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Vibrio fischeri: a model for host-associated biofilm formation

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ABSTRACT Multicellular communities of adherent bacteria known as biofilms are often detrimental in the context of a human host, making it important to study their formation and dispersal, especially in animal models. One such model is the symbiosis between the squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*. Juvenile squid hatch aposymbiotically and selectively acquire their symbiont from natural seawater containing diverse environmental microbes. Successful pairing is facilitated by ciliary movements that direct bacteria to quiet zones on the surface of the squid's symbiotic light organ where *V. fischeri* forms a small aggregate or biofilm. Subsequently, the bacteria disperse from that aggregate to enter the organ, ultimately reaching and colonizing deep crypt spaces. Although transient, aggregate formation is critical for optimal colonization and is tightly controlled. *In vitro* studies have identified a variety of polysaccharides and proteins that comprise the extracellular matrix. Some of the most well-characterized matrix factors include the symbiosis polysaccharide (SYP), cellulose polysaccharide, and LapV adhesin. In this review, we discuss these components, their regulation, and other less understood *V. fischeri* biofilm contributors. We also highlight what is currently known about dispersal from these aggregates and host cues that may promote it. Finally, we briefly describe discoveries gleaned from the study of other *V. fischeri* isolates. By unraveling the complexities involved in *V. fischeri*'s control over matrix components, we may begin to understand how the host environment triggers transient biofilm formation and dispersal to promote this unique symbiotic relationship.

KEYWORDS *Vibrio*, biofilms, sensory transduction processes, *Vibrio fischeri*, symbiosis, regulation of gene expression, SYP, cellulose, large adhesive protein

Biofilms are communities of bacteria encased in an extracellular matrix that may include polysaccharides, proteins, and/or DNA. The matrix helps to protect the cells within from outside harm, for example, antibiotics and shear force, and also allows them to adhere to each other and a variety of surfaces (1–3). Subsequently, the bacteria within these aggregates can disperse and seed biofilms in new locations, contributing to bacterial spread in various environments (4). There are many examples in which biofilms are detrimental to the bacterium's host. One of the most prominent examples is in cystic fibrosis patients, where *Pseudomonas aeruginosa* can form biofilms in the upper and lower airways to cause disease (5). Skin infections can also be caused by biofilms composed of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and/or *P. aeruginosa* (6). In addition, biofilms that form on medical devices, such as catheters and implants, are a major concern in the healthcare field because these surfaces are attractive to bacteria that are primed for adhesion (7).

Various pathogenic *Vibrio* species can also aggregate into biofilms. *Vibrio cholerae*, the causative agent of cholera, forms biofilms during its infection cycle. These *V. cholerae* biofilms can be found attached to the intestinal mucosa within the body and in the stools of cholera patients (8). *Vibrio vulnificus* is an opportunistic pathogen that can cause septicemic and/or wound infections following consumption of or contact with contaminated raw oysters (9, 10). Biofilm formation is hypothesized to be important in

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multiple steps of the *V. vulnificus* lifestyle; it has been suggested that *V. vulnificus* relies on biofilm formation to colonize its oyster host, persist in the surrounding seawater, and cause pathogenesis in humans (11–13). Similarly, another oyster-residing *Vibrio* pathogen, *V. parahaemolyticus*, utilizes biofilm formation to adhere to its seafood host (14). In addition, every *V. parahaemolyticus* clinical isolate analyzed in one study was a biofilm former, suggesting that there is a correlation between the ability to form a biofilm and virulence in humans (15). It is clear that biofilm formation is an aspect of the infectious lifestyle of all three of these *Vibrio* pathogens.

Biofilms can also be beneficial to a host. This is the case for the symbiotic relationship between the marine bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Fig. 1A) (16, 17). This symbiosis, which has informed multiple fields of study [e.g., quorum sensing, motility, Type VI secretion, and bacterial competition (18–20)], is promoted by *V. fischeri*'s ability to form a transient biofilm (21). Cilia present on the surface of the symbiotic light organ direct the flow of seawater across the organ and facilitate the earliest interactions between the host and the symbiont (22). Bacteria and similarly sized particles land in host-derived mucus within sheltered zones where *V. fischeri* cells attach to cilia and build a biofilm-like aggregate (Fig. 1B) (23, 24). Even when outnumbered by other bacteria, *V. fischeri* rapidly becomes the dominant microbe in these host-associated aggregates, indicating that it has a superior ability to form aggregates and/or compete within that environment (25). These aggregates, although often very small, can be considered biofilms because they are groups of surface-associated bacteria whose ability to cluster together depends upon the expression of polysaccharide biosynthesis proteins, and, presumably, the resulting polysaccharide. In support of this conclusion, when these biosynthetic proteins are overproduced, there are corresponding increases in both *in vitro* biofilms and aggregate formation as well as symbiotic competence, as we discuss in detail below (22, 26, 27). Finally, some natural isolates shown to be superior colonizers can also form stronger biofilms *in vitro*, providing another connection between biofilm formation and squid colonization (28–30).

After a short period of time, *V. fischeri* cells disperse from the biofilm and migrate into the light organ, ultimately reaching deep crypt spaces, the sites of colonization (Fig. 1C) (21). There, they grow using squid-supplied nutrients and induce bioluminescence (32, 33), which the squid uses for camouflage while hunting in the moonlight (34). This symbiosis is highly specific, as only *V. fischeri* can colonize the light organ of the squid. Non-colonized squid are not found in their natural environment, suggesting that

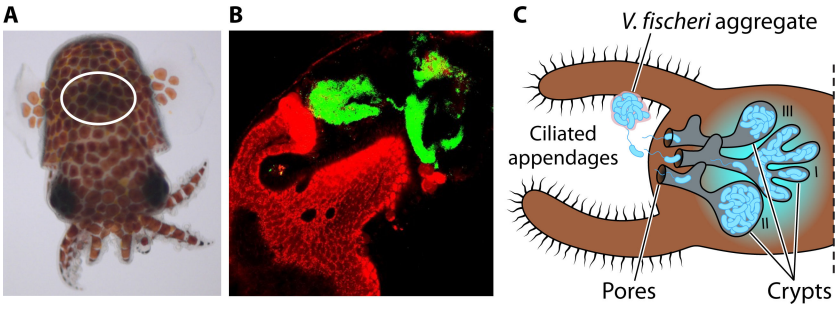

FIG 1 The *Euprymna–V. fischeri* symbiotic relationship. (A) *E. scolopes*, the Hawaiian bobtail squid, forms a symbiotic relationship with the bacterium *V. fischeri*. A newly hatched juvenile squid is shown with a circle indicating the internal location of the symbiotic light organ where *V. fischeri* colonizes. (Image courtesy of Val A. Ray, reproduced with permission.) (B) *V. fischeri* cells (green) formed a biofilm-like aggregate on the surface of the squid (red) light organ. (Image republished from reference 31 with permission.) (C) This cartoon depicts one-half of the bi-lobed light organ of a juvenile squid. Cilia present on surface appendages direct bacteria to sheltered zones where *V. fischeri* cells (blue curved rods) form a transient aggregate or biofilm. Subsequently, the cells disperse from the biofilm, enter the organ through pores, and migrate into the deep crypt spaces where colonization and luminescence (teal shading) occur.

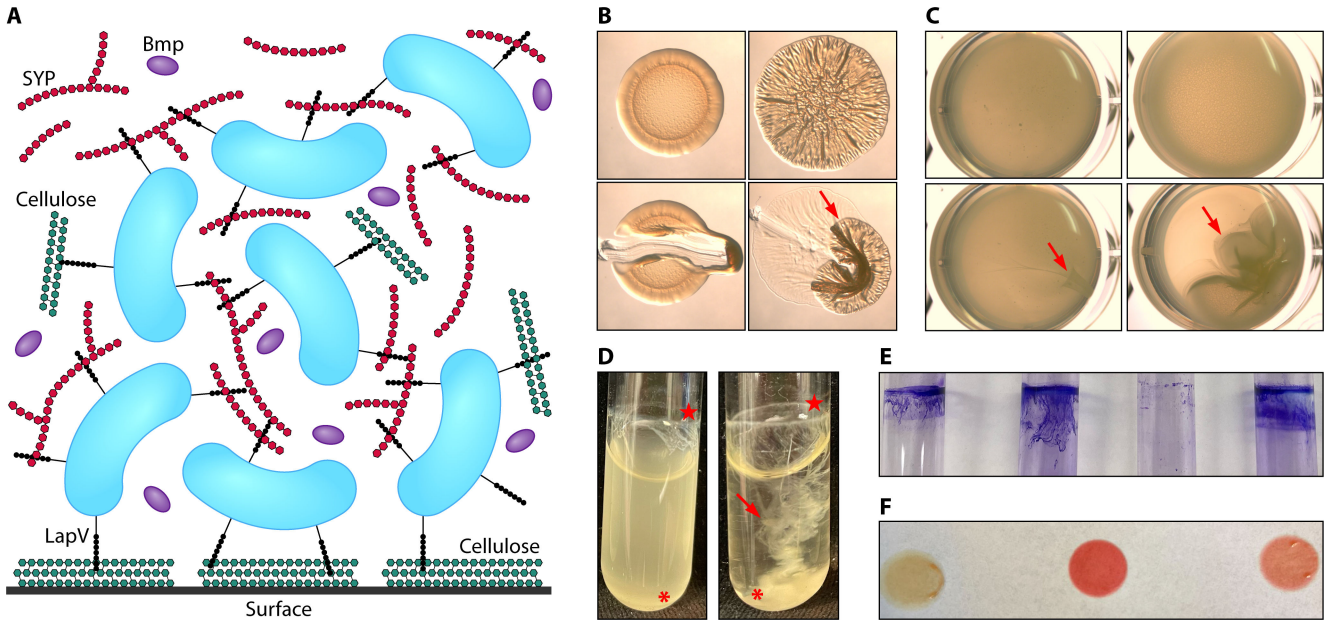

FIG 2 *V. fischeri* biofilm model and phenotypes. (A) Model depicting the best understood components of the *V. fischeri* biofilm. Cellulose polysaccharide allows for the attachment of *V. fischeri* cells to surfaces while SYP is required for cell-cell interactions. LapV is an adhesin that is also required for biofilm formation, potentially, as implied here, by binding one or more of the polysaccharides. Lastly, Bmp assists with the maturation of the biofilm. (B-F) *V. fischeri* biofilms can be studied using various *in vitro* assays. Strains are listed from left to right. (B) Spot plate assays allow for the assessment of wrinkled colony formation and stickiness. Wild-type ES114 and a hyper-biofilm former are shown as representatives (top). Toothpick-mediated disruption (bottom) permits assessment of the stickiness of the colonies. Red arrows indicate where stickiness can be observed. (C) Static liquid conditions result in the formation of pellicles at the air/liquid interface (top). Visualization is facilitated by toothpick disruption, which also permits assessment of stickiness (indicated by red arrows at the bottom). Featured here are ES114 and a hyper-biofilm former. (D) Complex biofilms can also form in shaking liquid conditions. The various biofilm structures—ring formation (seen just above the liquid) indicated by red stars, strings (extending from the rings and into the liquid) denoted by red arrows, and clumps (generally at the bottom of the tube but often also attached to the strings) indicated by red asterisks—can be analyzed to determine the extent of biofilm formation. ES114 and a hyper-biofilm former are shown. (E) The crystal violet assay is used to stain surface-attached biofilms. Shown are the tops of crystal violet stained tubes from cultures grown in shaking conditions: ES114, a hyper-biofilm former, and the hyper-biofilm forming strain that contains a mutation that disrupts the production of cellulose or SYP. (F) Congo red can bind to cellulose and other amyloids, leading to a red color. The Congo red assay is performed by spotting or streaking strains onto agar plates that have Congo red and Coomassie blue. Featured in this image are a strain lacking cellulose, a strain with increased cellulose production, and a strain with wild-type levels of cellulose.

colonization and, likely, light production are keys to squid survival (17). Since efficient colonization depends on biofilm formation (26, 35–42), this characteristic of *V. fischeri* may be beneficial for squid survival in the wild.

Due to (i) the importance of biofilm formation in host colonization, (ii) the ready ability to visualize the dynamics of bacteria-host interactions, (iii) the ease of manipulating and handling the bacterium and assessing biofilm phenotypes and underlying mechanisms in culture (Fig. 2; Table 1) (43, 44), and (iv) the strong correlations between biofilm competence *in vitro* and both *in vivo* aggregate formation and colonization, the *V. fischeri–E. scolopes* partnership makes an excellent model for studying biofilm formation in the context of an animal host. In this review, we will discuss the *V. fischeri* biofilm matrix components in detail, including their nature and regulation, and other lesser studied components that require further research. We also describe the limited body of knowledge with respect to biofilm dispersal as well as insights gleaned from studying an increasing number of *V. fischeri* isolates.

TABLE 1 *In vitro V. fischeri* biofilms under different conditions

Condition	Phenotype	Example	Matrix component
Agar plates	Colony bumpiness	Fig. 2B left top	Cellulose
	Colony wrinkling	Fig. 2B right top	Bmp, SYP
	Cohesion	Fig. 2B right bottom	SYP
	Congo red binding	Fig. 2F middle, right	Cellulose
Static liquid	Pellicle	Fig. 2C	SYP, cellulose, LapV
	Cohesion	Fig. 2C (arrows)	SYP, LapV
Shaking liquid	Surface attachment/ring	Fig. 2D (star), E	Cellulose, LapV
	Strings	Fig. 2D right (arrow)	SYP, cellulose, LapV
	Clump	Fig. 2D (*)	SYP, LapV

Known and putative polysaccharides of the *V. fischeri* biofilm

The symbiosis polysaccharide, SYP

First identified in *V. fischeri* as critical for host-associated biofilm formation and colonization, Symbiosis Polysaccharide, or SYP, is the best-studied component of the *V. fischeri* biofilm (35, 36). SYP production depends on the 18-gene *syp* locus (26, 35, 45), which encodes products ranging from regulators to glycosyltransferases and export proteins (Fig. 3). Loci homologous to *syp* have been identified and subsequently shown to be important for biofilm formation in other *Vibrio* species, such as *Aliivibrio salmonicida*, *V. vulnificus*, and *V. parahaemolyticus* (35, 46–48). In *V. fischeri*, deletions of individual

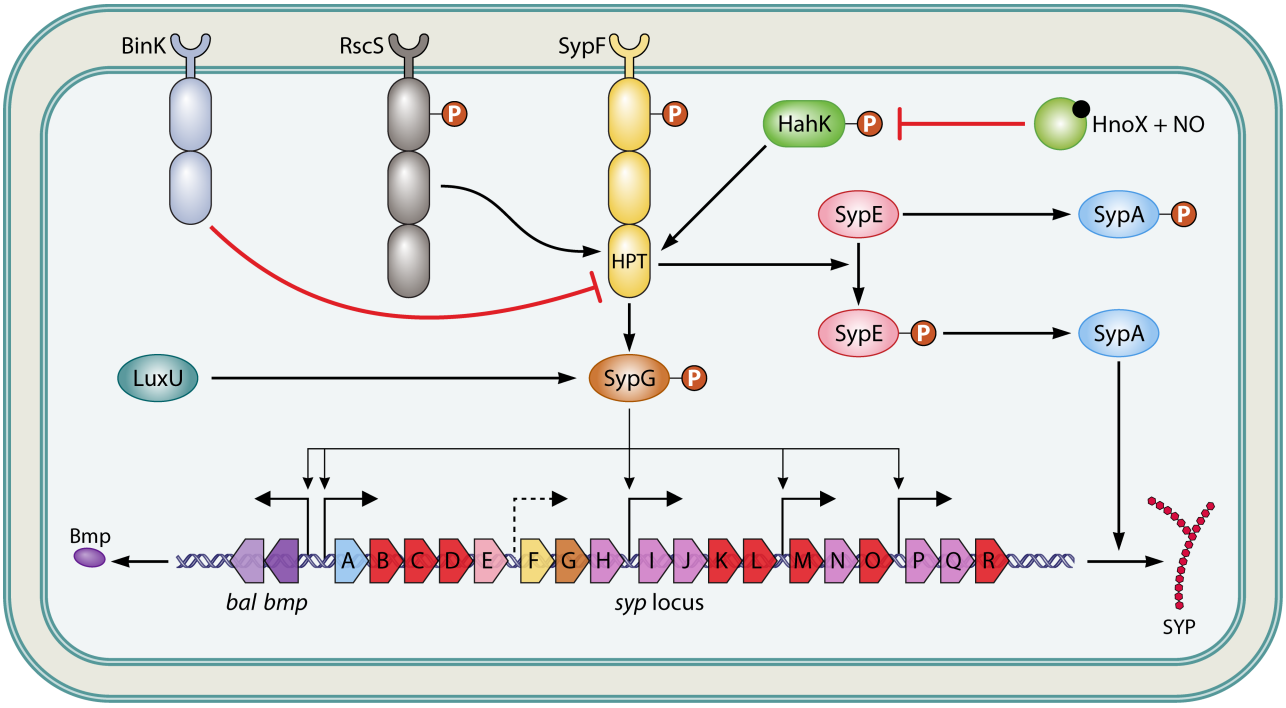

FIG 3 Model of SYP regulation. The current model for control of SYP production is shown. SYP is produced and exported by proteins encoded by the *syp* genes (bottom). The individual *syp* genes are color-coded to reflect the function of their respective protein product (light purple: glycosyltransferases, red: export proteins/polysaccharide modification), with the regulators *sypA*, *sypE*, *sypF*, and *sypG* colored to correspond to the protein shown in the model. The solid arrows within the locus represent known promoters. The dashed arrow depicts a putative promoter upstream of *sypF*. Transcription of the *syp* locus is activated by the response regulator SypG, whose activity is dependent on phosphorylation via SypF's HPT domain. SypF's HPT domain may be phosphorylated by SypF itself, RscS, or HahK, while BinK is predicted to remove phosphoryl groups. HahK can be inhibited by the NO-binding protein, HnoX. LuxU is another HPT that is predicted to activate SypG under specific conditions. In addition to inducing *syp*, SypG also promotes transcription of three sets of *bmp* and *bal* genes (A, B, and C); only the *bmpA-balA* operon, which is adjacent to the *syp* locus, is shown. SYP production is positively regulated at an unknown post-transcriptional level by SypA, whose activity is inhibited by phosphorylation. SypE inactivates and activates SypA by phosphorylating and dephosphorylating it, respectively.

syp genes caused severe squid colonization defects for a majority of the 18 mutants, underscoring the importance of SYP for the *V. fischeri –E. scolopes* symbiosis (26, 37, 38, 45, 49). Furthermore, strains engineered as SYP-overproducers exhibited enhanced symbiotic aggregation and colonization phenotypes, correlating with increased biofilm formation *in vitro* (e.g., wrinkled, cohesive colonies on agar plates and pellicles in static liquid media) (Fig. 2B and C) (35, 36, 39, 42). Subsequent studies found that SYP was responsible for biofilm stickiness or cohesion (50, 51), indicating that SYP allows for cell-cell interactions (Fig. 2A; Table 1).

In a hyper-biofilm forming background, most individual *syp* deletions resulted in a loss of wrinkled colony and pellicle formation *in vitro*, suggesting that these genes contribute to overall biofilm formation. Three exceptions were mutants defective for *sypB, sypI*, and the negative regulator *sypE* (see below), which were (i) no different from their parent in the pellicle assay, (ii) exhibited only slight decreases in wrinkled colony formation, and (iii) largely colonized to the same extent as the wild-type parent (26, 38, 39, 45). Together, these findings were foundational in demonstrating strong correlations between *in vitro* biofilm formation and the *in vivo* phenotypes of symbiotic aggregation and colonization, permitting the use of *in vitro* studies to identify symbiosis-relevant mechanisms.

Transcriptional regulation of the syp locus

V. fischeri tightly controls *syp* transcription. SypG, a σ^{54}-dependent response regulator, activates *syp* transcription by binding to *syp* enhancer sequences upstream of *sypA*, *sypI*, *sypM*, and *sypP* (35, 52). An additional non-SypG-dependent promoter likely exists upstream of *sypF* (49, 53). The activity of SypG is controlled by multiple two-component sensor kinases (Fig. 3). Specifically, SypG is activated by phosphorylation via SypF, a hybrid sensor kinase that, under *in vitro* conditions, appears to function primarily as a phosphatase (41, 54). Two other hybrid sensor kinases, RscS and HahK, indirectly activate SypG and thus *syp* transcription via SypF's histidine phosphotransferase (HPT) domain, presumably by donating phosphoryl groups (Fig. 3) (36, 41, 45, 49, 55). RscS is of particular note because its initial identification as a colonization determinant in a random mutant screen (56) fueled the discoveries of the importance of *syp*-dependent biofilm formation in the squid symbiosis and RscS's role both as a biofilm regulator (36) and as a specificity factor (57). Indeed, most early discoveries of *syp* regulation and function were made in the context of *rscS* overexpression strains because conditions under which the wild-type strain (ES114) formed biofilms *in vitro* were unknown. Together with the identification of the *syp* locus (35), this work on *rscS* provided the foundation for investigating symbiosis-relevant biofilm formation by *V. fischeri*.

In addition to the apparent function of SypF as a negative regulator, the hybrid sensor kinase BinK also negatively regulates *syp* transcription. BinK was initially discovered as an inhibitor of colonization through two large-scale gene discovery experiments: (i) an insertion-sequencing analysis, in which recovery of a *binK* transposon mutant following squid colonization was increased fourfold compared to the starting pool and (ii) an evolution experiment involving multiple rounds of passaging a non-colonizing *V. fischeri* fish isolate through squid that resulted in nine independent *binK* mutants with enhanced colonization (40, 42, 58). BinK was subsequently identified as critical for preventing the induction of *syp* in *in vitro* studies with *rscS* overexpression (42), in the context of the squid crypts (59), and in response to calcium (55). With respect to the latter, it was found that loss of BinK alone (without the need for *rscS* overexpression) permitted an otherwise wild-type strain to produce robust biofilms in response to seawater-relevant levels of calcium. Upon alteration of either of BinK's two predicted sites of phosphorylation, H362 and D794 within the histidine kinase and receiver domains, respectively, inhibition of biofilm formation was lost, indicating that these two sites are required for BinK to function (59). It has been suggested that BinK functions as a phosphatase to remove phosphoryl groups from SypF's HPT domain to inhibit *syp* transcription (Fig. 3) (42, 55, 59).

BinK and RscS appear to have opposing functions, with BinK inhibiting squid aggregate formation and biofilms *in vitro* and RscS promoting them. Loss of BinK overcame the requirement for RscS in symbiotic aggregation and biofilm formation (36, 59). However, RscS plays an as-yet undetermined role distinct from BinK, as competitive colonization experiments revealed a substantial defect for the Δ*rscS* Δ*binK* mutant relative to the single Δ*binK* mutant, suggesting that this factor controls other important symbiosis traits beyond *syp* (59).

The identification of BinK permitted the subsequent discovery of a missing player in the *syp* regulatory pathway, now known to be HahK (55). In turn, uncovering HahK as a regulator led to the understanding that HahK's biofilm-promoting activity is inhibited by HnoX, a nitric oxide (NO) sensing protein (55, 60); increasing amounts of NO prevented biofilm formation in an *hnoX*-dependent manner (Fig. 3) (61).

In addition to calcium and NO, the vitamin para-aminobenzoic acid (pABA) also influences *syp* regulation. pABA and calcium addition increased *syp* transcription and cohesive colony formation by ES114 (51). Both signals seem to be required for optimal *syp* induction, as the two signals individually did not promote colony cohesion. This phenotype was dependent on the presence of RscS and SypF's HPT domain (30). It remains unclear whether this effect results from a direct interaction of pABA and calcium with the sensory domains of RscS and/or SypF or if these histidine kinases sense downstream consequences of these signals (30, 51). Although pABA can appear to be an unusual signaling molecule, it has been shown to induce biofilm formation in one other model system (62). Whether pABA or a pABA derivative is present in the squid or the seawater remains unknown.

Another arm of *syp* transcriptional control seems to stem from the quorum sensing pathway, which has been heavily studied for its role in luminescence control in *V. fischeri* (63). LuxQ, one of the sensor kinases responsible for recognizing autoinducers produced by *V. fischeri*, positively impacts biofilm formation through the histidine phosphotransferase LuxU. Results to date suggest that it functions by facilitating activation of SypG, at least when *sypG* is overexpressed (Fig. 3) (64). This regulation appears to be reciprocal as SypG can also induce expression of the quorum regulator *qrr1* (65, 66). This apparent connection between quorum sensing and biofilm formation needs to be investigated further.

Other ways SYP production is modulated have been uncovered, adding to its already complex regulatory control. High levels of the second messenger cyclic diguanylate (c-di-GMP) following the deletion of genes for six phosphodiesterases (PDEs), enzymes that break down c-di-GMP, resulted in decreased *syp* transcription *in vivo* but not *in vitro* in two different media conditions (67). This distinction suggests that more factors affect *syp* regulation in squid that are not active in current laboratory conditions. In addition, cysteine, or more specifically CysK, which encodes O-acetylserine sulfhydrolase in the cysteine biosynthetic pathway, contributes positively to SYP-dependent biofilm formation and squid colonization (68, 69). However, the mechanism by which CysK affects SYP production—and whether it occurs at the level of *syp* transcription—remains unknown. Taken together, biofilm formation via *syp* activation involves a complex set of regulatory events, some of which cannot yet be replicated *in vitro*. The intense control may indicate that multiple signals are necessary for *V. fischeri* to appropriately produce *syp*-dependent aggregates at the right time and place and successfully depart from them to colonize its host.

Additional control of SYP production

SYP production is controlled post-transcriptionally by the proteins SypE and SypA (Fig. 3). SypE is a response regulator with serine kinase and phosphatase domains. Under *sypG* overexpression conditions, SypE functions as a strong negative regulator of biofilm formation, while in the context of *rscS* overexpression, it serves as a positive regulator (39, 49). It is possible that RscS via SypF, but not SypG, can facilitate phosphorylation of SypE, thereby shifting its activity to a biofilm-promoting state (Fig. 3) (41). SypE's

enzymatic activities target SypA, a STAS (sulfate transporter and anti-sigma antagonist) domain protein. In its inhibitory role, SypE phosphorylates SypA at amino acid S56, preventing biofilm formation and colonization. SypE can also activate SypA, and thus biofilm formation and colonization by dephosphorylating SypA at the same position (38, 39, 70). How SypA controls *syp*-dependent biofilms, and thus, squid colonization, is still unknown but it appears to function at a level below *syp* transcription.

Cellulose

Like many bacteria, including the pathogenic *V. parahaemolyticus* (71), *V. fischeri* produces cellulose polysaccharides as part of its extracellular matrix during biofilm formation *in vitro*. Studies in *Salmonella enterica* Typhimurium, *Escherichia coli*, and *Komagataeibacter xylinus* have revealed that a complex of proteins designated Bcs function to synthesize cellulose, a glucose polymer linked via 1,4-β glycosidic bonds that, in some cases, is modified to make phosphoethanolamine cellulose (72–74). Cellulose synthase, comprised of a BcsA and BcsB heterodimer, spans the inner membrane, while BcsC spans the outer membrane (75). BcsA binds to and is activated by c-di-GMP (76, 77).

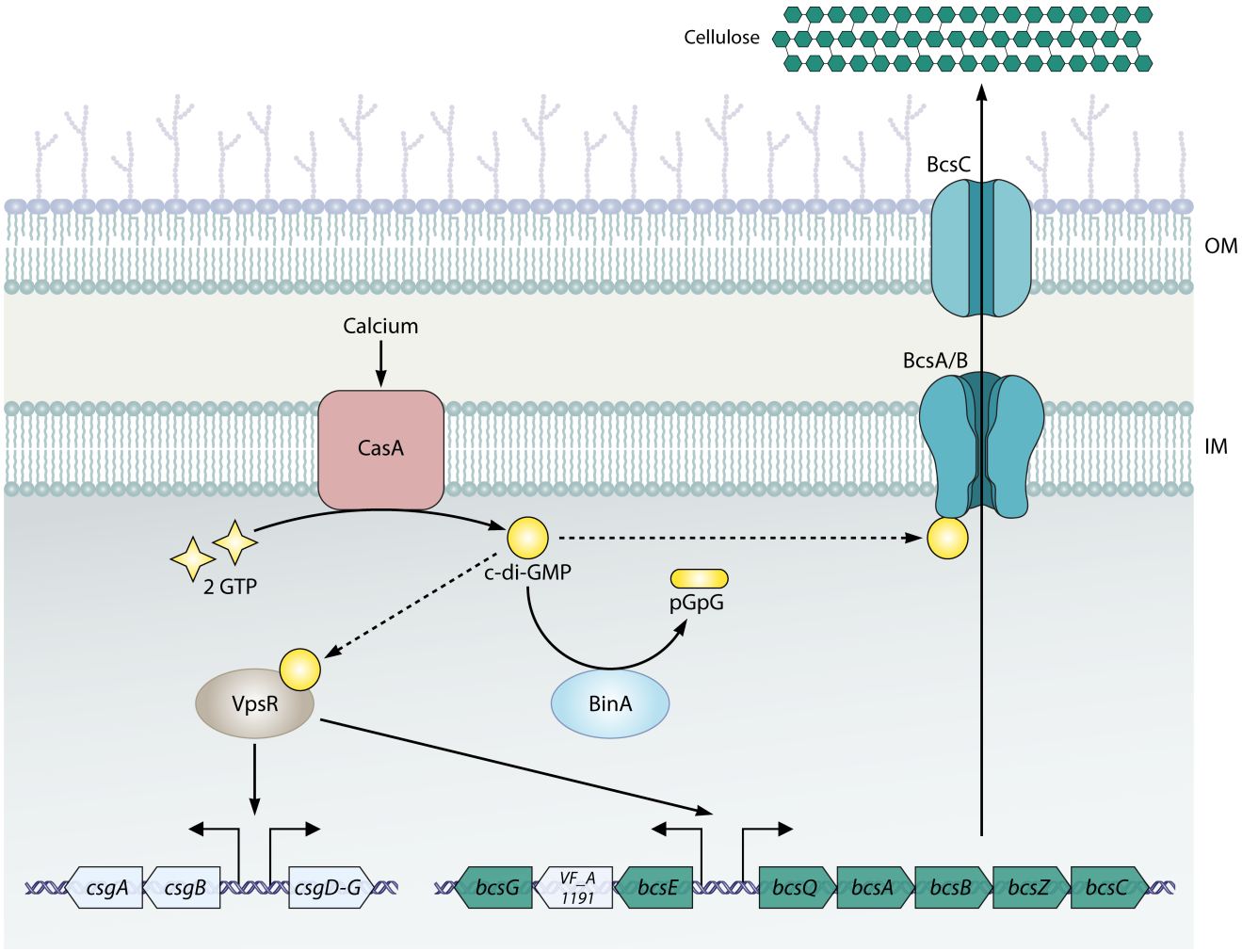

FIG 4 Cellulose polysaccharide regulation. Cellulose polysaccharide is synthesized and exported by the protein products encoded by the *bcs* locus, with BcsA-C likely responsible for the export. Both calcium and high c-di-GMP levels promote cellulose production. Calcium does so by activating CasA to increase c-di-GMP levels, which, in turn, induces *bcs* transcription via VpsR. The dashed arrow indicates that it is unknown if the c-di-GMP produced by CasA affects the BcsA function. VpsR also induces transcription of the *csg* genes, which encode curli. The PDE BinA negatively regulates cellulose production, although the level at which BinA controls cellulose production is unknown. BinA may degrade the c-di-GMP used by either VpsR or BcsA or both, as these proteins both likely bind c-di-GMP.

Other proteins contribute to modification and export; for example, BcsG is necessary for phosphoethanolamine modification (72). *V. fischeri* encodes cellulose synthesis proteins similar to those of *Salmonella* (Fig. 4) (45, 78), suggesting that function may be conserved between these organisms.

A limited number of studies have focused specifically on the role of cellulose in *V. fischeri* biofilm formation. Based on these studies, cellulose promotes attachment of *V. fischeri* to abiotic surfaces such as test tubes *in vitro* (Fig. 2; Table 1). For example, when the Δ*binK* mutant, a hyper-biofilm forming strain, was grown with shaking in the presence of calcium, biofilms accumulated in the "splash zone" above the liquid, denoted as "ring formation," in a cellulose-dependent manner (Fig. 2D and E) (55). Another study analyzing three-dimensional biofilm formation on a polypropylene membrane showed that a cellulose synthesis-deficient *V. fischeri* mutant formed fewer biofilms but each biofilm was larger (79). In contrast, a mutant with increased cellulose production formed a greater number of smaller biofilms that together had overall greater biomass than that of the parent strain. Cellulose also appears to contribute to biofilm architecture as disrupting cellulose production (a Δ*bcsA* mutation) resulted in a loss of visible colony bumpiness (51, 80). Together, these studies demonstrate that cellulose contributes to surface attachment and colony architecture (Fig. 2A; Table 1).

Cellulose production in *V. fischeri* is controlled by (i) an inducing signal (calcium), (ii) a positive transcriptional regulator (VpsR), and (iii) enzymes that increase or decrease cellular levels of c-di-GMP (CasA and BinA, respectively) (Fig. 4) (45, 55, 78, 81). The addition of increasing amounts of calcium to shaking liquid cultures caused a correlative increase in ring formation similar to the cellulose-dependent biofilms observed with the Δ*binK* mutant (55, 81). This effect occurs at the level of *bcs* transcription, as calcium addition caused an increase in transcription of *bcsQ*, the first gene in one of the two *bcs* operons in *V. fischeri* (Fig. 4) (81).

VpsR and Bcs were initially identified as required for biofilm phenotypes induced by overproduction of an active allele of the *syp* regulator SypF (45). VpsR shares homology with a transcription factor of the same name in *V. cholerae* where it upregulates the production of Vibrio polysaccharide, VPS (45, 82). In *V. fischeri*, VpsR controls *bcs* transcription and *bcs*-dependent phenotypes (Fig. 4). Specifically, the overexpression of *vpsR* from a plasmid caused a *bcsA*-dependent increase in biofilm production in static liquid culture (45). Correspondingly, loss of VpsR blocked calcium-induced *bcsQ* transcription (81). A recent study using an *rscS* overexpression strain showed that VpsR is responsible for the majority of *bcs* upregulation in a SypF- and SypG-independent manner (53), further indicating its importance in cellulose phenotypes and underscoring the potential of RscS to control other factors.

The calcium-induced increases in *bcsQ* transcription and biofilm ring formation are both reliant on the amount of available c-di-GMP, which induces biofilm formation when levels are high (83). CasA, a calcium-sensing transmembrane diguanylate cyclase (DGC), increases the levels of c-di-GMP in response to calcium to induce cellulose production (81). Without this CasA-mediated increase in c-di-GMP, VpsR can no longer upregulate *bcsQ* transcription in response to calcium (Fig. 4) (81). It is possible that CasA-produced c-di-GMP could also bind BcsA to post-transcriptionally facilitate cellulose synthesis based on what is known in other bacteria [e.g., (76)] but this remains to be determined. When overexpressed, 16 of the other 25 DGCs encoded by *V. fischeri* and two of its five dual-function enzymes also contribute to cellulose production as assessed by Congo red binding (Fig. 2F; Table 1) (84), highlighting the connection between c-di-GMP and cellulose, as has been seen in other systems [e.g., (85)].

BinA is a PDE that negatively regulates the *bcs* locus, and in turn, cellulose-dependent biofilms (Fig. 4) (78). Deletion of *binA* led to increased (i) static biofilm formation, (ii) binding of Congo red and Calcofluor, (iii) colony architecture on solid agar, and (iv) levels of c-di-GMP (78). Disruption of cellulose production eliminated the increase in biofilm formation. It is unknown how BinA mechanistically inhibits cellulose synthesis but we predict that BinA decreases the available levels of c-di-GMP needed for BcsA and/or

VpsR function. The overexpression of 11 of the other 13 *V. fischeri*-encoded PDEs and the remaining three dual-function proteins diminished Congo red binding, and thus cellulose production (84). As with the DGCs, these results demonstrate the link between c-di-GMP, cellulose production, and biofilm phenotypes.

Connections between SYP and cellulose have been observed in multiple *in vitro* studies. One study revealed that *sypG* overexpression induces *binA* transcription, suggesting that activation of the *syp* locus may result in diminished cellulose production (78). In addition, a recent publication showed that mutating *bcsA* resulted in increased colony "stickiness" on solid agar, a phenotype that was dependent on SYP production (51). Finally, a strain that produced high levels of c-di-GMP exhibited increased *bcs* transcription and reduced *syp* transcription *in vivo* (67). Despite these changes, the strain formed more aggregates on the surface of the squid light organ that were, on average, larger than those produced by the wild type or a strain with low levels of c-di-GMP. These aggregates were dependent on SYP, but not cellulose polysaccharide, further demonstrating the importance of SYP in the squid. However, despite the larger SYP-dependent aggregates, this strain was a poor colonizer, potentially because it also exhibited decreased motility, a trait that is required for colonization (18, 67). While there seems to be underlying connections between SYP and cellulose polysaccharides, neither the specific regulatory connections nor how the production of one impacts that of the other is understood, and thus represents an area ripe for more research.

Other polysaccharides

The *V. fischeri* genome contains other loci encoding glycosyltransferases that could contribute to biofilm formation. For example, one less studied polysaccharide is produced by enzymes encoded by the *VF_0157–180* locus. These genes are upregulated by the quorum sensing protein LitR and negatively regulated by HbtR, a transcription factor with homology to the *V. cholerae* virulence factor TcpP (86). This locus is responsible for the majority of the exopolysaccharide produced by *V. fischeri* grown in minimal-salts medium supplemented with casamino acids and *N*-acetylneuraminic acid, as measured by alcian blue staining. Despite this, its function in the *V. fischeri* biofilm remains elusive, potentially due to the diminished transcription of these genes in the *rscS* overexpression and calcium-induced Δ*binK* mutant biofilm models (53). This polysaccharide locus may be important in an as-yet-undiscovered biofilm condition.

Recently, VF_0133, a putative glycosyltransferase, was implicated in inhibiting biofilms that are independent of *syp* function (87). Passaging *V. fischeri* in bioluminescence-inducing conditions yielded an isolate with increased *in vitro* glass attachment, decreased motility, and impaired squid colonization. These phenotypes were attributed to a point mutation in *VF_0133,* as they could be largely or fully complemented by a wild-type copy of *VF_0133*. Subsequent analysis demonstrated that VF_0133 is necessary for the addition of O-antigen to lipopolysaccharide (LPS) (87). These results, together with data that demonstrate a role for cellulose in glass attachment [e.g., (Fig. 2E) (55, 78)], implicate LPS in the inhibition of biofilms that are, potentially, cellulose-dependent (87).

Known or potential contributions of proteins to *V. fischeri* biofilms

In addition to the polysaccharide components listed above, *V. fischeri* encodes proteins and protein complexes that are known to contribute to biofilm formation (LapV, Bmp, and flagella) and others that may contribute (pili).

Analogous to large adhesive proteins found in *Pseudomonas* (LapA, CdrA) (88, 89) and *V. cholerae* (FrhA, CraA) (90), *V. fischeri* encodes a large adhesive protein, LapV, that promotes biofilm formation (Fig. 2A). LapV contains multiple VCBS/cadherin-like repeats along with a Type I secretion signal sequence (91). VCBS repeats, thought to be involved in adhesion, are found in large proteins from *Vibrio*, *Colwellia*, *Bradyrhyzobium*, and *Shewanella* genera (91). Disruption of the *lapV* gene in *V. fischeri* resulted in loss of biofilm formation in shaking liquid conditions and diminished architecture in a pellicle assay (91). Due to these phenotypes, as well as what is known from analogous systems [e.g.,

(92)], we hypothesize that LapV binds to SYP, cellulose, and/or other polysaccharides in the *V. fischeri* biofilm matrix (Fig. 2A; Table 1). In addition to LapV, *V. fischeri* encodes a second potential adhesin, LapI. The *lapI* gene is positioned within a locus containing genes known to control the surface localization of LapV. However, a *lapI* deletion mutant had no detectable phenotype.

The *bmp* genes encode a group of proteins that contribute to the *V. fischeri* biofilm (50). Initially discovered due to the location of *bmpA* adjacent to the *syp* locus, the *bmpA*, *bmpB*, and *bmpC* operons were shown to be part of the SypG regulon (Fig. 3) (50). The three *bmp* genes encode proteins that appear to contribute redundantly to biofilm architecture (Fig. 2A; Table 1). A triple mutant defective for the three genes failed to produce wrinkled colonies but these colonies retained cohesiveness, suggesting that SYP was still produced and functional. Indeed, when a *sypL* mutation was introduced into the triple mutant, the resulting strain exhibited the null phenotype of *syp* mutants, which produce non-wrinkled, non-cohesive colonies. BmpA is secreted into the extracellular space, where presumably it functions within the biofilm matrix; it is sufficiently diffusible on agar plates that a producer strain can promote wrinkling of an adjacent *bmp* mutant colony. Two conclusions that can be drawn from these data are (i) Bmp is not required for biofilm formation but appears to contribute to biofilm maturation and (ii) Bmp and SYP may work together in wild-type *V. fischeri* biofilms (Fig. 2A and 3). The *bmp* genes are conserved in other members of the *Vibrionaceae* and marine bacteria, but because the protein domains have unknown functions, not much else is known about these matrix components.

In a variety of bacteria, including *Vibrio* species, flagella and pili are connected to biofilm formation and/or development [e.g., (93, 94)]. In *V. fischeri*, the flagellar transcription factor, FlrC, contributes to glass attachment and pellicle formation under static growth conditions (49); however, these results could be due to the requirement for motility for cells to reach the biofilm location rather than a specific requirement for biofilm development itself (49). A non-flagellated mutant, *flgF*, was also significantly impaired for biofilm formation when assessed in two ways. The first was by scanning electron micrograph (SEM) of coverslips incubated with the *flgF* mutant or its parent strain under static growth conditions to assess binding. The second method utilized 96-well plates for static biofilm growth, which was quantified by crystal violet staining (95). Because those experiments both assessed surface attachment, a phenotype that in *V. fischeri* depends heavily on cellulose [e.g., (Fig. 2E) (55, 96)], these results suggest that flagella could be a key aspect of cellulose-dependent biofilm development. When evaluated for colonization, the *flgF* mutant was unable to colonize the squid (95), as has been seen for other flagellar mutants [reviewed in (18)].

Genes for curli (*csg*), an amyloid matrix adhesin produced by bacteria such as *E. coli* (97), are present within the *V. fischeri* genome (98). The *csg* genes are highly upregulated in two of the relevant *V. fischeri* biofilm models, *rscS* overexpression and calcium-induced Δ*binK* mutant biofilms, by the transcription factor VpsR (53), which also promotes cellulose production at the transcriptional level as discussed above (Fig. 4) (45, 81). One of the genes in the *csg* locus, *csgD* (*vpsT*), is homologous to the similarly named genes in *Salmonella enterica* and *V. cholerae*. In these organisms, the products downstream of CsgD and VpsT regulatory control, curli and VPS, respectively, make substantial contributions to biofilm formation (99, 100). Although the regulatory similarity and the increase in transcription in the above-mentioned contexts suggest that curli in *V. fischeri* would function in biofilm formation, no such phenotype has been discovered. It seems likely that appropriate conditions for curli function have yet to be identified and/or that their function is masked by other pili. In summary, the addition of *csg* to VpsR's regulon, which previously included only *bcs*, expands our understanding of this transcription factor.

Other pili have also been implicated in enhancing biofilm formation by promoting adherence of bacteria to a surface or aiding in twitching motility to reach those surfaces (31, 101). While *V. fischeri* carries many putative pili genes (98, 102), only two, *pilT*

and *pilU*, have been analyzed for their contribution to biofilm formation, and only in ETJB1H, a *V. fischeri* strain isolated from the squid *Euprymna tasmanica*. Assaying mutants for these two genes resulted in varying phenotypes: no difference from the wild-type strain when grown statically on coverslips and analyzed by SEM but decreased biofilm formation when analyzed in a dynamic flow chamber by confocal microscopy (95). These two mutants were also deficient in colonization compared to the parent strain. These findings highlight potential differences in the regulation and importance of these pili genes under static vs dynamic motion for biofilm development and thus, colonization. One other pili mutant, *pilA2* in ES114, had a competitive disadvantage in colonization (103). Although no biofilm studies were performed, these data suggest that *pilA2* plays some role in early colonization of the squid.

Dispersal

During *V. fischeri* colonization of the squid host, the symbiont must disperse from the aggregate just outside the pores of the light organ and migrate into the crypt spaces (Fig. 1C) (16, 17). We understand very little about how *V. fischeri* dispersal is regulated. Here, we explore signals that appear to contribute to dispersal in the context of symbiosis and describe mechanisms uncovered in laboratory culture that may be responsible.

Two potential signals for dispersal, NO and low levels of c-di-GMP, were briefly described earlier in the context of biofilm formation. NO is produced by the squid in areas where *V. fischeri* cells aggregate (104). NO can be detrimental to other bacterial species, but *V. fischeri* can protect itself from effects caused by NO through the flavohemoglobin Hmp (105). This protection allows *V. fischeri* to form biofilms on the surface of the light organ even in the presence of NO. Adding NO inhibitors, such as rutin hydrate, allowed *V. fischeri* and a non-symbiont, *V. parahaemolyticus*, to form larger aggregates on the light organ (104). These data suggest that NO prevents symbiotic biofilm formation and/or promotes dispersal. This effect likely occurs, at least in part, via the NO-binding protein HnoX, as a Δ*hnoX* mutant exhibited increased colonization (60) and symbiotic aggregation (61). One possible scenario is that *V. fischeri* cells newly arriving in the sheltered zones on the light organ surface experience low levels of NO, permitting HahK to contribute to the activation of *syp* transcription, polysaccharide production, and symbiotic biofilm formation. As the levels of NO increase, more will bind HnoX, which may negatively regulate HahK to inhibit *syp* transcription (61), increasing the likelihood of dispersal. In any case, NO's influence on *V. fischeri* aggregation is clearly nuanced, and more research is needed to determine if NO shifts *V. fischeri* aggregates to a planktonic lifestyle to initiate dispersal.

The second potential signal is the intracellular signaling molecule c-di-GMP. Low levels of c-di-GMP diminish biofilm formation through the previously discussed *lap* system (Fig. 5). This pathway was first uncovered as a dispersal mechanism in *Pseudomonas fluorescens* (106–108), where surface localization of the analogous adhesin, LapA, is controlled by the periplasmic protease LapG, which cleaves the adhesin, promoting dispersal (106, 107). LapG can also associate with and be sequestered by the inner membrane protein LapD, preventing LapG's protease activity. The local levels of c-di-GMP control the activity of LapD: when levels are high, c-di-GMP-bound LapD binds and sequesters LapG, preventing protease activity; when the levels are low, dispersal is facilitated (107).

In *V. fischeri*, among the 50 proteins predicted to control levels of c-di-GMP, a single one, PdeV, contributes to *lap* system control. When PdeV, a predicted PDE, is expressed at high levels from a multicopy plasmid, biofilm formation is abrogated (91). Moreover, when *pdeV* is deleted from the genome, biofilms form readily in shaking conditions in a LapD- and LapV-dependent manner. Similar results were seen for the deletion of *lapG*: biofilms formed and were dependent on *lapV* but independent of *lapD*. Furthermore, the robust biofilm formation of a Δ*binK* mutant was diminished by the deletion of *lapD*. The diminished biofilm phenotype was reversed by either complementation with *lapD*

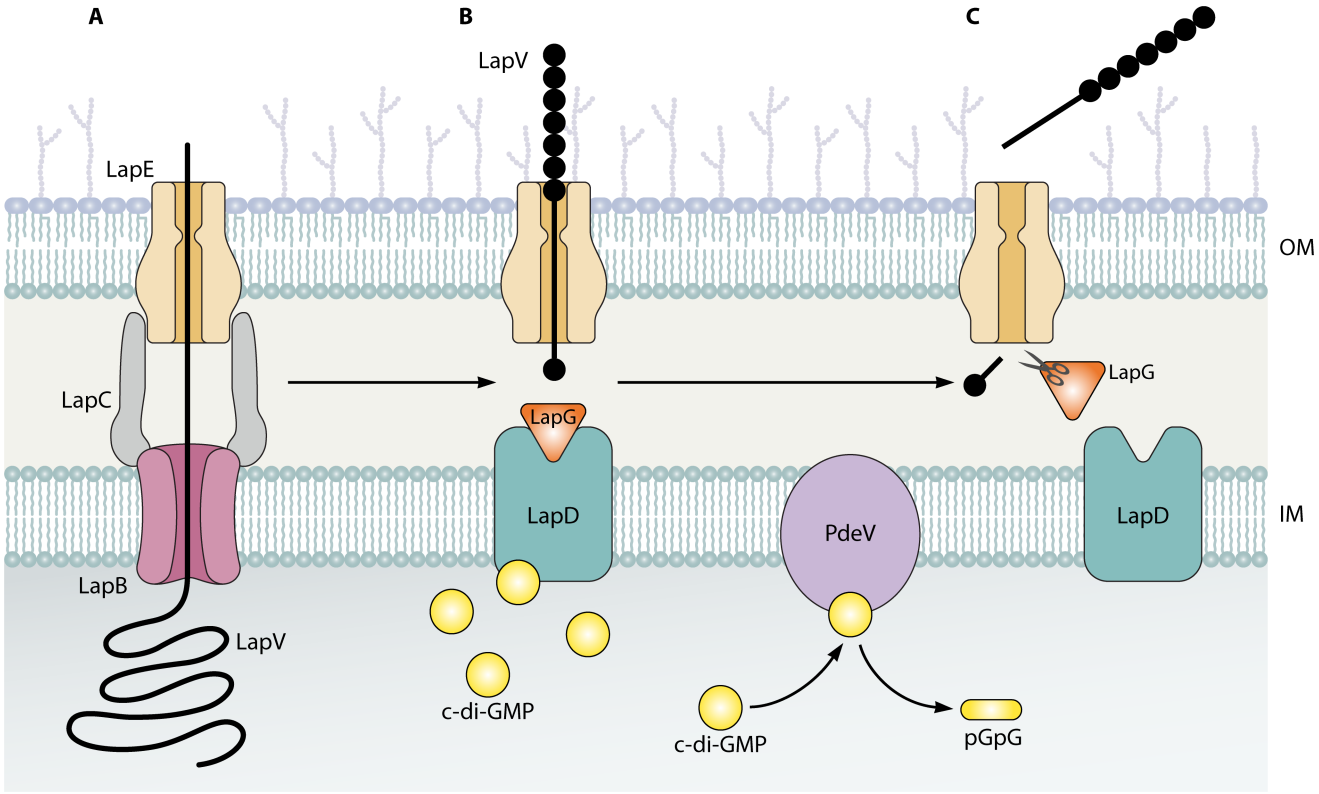

FIG 5 Role of LapV in *V. fischeri* biofilm formation and dispersal. The current model for the role of the LapV adhesin in *V. fischeri* biofilm formation and dispersal is depicted. (A) The LapV adhesin is exported to the cell surface dependent on the activities of LapB, LapC, and LapE, the latter of which may serve to anchor it in the outer membrane. (B) The presence of LapV is controlled by the local levels of c-di-GMP. When there are high levels of c-di-GMP, c-di-GMP binds to the inner membrane protein LapD, permitting it to negatively control LapG. This promotes biofilm formation due to the accumulation of LapV on the surface of *V. fischeri*. (C) When the levels of c-di-GMP are lowered by the PDE PdeV, LapG is active to cleave LapV from the cell surface, resulting in biofilm dispersal.

or deletion of both *lapD* and *lapG* (91). These data suggest that the *V. fischeri* pathway functions like that of *Pseudomonas*, with c-di-GMP degradation, in this case via PdeV, disrupting the activity of LapD, releasing LapG, which, in turn, cleaves LapV to permit dispersal (Fig. 5).

The *lap* pathway was also shown to influence symbiotic colonization. Because mutant *lapD* strains have reduced biofilm formation, they were tested for impairment in squid colonization. Δ*lapD* mutants colonized one-third of the animals compared to the wild-type strain. A subset of animals displayed an unusual phenotype with the *lapD* mutant colonizing only some of the light organ crypts (91). These data indicate that the *lap* pathway is relevant for symbiotic colonization but may only represent one of several pathways that control dispersal.

The transition from biofilm to dispersal necessitates that the bacteria become flagellated, as subsequent colonization relies on chemotaxis and flagellar motility. With respect to chemotaxis, movement from the symbiotic biofilm into the light organ is signaled by squid-produced chitobiose, a chitin derivative that serves as a chemoattractant for *V. fischeri* (109, 110). In contrast, essentially nothing is known about how *V. fischeri* switches from the sessile form to a motile, planktonic form. It has been documented that biofilm overproducers often have mild motility defects, suggesting an inverse correlation between these two states (53, 91). In addition, the quorum sensing regulator Qrr1 promotes bacterial motility and is activated by SypK and SypG, which, in turn, are inhibited by BinK (65, 66). Potentially, activation of the *syp* locus and biofilm formation could subsequently signal dispersal through Qrr1-mediated control of motility. However,

other factors must be necessary as a *qrr1* mutant retains the ability to colonize squid (111). Clearly, additional work is needed to probe this key developmental switch.

Insights from other *V. fischeri* isolates

Most of the work described above was performed using strain ES114 and its hyper-biofilm derivatives. However, there are many other isolates whose study has the potential to enhance our understanding of *V. fischeri* biofilm formation and aggregation. For example, we learned that *rscS* is a specificity factor through the study of strain MJ11, a fish symbiont that lacks *rscS* and cannot colonize squid. Introducing the ES114 *rscS* allele into MJ11 allowed this isolate to colonize squid (57, 112).

The *V. fischeri* isolates that can colonize the squid light organ have been grouped into two categories: sharing and dominant strains (28). Sharing strains are so named because in squid co-colonization experiments, they co-inhabit the crypts with other strains. Dominant strains, on the other hand, fail to share, instead dominating the crypt spaces as clonal populations, likely for a variety of reasons [see, e.g., (19, 28, 113)]. For some of the dominant strains, colonization dominance may result from their ability to rapidly form and disperse from larger *syp*-dependent aggregates on the light organ (29, 114). Correspondingly, the *in vitro* static biofilm phenotypes of the dominant strain, MB13B2, and the sharing strain, ES114, correlate in that MB13B2 more rapidly formed robustly cohesive pellicles (29).

Both sharing and dominant strains require *syp* for efficient squid colonization. Genome analysis of various isolates revealed that one of the major determinants of sharing vs dominance was the functionality of individual components in the upstream regulatory network for *syp* transcription and, specifically, the *rscS* allele (112). Sharing strains all have an intact ES114-like *rscS* allele that is required for squid colonization. Dominant strains MB11B1, ES213, and KB2B1 do not need their *rscS* allele due to a frameshift mutation in the negative regulator *sypE* (112). This frameshift mutation bypassed the requirement for *rscS* for both biofilm formation and colonization; repairing the frameshift mutation resulted in poorer biofilm formation. However, this mutation alone was not sufficient to convert sharing strains into dominant ones, indicating that there are other mutations/genes that are needed to confer this attribute on sharing strains.

The *V. fischeri* isolate SR5 presents a unique case, in that SR5 does not have an *rscS* gene and there is no frameshift mutation in its *sypE* allele to counteract the loss of *rscS* (112, 115). Despite this, SR5 formed robust biofilms and could colonize squid (57, 112). Although not much is known about how SR5 bypasses the requirement for RscS, it still needs an intact *syp* locus for efficient colonization (112), highlighting the importance of SYP for symbiosis.

CONCLUDING REMARKS

Of all the components discussed, the roles of SYP, cellulose polysaccharide, and LapV are the most well understood (Fig. 2A) but more work is still needed to fully understand the nature of *V. fischeri* biofilms. Broadly, there are gaps in knowledge regarding the composition and structure of SYP, the role of SypA, the specific contributions of the less-studied components (e.g., Bmp, curli, and the polysaccharide produced by *VF_0157–180*), dispersal, and the connections between biofilm regulation and other processes such as quorum sensing, motility, and competition. Despite these unknowns, it is clear that the mechanisms underlying the control over symbiotic biofilm formation and dispersal are complex. Although the focus here was on the symbiont, the squid host will also transcriptionally and biochemically change upon exposure to the aggregates, requiring *V. fischeri* to adapt to a changing environment in a way that is distinct from the more controlled *in vitro* experiments. Thus, incorporating squid-centric approaches permits us to garner a more wholistic view of the biofilm- and dispersal-promoting interactions (109). This model has the added advantage that it avoids the confounding effects of tissue destruction and immune reactions that frequently occur in pathogenesis

models that may complicate interpretations. As a result, the *V. fischeri*–squid model system may also permit insight into other biofilm-forming pathogenic members of the *Vibrionaceae*, making it an excellent tool for studying host-associated biofilms.

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