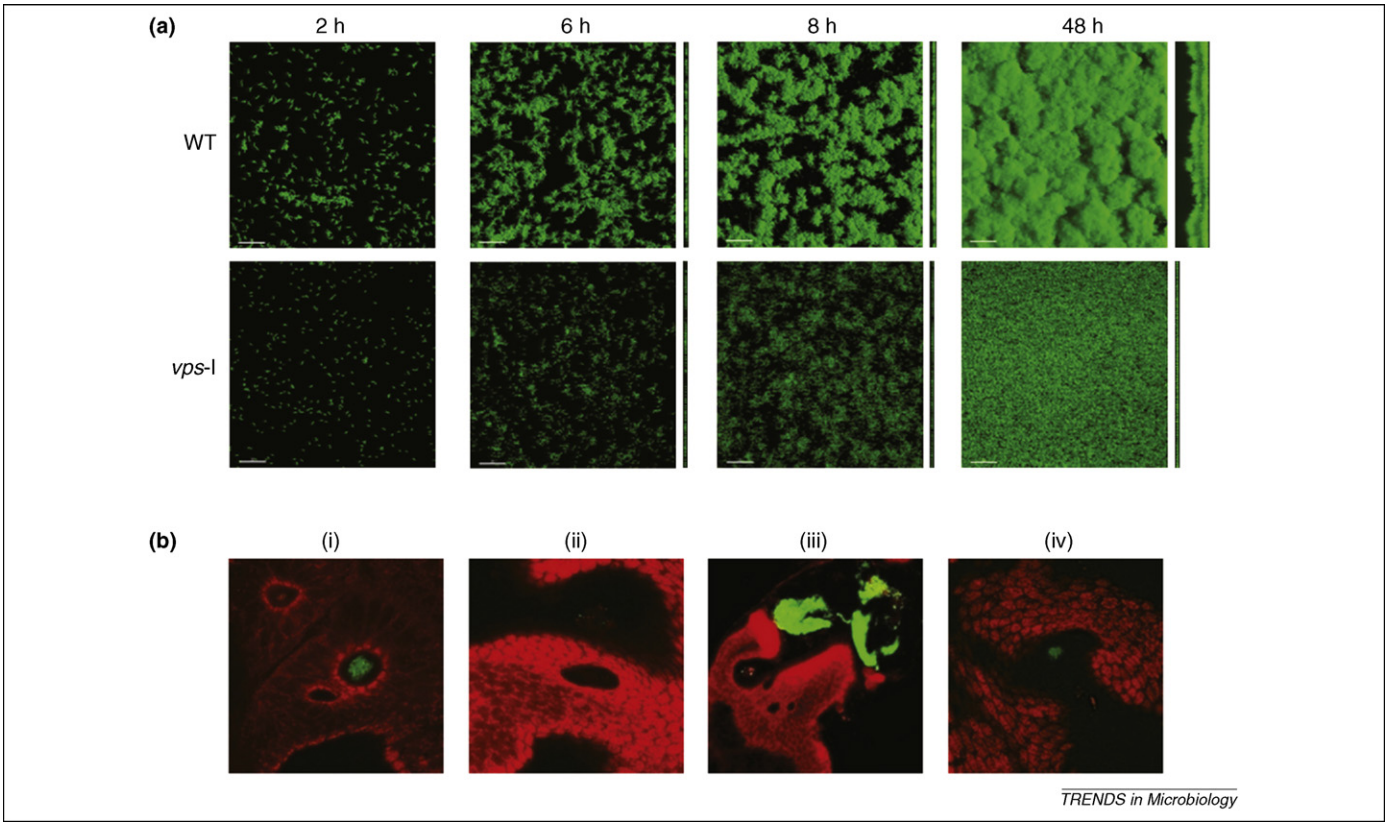


Figure 1. Biofilms of *V. cholerae* and *V. fisheri*. **(a)** 3D biofilm structures of green fluorescent protein (GFP) tagged wild-type *V. cholerae* and a *vps*-I cluster mutant (unable to produce VPS) formed after 2, 6, 8 and 48 h post-inoculation in once-through flow cell. Images were acquired with confocal laser scanning microscopy (CSLM) and top-down and side views of biofilms are shown. Scale bar indicates 30 μm. Data (but not images) are from Ref. [66]. **(b)** Biofilm-like aggregate formation on the light organ of squid. Newly hatched squid were inoculated with GFP tagged wild-type cells (i) or *sypN* polysaccharide mutant carrying vector control (ii), and wild-type cells (iii) or *sypN* mutant overexpressing the histidine kinase RscS (iv). Between 2–6 h post inoculation, squids were stained with Cell Tracker Orange (red color) and aggregate formation by *V. fisheri* strains was analyzed by CSLM. Data (but not images) are from Ref. [11].

O139, time lapse video microscopy [13] revealed that initial interactions between wild-type cells and a surface occurred rapidly: bacteria near the surface swam in circles, then exhibited more restricted movement. Some cells, tethered to the surface, alternated between short periods of jerky movement and no movement. The numbers of surface associated and immobilized bacteria increased over time, with subsequent formation of microcolonies and classical 3D structures. A *V. cholerae* O139 *flaA* mutant (lacking the major flagellin subunit), however, did not undergo this pattern of biofilm formation. Instead, the mutant aggregated in liquid culture, then subsequently settled onto the surface as immobilized clusters of cells [13]. After this settling, the biofilms developed relatively normally, indicting that the *flaA* mutant could form biofilms if it was allowed sufficient time.

Box 1. Experimental analysis of biofilm formation

There is a correlation between biofilm matrix production and colony morphology. Cells able to produce exopolymers have corrugated (also termed wrinkled or rugose), or in some cases mucoid, colony morphologies. Thus, changes in colony morphology have been extensively utilized to identify biofilm matrix components. For example, *V. parahaemolyticus* undergoes a reversible phase variation between opaque (OP) and translucent (TR) colony types (Figure I). OP colonies tend to result from increased polysaccharide production, and loss of the involved polysaccharide locus results in colonies that are TR [37,51]. Besides OP and TR morphotypes, *V. parahaemolyticus* forms rugose colonies that exhibit increased CPS production compared to parental TR or OP strains [69]. *V. vulnificus* produces OP, TR and rugose colony morphotypes [33,61,72], whereas *V. cholerae* undergoes phase variation to switch between the smooth and rugose colony morphotypes [7].

Biofilms formed at solid-liquid interfaces have been analyzed under static or flow conditions [66]. For static culture conditions, microorganisms are grown in microtiter dishes or test tubes and the extent of biofilm formation is followed by staining of the surface-associated biofilm with crystal violet. Pellicle formation has also been used to analyze biofilms formed at air-liquid interfaces under static conditions. To evaluate biofilm architecture, biofilms that form in static cultures or in the 'once-through' flow cell reactor can be analyzed using confocal laser scanning microscopy (CSLM), usually utilizing strains that express fluorescent proteins.

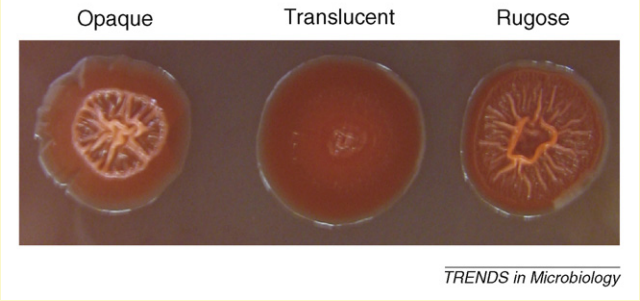


Figure I. Representative colony phenotypes of *V. parahaemolyticus*. Colony phenotypes of opaque, translucent and rugose strains of *V. parahaemolyticus* grown on Congo red plates are shown.

The planktonic aggregation of *flaA* mutants was subsequently attributed to increased production of an exopolysaccharide termed vibrio polysaccharide (VPS). Consistent with this result, these mutants formed rugose colonies. Surprisingly, any of several paralyzed (*mot*) mutants, which produce a flagellum but cannot rotate it, formed smooth colonies and poor biofilms [13,14]. A *flaA motX* double mutant also produced smooth colonies, indicating that disruption of *mot* can eliminate the VPS-inducing signal caused by loss of the flagellum [14]. Thus, *V. cholerae* could use the flagellar motor not only to promote motility but also to transmit a signal indicating interaction with a surface [14].

Such a possibility would not be unprecedented: *V. parahaemolyticus* uses its motor to decide when to switch from a swimming cell (polarly flagellated) to a swarmer cell (with lateral flagella) capable of moving on surfaces or in highly viscous media [15]. *V. parahaemolyticus* also uses its polar flagellum to promote biofilm formation [16]. *flgE* and *flgD* (hook) mutants are defective in attachment, pellicle formation (forming 'speckled' pellicles) and biofilm formation [16]. The severity of the defect depended on the strain background. For example, whereas TR *fla* mutants in submerged cultures could not adhere, OP *fla* mutants could adhere, but could not form complex 3D structures, even with prolonged incubation. These data indicate a role for the polar flagellum beyond biofilm initiation and/or in controlling other factors [16].

In *V. vulnificus* and *V. fischeri*, flagellum-mediated motility also promotes biofilm formation. For example, a *V. vulnificus flgE* (hook) mutant is defective for attachment both to polystyrene and to glass wool [17]. In *V. fischeri*, the *flrC* regulator is also required for biofilm formation [18]; prolonged incubation could overcome some, but not the entire defect.

Thus, motility has a key role in biofilm formation in the vibrios, as has been seen for other biofilm models. Interestingly, the role of motility seems to extend beyond simply allowing the cell to reach the right location. Understanding other role(s) for motility and/or the motility apparatus during biofilm development remains an important area of investigation.

Pili promote cell-surface and/or cell–cell interactions

In *V. cholerae*, at least three types of pili contribute to biofilm formation: mannose-sensitive haemagglutinin type IV pili (MSHA), toxin co-regulated pili (TCP) and chitin-regulated pili (ChiRP; formerly termed PilA) [19–22]. The relative importance of these pili varies under different conditions, and from strain to strain. MSHA, for example, is crucial for early attachment to abiotic surfaces in *V. cholerae* O1 El Tor strains, yet initial studies revealed no role in strain O139 [13]. Subsequent work revealed a role for the O139 MSHA pilus structural gene *mshA* in monolayer formation, and demonstrated that the *mshA* mutant could bypass this stage by aggregating as planktonic cells and subsequently settling and forming 3D biofilms [23]. Similarly, conflicting results for the importance of MSHA pili have been obtained for biofilm formation on various chitin substrates [19,20,22]. Thus, the contribution of MSHA to biofilm formation is impacted by both environmental and genetic factors.

The classic, virulence-associated pilus of *V. cholerae*, TCP, is involved in microcolony formation on an environmentally relevant substrate, chitin. A TCP pili mutant of El Tor N16961 formed monolayers, but not microcolonies, on a chitin substrate [21]. Recent work revealed that MSHA and TCP pili are inversely controlled at multiple levels [24], indicating the possibility that the two pilus types sequentially promote monolayer and 3D biofilm formation.

A role for ChiRP is less clear. ChiRP was required for competitive attachment to a chitin surface, crab shell, but largely unnecessary for individual attachment to chitin particles [20]. It is speculated that ChiRP might have a role other than adherence, such as orienting the cell optimally for chitin degradation [20].

V. parahaemolyticus also employs MSHA and ChiRP for biofilm formation. MSHA mutants form substantially reduced biofilms – a defect that could be overcome by prolonged incubation – and defective, speckled pellicles [16]. Like *V. cholerae*, strain background influenced the severity of the defect. ChiRP mutants fail to progress past monolayer formation [25]. In addition, both MSHA and ChiRP contribute to attachment to chitin particles [25].

In *V. vulnificus*, the type IV pilus structural protein PilA and, to a greater degree, the pre-pilin peptidase PilD, contribute to binding both to abiotic surfaces and to human epithelial cells [26,27]. The difference in relative importance of the two genes could be attributed to the retention by the *pilA* mutant of other types of pili, and/or to the role of PilD in processing other secreted proteins [26,27]. PilA and PilD are also necessary for prolonged attachment to oysters [28].

A role for pili in biofilm formation in *V. fischeri* has not been determined but is expected, given that the genome encodes eight putative pili loci, two of which contribute to efficient symbiotic colonization [29,30].

In general, although similar pili are used by vibrios for attachment, it seems that the genetic context of the cell and the type of surface it encounters (and/or other clues from the environment) can substantially influence the relative importance and thus usage of a particular type of pili for attachment.

Polysaccharides are the most prevalent component of *Vibrio* biofilms

Production of mature biofilms requires extracellular matrix components that hold the cells together and keep the biofilm attached to the surface. The capsular polysaccharide (CPS) or exopolysaccharide (EPS, or VPS in *V. cholerae*) loci involved in biofilm formation have been identified from numerous *Vibrio* spp. Expression of these loci is frequently correlated with biofilm-associated changes in colony morphology, in particular OP, rugose or wrinkled colonies (Box 1).

vps and vps-like loci

In *V. cholerae* O1 El Tor A1552, biofilm formation depends upon two linked loci, *vps*-I and *vps*-II (collectively termed *vps*), which encode structural proteins responsible for EPS production [7] (Figure 2a). *vps*-I and *vps*-II are separated by six genes (*rbmA-F*) that also are involved in biofilm

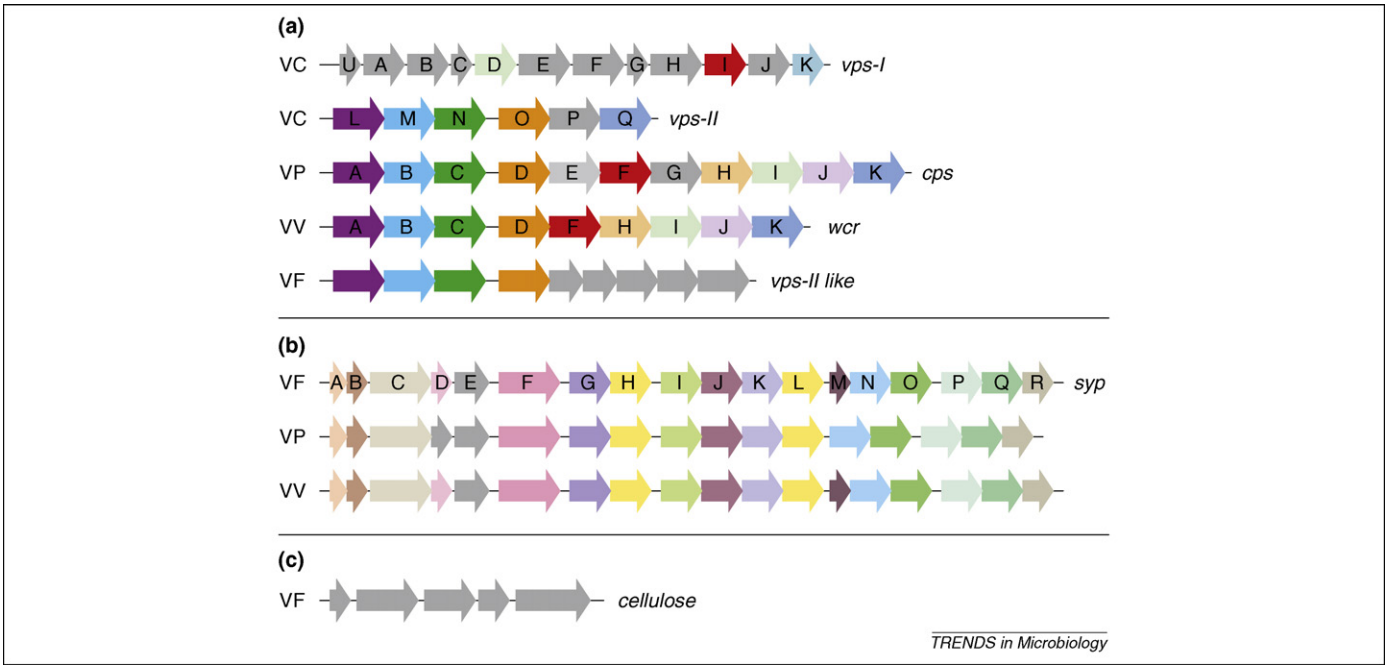


Figure 2. Polysaccharide loci in *Vibrio* spp. The three most prevalent loci with established roles in biofilm formation in *Vibrio* spp. are shown: **(a)** *vps* and *vps*-like, **(b)** *syp* and *syp*-like and **(c)** cellulose. In *V. cholerae*, the large *vps* locus encompasses 2 sub-loci, *vps*-I and *vps*-II; the polysaccharide loci of other vibrios is more similar to *vps*-II than to *vps*-I and thus for clarity these sub-loci are separated. (a) *V. cholerae* (VC) *vps* locus (*vps*-I (VC0916-VC0927) and *vps*-II (VC0934-VC0938*), V. parahaemolyticus* (VP) *cps* locus, *V. vulnificus* (VV) *wcr* locus (VVA0395-VVA0387; VV21582-VV21574) and a *vps*-II-like locus in *V. fischeri* (VF) (VF0352-VF0344). (b) *V. fischeri syp* locus (VFA1020-VFA1037) and similar loci in *V. parahaemolyticus* (VP1476–1458) and *V. vulnificus* (VV12658-VV12674). (c). *V. fischeri* cellulose locus (VFA0885-VFA0881). Genes (not drawn to scale) are represented by arrows. Gray arrows represent genes that are dissimilar to others in the same panel, whereas those with the same color exhibit sequence similarity. Genes are named as labeled.

formation but not polysaccharide production, with one exception: disruption of *rbmB* (a predicted VPS lyase) leads to an accumulation of polysaccharide [31]. VPS from *V. cholerae* O1 El Tor A1552 primarily contains glucose and galactose [7], with N-acetyl glucosamine, mannose and xylose representing minor constituents. This VPS is associated with rugose colony formation, matrix production, pellicles, 3D biofilms and resistance to chlorine [7,32]. Loss of *vps* eliminates these phenotypes.

The *vps* locus is conserved, in part, in other vibrios [29,33,34] (Figure 2a). No role in biofilm formation for the *vps*-like locus of *V. fischeri* has been observed to date [35]. Similarly, little is known about the *V. vulnificus* locus, *wcr*, other than that it is associated with the formation of both OP and rugose colonies [33,36]. *wcr* most resembles the *V. parahaemolyticus* locus, *cps*, which produces a CPS (CPSA), rather than an EPS like *V. cholerae* [37]. *cps* is required for OP colony morphology and pellicle formation, as its loss disrupts these phenotypes [34]. OP colonies have increased *cps* gene expression and produce more CPSA [34,37]. CPSA contains approximately equal amounts of fucose, galactose, glucose and *N*-acetylglucosamine, making it distinct from *V. cholerae* EPS [16,37]. Its production seems to require activated sulphur, as cysteine mutants fail to synthesize CPSA [16].

syp and syp-like loci

Biofilm formation in *V. fischeri* depends upon an 18 gene cluster of polysaccharide biosynthetic genes (*syp*) and associated regulators [11,38]. This locus is lacking in *V. cholerae*, but is conserved in *V. parahaemolyticus* and *V. vulnificus* (Figure 2b). Induction of *syp* expression results in wrinkled colonies, pellicle formation and matrix production; loss of specific *syp* genes largely restores the wild-type phenotype [11,18]. The Syp polysaccharide has not been purified, but seems to contain glucose or α-linked mannose [11]

Other polysaccharide loci

Biofilm formation by *V. fischeri* also depends upon a cellulose gene cluster similar to that found in *Salmonella* but absent in other vibrios [35] (Figure 2c). Numerous other polysaccharides and polysaccharide loci have been uncovered in *Vibrio* spp. (e.g. Refs [39,40]), although few have been investigated for roles in biofilm formation. Where investigated, a surprising number seem to have negative roles. For example, the group 1 CPS in *V. vulnificus* is associated with the production of OP colonies, but the loss of a representative gene increases attachment [41,42]. *V. cholerae* O139 contains a locus with genes for CPS and lipopolysaccharide (LPS) O antigen biosynthesis that also has a negative role in biofilm formation [43]. A third example is a putative O antigen CPS locus of *V. parahaemolyticus*, VP0214-VP0237, the loss of which increases attachment [16]. A deeper understanding of the roles of these possible polysaccharide loci awaits further investigation.

The *Vibrio* species studied to date produce distinct EPSs, and some if not all have the potential to produce multiple types of polysaccharides. Because biofilms gain their structural integrity largely from the EPS matrix component, these studies indicate a diversity of biofilms are produced. It is possible that these biofilms, thus, could have an important role in niche selection.

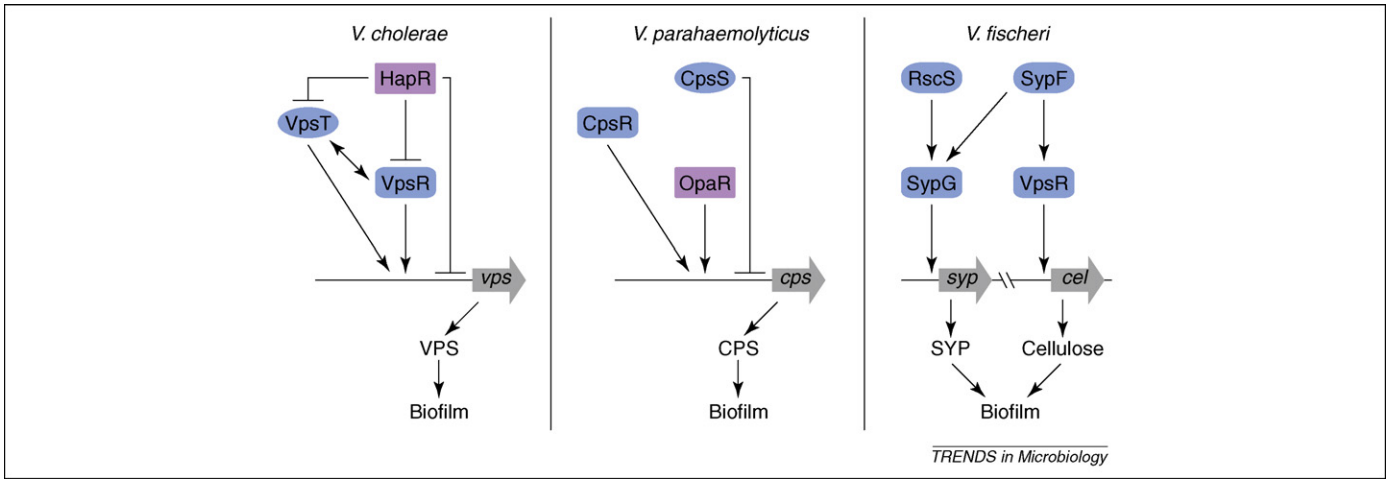


Figure 3. Regulation of biofilm formation in *Vibrio* spp. Biofilm formation in *V. cholerae* is positively regulated by the regulators VpsR and VpsT. The magnitude of transcriptional control of *vps* genes by VpsR is greater than that of VpsT. Expression of *vps* genes and the regulators VpsR and VpsT are negatively controlled by the HapR regulator. In *V. parahaemolyticus* expression of *cps* genes is negatively regulated by a homolog of VpsT, CpsS. In the absence of CpsS, OpaR and CpsR (HapR and VpsR homologs, respectively) positively control *cps* gene expression and biofilm formation. In *V. fischeri* transcription of *syp genes* and biofilm formation are positively controlled by the histidine kinases RscS and/or SypF, which act through response regulator SypG. SypF also positively regulates production of cellulose (*cel*), acting although the VpsR homolog of *V. fischeri*. The roles of VpsR and SypE as inhibitors in *V. fischeri* are poorly understood and thus these are omitted from this figure.

Transcriptional control of EPS production genes

In *Vibrio* species, regulation of exopolysaccharide production and biofilm formation is complex, and involves numerous transcriptional regulators, particularly two-component signal transduction and quorum sensing regulators (Figure 3). In a typical two-component system, a stimulus detected by a sensor histidine kinase (HK) is transformed into a cellular signal by a phosphorelay event that involves autophosphorylation of the HK at a conserved histidine residue. The phosphoryl group is then passed to a response regulator (RR) at a conserved aspartate residue, leading to activation and altered gene expression or protein function. Quorum sensing involves sensing and responding to population density (Box 2). Global regulators, such as CRP [44,45] and sigma factors RpoS and RpoN [32], have a role in biofilm formation in the vibrios, but they are not discussed here.

Two-component regulators

VpsR and VpsR-like regulators. In *V. cholerae*, the RR VpsR is a key regulator of biofilm formation. VpsR promotes transcription of the *vps* genes and formation of typical 3D biofilm structures [32,46]. Mutations that alter the residue predicted to be phosphorylated (D59) either inactivate this protein (D59A) or increase its activity (D59E) [14], thus supporting that phosphorylation on this residue is required for activation. However, the HK responsible for phosphorylation of VpsR has not been identified, as it is not physically linked to *vpsR*. Identifying the cognate HK will facilitate understanding the regulatory network controlling *vps* gene expression and how environmental signals regulate VPS production.

Under specific genetic conditions, *V. parahaemolyticus* also produces high levels of CPS and thus rugose colonies. Disruption of the *vpsR* homolog, *cpsR*, yields smooth colonies and decreases transcriptional activity of *cpsA* fused to a *lacZ* reporter in the rugose background [34]. However, CpsR is not required for basal levels of *cps* expression in TR or OP strains, as disruption of *cpsR* in these strains had no effect on *cps* transcription [34]. This contrasts with the role of VpsR, which is essential for transcription of *vps* genes in all forms of *V. cholerae*. It remains to be determined whether CpsR directly regulates transcription of *cps* genes.

V. fischeri also encodes a VpsR homolog. Disruption of *vpsR* leads to formation of mucoid colonies, which are indicative of enhanced polysaccharide production, indicating that VpsR might be a repressor [35]. When overexpressed, however, VpsR induces biofilm formation, indicating that it could also be an activator. Surprisingly, VpsR-induced biofilms depended not on a *vps*-like locus but on a putative cellulose biosynthesis cluster not found in other characterized vibrios (Figure 2c) [35]. Thus, VpsR seems to have a novel role in *V. fischeri*.

VpsT and VpsT-like. In *V. cholerae*, a second positive regulator is VpsT, a member of the UhpA (FixJ) family of transcriptional regulators [47]. VpsT shows homology to CsgD, which positively controls curli and cellulose production in *Salmonella enterica*. Disruption of *vpsT* yields smooth colonies and reduces *vps* expression, and biofilm formation [47]. Although the putative phosphorylation site in the receiver domain of VpsT is conserved, it is not necessary for the *in vivo* function of VpsT (J. Meir and F. Yildiz, unpublished). Thus, whether VpsT functions as a canonical RR remains unclear.

Characterization of *vpsT* and *vpsR* mutants in *V. cholerae* revealed different roles for these regulators in determining biofilm architecture. The *vpsT* mutant is still able to form biofilms (albeit distinct from its rugose parent), whereas *vpsR* and *vpsT vpsR* mutants produce only single cells or small microcolonies attached to the substratum [48]. The VpsR and VpsT regulons are largely identical, although VpsR exerts a larger impact on expression [48]. Thus, VpsR is essential for VPS production and biofilm formation, whereas VpsT seems to have an accessory role, possibly by increasing the level or activity of VpsR. Whether VpsT and VpsR serve as direct regulators of *vps* remains unknown.

Box 2. Quorum sensing

Quorum sensing is a mechanism that cells use to determine their population density. Signal synthases produce a diffusible molecule (autoinducer [AI]) that can accumulate in the extracellular environment. When the cell population is sufficiently high, and enough signal accumulates, this molecule binds to and activates cellular receptors. In *Vibrio* species, one consequence of this signaling is a phosphorelay that ultimately controls the synthesis of a global regulator and phenotypes that are more useful to a group of cells, such as luminescence, rather than to individuals [50]. Because biofilm formation is a behavior that depends upon a group of cells, it makes sense for bacteria to rely on quorum sensing regulators to control biofilm formation, and indeed they do. In *V. cholerae*, however, the sense of this control is not as might be expected: *V. cholerae* mutants, which are 'locked' into a regulatory state mimicking high cell density, are impaired in biofilm formation [54].

V. cholerae produces two AIs [50]. CAI-1 [(S)-3-hydroxytridecan-4-one] [73] is synthesized by CqsA and detected by CqsS. AI-2 [the furanosyl borate diester (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate] is synthesized by LuxS and detected by the LuxQP receptor [50]. Information from the sensors is transduced through a phosphorelay, first to the LuxU phosphotransfer protein and then to LuxO, which is an RpoN dependent response regulator. At low cell density, when the concentrations of AIs are low, LuxO is phosphorylated and activates expression of a set of small regulatory RNAs (Figure Ia) [74]. The result is inhibition of expression of the major quorum sensing regulator, HapR. At high cell density, LuxO is dephosphorylated and expression of HapR is increased. Under these conditions, the *vps* genes and virulence-associated genes (*aphA*) are repressed (Figure Ib).

This regulation seems to be both direct and indirect as the empirically defined HapR binding motif was found in the promoter regions of both the *vps* locus and its regulator *vpsT* [32]. Mutants in *cqsA* that cannot produce the CAI-1 signal, a condition that should mimic low cell density, form thicker biofilms [55]. This indicates that CAI-1 signals are crucial for repression of biofilm formation via the quorum sensing regulatory circuitry. It is thought that quorum sensing ensures development of 'normal' biofilm structures that permit rapid dispersion of bacteria from the biofilm, thereby facilitating transmission.

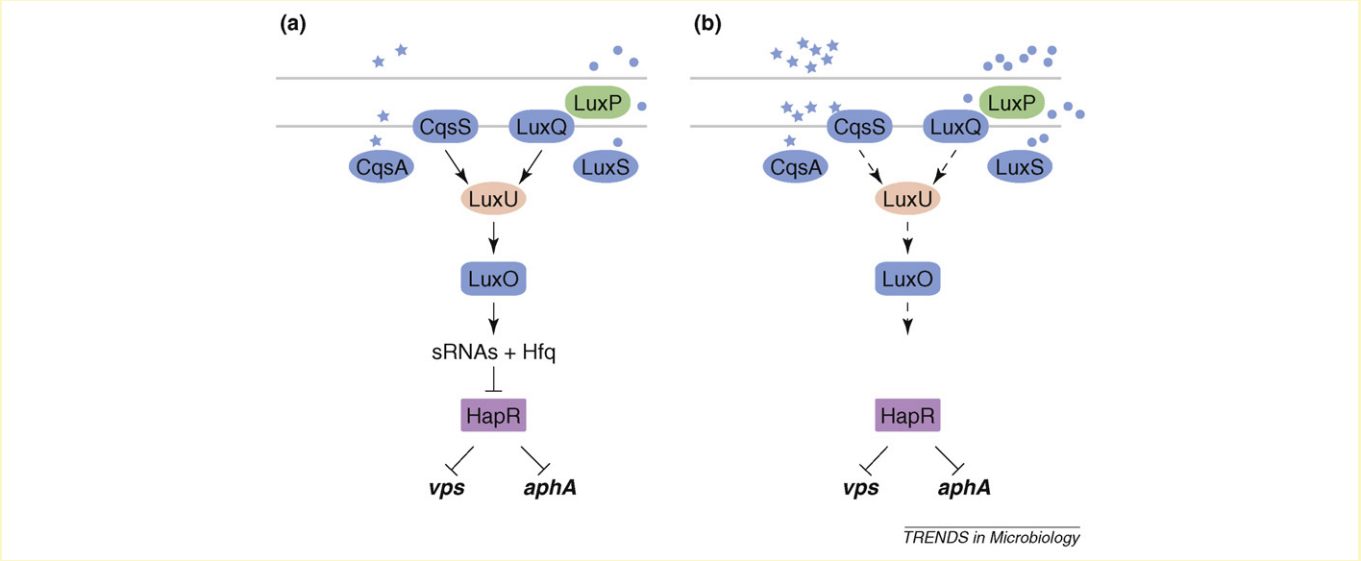


Figure I. *V. cholerae* quorum sensing. *V. cholerae* synthesizes two AIs, CAI-1 (stars) made by CqsA and AI-2 (circles) made by LuxS, that are detected by sensor kinases CqsS and LuxQ (working with periplasmic protein LuxP), respectively, which control a phosphorelay system. Low **(a)** and high **(b)** levels of autoinducers alter the signal transduction cascade, ultimately impacting biofilm formation and virulence gene expression. Abbreviations: Hfq, RNA binding protein; sRNAs, small regulatory RNAs.

A VpsT homolog, CpsS, also controls biofilm formation in *V. parahaemolyticus*, but as a negative regulator [34]. Introduction of a *cpsS* mutation into TR or OP strains derepresses *cpsA* transcription, resulting in rugose colonies. In TR strains, derepression is mediated through CpsR, whereas in OP strains, CpsR has an accessory role [34]. Thus, *V. cholerae* and *V. parahaemolyticus* use similar proteins, but they function in the opposite sense and to different degrees: CpsS is the dominant negative regulator in *V. parahaemolyticus*, whereas VpsT is a positive co-regulator in *V. cholerae*.

Other two-component regulators. In *V. vulnificus*, the RR NtrC contributes to biofilm formation through its transcriptional control of *gmhD* [49]. The GmhD protein, an ADP-l-glycero-D-manno-heptose-6-epimerase, is required for LPS and EPS production and biofilm formation [49]. The relative roles of LPS and EPS in biofilm formation, in addition to the identity of the EPS involved in this process, have yet to be determined.

In *V. fischeri*, two-component regulators have crucial roles in the control of biofilm formation, primarily through their activation of the *syp* polysaccharide locus. For example, the orphan HK RscS, when overexpressed, induces *syp* transcription, wrinkled colony and pellicle formation, and symbiotic biofilm formation [11]. RscS acts upstream of SypG, a *syp*-encoded RR that is proposed to be the direct activator of *syp* transcription [18].

Surprisingly, overexpression of SypG induced *syp* transcription but not the formation of wrinkled colonies or strong pellicles. However, overexpression of SypG in a *sypE* mutant, permitted wrinkled colony and pellicle formation [18]. Thus, SypE, a putative RR that is not predicted to bind DNA, seems to have an inhibitory role. RscS might therefore both activate SypG and inactivate SypE. Homologs of SypG are present in *V. vulnificus* and *V. parahaemolyticus*, but RscS and SypE are unique to *V. fischeri*.

Overexpression of a third *syp*-encoded regulator, SypF, also induces biofilm formation in *V. fischeri* [35]. This

regulator seems to function upstream of both SypG and VpsR: mutation of both regulators was necessary to eliminate all the biofilm phenotypes induced by SypF overexpression. The SypF-VpsR path seems to promote cell-surface attachment, whereas the SypF-SypG path seems to be responsible for cell–cell attachment [35].

Quorum sensing regulators

Vibrio species use quorum sensing (Box 2) to control numerous traits, including luminescence, virulence and biofilm formation. Best studied in *Vibrio harveyi*, in which the endpoint regulator is termed LuxR (reviewed in Ref. [50]), similar pathway components are found in all other vibrios studied to date.

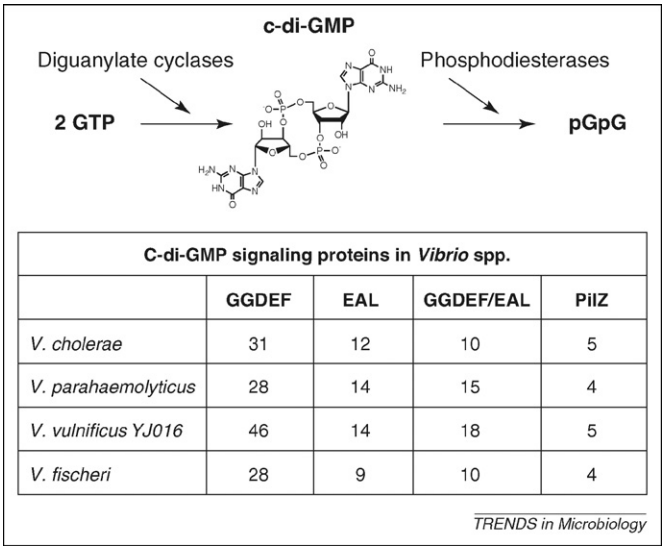
The *V. parahaemolyticus* LuxR homolog, OpaR, positively regulates opacity, *cps* gene expression and biofilm formation [51]. Disruption of *opaR* in an OP strain yielded TR colonies, and overexpression of *opaR* in a TR strain increased *cps* expression and colony opacity [37]. A similar phenomenon is seen in both *V. vulnificus* and *V. fischeri*: mutants defective for the LuxR homologs, SmcR [52] and LitR [53], respectively, form TR colonies instead of the parental OP colonies; in the case of *V. vulnificus*, this disruption is associated with decreased biofilm formation. However, the molecular mechanisms causing decreases in colony opacity and biofilm formation and their connections to CPS or EPS production by *V. vulnificus* and *V. fischeri* are unknown.

In *V. cholerae*, the story is different (Figure 3). Mutants of the LuxR homolog, HapR, exhibit increased rugosity and increased *vps* expression [32,54,55]. These data indicate that HapR is a negative regulator of biofilm formation. HapR can directly bind DNA and repress expression of *vpsT* [56]. It can also control *vpsR* expression in some strains [32]. Thus, quorum sensing control of cell surface properties and biofilm formation is opposite in *V. cholerae* relative to the other vibrios. The ecological importance of this regulation is yet to be determined.

C-di-GMP signaling and biofilm formation

C-di-GMP is a ubiquitous second messenger that controls the transition from a free-living, motile lifestyle to a biofilm lifestyle in many bacteria (reviewed in Ref. [57]), including vibrios [58–62]. Increased c-di-GMP levels tend to promote biofilm formation and/or inhibit flagellar motility. C-di-GMP production and degradation is controlled by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively [57] (Figure 4). Overexpression of these regulators tends to cause global effects. Intriguingly, vibrios contain much larger numbers of DGCs and PDEs than other bacteria [63]. The abundance of enzymes controlling synthesis and degradation of c-di-GMP in vibrios indicates the importance of c-di-GMP signaling to the biology of vibrios. Because different types of sensory domains are found in proteins predicted to function as DGCs or PDEs [63], one possibility is that cells adjust their c-di-GMP levels in response to environmental and intracellular signals and that c-di-GMP signaling has an important role in adaptation of vibrios to different environments.

In *V. cholerae*, c-di-GMP increases biofilm formation by stimulating transcription of *vps* genes and the positive transcriptional regulators *vpsR* and *vpsT* [60,64]. Mutants of the known or putative PDE genes, *mbaA*, *rocS*, *cdgC*, *cdpA* and *vieA*, exhibit enhanced biofilm formation, presumably because of increased c-di-GMP levels [65,66].



C-di-GMP signaling proteins in *Vibrio* spp.

	GGDEF	EAL	GGDEF/EAL	PilZ
V. cholerae	31	12	10	5
V. parahaemolyticus	28	14	15	4
V. vulnificus YJ016	46	14	18	5
V. fischeri	28	9	10	4

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Figure 4. C-di-GMP signaling proteins in *Vibrio* spp. Cyclic di-guanosine-monophosphate (c-di-GMP) controls cell surface structures and biofilm formation in a diverse group of microorganisms. C-di-GMP is created from GTP (guanosine-5′-triphosphate) by diguanylate cyclase proteins that bear a GGDEF amino acid motif and degraded to the dinucleotide pGpG by phosphodiesterase proteins with EAL domains. C-di-GMP can be sensed by proteins with a PilZ domain. Numbers of genes encoding GGDEF, EAL, dual GGDEF and EAL or PilZ domain proteins in different *Vibrio* species are shown.

Recently, c-di-GMP also has been linked to the natural capacity of *V. cholerae* to generate rugose variants. For example, the prototype rugose strain A1552 expresses elevated c-di-GMP levels caused by a single amino acid change in a DGC protein, VpvC, relative to the smooth variant [67]. Disruption of *vpvC* in this rugose variant reduces overall c-di-GMP levels and causes cells to become similar to the smooth variant with respect to biofilm formation and *vps* transcription. Rugosity can also be generated by deletion of the master quorum sensing regulator *hapR*. This effect occurs through CdgA, a DGC whose mRNA abundance is increased in the *hapR* mutant; this increased CdgA presumably increases cellular c-di-GMP. Deletion of *cdgA* decreased *vps* transcription and restored smooth colony formation to the rugose *hapR* mutant [48]. Subsequent studies revealed that HapR serves as a direct regulator of *cdgA* [56].

Increased c-di-GMP level leads to a decrease in motility. In *V. cholerae*, as expected, mutations in DGC genes *cdgD* [60], *cdgH* [68] and *vpvC* [67] lead to an increase in motility, whereas mutations in PDE genes *vieA*, *rocS*, *cdgC* and *mbaA* lead to a decrease in motility relative to wild-type, when tested on Luria-Bertani medium (LB) soft agar motility plates [66].

In *V. parahaemolyticus*, increases in cellular c-di-GMP levels prevent swarming motility and promote biofilm formation. Two genes involved in c-di-GMP control, *scrG* and *scrC*, have been extensively characterized [58,59,69]. ScrG functions as a PDE: null mutants increase c-di-GMP and exhibit increased *cps* and decreased lateral flagellar

Box 3. Questions for future research

- What combination(s) of polysaccharides are being produced under various laboratory and environmental conditions?
- What are the constituents (protein or DNA) of biofilm matrices under laboratory, environmental and/or disease conditions?
- What other structural and regulatory factors are involved in biofilm formation?
- What are the environmental conditions that promote formation and dissolution of biofilms?
- What are the stimuli sensed by two-component systems regulating biofilms formation?
- What is the mechanism of c-di-GMP signaling and how is it connected to the regulatory network controlling biofilm formation?
- Do differences in regulation of biofilm formation reflect the importance of the biofilm lifestyle to each *Vibrio* spp. during their *in vivo* and *ex vivo* life cycles?

gene (*laf*) expression and, thus, enhanced biofilm formation and reduced swarming motility [59]. Null mutants defective for the *scrABC* operon behave similarly, whereas overexpression of *scrABC* yields the opposite results [58]. Interestingly, however, overexpression of *scrC* in the absence of *scrAB* (encoding putative pyridoxal-phosphate-dependent and extracellular solute-binding proteins, respectively) induces *cps*, not *laf*, expression [58]. Subsequent work revealed that ScrC is a bifunctional protein that functions as a DGC to synthesize c-di-GMP, but when co-produced with ScrAB, functions as a PDE and degrades c-di-GMP. Epistasis analysis indicates that ScrG and ScrABC act in the same regulatory circuitry and that *scrG* and *scrABC* double mutants show a cumulative effect at the level of *laf* and *cps* gene expression [58].

Relatively little is known about c-di-GMP and biofilm formation in *V. vulnificus* and *V. fischeri*. In *V. vulnificus*, expression of the DGC DcpA converted TR colonies of an acapsular mutant into OP colonies, but did not impact motility [61]. Overexpression of *dcpA* induced production of an EPS that was structurally distinct from the CPS, rugose colony formation and biofilm formation [61]. In *V. fischeri*, overexpression of the putative DGC MifA promotes cellulose biosynthesis and biofilm formation, indicating that c-di-GMP is a player in biofilm formation in this microbe as well [70].

How are c-di-GMP levels sensed by the cell? One protein domain that binds c-di-GMP is the PilZ domain. Of the five PilZ domain proteins in *V. cholerae*, two of these, PlzC and PlzD, have been recently shown to bind c-di-GMP and are known to regulate biofilm formation and/or motility [71]. Thus, PilZ domain proteins can function as c-diGMP receptors and regulate c-di-GMP-dependent processes in *V. cholerae* and likely in other vibrios.

It is becoming clear that, although *Vibrios* share common regulatory proteins and signaling systems, the biofilm regulatory circuitry is unique to each *Vibrio* spp. Differences in regulation might reflect the importance of the biofilm life style to each *Vibrio* spp. during their *in vivo* and *ex vivo* life cycles, differences in niche occupation, differences in environmental parameters that they respond to and/or parameters driving evolution of the pathogens and symbionts.

Concluding remarks

Biofilm formation, particularly on a biotic, possibly nutritional surface, seems likely to provide a substantial survival advantage to aquatic organisms such as *Vibrio* species. That these organisms use similar traits and regulators to solve the problem of biofilm formation is not unexpected. That they use such diversity in approaches – the relative importance of the traits and regulators, and even the sense (positive or negative) of control – is surprising and thus has the potential to provide great insights into the peculiar lifestyles of these microbes. Some outstanding research questions are listed in Box 3. Because biofilm formation is also part of the pathogenic lifestyles of *Vibrio* spp., elucidation of the molecular mechanisms and regulation of biofilm formation will provide the foundation for developing novel treatments and prevention strategies against *Vibrio*-associated illnesses.

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