Inhibition of SypG-Induced Biofilms and Host Colonization by the Negative Regulator SypE in Vibrio fischeri

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Abstract

*Vibrio fischeri* produces a specific biofilm to promote colonization of its eukaryotic host, the squid *Euprymna scolopes*. Formation of this biofilm is induced by the sensor kinase RscS, which functions upstream of the response regulator SypG to regulate transcription of the symbiosis polysaccharide (*syp*) locus. Biofilm formation is also controlled by SypE, a multi-domain response regulator that consists of a central regulatory receiver (REC) domain flanked by an N-terminal serine kinase domain and a C-terminal serine phosphatase domain. SypE permits biofilm formation under *rscS* overexpression conditions, but inhibits biofilms induced by overexpression of *sypG*. We previously investigated the function of SypE in controlling biofilm formation induced by RscS. Here, we examined the molecular mechanism by which SypE naturally inhibits SypG-induced biofilms. We found that SypE’s N-terminal kinase domain was both required and sufficient to inhibit SypG-induced biofilms. This effect did not occur at the level of *syp* transcription. Instead, under *sypG*-overexpressing conditions, SypE inhibited biofilms by promoting the phosphorylation of another *syp* regulator, SypA, a putative anti-sigma factor antagonist. Inhibition by SypE of SypG-induced biofilm formation could be overcome by the expression of a non-phosphorylatable SypA mutant, indicating that SypE functions primarily if not exclusively to control SypA activity via phosphorylation. Finally, the presence of SypE was detrimental to colonization under *sypG*-overexpressing conditions, as cells deleted for *sypE* outcompeted wild-type cells for colonization when both strains overexpressed *sypG*. These results provide further evidence that biofilm formation is critical to symbiotic colonization, and support a model in which SypE naturally functions to restrict biofilm formation, and thus host colonization, to the appropriate environmental conditions.

Introduction

Bacterial biofilms, or surface-associated communities of cells encapsulated in an extracellular matrix, are ubiquitous in the environment and likely represent the preferred lifestyle mode for many bacterial species [1]. The formation of a biofilm can impact multiple aspects of a bacterium’s physiology, including sensitivity to antibiotics and other antimicrobials [2], cellular metabolism [3], and gene expression [4]. Biofilms have also been demonstrated to play critical roles in mediating interactions between bacteria and their eukaryotic hosts [5,6].

The symbiotic association between the marine bacterium *Vibrio fischeri* and its host the Hawaiian squid *Euprymna scolopes* provides a natural model to study the bacterial processes necessary to promote host colonization [7,8,9]. Importantly, one of the earliest stages of the colonization process involves the formation of a specific biofilm or bacterial aggregate on the surface of the squid’s symbiotic light organ [10]. Formation of this biofilm requires the symbiosis polysaccharide (*syp*) locus, which is regulated at the transcriptional level by the sensor kinase (SK) RscS and the *syp*-encoded response regulator (RR) SypG [5,11,12]. As a result, both *rscS* and *sypG* are required for initiation of host colonization [12,13]. The current model predicts that, upon detection of an as-yet unidentified signal(s), RscS autophosphorylates and serves as a phosphodonor to ultimately activate the RR SypG [12] (Fig. 1). Phosphorylated SypG is thought to directly promote transcription of the individual *syp* operons, which encode the structural genes necessary for polysaccharide production and thus biofilm formation [5,14].

The natural signal(s) that activate RscS remain unknown, and thus conditions under which wild-type cells form *syp*-dependent biofilms, other than during symbiosis, have not been established. However, overexpression of either *rscS* or *sypG* can promote the production of biofilms in laboratory culture, and provides a powerful tool to dissect the regulatory components involved in this signaling pathway. Overexpression constructs have been similarly employed in a variety of bacterial systems in which the natural signals remain unidentified, such as *Vibrio cholerae* and *Salmonella enterica* serovar Typhimurium [15,16,17]. The overexpression of *rscS* in wild-type cells results in the activation of *syp* transcription and *syp*-dependent biofilm formation, in particular the formation of wrinkled colonies on solid agar media and pellicles in static liquid cultures [11,12]. The formation of these biofilms depends on an intact, functional SypG [12].
overexpression also results in increased symbiotic aggregation, which correlates with a colonization advantage over wild-type cells [11].

Intriguingly, overexpression of *sypG* in wild-type cells similarly induces activation of the *syp* locus, but fails to promote biofilm phenotypes as observed for RscS [12]. In a previous study, Hussa et al. (2008) demonstrated that the inability of *sypG* overexpression to induce *syp* biofilm formation was due to the inhibitory activity of a second *syp*-encoded RR, SypE; overexpression of *sypG* in a Δ*sypE* mutant resulted in dramatic biofilm formation similar to that observed with *rscS*-overexpressing wild-type cells [12]. Complementation with a wild-type allele of *sypE* fully restored the wild-type phenotype.

Figure 1. Model of inhibition of SypG-induced biofilms by SypE. (A) Schematic of the domain structure of SypE and select SypE mutants. SypE contains a central regulatory receiver (REC) domain flanked by a N-terminal HPK-like serine kinase domain and a C-terminal PP2C-like serine phosphatase domain. The black lines represent select SypE mutants containing the indicated protein domains. (B) When *sypG* is overexpressed, transcription of the *syp* locus is activated, resulting in the production of Syp structural proteins necessary for polysaccharide production and biofilm formation, as well as the production of regulatory proteins SypA and SypE. Our data here show that, under these conditions, SypE functions as a serine kinase and phosphorylates the downstream target protein, the putative anti-sigma factor antagonist protein SypA. Phosphorylated SypA is inactive to promote biofilm formation and host colonization. In contrast, when *rscS* is overexpressed, the kinase activity of SypE is inactivated (presumably through phosphorylation of SypE’s REC domain); instead, it functions as a serine phosphatase to dephosphorylate SypA, which promotes biofilm formation and colonization through an unknown mechanism. Co-overexpression of *sypG* and *sypA* (not shown) leads to biofilm formation, presumably because high levels of SypA permit some SypA to escape phosphorylation and inactivation by SypE.
type, non-biofilm-forming phenotypes [12]. Two main conclusions can be drawn from these studies: 1) RscS and SypG both function to induce biofilm formation, and 2) RscS may have an additional function in controlling SypE activity that SypG lacks.

SypE is a multi-domain RR consisting of a central RR receiver (REC) domain flanked by an N-terminal, serine kinase domain and a C-terminal, serine phosphatase domain (Fig. 1A) [18,19,20]. The mechanism by which SypE naturally inhibits biofilms under SypG-inducing conditions has not yet been investigated. However, we recently characterized the role of SypE in biofilms formed when rscS is overexpressed. Under these conditions, deletion of sypE slightly, but reproducibly delayed the formation of wrinkled colonies. This phenotype could be complemented by expression of the full-length SypE protein from an exogenous location in the chromosome. In addition, it could be complemented by expression of SypE’s C-terminal, serine phosphatase domain alone. Surprisingly, however, expression of SypE’s N-terminal kinase domain alone not only failed to complement, but in fact fully inhibited biofilms induced by rscS overexpression [19]. These data support the idea that RscS must modulate the activity of SypE such that it functions primarily to promote biofilm formation.

These studies also predicted that SypE regulates biofilms by controlling the phosphorylation state of a downstream regulatory protein. That downstream regulator has since been identified as SypA, a putative anti-sigma factor antagonist (Fig. 1B) [20]. Under rscS-overexpressing conditions, SypA is largely unphosphorylated and promotes biofilm formation [20]. Furthermore, SypA is largely phosphorylated, even when rscS is overexpressed, when the cells express a mutant SypE protein (SypED192A) that constitutively inhibits biofilm formation. SypE thus mediates control over biofilms induced by rscS overexpression by controlling the phosphorylation state of SypA [20].

These previous studies investigated the regulation of biofilms induced by overexpression of rscS, a condition in which SypE functions as a positive regulator. Our goal here was to further our understanding of SypE function by determining whether its ability to naturally inhibit biofilm formation induced by sypG overexpression occurs through mechanisms similar to that observed with RscS overexpression. Indeed, we obtained compelling evidence that under sypG overexpressing conditions, SypE functions as a kinase to inhibit biofilm formation: (1) SypE’s N-terminal kinase domain was both required and sufficient to mediate inhibition of SypG-induced biofilms and (2) SypE inhibits biofilms in sypG-overexpressing cells by promoting phosphorylation, and thus inactivation, of the regulatory protein SypA. In addition, we further extend our understanding of SypE function by determining that the impact of SypE occurs at a level downstream of syp transcription. Finally, we find that the ability of SypE to inhibit biofilm formation in laboratory culture is mirrored in its ability to inhibit symbiotic colonization of the squid host. Together, these data demonstrate that the negative regulation of SypG-induced biofilms by SypE is a critical mechanism by which V. fischeri restricts host colonization.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Wild-type (WT) V. fischeri ES114 [21] was used as the parent strain for these studies. Strains derived from ES114 and plasmids utilized in this study are listed in Tables 1 and 2, respectively. E. coli strains TAM1 (Active Motif, Carlsbad, CA), TOP10 F (Invitrogen, Carlsbad, CA), and GT115 (InvivoGen, San Diego, CA) were used for cloning. To generate Tn7 insertions in V. fischeri, tetraparental matings were carried out as previously described [22]. To generate the sypG overexpression plasmid pCLD56, restriction digest was utilized to remove the CmR cassette from plasmid pEAH73 [12]. To generate the various sypE and sypA alleles, we performed PCR using the primers listed in Table 3. The resulting PCR products were cloned into the cloning vector pET1.2 (Fermentas, Glen Burnie, MD) and then subcloned into mobilizable plasmid pVS105 [23], pKV282 [19], or pEV5107 [22]. V. fischeri strains were grown in LBS [24] or sea water tryptone (SWT) [5]. Escherichia coli strains were grown in LB [25] or brain heart infusion medium (Difco, Detroit, MI). The following antibiotics were added to V. fischeri media, where necessary, at the indicated concentrations: chloramphenicol (Cm) 2.5 μg mL⁻¹, erythromycin (Em) at 5 μg mL⁻¹, and tetracycline (Tc) at 5 μg mL⁻¹ in LBS and 30 μg mL⁻¹ in SWT. The following antibiotics were added to E. coli media, where necessary, at the indicated concentrations: Cm at 25 μg mL⁻¹, kanamycin (Km) at 50 μg mL⁻¹, Tc at 15 μg mL⁻¹, or ampicillin (Ap) at 100 μg mL⁻¹. For solid media, agar was added to a final concentration of 1.5%.

Wrinkled Colony Assays

To observe wrinkled colony formation, the indicated V. fischeri strains were streaked onto LBS agar plates containing Tc and Cm overnight. Single colonies were subsequently cultured with shaking in LBS broth with Tc at 28°C overnight and then sub-cultured to an optical density at 600 nm (OD₆₀₀) of 0.2 in 5 mL of fresh medium. Cells were spun down, washed twice in 70% artificial seawater (ASW), and re-suspended in 70% ASW and diluted to an OD of 0.2. 10 μL of re-suspended cultures were spotted onto LBS agar plates and grown overnight at 28°C [26]. Images of spotted cultures were acquired at the indicated time points using the Zeiss Stemi 2000-C dissecting microscope as previously described [26].

Static Pellicle Assays

Strains were grown with shaking in LBS containing Tc and Cm at 28°C overnight and then subcultured to an OD of 0.1 in 1.5 mL of fresh medium in 24-well microtiter dishes. Cultures were then incubated at 28°C for 48 h. The strength of each

Table 1. V. fischeri strains used in this study.

| Strain | Relevant Genotype | Source or Reference |
|--------|-------------------|---------------------|
| ES114  | Wild-type V. fischeri | [21] |
| K3245  | attTn7::sypA-lacZ emR | This study |
| K3299  | ΔsypE | [12]. |
| K4389  | attTn7::emR | [19] |
| K4390  | ΔsypE attTn7::emR | [19] |
| K4717  | ΔsypA | [20] |
| K4716  | ΔsypA ΔsypE | [20] |
| K4819  | ΔsypE attTn7::sypA-lacZ emR | [19] |
| K4926  | ΔsypE attTn7::sypA-lacZ emR | This study |
| K4759  | ΔsypA attTn7::sypA-lacZ emR | [20] |
| K5481  | ΔsypA attTn7::sypAΔOSA emR | [20] |
| K5649  | ΔsypA ΔsypE attTn7::sypA-lacZ emR | [20] |
| K6578  | ΔsypE attTn7::sypA-HA emR | This study |
| K6579  | ΔsypA attTn7::sypAΔOSA-HA emR | This study |
| K6580  | ΔsypA ΔsypE attTn7::sypA-HA emR | This study |

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pellicle was evaluated by disrupting the air-liquid interface with a sterile pipette tip after 48 h of incubation. Cultures with little/no pellicle were scored as (–); cultures with a strong pellicle that remained intact following disruption were scored as (+). Images of pellicles were acquired at the indicated time points using a Zeiss Stemi 2000-C dissecting microscope.

SypE FLAG Protein Expression

FLAG epitope fusions were generated to the C-terminus of the sypEDNT and CTD mutants using the reverse primer 921 and the forward primers 868 and 910, respectively (Table 3). The FLAG-sypE alleles were cloned into the expression vector pVSV105 and subsequently conjugated into ΔsypE cells containing the sypG expressing plasmid (pCLD56). Strains were cultured in LBS containing Tc and Cm for 18 h at 28°C. 1 mL of overnight culture was pelleted by centrifugation, lysed in 500 μL 2X SDS

Table 2. Plasmids used in this study.

| Name      | Description                  | Relevant primers | Source or Reference |
|-----------|------------------------------|------------------|---------------------|
| pARM3     | pVSV105+1.2 kb SypEDNT, cmR  | 256, 868         | This study          |
| pARM4     | pVSV105+sypE NT2A, cmR        | 849, 876         | This study          |
| pARM7     | RscS overexpression construct; tetR | N/A              | [19]                |
| pARM9     | pVSV105+1.6 Kb sypG; cmR     | 393, 559         | This study          |
| pARM13    | pKV282+770 bp sypA; tetR     | N/A              | [20]                |
| pARM35    | pKV282+770 bp sypA-FLAG; tetR| N/A              | [20]                |
| pARM111   | pVSV105+700 bp sypEDNT-FLAG; cmR | 910, 921        | This Study          |
| pARM162   | pVSV105+1.2 kb SypEDNT-FLAG; cmR | 868, 921        | [20]                |
| pARM163   | pEV5107+1.1 kb sypA-HA; kanR, ermR | 1040, 806      | This study          |
| pARM164   | pEV5107+1.1 kb sypA NT2A-HA; kanR, ermR | 1040, 806      | This study          |
| pCLD48    | pVSV105+1.5 Kb sypE; cmR     | N/A              | [12]                |
| pCLD56    | pKV282+1.6 Kb sypG; tetR     | 393, 559         | This study          |
| pCLD64    | pVSV105+500 bp sypEDNT; cmR  | 461, 911         | This study          |
| pCLD65    | pVSV105+500 bp sypEDNT, N12A; cmR | 461, 911        | This study          |
| pCLD67    | pVSV105+700 bp sypEDNT; cmR  | 256, 910         | This study          |
| pEAH90    | pEV5107+ PsypA-lacZ           | N/A              | [26]                |
| pEV5104   | Conjugal helper plasmid (tra trb), kanR | N/A            | [36]                |
| pEV5107   | Mini-Tn7 delivery plasmid; mob; kanR, emR | N/A            | [22]                |
| pKV282    | Mobilizable vector; tetR     | N/A              | [19]                |
| pVSV105   | Mobilizable vector; cmR      | N/A              | [23]                |

1 Relevant primers for constructs generated in this study.

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Table 3. Primers used in this study.

| Primer name | Sequence 1 |
|-------------|------------|
| 256         | ttttctgcacTATTGATTCATCAATTACAGC |
| 393         | GCTACACTTTACATAGACGC |
| 461         | CATATGGCATGCTGATCC |
| 559         | ggtgcatCATCTTGAGTCATCTCATAG |
| 806         | AGCTTTCTCTATTAATGATAGT |
| 849         | CCTGTGAGAAATTATCCAGC |
| 868         | GTGGTGTAATCTGAGGCGTCTCCCTCCCAT |
| 876         | TCTGAATGGACGACGcTCTGTTGTCACCT |
| 910         | GTGGTGTAATCTGAGGCTACTTATTACACAA |
| 911         | CTTAATGGGAAAGGGACCTC |
| 921         | acccggttatattcattatcatcttattataATCTTTATCTCCTTATATTACAG |
| 1040        | acccggttatattcattatcatcttattataATCCGGTATTTATATTACAGG |

1 Non-native sequences are shown in lower case letters.

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sample buffer (4% SDS, 40 mM Tris pH 6.3, 10% glycerol), and resolved on 15% SDS-PAGE gels. Protein transfer to PVDF membrane was performed using standard Tris-Glycine transfer buffer (20% MeOH, 50 mM Tris, 40 mM glycine). Western blotting was performed using standard protocols with rabbit anti-FLAG primary antibody (Sigma-Aldrich, St. Louis, MO) and donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). Blots were imaged by chemiluminescent detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Inc., Rockford, IL).

Analysis of SypA Phosphorylation Using Phos-tag™ Acrylamide

Hemagglutinin (HA) and FLAG-epitope fusions to the C-terminus of SypA were generated using the primers listed in Table 3. The sypA-HA alleles were cloned into the Tn7 delivery plasmid pEV8107 [22] (Table 2). The resulting plasmids were used to introduce the sypA-HA alleles at the Tn7 site of the ΔsypA and ΔsypA ΔsypE strains. Subsequently, the sypG overexpression plasmid pCLD56 was introduced. The resulting V. fischeri strains were streaked onto LBS agar plates containing Tc and several colonies were then cultured overnight in LBS containing Tc at 28°C with shaking. Aliquots of cells (1 mL) were spun down, washed twice with 1X PBS, and standardized to the same amounts using OD600 measurements. Samples were lysed in 2X SDS sample buffer and resolved on SDS-PAGE gels containing 30 μM Phos-tag™ acrylamide (WAKO Chemicals, Richmond, VA) and 50 μM MnCl2. We observed some variation in the extent to which phospho-SypA migration was retarded relative to SypA, perhaps due to aging/integrity of the Phos-tag™ acrylamide. Gels were fixed for 15 min in standard transfer buffer containing 1 mM EDTA to remove Mn2+ from the gel. Gels were incubated for an additional 20 min in transfer buffer without EDTA. Proteins were transferred to a PVDF membrane and the proteins were detected by western blot analysis using an anti-HA antibody (Sigma-Aldrich, St. Louis, MO).

Similar approaches were used to assess SypA phosphorylation under overexpressing conditions with the exceptions that (1) two antibiotics were added (Cm and Tc) throughout to maintain the two sypG- and sypA-overexpressing plasmids and (2) the syp4 alleles were FLAG-tagged and thus detected using an anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO).

β-galactosidase Assays

The indicated strains were grown (triplicate) with shaking in LBS containing Tc and Cm at 28°C overnight and then subcultured into fresh medium and grown for up to 24 h. Aliquots (1 mL) of cells were removed at 8 h and 24 h post-inoculation, concentrated, resuspended in Z-buffer, and lysed. The β-galactosidase activity [27] and total protein concentration [28] of each sample were assayed. β-galactosidase units are reported as units of activity per mg of protein.

Squid Colonization Assays

Experiments involving E. scolopes animals were carried out using approaches described in an Animal Component of Research Protocol (ACORP) approved by Loyola University’s Institutional Animal Care and Use Committee (IACUC) (LU #107314, 201297). To perform competitive colonization assays, juvenile squid were placed into artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) containing approximately 1,000 V. fischeri cells per mL of seawater. Juvenile squid were inoculated with an approximate 1:1 ratio of mutant and wild-type cells, and colonization was allowed to proceed for 18 h. For these assays, ΔsypE cells were marked with an erythromycin resistance (EryR) cassette within the chromosome at the Tn7 site. Reciprocal experiments were also performed in which wild-type cells contained the EryR marker. The ratio of bacterial strains within the light organs of the animals was assessed through luminescence and homogenization/plating assays as described previously [11]. The competitive colonization data are reported as the Log-transformed Relative Competitive Index (Log RCI).

Results

SypE’s N-terminal Domain is Necessary and Sufficient to Inhibit SypG Biofilms

To better understand the mechanism by which SypE inhibits SypG-induced biofilm formation, we utilized a complementation approach. Briefly, we generated a set of plasmids with sypE alleles that contained or lacked the individual SypE domains (Fig. 1A). We then co-expressed sypG with the various sypE alleles in the ΔsypE mutant and assessed the resulting biofilm phenotypes. As previously reported, wild-type cells failed to produce SypG-induced biofilms, indicated by the smooth colony morphology on solid agar media and little to no pellicle formation in static liquid culture (Fig. 2A and 3A) [12]. In contrast, ΔsypE cells exhibited dramatic SypG-induced biofilm phenotypes, including wrinkled colony formation and robust pellicle formation (Figs. 2B and 3B, respectively) [12]. Co-expression of wild-type sypE fully complemented the sypE mutant and restored inhibition of biofilms, resulting in smooth colony morphology and lack of robust pellicle formation similar to wild-type cells (Fig. 2C and 3C, respectively).

We next examined the regulatory activity of a SypE variant (SypE<sup>NTD</sup>; Fig. 1A) that lacks the N-terminal 135 amino acids, and found that it failed to complement the sypE mutant, but instead permitted wrinkled colony formation (Fig. 2D) and pellicle formation similar to vector-containing cells (Figs. 2B and 3B, respectively). Similarly, expression of the C-terminal domain alone (SypE<sup>CTD</sup>; Fig. 1A) failed to complement the sypE mutant and inhibit biofilm formation (Figs. 2E and 3E). These results indicate that inhibition of SypG-induced biofilms requires the N-terminal domain of SypE. We previously reported that both the SypE<sup>NTD</sup> and SypE<sup>CTD</sup> alleles were capable of promoting biofilms induced by rscS overexpression, indicating the resulting proteins were produced and functional [19]. Western blot analyses also confirmed that the SypE<sup>NTD</sup> and SypE<sup>CTD</sup> proteins were indeed stably produced (Fig. 2f). These results indicate that inhibition of SypG-induced biofilms requires the N-terminal domain of SypE.

To further investigate the potential inhibitory role of the N-terminal domain, we expressed SypE<sup>NTD</sup>, which contains only the 140 amino acids of the N-terminal domain of SypE (Fig. 1A). We found that SypE<sup>NTD</sup> fully complemented the sypE mutant, restoring inhibition of both wrinkled colony and pellicle formation (Fig. 2F and 3F, respectively). Together, these results demonstrate that the N-terminal domain of SypE is both necessary and sufficient to inhibit SypG-induced biofilm formation.

Inhibitory Activity of the N-terminal Domain Requires Conserved Asparagine Residue N52A

The N-terminal domain of SypE exhibits sequence similarity to HPK (histidine protein kinase)-like serine kinases [18]. Importantly, SypE contains a conserved asparagine residue (N52) [18], which in HPK-like serine kinases participates in Mg<sup>2+</sup> ion coordination and the binding of ATP to the nucleotide pocket [29]. In several characterized HPK-like serine kinases, mutagenesis of this asparagine residue results in the loss of serine kinase activity [29]. In several characterized HPK-like serine kinases, mutagenesis of this asparagine residue results in the loss of serine kinase activity [29].
Similarly, we found that this conserved asparagine was required for a mutant SypE variant to inhibit biofilms induced by RscS [19]. To ask whether the ability of wild-type SypE to inhibit SypG-induced biofilms also required this conserved asparagine, we assessed the inhibitory activity of a SypE mutant carrying an alanine substitution at this site (SypEN52A). We found that expression of SypE N52A failed to complement the ΔsypE mutant: the colonies exhibited strong SypG-induced wrinkling and pellicle formation (Figs. 2G and 3G). Similarly, this mutation in the context of the inhibitory N-terminal domain alone (SypENTD, N52A) resulted in the complete loss of inhibitory activity: cells co-expressing ΔsypE NTD, N52A and sypG exhibited both wrinkled colony morphology and pellicle formation (Figs. 2H and 3H, respectively). The failure to complement cannot be attributed to protein instability, as epitope-tagged versions of both the SypEN52A and SypENTD, N52A proteins are stably expressed [19].

Figure 2. Regulation of SypG-induced wrinkled colony formation by SypE. The SypG expression plasmid (pCLD56) was introduced into either wild-type cells [A] or ΔsypE mutant cells [KV3299] carrying either empty vector (pVSV105) [B] or the indicated SypE-complementing plasmids: full-length SypE (pCLD48)[C], SypE<sup>NTD</sup> (pARM3)[D], SypE<sup>CTD</sup> (pCLD67)[E], SypE<sup>NTD</sup> (pCLD64)[F], SypE<sup>N52A</sup> (pARM4)[G], SypE<sup>NTD</sup>, N52A (pCLD65)[H]. Cultures were spotted onto agar plates and wrinkled colony morphology was assessed at 48 h post-spotting. Images are representative of at least three independent experiments. [I] Expression of FLAG epitope-tagged SypE mutant proteins was assessed by western blot analysis. Plasmids expressing FLAG-sypE<sup>CTD</sup> (pARM111; lane 1) or sypE<sup>NTD</sup> (pARM162; lane 2) alleles were introduced into the ΔsypE strain containing the sypG overexpression plasmid (pCLD56). Whole-cell lysates were resolved using SDS-PAGE and the FLAG-tagged proteins were detected by western blot analysis as described in the Materials and Methods. doi:10.1371/journal.pone.0060076.g002

activity [30,31]. Similarly, we found that this conserved asparagine was required for a mutant SypE variant to inhibit biofilms induced by RscS [19]. To ask whether the ability of wild-type SypE to inhibit SypG-induced biofilms also required this conserved asparagine, we assessed the inhibitory activity of a SypE mutant carrying an alanine substitution at this site (SypEN52A). We found that expression of SypEN52A failed to complement the ΔsypE mutant: the colonies exhibited strong SypG-induced wrinkling and pellicle formation (Figs. 2G and 3G). Similarly, this mutation in the context of the inhibitory N-terminal domain alone (SypE<sup>NTD</sup>, N52A) resulted in the complete loss of inhibitory activity: cells co-expressing ΔsypE<sup>NTD</sup>, N52A and sypG exhibited both wrinkled colony morphology and pellicle formation (Figs. 2H and 3H, respectively). The failure to complement cannot be attributed to protein instability, as epitope-tagged versions of both the SypEN52A and SypE<sup>NTD</sup>, N52A proteins are stably expressed [19]. From these
Co-overexpression of SypA and SypG Permits Biofilm Formation

Our results indicate that SypE inhibits SypG-induced biofilms through the activity of its N-terminal, serine kinase domain. These findings suggest that SypE primarily functions as a kinase under SypG-inducing conditions, and likely inhibits biofilms by phosphorylating a downstream target protein. We hypothesized that SypE exerts negative control over SypG-induced biofilms by phosphorylating the syp-encoded regulator SypA, which we recently identified as a downstream target of SypE (Fig. 1B) [20]. SypA, a putative anti-sigma factor antagonist, is required for biofilm formation, and phosphorylation of SypA inhibits its activity [20]. We reasoned that if SypE prevents SypG-induced biofilms by phosphorylation of SypA, then co-overexpression of sypG and sypA may result in sufficiently high levels of SypA such that some of it could escape phosphorylation and thus inhibition by SypE. To test this, we co-overexpressed sypA and sypG from compatible plasmids in a wild-type background and assessed biofilm formation. As controls, we first evaluated strains overexpressing either sypG or sypA alone, and found that biofilm formation was not induced (Fig. 4A and 4B, respectively). In contrast, cells co-overexpressing both sypG and sypA exhibited robust biofilm formation (Fig. 4C). These data indicate that providing excess SypA overcomes the inhibition of SypG-induced biofilms by SypE, and further suggest that SypA functions downstream of SypE and is likely the target for SypE’s inhibitory, kinase activity. In addition, the fact that biofilms were only induced upon co-overexpression of sypG and sypA, but not when sypA was overexpressed alone, indicates that sypG is still required to induce syp transcription and the production of the Syp structural proteins (Fig. 1B).

**Phosphorylation of SypA is Required for Inhibition of SypG Biofilms by SypE**

Our data predicted that the inhibition of biofilm formation by SypE depends upon its ability to phosphorylate SypA. If so, then it should be possible to overcome SypE’s inhibition of biofilm formation with a mutant of SypA that does not become phosphorylated. We thus assayed SypG-induced biofilm formation by cells that expressed the sypA<sup>S56A</sup> allele in single copy in the chromosome; the resulting mutant protein contains a substitution at a conserved serine required for phosphorylation, and fails to become phosphorylated either in vivo when rscS is overexpressed or in vitro [20]. We found that sypE<sup>C</sup> cells expressing SypA<sup>S56A</sup>, but not those that expressed wild-type SypA or contained the empty cassette, formed robust biofilms upon overexpression of sypG (Fig. 5). These data demonstrate that the non-phosphorylatable SypA<sup>S56A</sup> mutant is insensitive to the inhibitory activity of SypE, thus supporting the hypothesis that SypE inhibits the formation of SypG-induced biofilms via phosphorylation of SypA.

**SypE Promotes Phosphorylation of SypA in sypG-overexpressing Cells**

Our genetic data to date suggested that, in cells that overexpress sypG, SypE functions as a kinase to phosphorylate SypA. To assess this prediction directly, we evaluated the in vivo phosphorylation state of SypA under both biofilm-inhibitory conditions (i.e. sypG-overexpressing wild-type cells) and biofilm-permissive conditions (i.e. sypG-overexpressing ΔsypE cells). Briefly, we introduced an epitope-tagged wild-type sypA allele (containing a C-terminal HA tag) in single copy in the chromosome (at the Tn7 site) of either ΔsypA cells or ΔsypA ΔsypE cells. We then overexpressed sypG and

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**Figure 3. Inhibition of SypG-induced pellicle formation by SypE.** The sypG expression plasmid (pCLD56) was introduced into either wild-type cells [A] or ΔsypE mutant cells [KV3299] carrying either empty vector (pVSV105) [B] or the indicated SypE-complementation plasmids: full-length SypE (pCLD48)[C], SypE<sup>NTD</sup> (pARM3)[D], SypE<sup>CTD</sup> (pCLD67)[E], SypE<sup>NTD</sup> (pCLD64)[F], SypE<sup>S56A</sup> (pARM4)[G], SypE<sup>NTD, N52A</sup> (pCLD65)[H]. Strains were cultured statically in LBS medium and pellicle formation was assessed 48 h post-inoculation. A pipette tip was dragged over the surface of the air-liquid interface to visualize the pellicle. (–) denotes a weak, easily disrupted pellicle. (+) denotes a strong, detectable pellicle. Images are representative of at least three independent experiments. doi:10.1371/journal.pone.0060076.g003
assessed the in vivo phosphorylation state of SypA by resolving cell lysates on SDS-PAGE gels containing Phos-tag\textsuperscript{TM} acrylamide. This reagent permits the separation of phosphorylated and non-phosphorylated forms of proteins by preferentially binding to and retarding the migration of phosphorylated proteins [32,33]. SypA-HA proteins were detected by western blot analysis using an anti-HA antibody. As observed in figure 6A, sypG-overexpressing wild-type cells (lane 2) exhibited a single, upper band corresponding to phosphorylated SypA (SypA\textsuperscript{P}). These data indicate that in wild-type cells overexpressing sypG, the majority of, if not all, SypA protein is in the phosphorylated state. In contrast, sypG-overexpressing DsypE cells (which are competent to produce biofilms) exhibited a single, lower band representing unphosphorylated SypA (Fig. 6A, lane 3). Complementation with a wild-type allele of sypE (SypE\textsuperscript{+}) restored SypA phosphorylation as indicated by the presence of the shifted SypA band (Fig. 6A, lane 4). Finally, we confirmed that the SypA\textsuperscript{S56A} mutant fails to become phosphorylated in sypG-overexpressing cells (Fig. 6A, lane 5). These results demonstrate that SypE promotes SypA phosphorylation under SypG-inducing conditions, and corroborate our biofilm assays indicating that, under SypG-inducing conditions, SypE functions as a kinase. Importantly, they verify that phosphorylation of SypA is critical for biofilm inhibition.

To further test the hypothesis that biofilm formation requires unphosphorylated SypA, we examined the phosphorylation state of SypA in cells co-overexpressing sypG and sypA. We previously found that the co-overexpression of sypG and sypA promotes biofilm formation in wild-type (sypE\textsuperscript{+}) cells (Fig. 4). We hypothesized that the overexpression of sypA results in an excess of SypA protein, such that a pool of SypA escapes phosphorylation by SypE and is thus active to promote SypG-induced biofilms. We found that wild-type cells expressing sypA and sypG consistently exhibited two bands: a predominant, lower band corresponding to unphosphorylated SypA and a faint, upper band corresponding to phosphorylated SypA (Fig. 6B, lane 2). These data thus demonstrate that overexpression of sypA indeed provides an excess pool of unphosphorylated SypA protein. In agreement with our previous Phos-tag\textsuperscript{TM} experiments, SypA phosphorylation de-

**Figure 4. Co-overexpression of sypG and sypA permits biofilm formation.** Biofilm formation by wild-type *V. fischeri* cells carrying either the sypG overexpression plasmid (pARM9) [A], the sypA overexpression plasmid (pARM13) [B], or both [C]. For A and B, the indicated vectors are pKV282 and pSV105, respectively. The strains were cultured in LBS broth containing Tet and Cm. Cultures were spotted onto agar plates and wrinkled colony morphology was assessed at 48 h post-spotting. Images are representative of at least three independent experiments. doi:10.1371/journal.pone.0060076.g004

**Figure 5. A sypA\textsuperscript{S56A} mutant permits SypG-induced biofilm formation.** Assessment of SypG-induced wrinkled colony formation by sypG-overexpressing (pCLD56) wild-type cells [A], and sypG-overexpressing ΔsypA cells complemented with either wild-type sypA\textsuperscript{+} (KV5479) [B] or the sypA\textsuperscript{S56A} allele (KV5481) [C]. Cultures were spotted onto LBS medium containing Cm at 28 °C and wrinkled colony formation was assessed at 48 h post spotting. Images are representative of at least three independent experiments. doi:10.1371/journal.pone.0060076.g005
SypE Regulates SypG-induced Biofilm Formation Downstream of syp Activation

Although our data indicate that SypE-mediated phosphorylation of SypA inhibits biofilm formation upon sypG overexpression, it does not reveal the level at which SypE (via SypA) exerts its control. SypG regulates biofilm formation by inducing transcription of the syp locus, which is required for both biofilm formation in laboratory culture and the formation of bacterial aggregates on the surface of the squid light organ during colonization [3,12]. Therefore, one formal possibility is that SypE impacts transcription of the syp locus through SypA. To determine if SypE affects transcription, we utilized a sypA promoter-lacZ fusion inserted in single copy in the chromosome of the wild-type and ΔsypE strains. We then assessed sypA promoter activity by measuring β-galactosidase activity upon co-expression of sypG and sypE.

As previously observed [12], wild-type cells overexpressing sypG exhibited high levels of syp transcription, as indicated by the significant increase in β-galactosidase activity relative to vector control cells (Fig. 7). Compared to the wild type, cells deleted for sypE exhibited a moderate (~1.5-fold), but consistent, increase in SypG-induced syp transcription (Fig. 7). Co-expression of wild-type sypE and sypG in the ΔsypE mutant restored syp transcription to near wild-type levels (Fig. 7). These results suggest that SypE does have a minor effect on SypG-induced syp transcription.

To further determine whether SypE indeed controls biofilm formation at the level of syp activation, we asked whether inhibition of SypG-induced transcription required the SypE N-terminal serine kinase domain. We first tested whether expression of SypE<sup>ΔNTD</sup>, a sypE mutant that lacks the N-terminal domain and thus its ability to inhibit biofilm formation, impacted activation of syp transcription by SypG. Surprisingly, we found that co-expression of SypE<sup>ΔNTD</sup> and sypG in ΔsypE cells resulted in a decrease in syp transcription similar to cells expressing wild-type sypE, a result opposite to the observed biofilm phenotype (Fig. 7). These results suggested that while SypE may have a slight impact on syp transcription, it is not sufficient to account for the observed effects of SypE on SypG-induced biofilm formation. In support of this, we found that expression of the N-terminal domain alone (SypE<sup>ΔNTD</sup>), which is sufficient to inhibit SypG-induced biofilm formation, had no observable impact on syp transcription (Fig. 7; compare with ΔsypE vector control). From these data, we conclude that the minor effect of SypE on SypG-induced syp transcription is insufficient to account for the dramatic effects on biofilm formation, and thus SypE must exert its regulatory effect downstream of syp transcriptional activation.

Deletion of sypE Confers a Competitive Colonization Advantage

The ability of V. fischeri to produce syp-dependent biofilms in laboratory culture directly correlates with the ability of the bacteria to efficiently colonize host juvenile squid [11,14,19]. However, we previously observed that the deletion of sypE in otherwise wild-type cells did not significantly impact host colonization [19,34]. We therefore questioned whether the presence of sypE would impact colonization when sypG is overexpressed. In other words, could the ability of the sypG-overexpressing sypE mutant cells to produce robust biofilms also promote colonization competence, or would the absence of sypE have no impact? To assess this question, we examined the ability of sypG-overexpressing wild-type and ΔsypE strains to competitively colonize juvenile squid. Newly hatched juvenile squid were inoculated with a 1:1 mixture of the two strains, one of which carried an erythromycin resistance (Em<sup>R</sup>) cassette on the
chromosome. Upon colonization, the squid were homogenized and the relative amounts of the individual strains were determined by calculating the percentage of Em\textsuperscript{R} and Em\textsuperscript{S} colonies. As shown in Figure 8A, ΔsypE cells dramatically outcompeted wild-type cells for host colonization. These data suggest that upon overexpression of sypG, deletion of sypE confers a competitive colonization advantage. To determine whether the loss of sypE was responsible for the colonization advantage observed in the ΔsypE strain, we complemented the ΔsypE strain with a wild-type allele of sypE in trans in the chromosome. Indeed, the SypE-complemented (SypE\textsuperscript{+}) cells failed to outcompete wild-type cells, while both were overexpressing sypG (Fig. 8B). These results reveal a critical role for SypE in inhibiting both biofilm formation and host colonization. Furthermore, they provide additional correlations between syp-independent biofilm formation in culture and the ability to promote host colonization in vivo.

Discussion

In this study, we investigated the role of the SypE in the regulation of SypG-dependent biofilm formation and host colonization in V. fischeri. Our interest in this area was prompted by an earlier report demonstrating that SypE permitted RscS-induced biofilms, yet inhibited biofilms induced by sypG overexpression [12]. Thus, SypE appeared to function in distinct ways under the two conditions. SypE is an unusual response regulator with both serine kinase and serine phosphatase activities [18,19,20]. To better understand the role of SypE as a negative regulator of syp-dependent biofilm formation, and to determine the mechanism behind the distinct phenotypes displayed by the rscS and sypG overexpression strains, we assessed SypE function under sypG-overexpressing conditions.

First, we performed a structure-function analysis to probe the regulatory activities of this novel response regulator. Through complementation studies, we observed that the N-terminal serine kinase domain is both required and sufficient to inhibit SypG-induced biofilm formation. The inhibitory activity of the isolated N-terminal domain (SypE\textsuperscript{NTD}) absolutely required residue N52, a conserved asparagine that is necessary for ATP binding and kinase activity in other characterized serine kinases [29,30]. These results parallel those observed for the role of SypE in RscS-induced biofilm formation: expression of SypE\textsuperscript{NTD} alone also inhibited biofilm formation in a manner that depended on N52 [19]. Future efforts are required to determine what other residues contribute to SypE-mediated inhibition of biofilms. Unlike our results with the N-terminal domain, however, our results with respect to the C-terminal domain differed under rscS and sypG-overexpressing conditions: whereas the C-terminal domain promoted RscS-induced biofilms [19], we failed to observe any impact of this domain in promoting SypG-induced biofilm formation (Fig. 2 and data not shown). It remains possible that the C-terminal serine phosphatase domain does promote SypG-induced biofilms, but that this impact is not readily apparent under the current experimental conditions. Indeed, SypE-mediated induction of syp transcription is stronger than that induced by RscS, and correspondingly, biofilm formation occurs at an accelerated pace by sypG-overexpressing ΔsypE strains relative to rscS-overexpressing strains (Morris and Visick, unpublished data). Thus, it is more difficult to assess a subtle increase in biofilm formation under the SypG conditions.

Next, we used both genetic and biochemical approaches to assess whether SypE inhibited SypG-induced biofilms by promoting the phosphorylation, and thus inactivation, of SypA, a critical regulator required for biofilm development. Co-overexpression of sypG and sypA in sypE\textsuperscript{-} cells promoted biofilm formation, indicating that high levels of SypA can overcome the inhibitory activity of SypE, presumably because some SypA...
esences phosphorylation (Fig. 1B and 6B). In further support of this conclusion, expression from the chromosome of a sypE mutant that does not become phosphorylated (SypA^{NAGA}) also permitted the formation of SypG-induced biofilms in wild-type (SypE^+) cells. Finally, we found that SypA was predominantly phosphorylated under biofilm-inhibiting conditions (i.e., upon overexpression of sypG in wild-type cells), but unphosphorylated under biofilm-permissive conditions (i.e., upon overexpression of sypG in ΔsypE cells). The ability to evaluate the phosphorylation state of SypA in vivo provides a critical link between the biochemical proof of activity and the genetic and phenotypic analyses. Importantly, these findings demonstrate that SypE inhibits SypG-induced biofilm formation by functioning as a kinase to phosphorylate SypA (Fig. 1B). They also corroborate our previous study demonstrating that SypA is largely unphosphorylated under biofilm-permissive conditions, specifically in rscS-overexpressing wild-type cells [20].

Studies are underway to determine the mechanism by which the SypE-SypA pathway controls biofilm formation. Here, we asked whether SypE (via SypA) exerts any impact on biofilm formation through affecting syp transcription. We found that the deletion and/or overexpression of full-length sypE or a subset of mutant sypE alleles resulted in a slight, yet reproducible, impact on syp transcription. However, the observed effects on transcription were not sufficient to account for the observed biofilm phenotypes. For example, we found that a SypE mutant lacking the N-terminal domain (SypE^{NTD}) and, thus, lacking inhibitory activity retained the ability to decrease SypG-induced transcription. We hypothesize that SypE's REC domain may be responsible for the observed impact on syp transcription. SypE, like SypG, appears to function below RscS in the regulatory cascade [12,19]. If these two response regulators do indeed receive signals from the same upstream sensor kinase, then the REC domains of SypE and SypG may compete for phosphorylation, resulting in a decrease in SypG activation and syp transcription. In support of this possibility, expression of SypE^{NTD}, a SypE derivative that lacks the REC domain but retains biofilm-inhibitory activity, had no observable impact on syp transcription. Due to the lack of correlation between biofilm formation and the minor impacts of a subset of sypE alleles on syp transcription, we conclude that SypE functions at a level below syp transcription. In support of this conclusion, our preliminary studies indicate that expression of sypE, although critical for biofilm formation, exerts no impact on syp transcription (Morris and Visick, unpublished data).

Finally, we found that the ability of V. fischeri to form biofilms in culture directly correlates with colonization efficiency in vivo, a result that is consistent with previous studies [11,14,19]. In particular, we observed that the enhanced ability of the ΔsypE strain to form biofilms upon overexpression of sypG provides these cells with a competitive colonization advantage, presumably due to enhanced aggregation outside of the symbiotic light organ. We found that this colonization advantage, similar to biofilm formation, depended upon loss of sypG as complementation with sypG in trans in the chromosome abolished the colonization advantage. Since previous studies had found no substantial colonization phenotype upon the deletion of sypE [19,34], why was SypE so critical here? Our results to date indicate that wild-type cells inactivate SypE during the early stages of squid colonization, and thus its loss would have relatively little impact on colonization initiation [19]. We speculate that the levels of SypE produced under sypG-overexpression conditions are greater than can be inactivated through the mechanisms in place. In support of this idea, we have found that SypE expressed from a multi-copy plasmid inhibits biofilm formation, even under rscS-overexpression conditions (Morris and Visick, unpublished data). In any event, the significant inhibitory effect of SypE on colonization under SypG-inducing conditions underscores the critical importance of biofilm formation in promoting symbiotic host colonization.

This study also emphasizes the strict control V. fischeri exerts over biofilm formation (Fig. 1B). The finding that SypE inhibits biofilms under SypG-inducing conditions, yet permits biofilms
upon overexpression of \( \text{rscS} \), suggests that SypE could function to prevent aberrant biofilm formation induced by the phosphorylation, and thus activation, of SygG in the absence of RscS signaling/activation. It is currently unclear under what conditions SygG may become phosphorylated, and induce \( \text{syg} \) transcription, without signaling through RscS. It remains possible that SygG may receive phosphoryl groups and become activated through other regulatory inputs in addition to RscS. Recently, Ray and Visick (2012) demonstrated that regulatory components within the Lux phosphorelay controlling bioluminescence also impact the regulation of \( \text{syg} \) biofilm formation. Importantly, this impact on \( \text{syg} \) biofilms appears to occur at the level of SygG and \( \text{syg} \) locus activation [35]. Thus, it is possible that, under conditions in which the Lux phosphorelay promotes SygG activation and \( \text{syg} \) induction, SypE functions as a negative regulator to inactivate Syg and prevent biofilm formation. Alternatively, SypE may be important for preventing biofilm formation under conditions in which SygG might get phosphorylated due to inadvertent “cross-talk” from other sensor kinases that are activated. These possibilities remain to be investigated.

In summary, this work provides further insight into the mechanism by which the regulator SypE restricts biofilm formation and host colonization by \( \text{Vibrio fischeri} \). Furthermore, it emphasizes the critical role of SypE in regulating biofilm formation, particularly when RscS is not activating the pathway. Together, our data suggest that SypE may function to restrict biofilms to conditions in which RscS becomes activated (i.e., upon interaction with host squid), and thus to coordinate \( \text{syg} \) biofilm production with host colonization.

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Author Contributions

Conceived and designed the experiments: ARM KLV. Performed the experiments: ARM. Analyzed the data: ARM KLV. Wrote the paper: ARM KLV.

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