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RscS Functions Upstream of SypG To Control the *syp* Locus and Biofilm Formation in *Vibrio fischeri*[▽]

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Two-component signal transduction systems, composed of sensor kinase (SK) and response regulator (RR) proteins, allow bacterial cells to adapt to changes such as environmental flux or the presence of a host. RscS is an SK required for *Vibrio fischeri* to initiate a symbiotic partnership with the Hawaiian squid *Euprymna scolopes*, likely due to its role in controlling the symbiosis polysaccharide (*syp*) genes and thus biofilm formation. To determine which RR(s) functions downstream of RscS, we performed epistasis experiments with a library of 35 RR mutants. We found that several RRs contributed to RscS-mediated biofilm formation in *V. fischeri*. However, only the *syp*-encoded symbiosis regulator SypG was required for both biofilm phenotypes and *syp* transcription induced by RscS. These data support the hypothesis that RscS functions upstream of SypG to induce biofilm formation. In addition, this work also revealed a role for the *syp*-encoded RR SypE in biofilm formation. To our knowledge, no other study has used a large-scale epistasis approach to elucidate two-component signaling pathways. Therefore, this work both contributes to our understanding of regulatory pathways important for symbiotic colonization by *V. fischeri* and establishes a paradigm for evaluating two-component pathways in the genomics era.

Bacteria utilize two-component signal transduction pathways as "reflex" systems to sense and adapt to given environmental stimuli (9, 33, 38). Signaling via these systems is initiated by sensor kinase (SK) proteins, which autophosphorylate in response to a specific environmental cue. The phosphoryl group is subsequently transferred to a given response regulator (RR) or, in some cases, multiple RRs. The RR then promotes a given cellular output, often via the transcriptional activation of a subset of genes.

Two-component signaling systems are well suited to allow communication between symbiotic partners, mediating smooth transitions into, as well as maintenance within, such associations. Previous studies established that multiple two-component pathways are required for the initiation and maintenance of the symbiosis between the marine bioluminescent bacterium *Vibrio fischeri* and the Hawaiian squid *Euprymna scolopes* (19, 26, 36). At least 14 of the 40 putative RRs within the *V. fischeri* genome are required for efficient colonization. These include the luminescence regulator LuxO (15–17), the metabolic regulator ArcA (3), the global regulator GacA (39), the extracellular polysaccharide regulator SypG (10), and several less-characterized RRs (10).

Previous work also identified an SK protein, RscS (regulator of symbiotic colonization-sensor), that was required for symbiotic initiation (37). As an SK, RscS would be predicted to exert its influence through an RR protein. However, efforts to understand the RscS pathway were initially stymied by the lack of bioinformatic evidence. Frequently, sensor-regulator partners are encoded adjacently within the genome, often within or near the locus that they regulate. RscS is an orphan sensor, however, as it is not encoded adjacent to a predicted RR gene (37). In addition, while RscS is encoded within a locus of genes that function in glycerol metabolism, mutations in RscS do not alter the ability of *V. fischeri* wild-type strain ES114 to grow in media containing glycerol as the sole carbon source (37). Finally, general functional cues were also lacking; a disruption of *rscS* did not lead to defects in a number of phenotypes tested in culture.

Recently, however, we established that RscS activates the expression of the symbiosis polysaccharide (*syp*) cluster of genes (42). This cluster is composed of 18 genes organized into at least five putative operons (A to E, F to H, I to L, M to O, and P to R) (43; E. A. Hussa, K. Geszvain, and K. L. Visick, unpublished data). The *syp* gene products are predicted to function in polysaccharide synthesis and transport, and most are required for the initiation of symbiotic colonization (43). The overexpression of *rscS* results in the induction of *syp* transcription and *syp*-dependent biofilm formation (42).

With this discovery of culture phenotypes associated with RscS, it is now possible to use epistasis experiments to uncover the RR or RRs that function downstream of RscS. Recently, we identified and disrupted 35 of 40 putative RRs in the *V. fischeri* genome (10). Twelve of these genes were required for competitive colonization, a phenotype expected for the RR that functions downstream of RscS. However, many of these 12 RRs have established homologs and functions unrelated to those of RscS. For example, FlrC regulates flagellar synthesis (10, 12), whereas RscS does not appear to control motility (37). Furthermore, 9 of the 12 RRs are linked to a putative SK in the genome. These observations decreased, but did not eliminate, the potential of these RRs to relay the signal from RscS.

One candidate partner already known to affect *syp* transcription is SypG, a *syp*-encoded regulator that is also required for colonization (10, 43). SypG is predicted to be an RR in the

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pCLD1	Cm^r derivative of pEVS107; Kan^r Cm^r	This study
pCLD6	pCLD1 + 3.2-kb ApaI/SpeI fragment from pEAH41 containing the *psypA-lacZ* fusion; Kan^r Cm^r	This study
pCLD19	Delivery vector carrying sequences flanking *sypE*	This study
pCLD46	pVSV105 carrying the *rscS1* allele; Cm^r	This study
pCLD48	pVSV105 carrying wild-type *sypE*; Cm^r	This study
pCR2.1-TOPO	Commercial cloning vector; Ap^r Kan^r	Invitrogen, Carlsbad, CA
pEAH41	pTMO82 carrying *psypA* upstream of *lacZ*; Ap^r Kan^r	This study
pEAH73	pKV69 carrying wild-type *sypG*; Cm^r Tc^r	This study
pES420	Mobilizable suicide vector, Em^r	21a
pEVS107	Mini-Tn7 delivery plasmid; *mob*; Kan^r Em^r	18
pKG11	pKV69 carrying *rscS1* allele; Cm^r Tc^r	42
pKV69	Mobilizable vector; Cm^r Tc^r	37
pKV276	pEAH73 with D53E mutation in *sypG*; Cm^r Tc^r	This study
pTMO82	pCR2.1-TOPO carrying promoterless *lacZ*; Ap^r Kan^r	This study
pVSV105	Mobilizable vector; Cm^r	6

NtrC-like family of σ^{54}-dependent transcriptional activators. Like RscS, multicopy expression of *sypG* results in enhanced *syp* transcription and biofilm formation, as measured by glass attachment and pellicle formation (43); however, the pellicles are not as robust as those formed by RscS-overexpressing cells. In addition, the overexpression of SypG does not induce wrinkled-colony formation, another hallmark of RscS overexpression (42). Furthermore, the *sypG* gene is adjacent to two genes, *sypE* and *sypF*, that encode putative SK and RR proteins, respectively; thus, it is not clear whether SypG functions directly downstream of SypF or whether a more complicated regulatory scheme exists. Despite these complexities, the hypothesis that SypG functions downstream of RscS to control *syp* expression remained viable.

In this work, we surveyed putative *V. fischeri* RRs to identify those that function downstream of RscS. We report that several RRs contributed to RscS-mediated biofilm formation. However, the loss of *sypG* alone abrogated both RscS-mediated biofilm formation and *syp* transcription. These results thus identify SypG as being a critical link between the symbiosis regulator RscS and the processes that it controls and suggest that RscS and SypG function within the same signal transduction pathway.

MATERIALS AND METHODS

Strains and media. Plasmids and *V. fischeri* strains utilized in this study are listed in Tables 1 and 2, respectively. The parental *V. fischeri* strain used in this work was ES114, a strain isolated from *E. scolopes* (1). All derivatives were generated by conjugation, as previously described (4, 18, 37). *Escherichia coli* strains Tam1 λpir (Active Motif, Carlsbad, CA), DH5α, and Top10 (Invitrogen, Carlsbad, CA) were used for cloning and conjugative purposes. *V. fischeri* strains were grown in either the complex medium LBS (with 0.3% glycerol, where indicated) (8, 32) or HMM (27) containing either 0.3% tryptone (HMM-T) or 0.3% Casamino Acids and 0.2% glucose (43), where indicated. The following antibiotics were added to *V. fischeri* growth media, as necessary, at the indicated concentrations: chloramphenicol (Cm) at 2.5 μg/ml, erythromycin (Em) at 5 μg/ml, and tetracycline (Tc) at 5 μg/ml in LBS or 30 μg/ml in HMM (or HMM-T). The following antibiotics were added to *E. coli* growth media where necessary, at the indicated concentrations: Cm at 25 μg/ml, kanamycin (Kan) at 50 μg/ml, Tc at 15 μg/ml, or ampicillin (Ap) at 100 μg/ml.

Molecular techniques. The mini-Tn7-based reporters utilized within this study were generated by PCR amplification of the *sypA* promoter region using oligonucleotides VFA1019intR (TTTTTCGTACGTGATGGGAAATGACGTTGTG) and VFA1020per (CCGATGGCGTCCATATCAC) (MWG, High Point, NC). The product was then cloned into pTMO82, a derivative of pCR 2.1 TOPO (Invitrogen, Carlsbad, CA) carrying a promoterless copy of *lacZ*, via standard techniques. The resulting plasmid (pEAH41) expresses *lacZ* via the *sypA* promoter. The *sypA-lacZ* transcriptional fusion was then digested out of pEAH41 with ApaI and SpeI (New England Biolabs, Beverly, MA) and cloned into the mini-Tn7 transposon within similarly digested plasmid pCLD1, a Cm^r derivative of pEVS107 (18). SypG overexpression vector pEAH73 is a derivative of pKV69 (Table 1) carrying *sypG* amplified from the *V. fischeri* genome using primers VFA1025RTF (GCTACACTTTCACTAGACGC) and SypG His R (GGTACCTCATTCCGATTCTTCATAG), obtained from MWG (High Point, NC).

Generation of a *sypE* deletion. We constructed the *sypE* deletion strain (Δ*sypE*) as follows: we amplified and cloned sequences 2 kb upstream and downstream of *sypE* into pCR2.1-TOPO and pESY20, respectively. The resulting plasmids were ligated together to produce a composite plasmid, pCLD19, that contained sequences flanking *sypE* but that lacked *sypE* itself. pCLD19 was introduced into ES114 by conjugation and selection for Em resistance (Em^r). Subsequent passage of the resulting colonies on Em allowed the identification of Em^r stable cells; in these cells, a single recombination event had occurred, promoting the integration of the entire plasmid into the chromosome. These cells were subsequently passaged nonselectively to identify Em-sensitive cells in which a second recombination event had occurred, leaving behind either Δ*sypE* or wild-type sequences. One Δ*sypE* strain, KV3299, was identified using a PCR approach and subsequently confirmed by Southern analysis using *sypE* and flanking DNA as a probe.

Generation of the D53E mutation in *sypG*. To obtain the D53E mutation in SypG, we performed site-directed mutagenesis using the Change-IT kit (USB, Cleveland, OH). Plasmid pEAH73 (Table 1) served as the template for primers SypG D53E-phosph (CCACATTTGGTGATTCTCGA**G**TTGAAACTGCCAGATATGTCAG) and Phos-lacZ-up-rev (CCTGTGTGAAATTGTTATCCG). Because the base change (in boldface type) introduces an XhoI site, we screened clones resulting from the mutagenesis with XhoI. A plasmid containing the XhoI site, pKV276, was subsequently identified, and the mutation was confirmed through sequence analysis of the *sypG* coding region using the Genomics Core Facility at the Center for Genetic Medicine at Northwestern University.

Glass attachment and pellicle assays. Strains were grown overnight in HMM containing 0.3% Casamino Acids and 0.2% glucose as well as 30 μg/ml Tc to select for plasmid pKV69 or pKG11. Cultures were then diluted to an optical density at 600 nm (OD_{600}) of 0.1 in the same medium and allowed to grow in static culture in either borosilicate glass culture tubes (for glass attachment assays) or 12-well culture plates (for pellicle assays) for 48 h at 22°C (or 28°C, in the case of experiments conducted with pEAH73). To assay attachment to glass, cultures were stained for 15 min with a 1% crystal violet (CV) solution and subsequently rinsed with deionized water, dried by aspiration, and photographed. Staining was then quantitated by adding 2 ml of 100% ethanol to stained tubes containing 1g of 1-mm glass beads. The tubes were then vortexed until stained material was completely removed from the tube surface. Stained material was quantitated by measuring the OD_{600} (22). The strength of pellicle formation was assessed by drawing a sterile toothpick through the culture surface; pellicles were scored using a scale of "−" to "+++," representing the amount of resistance encountered by the toothpick. A score of "−" was assigned

TABLE 2. *V. fischeri* strains used in this study

Strain[a]	Characteristic(s)	Reference or source
Without reporter		
ES114	Wild type	1
KV1548	VF2120 (*arcA*)::pEAH1	10
KV1585	VF1570::pKV174	10
KV1592	VFA1024 (*sypE*)::pEAH7	10
KV1593	VFA0179::pKV178	10
KV1594	VF1401::pKV177	10
KV1595	VF1396::pKV176	10
KV1596	VFA0561::pKV175	10
KV1612	VFA1017::pKV179	10
KV1640	VFA0041::pTMB26	10
KV1641	VF1054::pAIA1	10
KV1650	VFA0266::pTMB27	10
KV1651	VF1988::pTMB28	10
KV1654	VFA1012::pTMB31	10
KV1655	VF2343::pTMB32	10
KV1665	VF1909::pTMB33	10
KV1666	VFA1026 (*sypG*)::pAIA4	10
KV1668	VFA0211::pAIA6	10
KV1672	VFA0181::pTMB34	10
KV1714	VFA0795::pEAH10	10
KV1715	VF0454::pEAH11	10
KV1727	VF0526::pEAH4	10
KV1730	VF0095::pKV180	10
KV1787	Δ*sypG*	10
KV1809	VF1854::pEAH26	10
KV2164	VF2374::pEAH24	10
KV2165	VFA0216::pEAH25	10
KV2191	VF0937 (*luxO*)::pAIA3	10
KV2501	VF1689::pAIA2	10
KV2503	VFA0103::pAIA5	10
KV2505	VFA0802::pKV207	10
KV2507	VF0114::pKV209	10
KV2509	VFA0698::pKV214	10
KV2510	VF1833::pKV215	10
KV2636	VF1148::pTMB30	10
KV2637	VF1879::pKV216	10
KV2874	VFA0732::pKV208	10
KV3299	Δ*sypE*	This study
With reporter[a]		
KV3001	ES114 *att* Tn7::*psypA-lacZ*	This study
KV3395	KV1548 *att* Tn7::*psypA-lacZ*	This study
KV3396	KV1585 *att* Tn7::*psypA-lacZ*	This study
KV3398	KV1592 *att* Tn7::*psypA-lacZ*	This study
KV3399	KV1593 *att* Tn7::*psypA-lacZ*	This study
KV3397	KV1594 *att* Tn7::*psypA-lacZ*	This study
KV3400	KV1595 *att* Tn7::*psypA-lacZ*	This study
KV3419	KV1596 *att* Tn7::*psypA-lacZ*	This study
KV3420	KV1612 *att* Tn7::*psypA-lacZ*	This study
KV3421	KV1640 *att* Tn7::*psypA-lacZ*	This study
KV3422	KV1641 *att* Tn7::*psypA-lacZ*	This study
KV3429	KV1650 *att* Tn7::*psypA-lacZ*	This study
KV3430	KV1651 *att* Tn7::*psypA-lacZ*	This study
KV3423	KV1654 *att* Tn7::*psypA-lacZ*	This study
KV3424	KV1655 *att* Tn7::*psypA-lacZ*	This study
KV3425	KV1665 *att* Tn7::*psypA-lacZ*	This study
KV3426	KV1666 *att* Tn7::*psypA-lacZ*	This study
KV3427	KV1668 *att* Tn7::*psypA-lacZ*	This study
KV3428	KV1672 *att* Tn7::*psypA-lacZ*	This study
KV3431	KV1714 *att* Tn7::*psypA-lacZ*	This study
KV3432	KV1715 *att* Tn7::*psypA-lacZ*	This study
KV3532	KV1727 *att* Tn7::*psypA-lacZ*	This study
KV3433	KV1730 *att* Tn7::*psypA-lacZ*	This study
KV3232	KV1787 *att* Tn7::*psypA-lacZ*	This study
KV3508	KV1809 *att* Tn7::*psypA-lacZ*	This study
KV3521	KV2164 *att* Tn7::*psypA-lacZ*	This study
KV3509	KV2165 *att* Tn7::*psypA-lacZ*	This study
KV3510	KV2191 *att* Tn7::*psypA-lacZ*	This study
KV3518	KV2501 *att* Tn7::*psypA-lacZ*	This study
KV3519	KV2503 *att* Tn7::*psypA-lacZ*	This study
KV3520	KV2505 *att* Tn7::*psypA-lacZ*	This study
KV3533	KV2507 *att* Tn7::*psypA-lacZ*	This study
KV3534	KV2509 *att* Tn7::*psypA-lacZ*	This study
KV3535	KV2510 *att* Tn7::*psypA-lacZ*	This study
KV3547	KV2636 *att* Tn7::*psypA-lacZ*	This study
KV3548	KV2637 *att* Tn7::*psypA-lacZ*	This study
KV3522	KV2874 *att* Tn7::*psypA-lacZ*	This study
KV3620	KV3299 *att* Tn7::*psypA-lacZ*	This study

[a] Derivatives of these strains containing either pKG11 or pKV69 (or pEAH73, pKV276, pVSV105, pCLD48, or pCLD46, where indicated) were constructed and utilized as a part of this study.

where no pellicle was detected; "+" represents a very thin, easily disrupted pellicle; "++" represents a more cohesive (less easily disrupted) pellicle; and "+++" represents a thick pellicle that was difficult to disrupt.

β-Galactosidase assays. Samples were grown for 21 h in HMM-T. β-Galactosidase assays were conducted as described previously (21). The total amount of protein in each sample was assayed using Lowry assays (14), and β-galactosidase activity per mg protein in each sample was calculated.

RESULTS

Dependence of RscS on *V. fischeri* RRs for surface attachment. To identify RRs that function downstream of RscS, we introduced the RscS overexpression plasmid pKG11 or a vector control (pKV69) into each of 35 different RR mutants (10). Because RscS mediates biofilm formation in *V. fischeri*, we first assayed these strains for their abilities to attach to a glass surface using a CV stain to visualize biofilm-associated cells and cellular materials (see Materials and Methods). In control experiments, we found that the overexpression of RscS in wild-type cells enhanced the appearance of CV-stainable biofilm material (Fig. 1A) by 10-fold relative to the vector control (Fig. 1B). Upon a similar examination of the 35 RR mutant strains, we found that 29 of these mutants exhibited no significant differences in RscS-mediated glass attachment compared to vector controls (data not shown).

Of the remainder, only the RscS-overexpressing *sypG* mutant exhibited a biofilm phenotype indistinguishable from that of the vector control (Fig. 1). Because this phenotype is similar to the loss of RscS-induced biofilm formation that occurs upon the disruption of structural genes such as *sypN*, which encodes a putative glycosyltransferase (42, 43), we repeated our experiments using an in-frame deletion strain, Δ*sypG*. We found that the Δ*sypG* mutation also eliminated RscS-mediated attachment (Fig. 1). Additionally, the co-overexpression of both SypG and RscS in the Δ*sypG* mutant strain restored the glass attachment phenotype (data not shown). Thus, SypG functions downstream of RscS to facilitate attachment to a glass surface.

RscS-mediated attachment to glass was also altered by the loss of any of five other RRs (Fig. 1A). The loss of either VF0454, a putative homolog of the polysaccharide regulator VpsR (41), or the flagellar regulator FlrC resulted in decreased CV staining (2.5- and 2.3-fold, respectively) (Fig. 1). Mutations in *arcA*, VF1401, or *sypE* altered the pattern, but not the overall level, of staining (Fig. 1).

Because *sypE* is embedded in the *syp* cluster two genes upstream of *sypG*, we further assessed its specific role by constructing an in-frame deletion. The overexpression of RscS in the Δ*sypE* mutant resulted in a slightly diffuse pattern of CV-stained material, which did not differ quantitatively from $sypE^+$ cells (Fig. 1B). These results confirmed a minor role for SypE in RscS-mediated attachment to glass. Throughout the remainder of this work, we limited our studies to the Δ*sypE* strain.

Dependence of RscS on *V. fischeri* RRs for pellicle formation. Wild-type *V. fischeri* strains carrying pKG11 produced strong pellicles at the air-liquid interface of statically grown minimal medium cultures, while vector controls formed no detectable pellicle (Fig. 2A) (42). To identify the *V. fischeri* RRs that promote RscS-mediated pellicle formation, we grew the RR mutant strains carrying pKG11 or the vector control in minimal medium (HMM) for 48 h in static culture and assessed surface aggregation by dragging a sterile toothpick

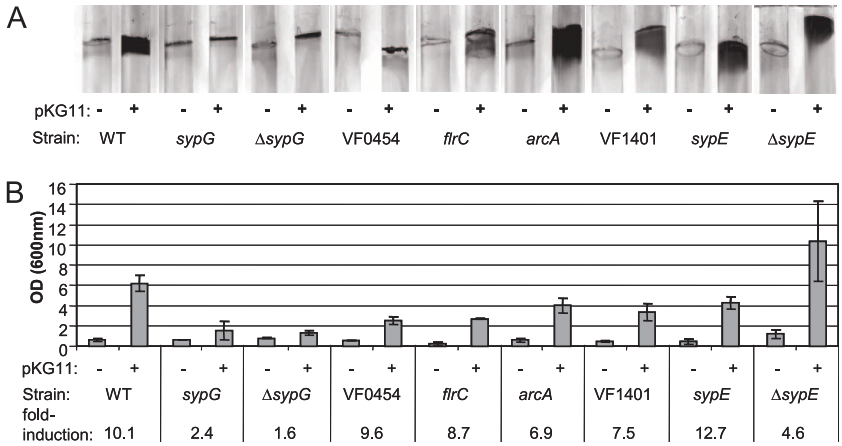

FIG. 1. RscS-mediated attachment to a glass surface in RR mutants. (A) Wild-type (WT) and RR mutant strains of *V. fischeri* carrying either RscS overexpression vector pKG11 (+) or the vector control, pKV69 (−), were grown statically in HMM containing glucose and Casamino Acids and stained with CV to visualize surface-attached material (representative photographs from an experiment conducted in triplicate). (B) Stain was removed by agitation with 1-mm glass beads and ethanol and quantitated by spectrophotometry. Induction represents the OD_{600} of a given strain carrying pKG11 divided by that of the same strain carrying the vector control.

through the culture surface (see Materials and Methods for a description of scoring). While 29 of the 35 RR mutant strains exhibited strong pellicle formation similar to that of the wild-type strain when multicopy RscS was present (data not shown), 6 displayed decreased pellicle formation or lacked pellicles entirely (Fig. 2). In particular, as observed with glass attachment assays, both *sypG* mutations (vector integration and in-frame deletion) abrogated RscS-mediated pellicle formation (Fig. 2A).

Disruption of five additional putative RRs resulted in decreased pellicle formation in RscS-overexpressing strains. Mutations in VF1401, *arcA*, VF0454, and *sypE* resulted in pellicles that were less dense and/or cohesive than those of RscS-overexpressing wild-type cells (Fig. 2A). The *flrC* mutant strain exhibited no detectable pellicle formation after 48 h (Fig. 2A). Previous reports suggested that biofilm formation is often delayed in nonmotile strains of bacteria (13). We therefore assayed pellicle formation after 72 h of incubation and found that pellicle formation by the *flrC* mutant increased; however, these pellicles remained less dense and less cohesive than those formed by the wild-type strain after either 48 or 72 h of incubation (Fig. 2B). These data indicate that *flrC* and/or motility influences RscS-mediated pellicle formation. Increased incubation time did not enhance pellicle formation by other RR mutant strains. Importantly, the *sypG* mutants did not form pellicles regardless of incubation time,

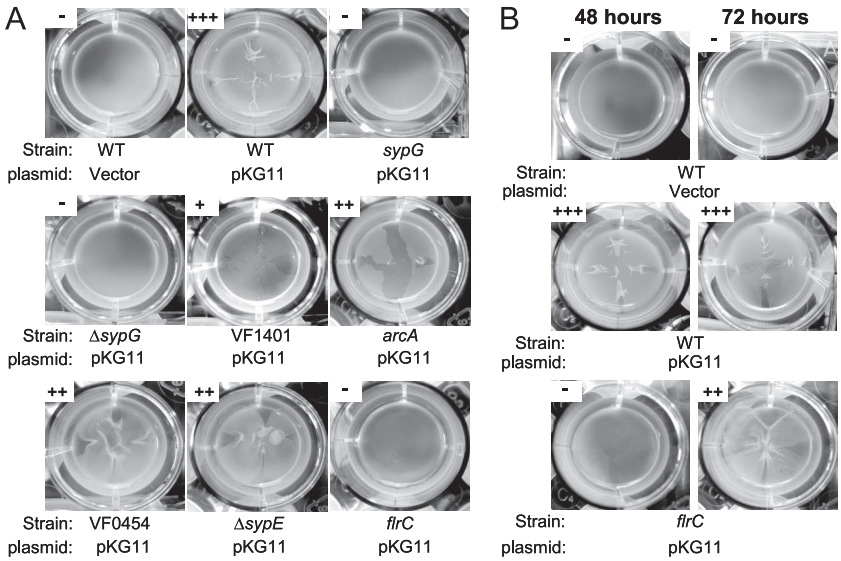

FIG. 2. RscS-mediated pellicle formation in RR mutants. Wild-type (WT) and RR mutant strains of *V. fischeri* carrying either pKG11 or a vector control (pKV69) were grown statically in HMM containing glucose and Casamino Acids for 48 h (A and B, as indicated) or 72 h (B). Pellicle formation was assessed by dragging a sterile toothpick through the culture surface and scored as described in Materials and Methods. Photographs are representative of samples from experiments conducted in triplicate.

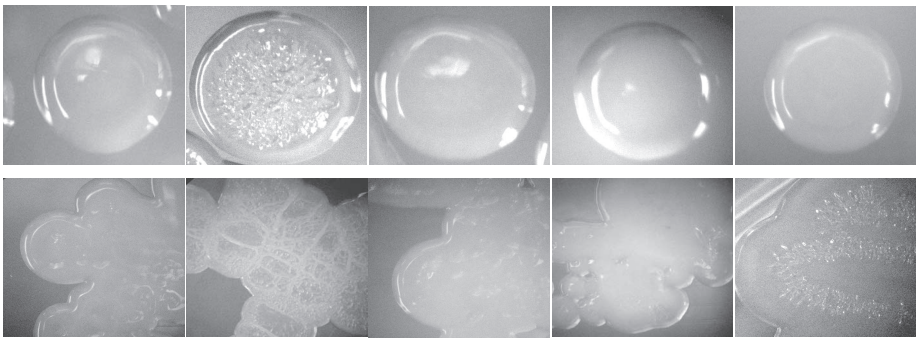

FIG. 3. RscS-mediated wrinkled-colony morphology in RR mutants. Wild-type (WT) and RR mutant strains of *V. fischeri* carrying either pKG11 or a vector control were streaked onto solid, complex medium (LBS with 0.3% glycerol and Tc) and allowed to grow for 3 days at room temperature. Photographs of individual colonies (top row) and also the heavy part of the streak (bottom row) were taken. Photographs are representative of at least three independent platings.

suggesting that *sypG* is absolutely required for this RscS-induced phenotype.

Dependence of RscS on *V. fischeri* RRs for wrinkled-colony morphology. Previous studies indicated that RscS-overexpressing *V. fischeri* cells formed colonies with a dry, wrinkled morphology (42). To identify the RR or RRs that control RscS-mediated wrinkled-colony formation, we examined the colonies formed on solid medium by each RR mutant carrying the vector control or pKG11. All mutants carrying the vector control formed smooth colonies that resembled those formed by the wild-type strain (Fig. 3 and data not shown). Wrinkled-colony formation induced by pKG11 occurred normally in 33 of the 35 RR mutants (data not shown). Only two mutations, in *sypG* and *sypE*, abrogated or reduced RscS-mediated wrinkled-colony formation.

Both *sypG* mutants exhibited completely smooth-colony morphology (Fig. 3). The wrinkling phenotype was restored when RscS and SypG were coexpressed in the Δ*sypG* mutant strain (data not shown), indicating that this RscS-mediated phenotype requires SypG. In contrast, the Δ*sypE* mutation resulted in a partial loss of RscS-mediated wrinkling. Individual *sypE* mutant colonies appeared smooth; however, the heavy part of the streak exhibited some dryness and wrinkling (Fig. 3). Thus, *sypE* plays an important role in, but is not completely required for, RscS-dependent wrinkling.

Dependence of RscS on *V. fischeri* RRs for *syp* transcription. All of the RscS-mediated biofilm phenotypes described above require the *syp* locus (42). Therefore, we assessed the effects of individual RR mutations on RscS-mediated *syp* transcription, measured via single-copy *sypA* promoter-*lacZ* fusions. Consistent with our previous studies (42), the multicopy expression of RscS from pKG11 caused a significant (75-fold) increase in *sypA* reporter activity in an otherwise wild-type background (Fig. 4).

Of the 35 RR mutants tested, 34 exhibited RscS-induced reporter activity at or above the level of the wild-type control (data not shown). The only exception was *sypG*: when RscS was overexpressed, the *sypG* vector integration and deletion mutants both exhibited significantly less reporter activity than did the wild-type strain (Fig. 4). Thus, not only is SypG the only RR required for all RscS-mediated biofilm phenotypes, it is also the only RR required for RscS to induce the expression of the *syp* cluster.

***sypG* overexpression in a *sypE* mutant mimics *rscS* overexpression.** Our results are consistent with the hypothesis that RscS functions upstream of SypG to induce *syp* transcription and biofilm formation. If the two regulators function together, then it might be expected that the two genes would induce similar phenotypes. Indeed, when overexpressed, SypG and RscS each induce *syp* transcription (43, 44). However, SypG overexpression, while promoting attachment to test tubes following growth under either static or shaking conditions (43), does not appear to cause wrinkled-colony formation or the production of strong pellicles (Fig. 5A and B, respectively). Based on these results, we formulated two hypotheses. First, SypG overexpressed in the absence of its SK might not be sufficiently activated (via phosphorylation) to induce the tran-

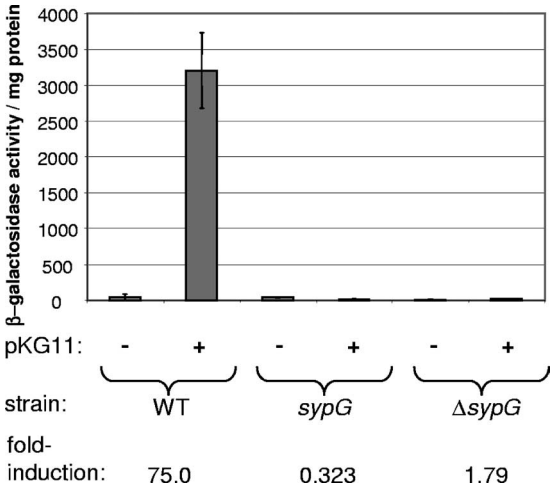

FIG. 4. RscS-mediated induction of *syp* transcription in RR mutants. Wild-type (WT) and RR mutant *sypA* reporter strains carrying either pKG11 or a vector control were grown with shaking in HMM-T at 22°C overnight. The level of transcription of the *sypA* reporter is reported as units of β-galactosidase activity per mg protein.

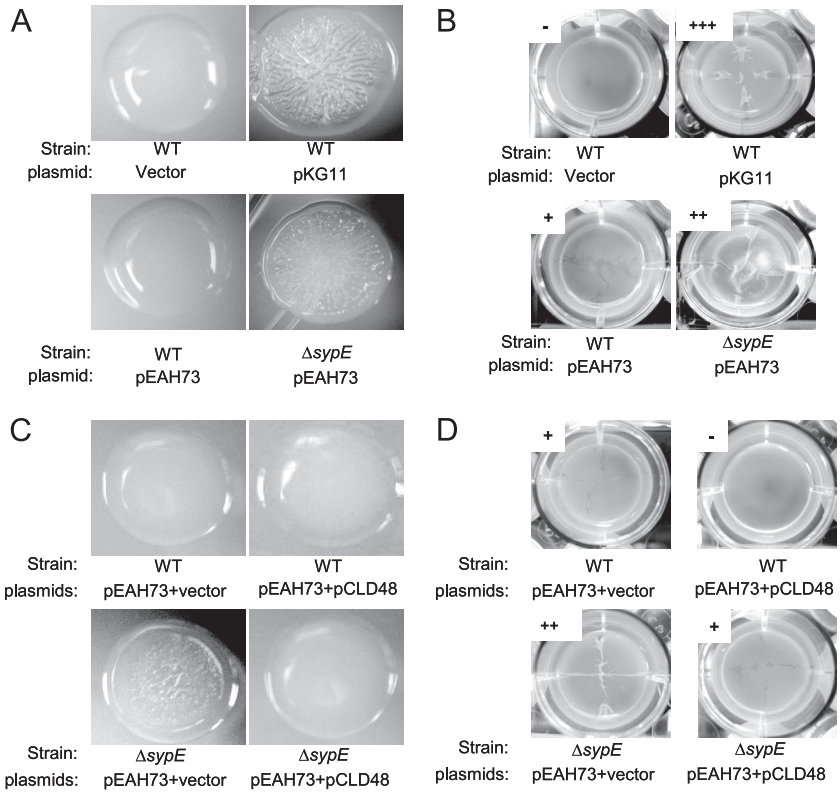

FIG. 5. SypG-mediated phenotypes in the absence of SypE. (A and C) Wild-type (WT) and Δ*sypE* mutant strains carrying the indicated plasmids were streaked onto solid, complex medium (LBS with 0.3% glycerol and Tc) and allowed to grow for 3 days at room temperature. Photographs are representative of samples from at least two independent platings. (B and D) Wild-type and Δ*sypE* mutant strains carrying the indicated plasmids were grown statically in HMM containing glucose and Casamino Acids at either 22°C (strains carrying the vector control and pKG11) or 28°C (strains carrying pEAH73). Pellicle formation was assessed by dragging a sterile toothpick through the culture surface and scored as described in Materials and Methods. Experiments were conducted in triplicate.

scription of the genes necessary for these phenotypes. Second, RscS could signal through more than one RR, either by activating an additional positive regulator or by inactivating a negative regulator, to induce the observed biofilm phenotypes. We favor the latter possibility, as our data thus far do not reveal another strong positive regulator of biofilm formation.

To distinguish these hypotheses, we sought a constitutively active allele of *sypG* through mutagenesis of the putative site of phosphorylation, aspartate 53. In other RRs such as CheY, NtrC, and LuxO, the substitution of Glu for Asp at that position has resulted in enhanced activity (7, 29, 30). The D53E substitution in SypG in fact resulted in a 3.5-fold increase in the SypG-mediated induction of the *sypA* promoter-*lacZ* reporter relative to that of wild-type SypG when expressed from a multicopy plasmid (data not shown). This allele did not, however, result in the appearance of wrinkled colonies or enhanced pellicles in wild-type *V. fischeri*.

Further attempts to clarify the roles of the various *syp* regulators, however, yielded an unexpected result: the overexpression of wild-type SypG (from pEAH73) in a Δ*sypE* mutant strain resulted in wrinkled colonies (Fig. 5A). These wrinkled colonies resembled those formed by RscS-overexpressing wild-type cells. Furthermore, we found that the Δ*sypE* strain carrying pEAH73 was capable of forming thick pellicles (Fig. 5B). To confirm that SypE inhibits SypG-mediated phenotypes, we complemented the Δ*sypE* mutation with *sypE* expressed from pCLD48, which is compatible with the SypG expression vector pEAH73. The co-overproduction of SypG and SypE in the Δ*sypE* strain restored smooth-colony morphology and weak pellicle formation (Fig. 5C and D, respectively), phenotypes similar to those of wild-type *V. fischeri* carrying pEAH73 and a vector control (pVSV105). Furthermore, the co-overproduction of SypE and SypG in the wild-type strain eliminated the formation of weak pellicles induced by the overexpression of SypG alone (Fig. 5D). These data reveal that phenotypes induced by SypG overexpression can mimic those induced by RscS, a result fully consistent with the hypothesis that the two regulators function together. They also support a model in which SypE is antagonistic to SypG, demonstrating the complexity of control over *syp*-dependent biofilm formation in *V. fischeri*.

DISCUSSION

The *V. fischeri* SK RscS was discovered as a determinant of colonization of the host squid, *Euprymna scolopes* (37). RscS regulates the expression of the *syp* cluster of genes and promotes cell-cell aggregation outside the squid light organ, an early event in colonization (42). Importantly, this biofilm-like behavior correlates with cell-cell aggregation phenotypes ob-

served in cells overexpressing RscS in culture (42). Until now, the identity of the downstream RR(s) in the two-component pathway represented by RscS has remained unclear.

To address this problem, we conducted epistasis experiments to identify the regulator(s) that functions downstream of RscS. Our data indicate that a mutation of six different RRs diminished RscS-mediated liquid biofilm phenotypes (i.e., pellicle formation and attachment to a glass surface): *arcA*, *sypE*, *sypG*, *flrC*, VF1401, and VF0454. Of these, only the loss of *sypG* completely eliminated the enhancement of liquid biofilms by RscS. The disruption of *sypG* also eliminated RscS-mediated wrinkled-colony formation. Only one other mutation, in the other *syp* cluster RR gene, *sypE*, diminished (but did not eliminate) the ability of RscS to promote wrinkling. Finally, the mutation of *sypG* alone eliminated the ability of RscS to induce the expression of a *syp* reporter.

Our data thus provide compelling genetic evidence that RscS and SypG function within the same two-component pathway. First, both RscS and SypG are required for the initiation of squid colonization (10, 37). Second, the overexpression of either RscS or SypG induces *syp* transcription and biofilm phenotypes (42, 43). Third, SypG is the only one of the 35 putative RRs tested that is required for all known functions of RscS.

Very few studies have established partnerships between orphan SKs and RRs. Some examples include UvrY and BarA in *E. coli* (23), ArcB and RssB in *E. coli* (20), DosT and DosR in *Mycobacterium tuberculosis* (25, 28), and CenK and CenR in *Caulobacter crescentus* (31). Often, in vitro phosphotransfer studies are utilized as evidence for such partnerships (31). Despite repeated attempts, both RscS and SypG proved difficult to purify, resulting in either low yields of protein (in the case of RscS) or aggregated, possibly unfolded protein (SypG) (E. A. Hussa and K. L. Visick, unpublished data). Thus, phosphotransfer experiments could not be performed. Due to the lack of biochemical data, it remains formally possible that RscS does not directly donate a phosphoryl group to SypG. There are at least four additional putative *V. fischeri* RRs that we were unable to disrupt in our previous mutagenesis study (10), one or more of which may function in the RscS pathway. Also, there is an additional *V. fischeri* RR, GacA, which was not considered in this study; mutation of GacA results in a growth yield defect that makes biofilm-related phenotypes difficult to study (39). However, the genetic evidence presented here demonstrates that RscS activity requires SypG; therefore, we assert that these additional RRs are likely not major components of the RscS pathway. There are also examples in which alternative paths of phosphotransfer are followed, such as the *E. coli* Rcs system, in which the RcsC kinase donates a phosphoryl group to an Hpt domain within an intermediate protein, RcsD (34). Intriguingly, encoded just upstream of *sypG* is a hybrid SK, SypF. However, unlike the loss of *sypG*, the disruption of *sypF* does not eliminate RscS-mediated wrinkled-colony formation (C. L. Darnell and K. L. Visick, unpublished data). Therefore, we think that it is unlikely that a substantial amount of RscS-initiated activation occurs via SypF.

It is abundantly clear, however, that the regulation of *syp*-dependent biofilms is complex. In support of this idea, in this study, we determined that the overexpression of SypG in a *sypE* deletion strain results in the formation of wrinkled colonies and pellicles mimicking those produced by wild-type strains overexpressing RscS. We interpret these data as further support of our model that RscS and SypG function in the same pathway. However, these results generate additional questions. For example, if SypE is required for the full expression of RscS-mediated biofilm phenotypes, then why does its loss allow biofilm formation to be induced by SypG overexpression?

SypE is not a typical RR: its phosphate-accepting receiver (REC) domain is centrally located and is not adjacent to a known DNA binding domain. Instead, C terminal to the REC domain is a putative protein phosphatase 2C domain (2) predicted to function as a serine phosphatase. N terminal to the REC domain is a region with weak similarity to the *Bacillus subtilis* RsbW protein, which acts as a serine kinase (5). If these domains function as predicted, it is possible that the phosphorylation of SypE could modulate its ability to serve as either a phosphatase or a kinase. Thus, we hypothesize that RscS may also function upstream of SypE in a manner that negates its antagonism of SypG-mediated phenotypes. In this model, the overexpression of RscS would both activate SypG and inactivate SypE, ultimately inducing wrinkled-colony formation. In contrast, the overexpression of SypG alone would not be sufficient to prevent SypE-mediated antagonism; thus, wrinkled-colony formation occurs only in the absence of SypE. A further understanding of how SypE functions awaits specific, mechanistic characterization of this protein and its unusual domains through mutagenesis studies.

This work also revealed roles for four additional RRs in *V. fischeri* biofilm formation (i.e., glass attachment and pellicle formation): FlrC, ArcA, VF0454, and VF1401. The first three of these RRs were previously shown to be involved in biofilm formation in other bacteria (10, 11, 13, 24, 35). In particular, VF0454 encodes a protein with high sequence identity to the *Vibrio cholerae* exopolysaccharide regulator VpsR; a distinct study from our laboratory has also uncovered an important role for VF0454 in biofilm formation (3a). Little is known about the final *V. fischeri* biofilm regulator, VF1401, except that it is required for competitive colonization and belongs to the family of σ^{54}-dependent transcriptional activators (10). At this time, we cannot determine whether these four RRs function specifically downstream of RscS or whether they have independent effects on biofilm formation, as wild-type strains of *V. fischeri* (i.e., strains not overexpressing *syp*) do not form robust biofilms in culture (40, 42, 43).

In summary, we have shown that the SK RscS functions primarily upstream of the RR SypG as a major two-component pathway involved in *V. fischeri* biofilm formation. This RscS/SypG-mediated signaling, however, is likely to be complex, involving at least one additional *syp* regulator, SypE, which appears to severely inhibit SypG-mediated biofilm formation. In addition, we have uncovered at least four other *V. fischeri* two-component regulators that feed into biofilm formation: FlrC, VF0454, ArcA, and VF1401. This work has thus provided a basis for understanding the complex control of biofilm formation in *V. fischeri*.

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