

The Putative Hybrid Sensor Kinase SypF Coordinates Biofilm Formation in *Vibrio fischeri* by Acting Upstream of Two Response Regulators, SypG and VpsR[∇]

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Received 7 February 2008/Accepted 5 May 2008

Colonization of the Hawaiian squid *Euprymna scolopes* by the marine bacterium *Vibrio fischeri* requires the symbiosis polysaccharide (*syp*) gene cluster, which contributes to symbiotic initiation by promoting biofilm formation on the surface of the symbiotic organ. We previously described roles for the *syp*-encoded response regulator SypG and an unlinked gene encoding the sensor kinase RscS in controlling *syp* transcription and inducing *syp*-dependent cell-cell aggregation phenotypes. Here, we report the involvement of an additional *syp*-encoded regulator, the putative sensor kinase SypF, in promoting biofilm formation. Through the isolation of an increased activity allele, *sypF1*, we determined that SypF can function to induce *syp* transcription as well as a variety of biofilm phenotypes, including wrinkled colony formation, adherence to glass, and pellicle formation. SypF1-mediated transcription of the *syp* cluster was entirely dependent on SypG. However, the biofilm phenotypes were reduced, not eliminated, in the *sypG* mutant. These phenotypes were also reduced in a mutant deleted for *sypE*, another *syp*-encoded response regulator. However, SypF1 still induced phenotypes in a *sypG sypE* double mutant, suggesting that SypF1 might activate another regulator(s). Our subsequent work revealed that the residual SypF1-induced biofilm formation depended on VpsR, a putative response regulator, and cellulose biosynthesis. These data support a model in which a network of regulators and at least two polysaccharide loci contribute to biofilm formation in *V. fischeri*.

In nature, the preferred lifestyle of many bacterial cells is growth within a biofilm, a community of microbes attached to a surface and/or to each other and embedded in a matrix consisting primarily of secreted polysaccharides. Biofilm cells exhibit different properties than individual, planktonic cells, such as increased resistance to antimicrobial agents, altered gene expression, and reduced metabolic rates (reviewed in references 11, 13, 14a, and 40). Because of these properties, and the fact that many human infections occur in the context of a biofilm, a critical area of research is in understanding how biofilms form, persist, and disperse.

To date, a number of organisms have been intensively studied for their biofilm-forming properties, including *Pseudomonas aeruginosa* (17, 25, 36, 46), *Staphylococcus* spp. (1, 3, 30, 48), and *Vibrio cholerae* (23, 42, 45, 50, 51). In these model systems, it has become apparent that numerous traits, such as motility and polysaccharide production, contribute to optimal biofilm formation. It is further evident that the regulatory control over these processes can be quite complex. For example, *V. cholerae*, which forms biofilms in the natural environment and during intestinal colonization of individuals with cholera (14, 41), uses multiple regulators to control biofilm formation. One of these is VpsR, a putative two-component response regulator protein that alters the transcriptome to produce a biofilm-competent state under specific environmental conditions (5, 22, 49). Two important targets of VpsR are

the *vpsI* and *vpsII* (*vibrio* polysaccharide) loci, which produce VPS, the secreted polysaccharide that makes up the bulk of the *V. cholerae* biofilm matrix (49). Additional regulators, such as VpsT, a putative response regulator, HapR, a quorum-sensing regulator, and proteins that modulate the levels of the second messenger cyclic diguanylate (c-di-GMP), also control this locus (5, 9, 18, 54); thus, a complex network of regulation controlling biofilm formation exists.

While not traditionally considered an important model for biofilm formation, *Vibrio fischeri* contains a novel locus recently shown to be involved in polysaccharide production and biofilm formation. *V. fischeri* requires this 18-gene cluster, termed *syp* for symbiosis polysaccharide, to colonize its symbiotic host, the squid *Euprymna scolopes* (Fig. 1A) (53). In this highly specific model of symbiosis, *E. scolopes* acquires its bacterial symbiont soon after hatching (reviewed in references 29, 37, and 43). *V. fischeri* cells from seawater form a biofilm-like aggregate on the surface of the symbiotic organ before entering pores and migrating to the sites of colonization. The *syp* cluster and an unlinked regulator, RscS, appear to be critical for the initial aggregation outside the pores; disruption of *rscS* or a representative *syp* gene resulted in a loss of aggregate formation and a defect in colonization (44, 52, 53). Furthermore, induction of the *syp* cluster by multicopy expression of RscS resulted in substantially increased aggregation on the surface of the symbiotic organ; it also caused, in laboratory culture, a variety of cell-cell aggregation phenotypes, including wrinkled colony and pellicle formation (52). These data revealed a link between symbiotic aggregation and biofilm formation in culture and suggested that symbiotic aggregation represents a type of natural biofilm formation that is crucial for *V. fischeri* to colonize *E. scolopes*.

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[∇] Published ahead of print on 9 May 2008.

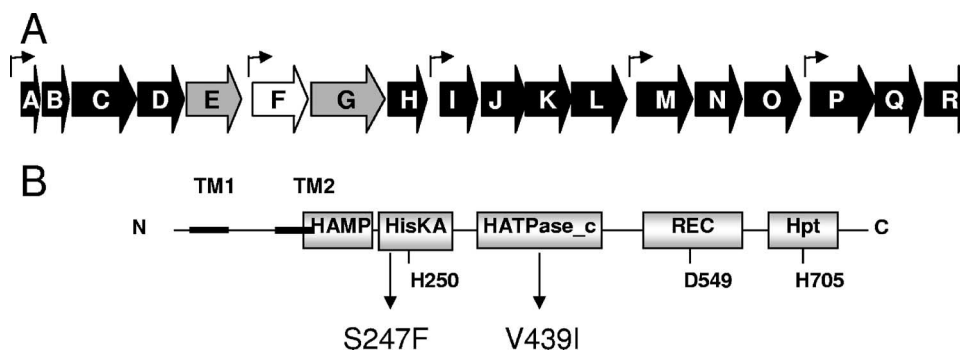


FIG. 1. The *syp* cluster and the SypF protein. (A) The *syp* cluster contains 18 genes (*sypA* to *-R*) that encode putative polysaccharide synthesis and transport proteins as well as four putative regulatory proteins, three of which are two-component regulators. The genes that are the subject of the manuscript are shown with gray (*sypE* and *sypG*) and white (*sypF*) arrows, while the remaining genes are shown with black arrows. The small bent arrows above the genes indicate known and putative promoters. (B) Domain structure of the hybrid sensor kinase SypF. SypF contains conserved histidine (H250, H705) and aspartate (D549) residues typical of hybrid sensor kinases. The putative SypF1 protein contains two substitutions, S247F in the HisKA domain and V439I in the H_ATPase domain, relative to that encoded within the annotated genome.

Control of *syp* expression appears complex. The unlinked regulator *rscS* encodes the sensor kinase portion of a two-component regulatory system. Two-component systems are widely used by bacteria to respond to their environment (reviewed in references 4, 7, and 20). A signal is sensed by the sensor kinase, resulting in autophosphorylation; the phosphoryl group is then passed to a response regulator. The activated response regulator then performs some function, such as inducing transcription, to elicit a cellular response. Indeed, we have found that RscS works upstream of SypG, a *syp*-encoded σ^{54} -dependent response regulator, to induce *syp* transcription and biofilm formation (21).

Located immediately upstream of *sypG* are genes for two other putative two-component regulators, *sypE* and *sypF* (Fig. 1A). The *sypE* gene encodes a putative response regulator that lacks a DNA-binding domain, while *sypF* encodes a putative sensor kinase. Our data to date suggest that SypE affects phenotypes induced by RscS and SypG; however, it appears to affect these phenotypes either positively or negatively, depending on which regulator is overexpressed, through an as-yet-unknown mechanism (21). A role for SypF has not yet been investigated. SypF appears to be a hybrid sensor kinase, with two putative transmembrane regions with a 120-amino-acid chain between them, a HAMP sensory domain, and putative domains typically involved in a phosphorelay (HisKA, HATPase_c, REC, and Hpt) (2, 35) (Fig. 1B).

Due to the complexity of the putative SypF protein and the intriguing location of its gene between two regulators required for biofilm formation, we sought a deeper understanding of its function. As part of this work we identified an increased activity allele, termed *sypF1*, that strongly induced biofilm-associated phenotypes. We found that these phenotypes depended in part on the *syp* locus and in part on another putative polysaccharide regulator, *vpsR*. These experiments thus reveal another layer in the complex control exerted by *V. fischeri* over polysaccharide production and biofilm formation.

MATERIALS AND METHODS

Media. *V. fischeri* strains were grown in SWT (53), LBS (15, 38), or HEPES minimal medium (HMM) (34) containing 0.3% Casamino Acids and 0.2% glucose (53). *Escherichia coli* strains were grown in LB (10) or brain heart infusion

medium (Difco, Detroit, MI). The following antibiotics were added as needed, at the final concentrations indicated: ampicillin (Ap), 100 $\mu\text{g ml}^{-1}$; chloramphenicol (Cm), 25 $\mu\text{g ml}^{-1}$ for *E. coli* and 2.5 $\mu\text{g ml}^{-1}$ for *V. fischeri*; erythromycin (Em), 150 $\mu\text{g ml}^{-1}$ for *E. coli* and 5 $\mu\text{g ml}^{-1}$ for *V. fischeri*; kanamycin (Kn), 50 $\mu\text{g ml}^{-1}$ for *E. coli* and 100 $\mu\text{g ml}^{-1}$ for *V. fischeri*; tetracycline (Tc), 15 $\mu\text{g ml}^{-1}$ for *E. coli* and 5 $\mu\text{g ml}^{-1}$ [in LBS] or 30 $\mu\text{g ml}^{-1}$ [in HMM] for *V. fischeri*. For solid media, agar was added to a final concentration of 1.5%.

Strain and plasmid construction. Wild-type *V. fischeri* ES114 (6) was used as the parent strain for these studies. Strains derived from ES114 and plasmids used in this study are described in Tables 1 and 2, respectively. *E. coli* strains TAM1 λ *pir* (Active Motif, Carlsbad, CA), TOP10 F' (Invitrogen, Carlsbad, CA), and DH5 α (47) were used for cloning, and *E. coli* CC118 (19) containing pEVS104 (39) was used to carry out triparental matings as described previously (44).

To overexpress *sypF*, we amplified the *sypF* gene using primers sypFexpF (AAGCGGGACACCACCC) and 1026RTR (GAGGATAGCCAGAGAAGTGG), cloned the fragment into PCR cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and then subcloned it into the mobilizable pKV69 (44) to obtain

TABLE 1. *V. fischeri* strains used in this study

Strain	Genotype	Reference
ES114	Wild-type isolate from <i>E. scolopes</i>	6
KV1715	<i>vpsR</i> ::pEAH11	22
KV1787	Δ <i>sypG</i>	22
KV1838	<i>sypN</i> ::pTMB54	53
KV2283	<i>sypL</i> ::pTMO89	This study
KV2481	<i>sypB</i> ::pESY33	This study
KV3001	Wild-type attTn7::P _{<i>sypA</i>} <i>lacZ</i>	21
KV3030	<i>sypB</i> ::pESY33 attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3031	<i>sypN</i> ::pTMB54 attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3232	Δ <i>sypG</i> attTn7::P _{<i>sypA</i>} <i>lacZ</i>	21
KV3291	Δ <i>sypG</i> <i>vpsR</i> ::pEAH11	This study
KV3299	Δ <i>sypE</i>	21
KV3378	Δ <i>rscS</i>	Gesvai and Visick ^a
KV3513	<i>vpsR</i> ::pEAH11 attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3514	<i>sypL</i> ::pTMO89 attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3586	Δ <i>sypE</i> <i>vpsR</i> ::pEAH11	This study
KV3619	Δ <i>sypG</i> <i>vpsR</i> ::pEAH11 attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3620	Δ <i>sypE</i> attTn7::P _{<i>sypA</i>} <i>lacZ</i>	21
KV3621	Δ <i>rscS</i> attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3769	<i>VFA0884</i> ::pCLD28	This study
KV3965	Δ <i>sypG</i> Δ <i>sypE</i>	This study
KV3968	Δ <i>sypG</i> Δ <i>sypE</i> attTn7::P _{<i>sypA</i>} <i>lacZ</i>	This study
KV3970	<i>VF0352</i> ::pCLD49	This study
KV4146	<i>VF0349</i> ::pCLD57	This study

^a K. Gesvain and K. L. Visick, submitted for publication.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pCLD6	Mini-Tn7 delivery plasmid; mob; Kn ^r Cm ^r attTn7::P _{sypA} -lacZ	21
pCLD19	pESY20 + 2 kb upstream and downstream of <i>sypE</i>	21
pCLD28	pESY20 + 5',3'-truncated <i>VFA0884</i>	This study
pCLD29	<i>sypF1</i> subcloned into pKV69; Cm ^r Tc ^r	This study
pCLD42	<i>vpsR</i> subcloned into pKV69; Cm ^r Tc ^r	This study
pCLD49	pESY20 + 5',3'-truncated <i>VF0352</i>	This study
pCLD54	Wild-type <i>sypF</i> subcloned into pKV69; Cm ^r Tc ^r	This study
pCLD57	pESY20 + 5',3'-truncated <i>VF0349</i>	This study
pCR2.1-TOPO	Commercial cloning vector; Ap ^r Kn ^r	Invitrogen
pEAH11	pEVS122 + 5',3'-truncated <i>vpsR</i> (VF0454)	22
pESY20	pEVS122 cut with EcoRI (partial), filled in, and self-ligated	31
pEVS107	Mini-Tn7 delivery plasmid; mob; Kn ^r Em ^r	27
pEVS122	R6Kγ <i>oriV oriTRP4</i> , Em ^r , <i>lacZα</i>	12
pJET1	Commercial cloning vector; Ap ^r	Fermentas
pKV69	Mobilizable vector; Cm ^r Tc ^r	44
pMAC3	pKV69 expressing SypF-V439I; Cm ^r Tc ^r	This study
pMAC4	pKV69 expressing SypF1; Cm ^r Tc ^r	This study

pMAC3. A similar approach was used to generate a *vpsR* overexpression construct pCLD42, using primers VpsRF (AAGGATCCCATTTGTGAGACGAGATAAGG) and VpsRR (AAGGATCCCATTTGTGAGACGAGATAAGG) and PCR cloning vector pJET1 (Fermentas, Glen Burnie, MD) as an intermediate cloning vector. The *sypF* overexpression plasmid pMAC3 was passed through *E. coli* mutator strain CC130 (26) and introduced into ES114 by conjugation. The resulting colonies were examined for wrinkled colony formation. We isolated the plasmid (pMAC4) from one wrinkled colony and subcloned the *sypF* portion into pKV69 to obtain the *sypF1* overexpression plasmid pCLD29. The sequences of the *sypF* and *vpsR* genes in these constructs were obtained using forward, reverse, and/or gene-specific primers using the Genomics Core sequencing facility at Northwestern University. Sequence analysis revealed a single point mutation in pMAC3 (resulting in a V439I substitution), so a wild-type copy of *sypF* was cloned using sypFexpF and VFA10265R (CTGCCCCTGACATATCTG), yielding pCLD54.

We constructed several *V. fischeri* mutants using a vector integration approach (8) as follows: we cloned an internal fragment of the target gene into the suicide vector pEVS122 (12) or its derivative pESY20 (31), generally by first cloning the appropriate PCR product into pCR2.1-TOPO. The resulting plasmid was introduced into *V. fischeri*. The resulting Em^r colonies were presumptive mutants, generated through homologous recombination to produce two truncated, non-functional copies of the gene. The mutants were subsequently verified by Southern analysis using the vector sequences as probe as described previously (53). The internal fragments for disrupting VFA0884, VF0349, and VF0352 were generated with primer sets VFA0884-c-F (CCGGTTGCAATTGAACTTTTAT)/VFA0884-c-R (GAGTTAAAGGAATGCTTGCG), VF0349-c-F (CGTC TGCGCGTGCTACTC)/VF0349-c-R (AGCTGCTGCTTCAATAACGG), and VF0352-c-F (GTACCCTAACAAAGGCATTG)/VF0352-c-R (CTGATACCGTAAGGAGTAAAG), respectively.

The $\Delta sypG \Delta sypE$ double mutant was constructed by allelic replacement, using the $\Delta sypG$ parent strain and the $\Delta sypE$ deletion construct pCLD19, as previously described (21).

β -Galactosidase assay. Strains were grown (in triplicate) with shaking in HMM with Tc at 28°C overnight and then subcultured into fresh medium and grown for 20 h. Aliquots (1 ml) of cells were removed, concentrated, resuspended in Z-buffer, and lysed. The β -galactosidase activity (28) and the protein concentration (24) of each sample were assayed. β -Galactosidase units are reported as units of activity per mg of protein.

Crystal violet assay of biofilm formation. Strains were grown with shaking in HMM with Tc at 28°C overnight and then subcultured into fresh medium to an optical density at 600 nm (OD₆₀₀) of 0.1. Cultures were then grown for 48 h under static or shaking conditions and stained with 1% crystal violet. Tubes were

rinsed with deionized H₂O and photographed. We quantified staining by adding 2 ml 100% ethanol and 1 g glass beads (1 mm), vortexing, and then measuring the OD₆₀₀.

Pellicle assay. Strains were grown with shaking in HMM with Tc at 28°C overnight and then subcultured to an OD₆₀₀ of 0.1 in 3 ml of fresh medium in 12-well microtiter dishes. Cultures were then grown at room temperature for 48 h. The strength of the pellicle was evaluated by disrupting the air-liquid interface with a sterile pipette tip after 48 h of incubation. Cultures with no pellicle were scored as -; cultures with an easily broken pellicle were scored as +; cultures with an intermediate strength were scored as ++; cultures with a strong pellicle that was able to be lifted from the culture intact were scored as ++++. To facilitate photography of the pellicles after they had formed, 25 μ l of 100 mg/ml Coomassie blue was added to provide contrast, and the pellicles were disturbed so that they could be visualized.

RESULTS

An increased activity allele of *sypF* induces wrinkled colony formation. Biofilm formation by *V. fischeri* is controlled by at least three regulators, a sensor kinase (RscS) and two response regulators (SypG and SypE) encoded within the *syp* locus (Fig. 1A) (53). Between the two *syp* genes lies *sypF*, which encodes a putative sensor kinase. The juxtaposition of these latter genes suggested a common function. Thus, we hypothesized that SypF might act upstream of SypG and/or SypE to control biofilm formation. To probe the role of SypF, we assayed the biofilm phenotypes of a *sypF* mutant, as well as a wild-type strain of *V. fischeri* overexpressing *sypF* (from the multicopy plasmid pCLD54). Like the wild type and vector control, these strains did not induce wrinkled colony formation, pellicle production, adherence to glass, or *syp* transcription, although overexpression of *sypF* caused a slight aggregation of cells during growth with shaking in a minimal medium (HMM) (data not shown).

We thus hypothesized that our culture conditions might not include a strong activating signal recognized by this regulator. Therefore, we sought a constitutively active allele of *sypF* through random mutagenesis experiments (see Materials and Methods). Of over 10,000 colonies screened, we found a single colony that exhibited a wrinkled colony phenotype (Fig. 2A). We verified through subcloning experiments that the *sypF* portion of the plasmid was responsible for the wrinkled colony phenotype. The sequence of the *sypF1* allele carried by pCLD29 differed from that of *sypF* in the annotated genome at two positions, resulting in protein changes S247F and V439I. S247F is located within the HisKA domain and is three residues N-terminal to the conserved histidine predicted to be the site of phosphorylation; a change at this position could impact the predicted kinase activity of SypF (Fig. 1). The latter substitution, V439I, appears unimportant, as a strain carrying plasmid pMAC3, which carries *sypF* with only this change, behaved like a strain carrying wild-type *sypF* on pCLD54 (i.e., no impact on biofilm formation) (Fig. 2A and data not shown; see also Fig. 3A, 4A, and 5A, below). For the purposes of these studies, we used pMAC3 to control for the impact of SypF1 (expressed from pCLD29) on biofilm formation by *V. fischeri*.

Wrinkled colony formation by SypF1 depends on multiple regulators. Because the *sypF1* allele mediated wrinkled colony formation, a phenotype previously associated with *syp* function, we asked whether SypF1-induced wrinkled colony formation required an intact *syp* cluster. We introduced pCLD29 or the vector control into mutants defective for representative *syp*

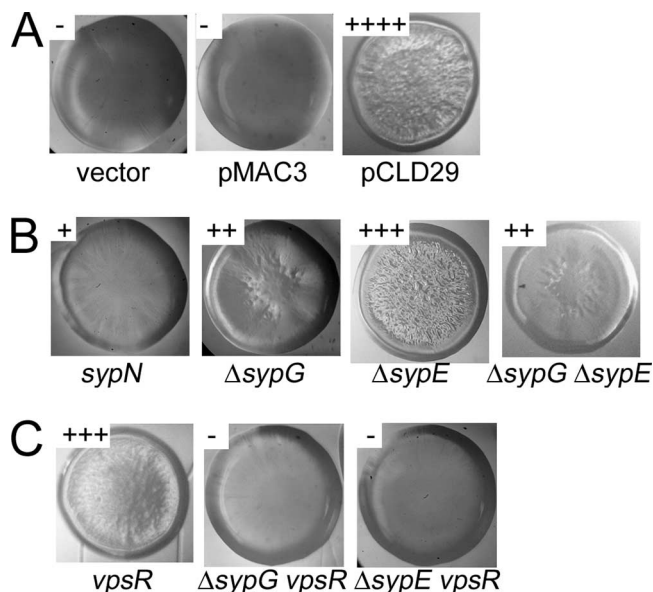


FIG. 2. Wrinkled colony morphology. Wrinkled colony formation was assessed by streaking strains on LBS with Tc and 0.3% glycerol. (A) Colonies formed by strains carrying the vector control, pMAC3 (*sypF* overexpression) or pCLD29 (*sypF1* overexpression). (B) Colonies formed by pCLD29-containing *syp* mutant strains, as indicated. (C) Colonies formed by pCLD29-containing *vpsR* mutant strains and *vpsR syp* double mutant strains.

structural genes: *sypB*, which encodes a putative OmpA-like outer membrane protein, and *sypI* and *sypN*, which encode putative glycosyltransferases. In these mutants, the ability of SypF1 to induce wrinkling was substantially reduced (Fig. 2B and data not shown). These data suggest that the *syp* cluster contributes to SypF1-induced wrinkling.

Our previous work revealed that wrinkled colonies induced by overexpressing the sensor kinase RscS were dependent upon the *syp*-encoded response regulator SypG, and to a lesser extent, SypE, a non-DNA-binding response regulator (21). To determine if these response regulators were required for SypF1-mediated wrinkled colony formation, we assayed the impact of SypF1 in mutants deleted for *sypG* or *sypE*. Deletion of either regulator caused a substantial reduction in, but not elimination of, wrinkling induced by SypF1 (Fig. 2B). To determine whether *sypE* and *sypG* together accounted for the ability of SypF1 to induce wrinkled colony formation, we constructed a strain in which both were deleted and then evaluated the resulting colony morphology induced by *sypF1*. Wrinkled colony formation in this strain was also substantially reduced, but again not eliminated (Fig. 2B). From these data, we conclude that SypE and SypG contribute to wrinkling induced by SypF1, but that another factor is also involved.

SypF1 induces pellicle formation. Another phenotype associated with the *syp* cluster is the formation of strong pellicles at the air-liquid interface of static cultures (52). We therefore assessed the ability of SypF1 to induce pellicle formation. We grew the *sypF1*-overexpressing strains statically in a minimal medium, HMM, for 48 h in a 12-well polystyrene microtiter dish. We found that overexpression of *sypF1* in the wild-type strain caused the formation of a strong pellicle that could be

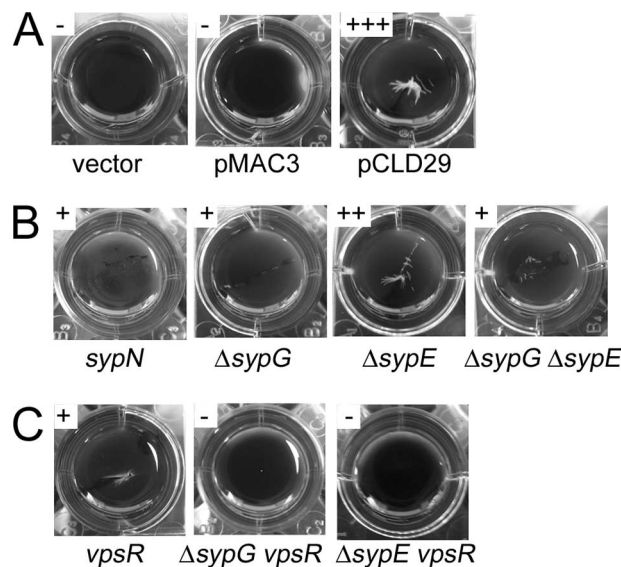


FIG. 3. Pellicle formation. Pellicles were assayed by culturing strains in HMM with Tc in 12-well microtiter dishes for 72 h. A pipette tip was dragged over the air-liquid interface to visualize the pellicle, and Coomassie blue was added to enhance the contrast. (A) Pellicle formation (or lack thereof) by strains carrying the vector, pMAC3, or pCLD29. (B) Pellicle formation by pCLD29-containing *syp* mutant strains. (C) Pellicle formation by pCLD29-containing *vpsR* mutant and *vpsR syp* double mutant strains. Scores were assigned as described in Materials and Methods.

lifted from the culture as an intact aggregate. To aid comparisons between this pellicle and those formed in other mutant backgrounds, we applied a sterile pipette tip to the surface of the pellicle and assigned a score (see Materials and Methods) to the resistance encountered; we scored the pellicles produced by pCLD29-containing wild-type cultures as +++ (Fig. 3A).

To determine whether these pellicles depended upon *syp* structural genes, we assayed pellicle formation by the SypF1-overexpressing *syp* mutants (*sypB*, *sypI*, and *sypN*). The *syp* structural mutations substantially disrupted pellicle formation induced by SypF1: the pellicles that formed were very thin and easily broken (scored as +) (Fig. 3B and data not shown). These data support the conclusion that the product of the *syp* locus makes an important contribution to cell-cell interactions under these conditions.

We next assayed the dependence of SypF1-induced pellicle formation on *sypG* and *sypE*. Deletion of *sypG* substantially reduced, but did not eliminate, pellicle formation induced by SypF1 (scored as +) (Fig. 3B); this pellicle was easily broken into segments, supporting the hypothesis that Syp is important for cell-cell interactions. Deletion of *sypE* also reduced pellicle formation, though not as severely as the *sypG* mutant (scored as ++). Finally, a strain in which both *sypG* and *sypE* were deleted still retained some SypF1-induced pellicle formation. These data further support the hypothesis that SypF1 may function through an additional regulator.

SypF1 increases adherence to glass. A third phenotype associated with the *syp* locus is adherence to a glass surface (21, 52, 53), as assayed by crystal violet staining of cultures grown in HMM. Therefore, we asked whether SypF1 overexpression caused an increase in crystal violet-stainable material of statically

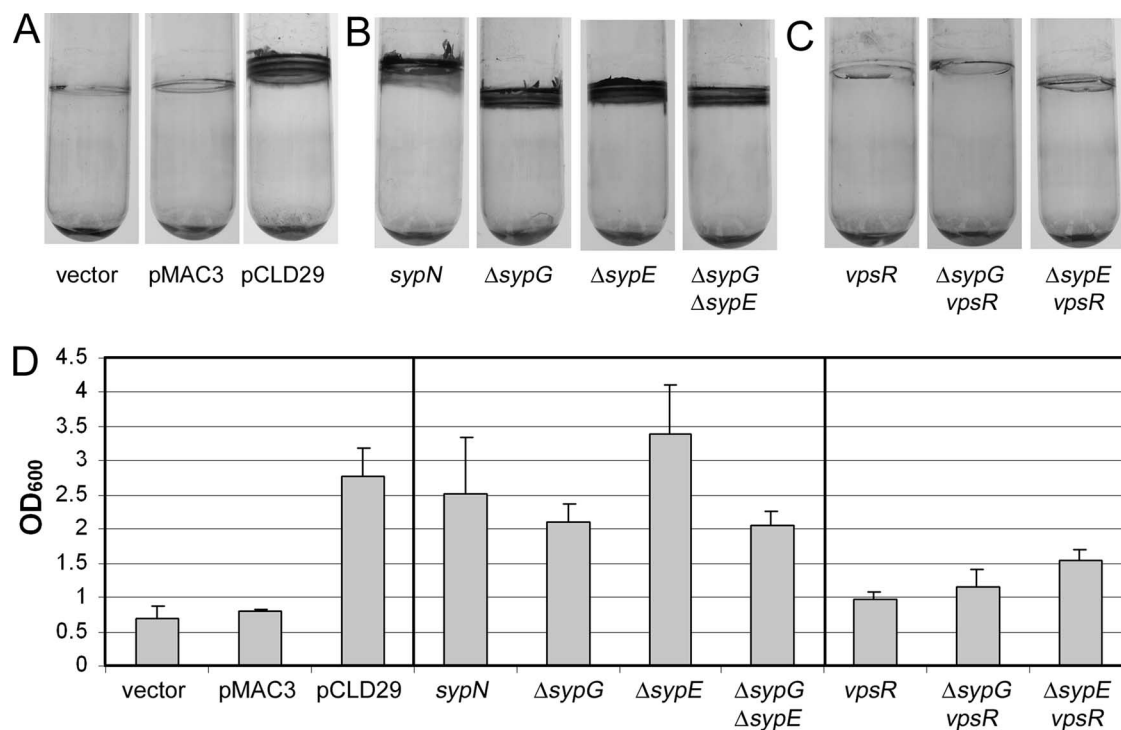


FIG. 4. Crystal violet staining of glass-attached material following static growth. Glass-attached materials following growth of cells under static conditions were assayed by subculturing into 2 ml HMM with Tc and incubating at room temperature for 48 h and then staining with 1% crystal violet. (A) Crystal violet staining of glass-attached material from cultures of vector-, pMAC3-, and pCLD29-containing cells. (B) Crystal violet staining of glass-attached material from cultures pCLD29-containing *syp* mutants. (C) Crystal violet staining of glass-attached material from cultures of pCLD29-containing *vpsR* mutant and *vpsR* *syp* double mutant strains. (D) Quantification of the crystal violet-stained material from tubes shown in panels A to C (and additional tubes not shown), solubilized as described in Materials and Methods. Error bars represent standard deviations.

grown *V. fischeri* cultures. We found that wild-type cultures carrying pCLD29 exhibited enhanced crystal violet staining at the air-liquid interface (Fig. 4A and D).

To determine if the SypF1-mediated enhancement of adherence to glass was dependent on *syp*, we evaluated attachment by pCLD29-containing *syp* mutants. We found that the *sypB*, *sypI*, and *sypN* genes were dispensable for this phenotype (Fig. 4B and data not shown). This result was somewhat surprising, since SypF1-mediated pellicle formation, a phenotype that occurs under similar static conditions, was disrupted by the loss of these genes. Like the structural genes, *sypG* and *sypE* did not appear to be important for the SypF1-induced adherence to glass under static conditions (Fig. 4B). Together with the pellicle data, our results suggested that the role of the *syp* cluster under these conditions involves promoting cell-cell, rather than cell-surface, interactions. They also supported the existence of a separate pathway, downstream from SypF, involved in mediating adherence to glass.

We also evaluated the ability of SypF1 to induce attachment to glass when cells were grown with shaking, a phenotype first observed in *V. fischeri* with the overexpression of the response regulator SypG (53). Under these conditions, SypF1 induced substantial cell clumping: the cells appeared aggregated, both at the air-liquid interface and the bottom of the tube, with the rest of the broth remaining relatively clear (Fig. 5A). Like SypG overexpression, SypF1 also induced a robust staining pattern with crystal violet (Fig. 5D and G).

Disruptions of *sypB*, *sypI*, and *sypN* each altered the appearance of the crystal violet-stained material and reduced aggregation (Fig. 5B and E and data not shown). Similarly, the loss of the regulators *sypG*, *sypE*, or both altered staining and decreased clumping (Fig. 5B, E, and G), though the *sypE* mutant had a less severe defect. Despite the different staining pattern, quantification revealed no significant differences in the amounts of crystal violet staining in these mutants relative to the wild type (Fig. 5G). We speculate that the qualitative difference in staining may result from apparent differences in the aggregation properties of the mutant cultures relative to the wild type (Fig. 5A). Taken together, these results further indicate that, under SypF1-overexpressing conditions, the *syp* regulators play more important roles in cell-cell adherence (as measured by aggregation) than in cell-surface attachment (as measured by crystal violet staining).

***sypF1* induces *syp* transcription.** To begin to understand the mechanism by which *sypF1* overexpression promotes the observed biofilm phenotypes, we asked whether pCLD29 induced transcription of the *syp* locus and, if so, whether induction depended upon SypG or SypE. We introduced pCLD29 and the vector control into strains that carried a P_{sypA} -*lacZ* transcriptional reporter (21) and measured the resulting β -galactosidase activity. Relative to the vector control, pCLD29 induced a 10-fold increase in β -galactosidase activity of the wild-type reporter strain (Fig. 6A). This induction was completely dependent on SypG, as its loss eliminated the observed induc-

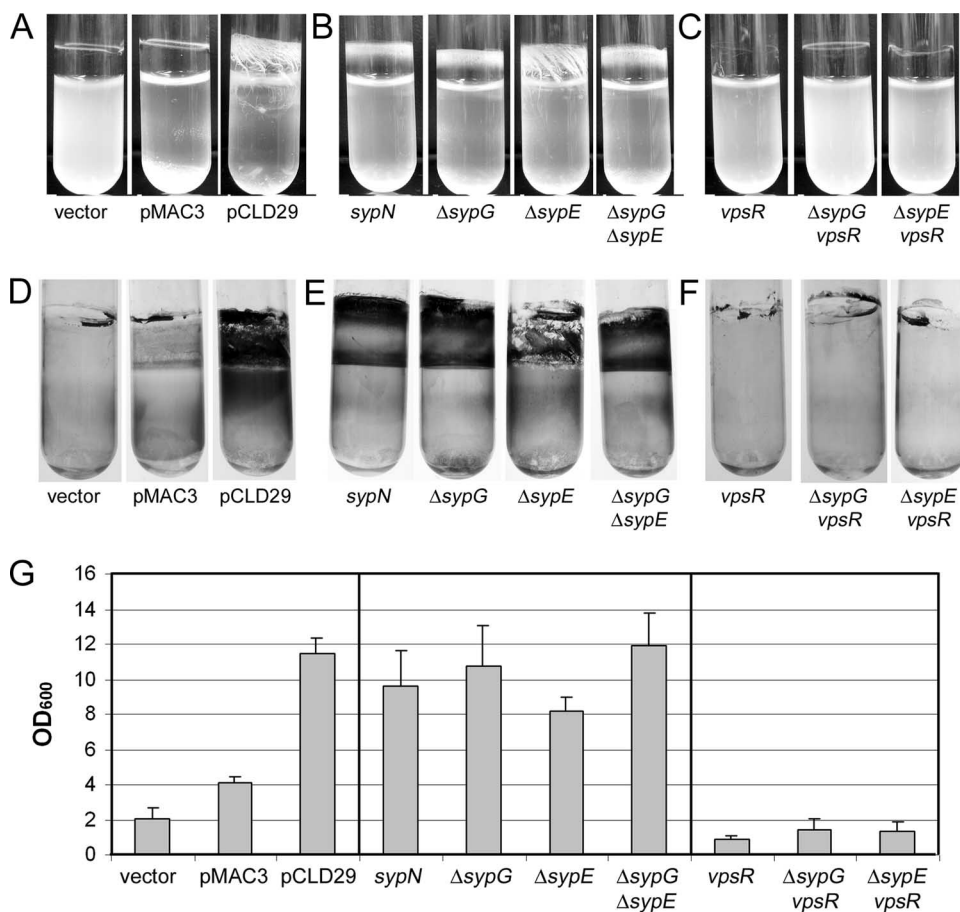


FIG. 5. Crystal violet staining of glass-attached material following growth with shaking. Glass-attached materials following growth of cells under shaking conditions were assayed by subculturing into 2 ml HMM with Tc and incubating at 28°C for 48 h (A to C) and then staining with 1% crystal violet (D to F). (A and D) Cultures of vector-, pMAC3-, and pCLD29-containing cells. (B and E) Cultures of pCLD29-containing *syp* mutants, as indicated. (C and F) Cultures of pCLD29-containing *vpsR* mutant and *vpsR syp* double mutant strains, as indicated. (G) Quantification of the crystal violet-stained materials from tubes shown in panels D to F (and additional tubes not shown), solubilized as described in Materials and Methods. Error bars represent standard deviations.

tion (Fig. 6B). The induction was also somewhat dependent on SypE, as a deletion of this gene reduced SypF1-mediated induction by sixfold (Fig. 6B). However, disruptions of other *syp* genes (*sypB*, *sypI*, and *sypN*) also caused reductions in *sypA*

transcription (Fig. 6B and data not shown), making it somewhat difficult to interpret the *sypE* results. In any event, because SypE has no predicted DNA-binding domain, the effect of SypE is likely to be indirect. Not surprisingly, induction of

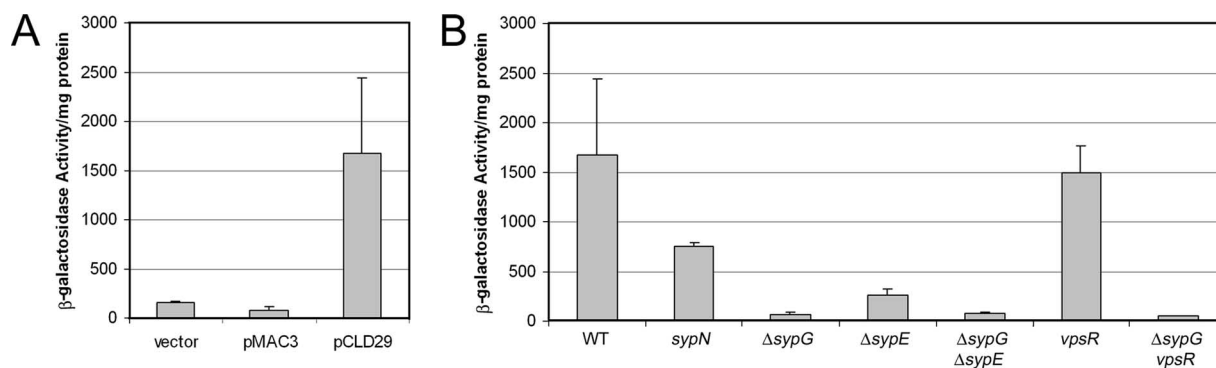


FIG. 6. β -Galactosidase activity. Transcription of the *syp* locus was monitored using a fusion of the *sypA* promoter region upstream of a promoterless *lacZ* gene, inserted in the chromosome at the Tn7 site of wild-type and mutant cells. Cells were grown in HMM with Tc for 24 h. (A) Transcription from P_{sypA} -*lacZ* in cells carrying the vector control, pMAC3, or pCLD29. (B) Transcription from P_{sypA} -*lacZ* in pCLD29-containing wild-type (WT), *syp*, or *vpsR* mutant cells, as indicated. Error bars represent standard deviations.

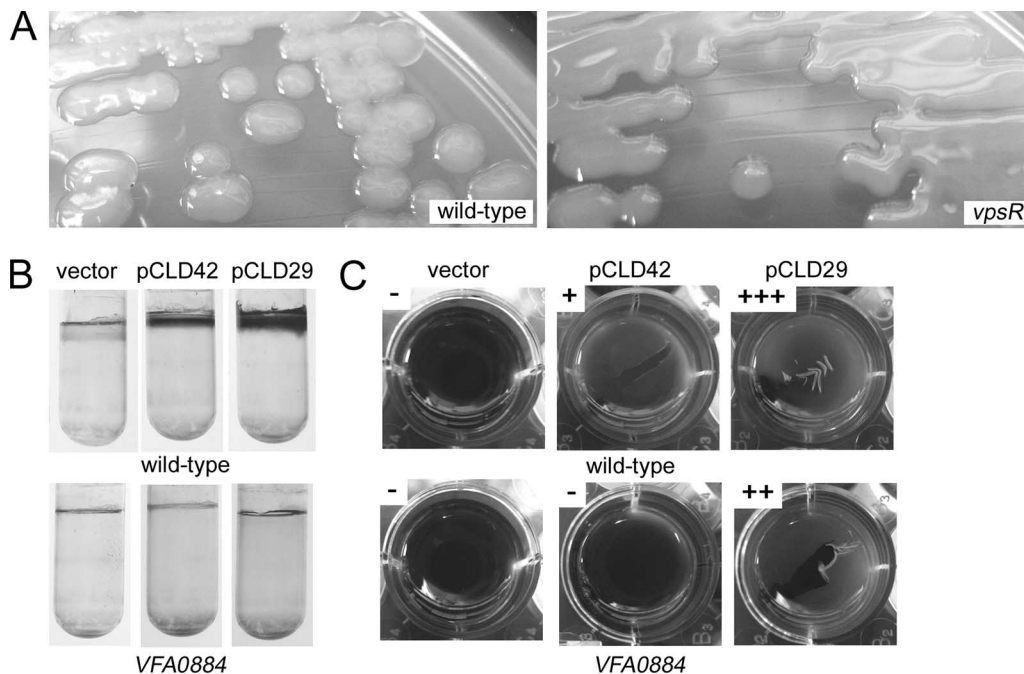


FIG. 7. Biofilm phenotypes of *vpsR* mutant and *vpsR* overexpression strains. Biofilms were produced upon disruption or overexpression of *vpsR*. (A) Colony morphology of vector (pKV69)-containing wild-type and *vpsR* mutant cells. (B) Crystal violet staining of glass-attached material from static cultures of wild-type or VFA0884 mutant cells carrying the vector control, pCLD42 (*vpsR* overexpressing), or pCLD29. (C) Pellicle formation by wild-type or VFA0884 mutant cells with vector, pCLD42, or pCLD29. Scores were assigned as described in Materials and Methods. Cells were grown as described in the legends for Fig. 2, 3, and 4.

the *syp* cluster was also eliminated in the *sypG sypE* double mutant (Fig. 6B). These data suggest that SypF1 primarily acts upstream of SypG in controlling *syp* transcription.

Some SypF1-induced phenotypes also depend upon *vpsR*. Together, our data support a role for *syp* and *syp* regulators in the phenotypes induced by SypF1, but it is clear that there are *syp*-independent effects. Because SypF is a sensor kinase, we hypothesized that it might signal through an additional response regulator(s) to promote the observed phenotypes. *V. fischeri* encodes 40 putative response regulators, including SypG and SypE. Of these, a prime candidate was VF0454. This gene encodes a protein with high sequence similarity to the *V. cholerae* gene *vpsR* (68% identity, 84% similarity over 441 amino acids [with one gap] of the *V. fischeri* ES114 protein with *V. cholerae* O1 biovar El tor [e-value, 6e-174]), which controls the *vps* polysaccharide locus (22, 35, 49).

Careful observation of a VF0454 vector integration mutant revealed that it formed colonies that were opaque and mucoid compared to the wild type (Fig. 7A and data not shown), suggesting that VF0454 may serve as a repressor of exopolysaccharide production. Therefore, we designated this gene *vpsR*, for vibrio polysaccharide regulator. Loss of *vpsR* also impacted the biofilm phenotypes induced by *sypF1* overexpression. Most significantly, the increase in crystal violet-stainable material produced under both static and shaking conditions appeared to be completely abrogated in the *vpsR* mutant (Fig. 4C and D and 5F and G). Loss of *vpsR* also altered wrinkled colony formation and reduced the strength of pellicle formed (Fig. 2C and 3C). The resulting pellicle was substantially defective in attaching to the surface of the well, suggesting a role for VpsR

in cell-surface attachment. Finally, despite the loss of *vpsR*, the pCLD29-containing cultures, when grown in HMM with shaking, retained their characteristic clumping, although the amount appeared somewhat reduced (Fig. 5C). Together these data support an important role for VpsR in some SypF-induced phenotypes, particularly the cell-surface phenotypes revealed by crystal violet staining, and a lesser role in others. These effects appeared to be independent of SypF1-mediated *syp* transcription, as loss of *vpsR* did not alter this activity (Fig. 6C).

Because most SypF-induced phenotypes were not abolished by loss of *sypG* and *sypE*, or of *vpsR*, we asked whether SypF worked through both pathways. To determine if these regulators together could account for all of the SypF-induced phenotypes, we constructed *sypG vpsR* and *sypE vpsR* double mutants. Like the single *vpsR* mutant, these double mutants exhibited a mucoid phenotype, though this phenotype was lost when SypF1 was overexpressed (data not shown). The formation of wrinkled colonies and pellicles was completely abrogated in both pCLD29-containing double mutants (Fig. 2C and 3C). Crystal violet staining of double mutant cultures grown under either static or shaking conditions looked similar to that of the *vpsR* single mutant, indicating VpsR is responsible for these phenotype (Fig. 4C and 5C). Furthermore, the shaken cultures themselves were substantially less aggregated, appearing more similar to the vector control cultures (Fig. 5C). We obtained similar results when we evaluated SypF1-induced biofilm formation in a mutant defective for both *vpsR* and a *syp* structural gene, *sypP* (data not shown). These data support the

hypothesis that *syp* and a *vpsR*-dependent pathway together account for the SypF1 overexpression phenotypes.

VpsR overexpression impacts biofilm formation. To further evaluate the role of VpsR in biofilm formation, we constructed a plasmid that overexpresses the *vpsR* gene. After introducing this plasmid, pCLD42, into wild-type cells, we evaluated biofilm-associated phenotypes. Overexpression of VpsR was not sufficient to induce wrinkled colony morphology or alter crystal violet staining resulting from cultures grown under shaking conditions (data not shown). However, VpsR-overexpressing cultures were able to form weak pellicles (Fig. 7). Additionally, pCLD42 caused an increase in crystal violet staining from cultures grown under static conditions (Fig. 7). Together, these data support VpsR as an activator of biofilm formation.

Cellulose plays a role in *V. fischeri* biofilm formation. In *V. cholerae*, VpsR controls a large polysaccharide gene locus, *vps* (49, 51). Only a portion of this locus is conserved in *V. fischeri* (16). To determine whether this *vps*-like locus was responsible for the phenotypes induced by either SypF1 or VpsR overexpression, we disrupted two representative genes, VF0349 and VF0352. However, neither of these mutations disrupted the observed biofilm phenotypes (data not shown).

Subsequent exploration of the biofilms induced by these regulators revealed an increase in Congo red binding, a phenotype indicative of, among other things, cellulose biosynthesis. *V. fischeri* contains a cellulose biosynthetic gene cluster (*VFA0881-885*) similar to that found in *Salmonella* (32, 33, 35). To evaluate the role of this locus in the observed phenotypes, we constructed a mutant defective for *VFA0884*, which encodes the catalytic subunit of a putative cellulose synthase, and introduced either pCLD29 (*sypF1*) or pCLD42 (*vpsR*). Loss of *VFA0884* disrupted the Congo red binding induced by these regulators (data not shown). Furthermore, this mutation eliminated glass attachment induced by SypF1 or VpsR (Fig. 7B). Finally, the pellicles formed by SypF1 in the *VFA0884* mutant were reduced to levels similar to the *vpsR* mutant; VpsR-induced pellicles were completely dependent on *VFA0884* (Fig. 7C). Together, these data support a role for cellulose in biofilm formation by *V. fischeri* under these conditions.

DISCUSSION

The significance of microbial biofilms as major survival strategies under both environmental and host conditions is well-accepted. Control of biofilm formation in numerous systems is complex (e.g., *V. cholerae* [5, 9, 50, 51, 54]). Here, we have shown that biofilm formation in *V. fischeri* also depends on multiple regulators. Our discovery of an increased activity allele, *sypF1*, revealed a role for this putative hybrid sensor kinase in the transcriptional control of *syp*, a polysaccharide locus necessary for symbiotic colonization. In addition, multicopy expression of *sypF1* induced biofilm phenotypes, including the formation of wrinkled colonies on solid medium and cell-cell aggregating, surface attachment, and pellicle formation in liquid medium. Surprisingly, we found that SypF1 appears to work through at least two response regulators, SypG and VpsR: disruption of both regulators was required for the complete loss of both wrinkled colony and pellicle formation induced by SypF1 overexpression. Multicopy expression of VpsR could also induce biofilm phenotypes (Fig. 7B and C),

and these were found to be dependent on cellulose biosynthesis. Together, these data extend our knowledge of the complex control of biofilm formation by *V. fischeri* and suggest that SypF may coordinate *syp* activity through SypG with cellulose through VpsR.

Perhaps the simplest phenotype induced by overexpression of *sypF1* was transcription of the *syp* locus, as monitored with a *lacZ* reporter fused downstream from the *sypA* promoter. Reporter activity was increased 10-fold by SypF1, and this induction depended completely on SypG, a putative DNA-binding response regulator thought to directly activate *syp* transcription (53) (E. A. Hussa and K. Visick, unpublished data). Transcription did not depend fully upon any other regulator, although loss of SypE impacted the overall levels of induction, as did, to a lesser extent, loss of representative Syp structural proteins (encoded by *sypB*, *sypI*, and *sypN*). Perhaps these results indicate a form of feedback control on *syp* transcription. Importantly, *syp* transcription did not depend on VpsR, a response regulator predicted to control polysaccharide production: loss of the *vpsR* gene did not impact *sypA* transcription induced by SypF1 overexpression. Finally, SypF1-mediated *sypA* transcription also did not depend upon RscS, a sensor kinase known to act upstream of SypG (21) (C. Darnell and K. Visick, unpublished data). Together, these data support the hypothesis that SypF1 activates *syp* transcription by working upstream of SypG.

Despite this apparent connection between SypF and SypG with respect to *syp* transcription, a number of biofilm phenotypes induced by overexpression of *sypF1* did not depend exclusively on SypG, or in the case of attachment to glass, on SypG at all. Biofilm phenotypes are complex, requiring many factors to produce a complete structure (including, for example, motility, pili, exopolysaccharide, secreted proteins, and in some cases DNA) (reviewed in references 11 and 13). Furthermore, different phenotypes require different subsets of these factors. Therefore, to evaluate the role of SypF in biofilm formation, we assessed three major phenotypes (wrinkled colony morphology, surface attachment, and pellicle formation). Through this comprehensive approach, combined with mutant analyses, we were able to uncover a role for VpsR. Whereas loss of either SypG or VpsR severely impacted only a subset of the SypF1-induced phenotypes, loss of both regulators abolished all of them. Surprisingly, although VpsR (but not SypG) was required for attachment to glass induced by SypF1 under shaking conditions (Fig. 5), VpsR overexpression did not induce a similar phenotype (data not shown). These results suggest the involvement of other factors controlled by SypF1.

SypF also appeared to work upstream of SypE, though the impact of *sypE* disruption was less severe than that of *sypG* disruption and similar to disruption of *syp* structural genes, suggesting a less important regulatory role for SypE. At this time, our data support the hypothesis that SypE contributes to SypF-mediated biofilm formation, but whether SypE can be considered a direct downstream regulator remains uncertain, as its loss eliminates none of the observed phenotypes. Indeed, our preliminary data suggest that an additional RR may also be involved in the complex phenotypes induced by SypF; this possibility is currently under investigation (Darnell and Visick, unpublished). However, from the data presented here, we con-

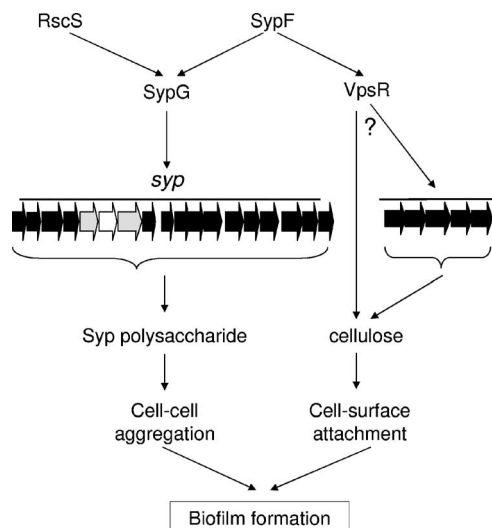


FIG. 8. Model for the roles of *syp* regulators in biofilm formation in *V. fischeri*. Transcription of the *syp* locus can be induced by overexpression of RscS or SypF (SypF1), acting through SypG. The Syp structural proteins contribute to the production of a Syp polysaccharide, involved in cell-cell aggregation phenotypes, such as wrinkled colonies and pellicles. SypE contributes by an unknown mechanism to these phenotypes; it also appears to inhibit SypG activity (not depicted). SypF overexpression may activate VpsR, ultimately promoting cellulose operon (VFA0881-885) expression or cellulose synthesis. The result of these interactions is enhanced cell-surface interactions. Block arrows indicate genes (depicted in gray for *sypE* and *sypG* and in white for *sypF*), and line arrows indicate activation. The question mark indicates that the level at which VpsR exerts its effect is not yet known.

clude that SypG and VpsR appear to represent major regulators involved in relaying the SypF-initiated signal.

Using these data and previous work (21, 52, 53) as a guide, we have developed a working model for the roles of the various *syp* regulators described to date (Fig. 8). SypF and RscS each have the potential to serve as phospho-donors to SypG, which we propose directly activates *syp* transcription. We previously reported that SypE may serve, under some circumstances, as an inhibitor of SypG activity, although SypE is required for full RscS and SypF activity (reference 21 and this work). As a result of these activities, a *syp*-dependent polysaccharide is produced, contributing to the formation of wrinkled colonies and pellicles and other phenotypes. In addition, SypF may directly or indirectly activate VpsR. Although the role of this protein as a response regulator is unclear, given the relative lack of conservation of key residues involved in phosphorylation (22, 49), genetic evidence supports the role of the *V. cholerae* homolog as a response regulator (23). Regardless of its specific activity, the *V. fischeri* protein apparently functions upstream of cellulose biosynthesis and thus biofilm formation. In other bacteria such as *Salmonella*, cellulose biosynthesis is controlled through posttranscriptional mechanisms, including through the availability of c-di-GMP (32, 33). Therefore, our simple model (Fig. 8) shows VpsR as a transcriptional regulator of the cellulose biosynthetic locus or as an indirect regulator of cellulose biosynthesis.

We propose that cellulose is responsible for initial attachment during static growth and that subsequent cell-cell interactions depend upon the Syp polysaccharide (Fig. 8). This

hypothesis is supported by the following data: loss of VpsR or VFA0884 (but not SypG) disrupted surface attachment induced by SypF1 during static growth. The subsequent development of pellicles depended heavily on SypG. Disruption of *vpsR* did not prevent pellicle formation, but the pellicles that formed failed to attach properly to the well. These data suggest different roles for the two polysaccharides.

Similar to VpsR in *V. cholerae* (VpsR_{VC}), in which this protein has been most thoroughly investigated (5, 49), VpsR in *V. fischeri* (VpsR_{VF}) appears to be an orphan response regulator encoded within an analogous region of the large chromosome. The two proteins exhibit high sequence similarity yet appear to play distinct roles. VpsR_{VF} controls cellulose biosynthesis; in contrast, *V. cholerae* doesn't contain a cellulose locus. While VpsR_{VC} activates transcription of the *vps* polysaccharide locus, VpsR_{VF} appears to serve both as a positive regulator of cellulose biosynthesis and as a negative regulator of an unknown polysaccharide, as evidenced by the increased mucoidy of a *vpsR* mutant. A further understanding of VpsR_{VF} function awaits a more thorough investigation of its regulon through array studies.

It is possible that by overexpressing an increased activity allele of *sypF*, we are creating artifactual phenotypes: an overabundance of active SypF may phosphorylate response regulators that are not biologically relevant. However, SypG is encoded directly downstream of *sypF*, suggesting a likely connection between these regulators. VpsR also seems to be a relevant target, given that it is unlinked to a known sensor kinase and it clearly plays a role in polysaccharide production in its own right. Finally, the *vpsR* mutant exhibited a small defect in colonization of the squid when competed with the wild-type strain, suggesting a biologically relevant role (22). Thus, while SypF1 may improperly donate phosphoryl groups to other response regulators that contribute to phenotypes not identified here, this work nonetheless reveals regulators and phenotypes involved in biofilm formation by *V. fischeri*.

Taken together, this work elucidates a role for SypF in controlling the *syp* locus and thus contributing to the production of a *syp*-generated polysaccharide. It also provides evidence for a second distinct arm for polysaccharide production coordinated by SypF through VpsR, resulting in cellulose production. These two polysaccharides make differential contributions to the observed SypF-induced biofilms. Thus, this work further demonstrates the complexity of biofilm formation and regulation in *V. fischeri*, a nontraditional model of biofilm formation.

ACKNOWLEDGMENTS

We thank Mark Clementz, Deborah Muganda, and Emily Yip for their experimental contributions to this work and Alan Wolfe and Christine Anderson for their review of the manuscript.

This work was supported by NIH grant GM59690 awarded to K.L.V.

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