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Signaling between two interacting sensor kinases promotes biofilms and colonization by a bacterial symbiont

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Summary

Cells acclimate to fluctuating environments by utilizing sensory circuits. One common sensory pathway used by bacteria is two-component signaling (TCS), composed of an environmental sensor (the sensor kinase, SK) and a cognate, intracellular effector (the response regulator, RR). The squid symbiont *Vibrio fischeri* uses an elaborate TCS phosphorelay containing a hybrid SK, RscS, and two RRs, SypE and SypG, to control biofilm formation and host colonization. Here, we found that another hybrid SK, SypF, was essential for biofilms by functioning downstream of RscS to directly control SypE and SypG. Surprisingly, although wild-type SypF functioned as a SK *in vitro*, this activity was dispensable for colonization. In fact, only a single non-enzymatic domain within SypF, the HPt domain, was critical *in vivo*. Remarkably, this domain within SypF interacted with RscS to permit a bypass of RscS's own HPt domain and SypF's enzymatic function. This represents the first *in vivo* example of a functional SK that exploits the enzymatic activity of another SK, an adaptation that demonstrates the elegant plasticity in the arrangement of TCS regulators.

Keywords

biofilm/*Euprymna*; *scolopes*/hybrid; sensor; kinase/two-component; signaling/*Vibrio fischeri*

Introduction

For organisms to survive, they must appropriately respond to the assorted environments they experience. To do this, they use signaling pathways that link environmental signals with relevant intracellular outputs. One type of cellular circuitry found in most bacteria, some archaea, and a few eukaryotic species, is the two-component signaling (TCS) pathway (reviewed in (Stock *et al.*, 2000, Wuichet *et al.*, 2010)). The basic TCS architecture consists of two types of proteins: a sensor kinase (SK) and a response regulator (RR). Typically, a specific environmental ligand binds a cell membrane-bound SK, which autophosphorylates on a conserved histidine within a HisKA domain using ATP as the phosphoryl donor. It then donates this phosphoryl group to a conserved aspartate in the REC (receiver) domain within a cognate RR, an event that is catalyzed by the enzymatic activity of the REC domain. Often

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Conflict of Interest

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the RR has an effector domain, such as a DNA binding or enzymatic domain, whose activity is activated or deactivated once the REC domains becomes phosphorylated (Galperin, 2010). This two-protein arrangement connected by a single His-Asp phosphotransfer event remains the most common TCS architecture found in bacteria; however, some TCS pathways consist of a complicated phosphorelay involving more than one phosphotransfer event (His-Asp-His-Asp) between two or more cognate TCS proteins. Often these phosphorelays include a "hybrid" SK, which contains a second site of phosphorylation within a covalently attached REC domain. Some hybrid SKs also possess a third site of phosphorylation, a histidine within a C-terminal histidine-containing phosphotransfer (HPt) domain. To date, most hybrid SKs with autokinase activity require these additional sites of phosphotransfer to effectively donate the phosphoryl group to their cognate RR (Hsu *et al.*, 2008, Jourlin *et al.*, 1997, Takeda *et al.*, 2001, Tsuzuki *et al.*, 1995, Uhl & Miller, 1996). It is believed that these extra phosphotransfer events represent checkpoints that control whether a cell initiates physiological changes under particular conditions (Jung *et al.*, 2012, West & Stock, 2001).

One developmental process in bacteria that is often governed by TCS circuits is the formation of biofilms, or matrix-encased communities of cells (Ferrieres & Clarke, 2003, Gooderham & Hancock, 2009, Hamon & Lazazzera, 2001, Huang *et al.*, 2013, Irie *et al.*, 2004, Li *et al.*, 2002, Petrova & Sauer, 2009, Stipp *et al.*, 2013, Su & Ganzle, 2014, Zhang *et al.*, 2004). It is believed that environmental signals activate or deactivate specific TCS pathways to disfavor the independent, planktonic state and favor the assembly of a community (McLoon *et al.*, 2011, Mulcahy & Lewenza, 2011, Ventre *et al.*, 2006). Environments that induce biofilm development can include host tissues, where these communities are implicated in initiation and persistence of colonization by both pathogenic and commensal bacteria (Reviewed in (Heindl *et al.*, 2014, Joo & Otto, 2012, Percival & Suleman, 2014, Ramey *et al.*, 2004, Yildiz & Visick, 2009)). One unique model system used to study biofilms in the context of a natural host is the symbiosis between the bacterium, *Vibrio fischeri*, and the Hawaiian bobtail squid, *Euprymna scolopes* (Reviewed in (McFall-Ngai, 2014, Stabb & Visick, 2013)). Successful colonization requires that *V. fischeri* cells form and disperse from a biofilm to enter the symbiotic organ, known as the light organ (Nyholm *et al.*, 2000, Yip *et al.*, 2006). This biofilm depends on the production of the symbiosis polysaccharide (Syp PS) generated by proteins encoded by the 18-gene *syp* locus (Shibata *et al.*, 2012, Yip *et al.*, 2006). Control over Syp production occurs via a complex TCS cascade. Previous work indicated that the hybrid SK, RscS, senses an unknown signal that leads to the phosphorylation of two downstream RRs, SypE and SypG (reviewed in (Visick, 2009)) (Fig 1A). SypG functions as a transcription factor to directly promote transcription of the *syp* locus, while SypE functions downstream of *syp* transcription to control production of Syp PS (Morris *et al.*, 2011, Morris & Visick, 2013, Ray *et al.*, 2013, Yip *et al.*, 2005). Both *sypE* and *sypG* are located within the *syp* locus, whereas *rscS* is located elsewhere in the chromosome and was proposed to be horizontally acquired (Mandel *et al.*, 2009, Visick & Skoufos, 2001, Yip *et al.*, 2005). The current model is that, after its acquisition, RscS evolved the ability to activate SypG and inactivate SypE, thus allowing *V. fischeri* to utilize Syp for colonization of *E. scolopes*.

RscS may not be the only SK that regulates the Syp pathway. Located between the RR genes *sypE* and *sypG* is an additional hybrid SK gene, *sypF* (Fig 1), a genetic configuration that is typical for TCS proteins that function together. Indeed, our previous work suggested that SypF could control biofilm formation: although overproduction of wild-type SypF had no effect on biofilms, overproduction of an active variant of SypF, termed SypF*, induced biofilm formation (Darnell *et al.*, 2008). This variant contained two mutations, one of which was located three amino acids upstream from the conserved site of phosphorylation (Fig 2A) (Darnell *et al.*, 2008). However, it remained unknown whether the phenotype of SypF* was physiologically relevant, whether SypF had any role in host colonization, or how input from two SKs (an unusual arrangement for TCS pathways), SypF and RscS, might dictate the control of biofilms.

In this study, we found that SypF is critical for biofilm formation and host colonization by functioning as the direct donor of phosphoryl groups to the downstream RRs SypE and SypG. Surprisingly, although SypF could autophosphorylate *in vitro*, only one non-enzymatic domain of SypF was required for biofilms and colonization. Instead of its own enzymatic domains, SypF relied on the catalytic activity of the upstream SK, RscS to control biofilms and colonization. SypF thus represents the first example of a hybrid SK that has the ability to function as a histidine kinase, yet, *in vivo*, it forfeits this activity to an upstream SK. This interaction between the recently acquired RscS protein and the more conserved SypF protein demonstrates the flexibility of TCS architectures, and provides insight into how these regulatory circuits might evolve to allow a bacterium to take advantage of a new niche, such as host tissues.

Results

SypF* functions as a canonical hybrid sensor kinase

In culture, biofilm formation by *V. fischeri* is induced upon overproduction of any of three TCS proteins: the SK RscS, the RR SypG (in the absence of inhibitory RR protein SypE), and a mutant version of the SK SypF (SypF*) (Darnell *et al.*, 2008, Hussa *et al.*, 2008, Yip *et al.*, 2006). Our long-standing model proposes that RscS directly controls SypG and SypE (Fig 1A). As a result, the role of SypF in controlling biofilm formation has been unclear, especially since overproduction of only SypF*, but not wild-type SypF, could induce biofilm formation (Darnell *et al.*, 2008). Within SypF*, two mutations exist (S247F and V439I). The former is located 3 residues away from the predicted site of autophosphorylation (H250) (Fig 2A). In this position, the substitution of the small serine side chain with the bulky phenylalanine side chain could affect the ability of H250 to be phosphorylated. Thus, it was inferred that SypF* exists in a kinase “active” conformation (Darnell *et al.*, 2008). This result, along with the strong conservation of sequences known to catalyze kinase and phosphotransfer reactions (Supplemental Fig S1A, B), suggested that SypF* functions as a SK. To test this hypothesis, we purified the cytoplasmic portion of SypF*, assessed whether it could autophosphorylate *in vitro*, and found that it could under the tested conditions (Fig 2B).

To determine if SypF* could function as a hybrid SK, we tested whether biofilm formation required predicted sites of phosphorylation in the REC (D549) and HPt domains (H705) (Fig

2A). We generated individual mutations of D549 and H705, overexpressed these mutant alleles in otherwise wild-type cells, and assessed the ability of the resulting strains to form wrinkled colonies, an *in vitro* indicator of biofilm formation. Whereas cells that overproduced SypF* (pSypF*) formed wrinkled colonies, those containing either pSypF*^{D549A} or pSypF*^{H705Q} formed smooth colonies (Fig 2C). Thus, similar to canonical hybrid SKs, SypF* required these sites of phosphorylation to function.

To confirm that the SypF* variants were produced, we generated constructs that produced FLAG epitope-tagged versions of SypF*, SypF*^{D549A} or SypF*^{H705Q}, as well as two additional mutants, SypF*^{H250Q} (in the HisKA domain) and SypF^{S247F} (containing only one of the two mutations present in SypF*). We then used western blot analysis to assess the levels of these proteins and colony morphology to assess their ability to induce biofilm formation. Importantly, we found that the steady-state levels of all these SypF variants were similar (Supplementary Fig S2B). However, the FLAG tag somewhat diminished the biofilm-inducing activity of SypF* (Supplementary Fig S2A, compare pSypF* to pSypF*-FLAG). Regardless, the H250Q, D549A, and H705Q mutants failed to induce the formation of wrinkled colonies. In contrast, the SypF^{S247F} mutant promoted wrinkled colony development to approximately the same extent as SypF*, demonstrating that this substitution was sufficient for the activity of SypF*. Together, our data support the hypothesis that SypF* functions as a canonical hybrid SK.

sypF is required for biofilm formation and *syp* transcription

We next asked where SypF might function in the Syp pathway to control biofilm formation. We first determined where it functioned relative to RscS, the other hybrid SK. To do this, we deleted *sypF* from the chromosome and assessed whether this affected the ability of RscS to induce wrinkled colonies. Whereas RscS overproduction induced the formation of wrinkled colonies by the wild-type strain, it failed to do so in the *sypF* mutant, which formed smooth colonies indistinguishable from the vector control (Fig 3A). Complementation of the *sypF* deletion with a wild-type copy of *sypF* in single copy restored wrinkled colony formation. These data suggest that SypF works below RscS in the regulatory hierarchy.

Because RscS-induced biofilm formation required *sypF*, we asked whether RscS-induced *syp* transcription would similarly require *sypF*. Thus, we evaluated the impact of the *sypF* deletion on the activity of a P_{sypA}-*lacZ* reporter. In the wild-type background, RscS induced expression of the P_{sypA}-*lacZ* reporter relative to the vector control. In the *sypF* deletion background, however, RscS failed to induce the reporter (Fig 3B). Finally, provision of the wild-type *sypF* allele *in trans* complemented the defect. We conclude that RscS requires SypF to induce *syp* transcription, and propose a model wherein SypF functions downstream of RscS in the Syp TCS pathway (Fig 1B).

SypF directly controls SypG and SypE

RscS is proposed to act upstream of two RRs, SypG and SypE (Hussa *et al.*, 2008, Morris *et al.*, 2011, Yip *et al.*, 2006). We thus asked whether SypF functioned between RscS and one or both of these downstream RRs (Fig 1B). Because RscS required SypF to promote *syp*

transcription, we first asked if SypF functions above SypG, the direct transcriptional activator of the *syp* locus (Ray *et al.*, 2013). If so, then it should be possible to bypass the requirement for *sypF* using an active SypG variant that no longer requires activation by an SK. We generated strains that produced SypG*, a SypG protein in which the conserved site of phosphorylation (aspartate 53) was converted to a glutamate. This mutation mimics the phosphorylated state of other RRs (Freeman & Bassler, 1999, Sanders *et al.*, 1992, Sanders *et al.*, 1989) and has been shown to increase the activity of SypG (Hussa *et al.*, 2008). Indeed, single-copy expression of *sypG** was sufficient to induce *syp* transcription in the wild-type background (Fig 4A) and in the absence of *sypF* (Fig 4A). These data support a model in which SypF functions between RscS and SypG to control *syp* transcription (Fig 1B).

RscS also functions upstream of SypE, the RR that controls biofilms below *syp* transcription; phosphorylation of SypE switches off its inhibitory activity, thus allowing biofilms to develop (Morris *et al.*, 2011) (Fig 1A). To determine if SypF also functions upstream of SypE, we evaluated RscS-induced wrinkled colony formation in a SypG*-producing *sypF* deletion strain like the one used above. Because expression of *sypG** overcomes the requirement for SypF in *syp* transcription, we anticipated that this strain would produce wrinkled colonies only if SypF is not also required to inactivate SypE. As controls, we evaluated the production of wrinkled colonies by $sypF^{+}$ cells. As predicted from previous work (Hussa *et al.*, 2008), single copy expression of *sypG** in an otherwise wild-type background failed to induce wrinkled colony formation due to inhibition by SypE; however, expression of both *rscS* and *sypG** in wild-type cells induced wrinkled colony formation (Fig 4B). This demonstrates that, in this strain background, *rscS* expression is sufficient to turn off the inhibitory activity of SypE. In contrast, expression of both *rscS* and *sypG** in the *sypF* mutant failed to induce this phenotype. This observation suggests that *sypF* has an additional role in promoting biofilms, potentially by inactivating SypE. Indeed, a *sypE sypF* double mutant formed wrinkled colonies with *rscS* and *sypG** expression (Fig 4B). We infer from these data that RscS works through SypF to control the activities of both SypG and SypE (See model in Fig 1B).

To more directly assess the ability of SypF to interact with and control SypG and SypE, we evaluated whether SypF could donate phosphoryl groups to these RRs *in vitro*. We purified the REC domain of SypG and the full-length form of SypE, and added these purified proteins to reactions containing phosphorylated SypF*. In support of the genetic data, we detected phosphorylated forms of SypE and SypG-REC after incubation with phospho-SypF* (Fig 4C). These data indicate that SypF can directly interact with and phosphorylate these two RR proteins.

RscS-induced biofilm formation does not require conserved SypF residues

The above evidence indicate that RscS functions through SypF to control the activity of SypG and SypE. This is an unusual regulatory set-up for TCS systems with multiple SKs; thus, the mechanism by which SypF functions after RscS to control biofilms remained unclear. Specifically, we wondered if wild-type SypF could function as a SK like SypF* and, if so, if that SK activity was necessary for RscS-dependent activation of the pathway.

To ask the first question, we purified the cytoplasmic portion of wild-type SypF and assessed whether it could autophosphorylate *in vitro*. Indeed, in the presence of radiolabeled ATP, SypF exhibited autophosphorylation activity (Fig 2B).

To determine whether RscS-induced biofilm formation requires SypF to function as a SK, we generated mutations in each predicted site of phosphorylation of wild-type SypF. We then assessed whether the mutant proteins could complement the *sypF* mutant for wrinkled colony formation. As shown previously (Fig 3A), overproduction of RscS in the *sypF* mutant failed to induce biofilm formation (Fig 5A), but this defect could be restored with a wild-type copy of *sypF* expressed in single copy from the chromosome. Surprisingly, mutating the first conserved histidine (H250Q), the conserved aspartate (D549A), or both together (H250Q D549A), did not negatively impact complementation: strains with these proteins retained their ability to form wrinkled colonies (Fig 5A). However, a SypF mutant disrupted for all three putative sites of phosphotransfer (H250, D549, and H705) failed to promote wrinkled colony formation, indicating that the last site of phosphotransfer may be required under these conditions. Indeed, $SypF^{H705Q}$, which contained a single substitution in the conserved site of phosphorylation within the HPt domain, did not complement the *sypF* deletion (Fig 5A). Analogous results were seen when assessing whether this mutant protein could complement a *sypF* deletion for *syp* transcription (Fig 3B). Finally, we observed similar steady-state levels for epitope-tagged versions of the wild-type and mutant SypF proteins via western blotting (Supplementary Fig S2C). Thus, the negative results for $SypF^{H705Q}$ and the triple mutant were not due to gross protein instability. Together, these data indicate that SypF does not function as a canonical hybrid SK under conditions that promote wrinkled colony development. Instead, SypF appears to require only H705 within its HPt domain to function.

RscS requires only the HPt domain of SypF

Because RscS-induced biofilm formation and *syp* transcription only required H705 in SypF, but not H250 or D549, we wondered whether the domain that contains H705, the HPt domain, was sufficient to promote these phenotypes. Indeed, *sypF* in other Vibrio species encodes a single HPt domain rather than a full-length SK (Supplementary Fig S3). We thus cloned this domain and assessed complementation. We found that the HPt protein alone permitted RscS-induced biofilm formation (Fig 5B) and *syp* transcription in a *sypF* deletion mutant (Fig 3B). In contrast, when the HPt domain contained a mutation in the site of phosphorylation, it did not complement the *sypF* deletion. These data suggest that the HPt domain in SypF is the sole domain to engage in phosphotransfer reactions that control biofilm formation induced by RscS.

RscS directly utilizes the HPt domain of SypF

The requirement for only the HPt domain of wild-type SypF was surprising because single domain HPt proteins do not exhibit enzymatic activity. Therefore, they must receive a phosphoryl group from an upstream protein to donate phosphoryl groups to downstream RRs. Interestingly, previous data suggested that RscS, a hybrid SK with three predicted sites of phosphorylation, did not require the last site of phosphorylation in its HPt domain to promote biofilms (Geszvain & Visick, 2008). Thus, we hypothesized that RscS donates

phosphoryl groups to the HPt domain of SypF, which then passes phosphoryl groups to the two downstream RRs, SypG and SypE (Fig 1B). To test this hypothesis, we generated a chimeric protein that contained the N-terminal portion of RscS (lacking its HPt domain) and the C-terminal HPt domain of SypF (Fig 6A.). We introduced the plasmid that produces this chimera into wild-type and *sypF* deletion backgrounds, and then assessed whether the chimeric protein was sufficient to induce biofilms even in the absence of *sypF*. In accordance with our hypothesis, the chimera induced wrinkled colonies in both backgrounds (Fig 6B). Together, these data suggest that neither RscS nor SypF require the full complement of their own phosphotransfer domains, but instead rely on each other for the signal transduction that leads to biofilm formation.

Requirement for SypF during host colonization

Our ability to assess the function of SypF in culture depends on the plasmid-based production of regulators such as RscS and SypF*. Use of those two different regulators, however, yielded conflicting results about how SypF regulates biofilms. More specifically, SypF* required all three sites of phosphorylation to induce wrinkled colony formation, whereas RscS-induced phenotypes only required a single conserved site of phosphorylation within the HPt domain of SypF. We thus wanted to define a clear role for SypF and its putative enzymatic domains during biofilm formation using a more physiologically relevant approach. To do this, we assayed the importance of *sypF* and its conserved sites of phosphorylation for *V. fischeri* to colonize its squid host. Importantly, colonization is an *in vivo* phenotype that requires biofilm formation, but does not rely on the overproduction of regulatory proteins.

We first assessed the requirement of *sypF* for this phenotype by incubating the *sypF* deletion mutant with aposymbiotic squid for 18 h and then determining the number of *V. fischeri* cells in each squid. As expected, wild-type *V. fischeri* could colonize; however, the *sypF* mutant exhibited a severe colonization defect that could be complemented by providing wild-type *sypF* in single copy *in trans* (Fig 7A, B). This evidence indicates that *sypF* is required for host colonization.

We next identified the domains/amino acids within SypF that are important for host colonization. We found that, similar to the RscS-induced wrinkled colony experiments, cells that produced $SypF^{H250Q}$ or $SypF^{D549A}$ successfully colonized the squid whereas cells producing $SypF^{H705Q}$ did not (Fig 7A, B). Additionally, production of the HPt domain of SypF alone allowed the *sypF* deletion mutant to colonize *E. scolopes* unless the HPt domain contained a mutation within the site of phosphorylation (Fig 7B). These results indicate that SypF does not function as an SK to promote colonization, and that the RscS-induced wrinkled colony phenotype is more physiologically relevant than the SypF*-induced phenotype.

Finally, to confirm our findings that RscS and SypF function in an unusual phosphorelay to promote biofilm formation, we asked whether the *rscS-sypF* chimera, expressed from the chromosome of a double *rscS sypF* mutant, was proficient to promote colonization. Because *rscS* and *sypF* are individually required for colonization ((Visick & Skoufos, 2001) (Fig 7A, B)), it was not surprising that the *rscS sypF* double mutant failed to colonize the squid, and

introducing either *rscS* or *sypF* alone into this strain did not restore host colonization (Fig 7C). However, in support of our model for biofilm regulation, the chimeric allele mostly complemented the *rscS sypF* mutant for colonization. Together, these data confirm that the HPt domain of SypF functions between RscS and SypG/SypE to control biofilms, and that the enzymatic activity of SypF is largely dispensable for this signaling cascade during host colonization.

Discussion

TCS is a universal mechanism that bacteria use to link environmental signals with an intracellular response. At the apex of these pathways is the SK, a receptor that senses an environmental ligand to initiate physiological changes within the cell. Bioinformatic analyses readily identify SK proteins based on highly conserved enzymatic residues involved in histidine autokinase activity (Kim & Forst, 2001, Kofoid & Parkinson, 1988, Nixon *et al.*, 1986, Stock *et al.*, 1988). Canonical SKs containing a single phosphorylatable residue, the site of histidine-autophosphorylation, are predicted in most bacterial genomes. In contrast, hybrid SKs are predicted in about 1/3 of bacterial genomes (Galperin, 2005, Zhang & Shi, 2005). Hybrid SKs enforce an extra level of regulatory complexity in TCS, as their additional sites of phosphorylation are thought to function as checkpoints that fine-tune whether a physiological output is instigated under particular environmental conditions. The vast majority of hybrid SKs that autophosphorylate require each additional phosphorylation site to promote effective regulation of downstream phenotypes (Goodman *et al.*, 2009, Hsu *et al.*, 2008, Jourlin *et al.*, 1997, Takeda *et al.*, 2001, Tsuzuki *et al.*, 1995, Uhl & Miller, 1996). SypF is an exception to this rule.

Our genetic and biochemical studies demonstrated that SypF controls biofilm formation by functioning directly above both SypG and SypE, confirming its importance in the Syp regulatory cascade. Complicating these results, however, was the irrefutable evidence that another hybrid SK, RscS, also controlled biofilms, an uncommon arrangement for TCS cascades. In other TCS pathways with multiple SKs, such as the *Vibrio harveyi* luminescence (Lux) and *Bacillus subtilis* sporulation cascades, these SKs function as separate inputs into downstream regulators (Henke & Bassler, 2004, Jiang *et al.*, 2000). Thus, we initially proposed that SypF and RscS, together, control the activity of the downstream RRs. This hypothesis was supported by the observations that in culture, overproducing either RscS or the SypF variant, SypF*, induced wrinkled colony formation (Darnell *et al.*, 2008, Yip *et al.*, 2006), and that both RscS (Geszvain & Visick, 2008) and SypF* (Fig 2) required sites of autophosphorylation to induce this phenotype. However, although SypF* could function as a hybrid SK in the cell, this activity seemed not to be physiologically relevant. In particular, only the single, non-enzymatic HPt domain of SypF was required to promote host colonization, an *in vivo* phenotype that does not require the artificial overexpression of regulatory genes. Similarly, we observed that RscS-induced wrinkled colonies required the HPt domain of SypF, but no N-terminal, enzymatic regions of SypF. Combined with our data that an RscS-SypF chimera is sufficient to promote colonization, we conclude that (1) SypF does not function as a SK under biofilm-promoting conditions, (2) SypF* activity is not physiologically relevant, and (3) SypF functions downstream of RscS and thus RscS and SypF do not provide separate inputs into the Syp

pathway. We propose a mechanism in which RscS bypasses its own HPt domain and preferentially hijacks the HPt domain of SypF to affect the activity of the downstream RRs, SypE and SypG, to control biofilms (Fig 1B).

Why might SypF*, but not SypF, function as an SK *in vivo*? We maintain our previous conclusion that, in the cell, SypF* is in a kinase "on" state (Darnell *et al.*, 2008). SKs generally function as homodimers, and histidine kinase activity requires that the ATP hydrolyzing domain (HATPase_c) interact with the HisKA domain, which contains the conserved, phosphorylatable histidine, *in cis* (Casino *et al.*, 2009, Pena-Sandoval & Georgellis, 2010) or *in trans* (Ashenberg *et al.*, 2013, Dago *et al.*, 2012, Marina *et al.*, 2005, Pan *et al.*, 1993). This histidine side chain is generally solvent exposed, allowing it to interact with and receive phosphoryl groups from the HATPase_c domain. Our observation that the S247F mutation within wild-type SypF generates the SypF* phenotype confirmed that this mutation is sufficient to alter the enzymatic activity of SypF within the cell. Serine 247 is located three amino acids away from the site of phosphorylation. Perhaps this mutation changes the position of the downstream histidine, placing it in a location to be more readily phosphorylated by the HATPase_c domain. Although our genetic data support this conclusion, it remains to be determined whether SypF* has higher catalytic activity than SypF in the cell.

What is unprecedented about the Syp pathway is that wild-type SypF apparently relies on the enzymatic activity of a different SK as a source of its phosphoryl group *in vivo*. This result is especially surprising considering the evidence that SypF exhibits autokinase activity *in vitro.* Similarly, the Eps pathway in *Myxococcus xanthus* contains a hybrid SK, EpsC, that exhibits SK activity *in vitro*, but does not require residues involved in autophosphorylation *in vivo* (Schramm *et al.*, 2012). *In vitro* evidence suggested that another hybrid SK, EpsA, could phosphorylate the REC domain of EpsC, but whether this mechanism occurs *in vivo* remains to be determined (Schramm *et al.*, 2012). Together, SypF and EpsC contradict the assumption that an enzymatically-competent SK must function as so *in vivo*. Furthermore, the fact that SypF instead uses the enzymatic activity of RscS is a unique result. We propose that this may be a mechanism more common than is currently appreciated; there are examples of SKs that do not require all sites of phosphorylation to promote a phenotype [*e.g.*, (Chand *et al.*, 2011, Laskowski & Kazmierczak, 2006)], but it remains to be tested whether they have histidine kinase activity or whether an interacting partner exists to supply phosphates.

If *V. fischeri* does not require SypF to function as an SK to promote biofilms, then why is full-length *sypF* maintained in the genome? This question is especially perplexing given the observation that the *syp* locus in some other species of Vibrio encodes SypF as a single HPt domain (Supplementary Fig S3). One explanation is that, in *V. fischeri*, *sypF* is fated toward degeneracy, but the 5′ sequences have not had sufficient time to be negatively selected for and lost. If this is *sypF*'s fate, then the Syp TCS would end up similar to the Rcs pathway in *Escherichia coli*, where the hybrid SK, RcsC, donates phosphoryl groups to the HPt domain in a degenerate SK, RcsD (Takeda *et al.*, 2001). Alternatively, conditions found in later stages of colonization or outside of squid colonization could require that SypF utilize its enzymatic domains. *V. fischeri* is a marine organism found on ocean sediment and in

association with a number of aquatic animals besides *E. scolopes* (Haygood, 1993, Lee & Ruby, 1992, Mandel *et al.*, 2009, Ortigosa *et al.*, 1994, Ramesh *et al.*, 1989, Ruby & Lee, 1998, Ruby & Nealson, 1976, Yetinson & Shilo, 1979). Perhaps in these other contexts SypF functions as a bona fide SK to induce formation of the Syp or a Syp-like biofilm. With this hypothesis in mind, the RscS-SypF interaction brings to light the intriguing possibility that domains within the same signaling network could have discrete roles depending on environmental conditions surrounding the cell. It should be noted that, although the HPt domain of SypF alone and the RscS-SypF chimera allowed for *V. fischeri* to colonize *E. scolopes*, these proteins did not promote the same efficiency of colonization as seen with wild-type *V. fischeri*. This suggests that there may be other, more subtle roles for the N-terminal domains of SypF or the HPt domain of RscS during colonization. For example, many SKs exhibit both kinase and phosphatase activity (Aiba *et al.*, 1989, Casino *et al.*, 2009, Freeman *et al.*, 2000, Huynh *et al.*, 2010, Ninfa & Magasanik, 1986, Yang & Inouye, 1993), so SypF could utilize both of these activities to permit fine-tuning of the Syp phosphorelay. Similarly, perhaps the transmembrane regions within SypF allow for membrane localization, which may be important for efficient signaling in the Syp pathway. The relative importance of these additional domains during colonization awaits exploration.

Continued research into TCS has unveiled an increasing number of TCS architectures with two or more interacting SKs [*e.g.*, (Goodman *et al.*, 2009, He *et al.*, 2013, Kong *et al.*, 2013, Schramm *et al.*, 2012)]; however, the environmental pressures that selected for these interactions remain unknown. Conversely, *V. fischeri* has given researchers some clues as to how the complex Syp pathway may have evolved. In *V. fischeri*, there are at least two genetic loci required for *in vivo* biofilms: the *syp* locus and *rscS*. Whereas the *syp* locus is conserved in *V. fischeri*, only a subset of *V. fischeri* strains contains *rscS* (Mandel *et al.*, 2009). This suggests that the acquisition of *rscS* eventually granted *V. fischeri* access to the light organ of *E. scolopes. sypF* is conserved in *V. fischeri*, but perhaps for colonization purposes, RscS functionally replaced the enzymatic activity of SypF, and the HPt domain of SypF was positively selected for to provide an additional regulatory checkpoint. If only a small number of environments require the Syp biofilm, then it seems reasonable that this intricate TCS arrangement evolved to prevent inappropriate activation of a complex developmental process.

Flexibility in the arrangement of TCS allows all domains of life to precisely regulate their physiology to manage a vast repertoire of environments. The unique architecture of Syp, for example, has allowed *V. fischeri* to expand its niche to include the light organ of *E. scolopes*, thus outcompeting all other bacterial strains found in the local environment. Therefore, Syp demonstrates not only the plasticity of TCS pathways, but also provides a potential model for how a bacterium may adapt to conquer new environments and guarantee proliferation of its progeny.

Experimental Procedures

Bacterial strains and media

The bacterial strains used in this study are listed in Table 1 and were derived from ES114, a wild-type *V. fischeri* strain isolated from *E. scolopes* (Boettcher & Ruby, 1990). *V. fischeri*

derivatives were generated using previously described conjugation (Visick & Skoufos, 2001) mutagenesis (Le Roux *et al.*, 2007, Shibata *et al.*, 2012), and transposon (Tn*7*) chromosomal-insertion (McCann *et al.*, 2003) methods. *V. fischeri* cells were grown in Luria-Bertani Salt (LBS) media (Graf *et al.*, 1994), Seawater Tryptone (SWT) media (Boettcher & Ruby, 1990), or HEPES Minimal Media (HMM) (Ruby & Nealson, 1977). *E. coli* strains used for molecular genetics in this study include: ER2508 (NEB), TAM1 λ *pir* (Active Motif), π3813 (Le Roux *et al.*, 2007), CC118 λ *pir* (Herrero *et al.*, 1990), and GT115 (Invivogen). *E. coli* strains were grown in LB (Davis *et al.*, 1980). Solid media contained 1.5% agar. For *V. fischeri*, antibiotics were added to the following concentrations when necessary: erythromycin (Erm) at 5 μg ml^{-1}, tetracycline (Tet) at 5 μg ml^{-1} in LBS or 30 μg ml^{-1} in SWT and HMM, or chloramphenicol (Cm) at 2.5 μg ml^{-1}. The following antibiotics were added to *E. coli* media where appropriate: Cm at 25 μg ml^{-1}, Tet at 15 μg ml^{-1}, kanamycin (Kan) at 50 μg ml^{-1}, or ampicillin (Ap) at 100 μg ml^{-1}.

Plasmid construction

Plasmids used in this study are indicated in Table 1 and Table S1. Plasmids were generated using either restriction digest-based cloning or Gibson assembly cloning [New England Biolabs (NEB)]. In some cases, DNA sequences of interest were amplified via PCR using the indicated primers and inserted into the pJET1.2 cloning vector. DNA sequences were subcloned into the pKV363 suicide vector used for gene deletions, the pKV69 overexpression plasmid, or the pEVS107 mini-Tn*7* delivery vector using standard molecular techniques. Alternatively, sequences were amplified using the indicated primers and then inserted into a mobilization vector using the Gibson Assembly approach (NEB). For site-directed mutagenesis of *sypF*, *sypG*, or *sypF**, either Gibson Assembly or the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) with the primer(s) indicated in Table S2 was used.

Wrinkled colony assay

V. fischeri cells were grown overnight shaking at 28°C in LBS Tet and then subcultured and grown to an optical density of 600 nm (OD_{600}) of 0.2. 10 μl of the culture were spotted on LBS plates containing Tet to maintain plasmid selection. All spots were grown at room temperature (24°C) and images were captured at the indicated time points using a Zeiss stemi 2000-C dissecting microscope.

β-galactosidase measurements

V. fischeri strains were grown overnight in triplicate at 24°C with shaking in HMM with Tet. Cultures were back-diluted into fresh medium to an OD_{600} of 0.2 and then grown for 24 hours. 1 ml was removed and β-galactosidase activity was measured as described (Miller, 1972). Protein levels were assessed using previously described methods (Lowry *et al.*, 1951) and the data are reported as β-galactosidase activity mg^{-1} of protein.

Western blot procedure

Overnight samples of *V. fischeri* cells were standardized by OD_{600} and lysed with 2X sample buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol, 12% β-mercaptoethanol, 0.01%

bromophenol-blue). When higher concentrations of cells were needed to assess SypF-FLAG levels expressed in single copy, samples were lysed with B-PER (Thermo Scientific) with 10 mg mL^{-1} DNase. Lysates were resolved on an SDS-polyacrylamide gel (10%, 29:1 acrylamide: *N, N′*-methylene-bis-acrylamide, 375 mM Tris pH 8.6, 0.1% SDS), transferred to a PVDF membrane, and subjected to standard western blot procedures using an anti-flag primary antibody (Sigma-Aldrich) and a HRP conjugated secondary antibody (Sigma-Aldrich). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate with subsequent exposure to film.

Protein production

Sequences encoding the REC domain of SypG and the cytoplasmic form of SypF were amplified by PCR and cloned into pMAL-c5x using Gibson Assembly to generate N-terminal maltose binding protein (MBP) fusion proteins. Plasmids were transformed into the ER2508 strain (NEB), a BL21 derivative that does not express native MBP. To purify cytoplasmic MBP-SypF (pANN48) and MBP-SypF* (pANN74), 1 liter of Amp-containing LB was inoculated with the appropriate *E. coli* strain and grown to an OD_{600} of 0.7 at 37°C. Protein production was induced with 0.1 mM IPTG at 18°C overnight. Cells were harvested by centrifugation (10,000 × g) for 10 min and lysed using B-PER detergent (Thermo Scientific) with 100 µl 20 mg ml^{-1} lysozyme (Thermo Scientific), 20 µl 10 mg ml^{-1} DNase (Sigma) and 50 µl 100 µM PMSF (Sigma). Lysates were cleared by centrifugation at 16,000 × g for 20 min. Supernatant was applied to an amylose-resin column (NEB), washed three times with 1X Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2CO_4, 1.8 mM KH_2PO_4, pH 7.4), and eluted with 10 mM maltose. An Amicon 30k filter device (Millipore) equilibrated with storage buffer (50 mM Tris pH 8, 50 mM KCl, 50% glycerol) was used to exchange the elution buffer with storage buffer and to concentrate the purified protein. To purify MBP-SypG-REC (pANN49), a similar approach as above was taken, except 500 ml of cells at an OD_{600} of 0.5 were induced with 0.5 mM IPTG at 24°C overnight. To purify GST-SypE (pARM141) we modified the methods from Morris and Visick (Morris & Visick, 2013) as follows: briefly, pARM141 expressed from the ER2508 strain was used because this improved solubility of GST-SypE. This *E. coli* strain was grown to an OD_{600} of 0.5 and then induced with 0.4 mM IPTG overnight. Cells were harvested and lysed with Bugbuster (Novagen), and the supernatants were applied to Glutathione Sepharose 4B columns. Bound proteins were eluted with 10 mM glutathione. GST-SypE was concentrated and the elution buffer was exchanged with storage buffer using an Amicon 30k filter device (Millipore). Purified proteins were assessed by resolving samples on a 10% SDS-polyacrylamide gel with subsequent Coomassie Brilliant Blue R-250 protein staining (Thermo-Scientific) or western immunoblotting procedures as described above using anti-GST or anti-MBP primary antibodies (Sigma).

in vitro assays

Autokinase reaction: 2 µg/µL of purified MBP-SypF or MBP-SypF* were incubated in kinase buffer [50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM $MgCl_2$, and 5 µCi [γ^{32}P]-ATP (3000 Ci $mmol^{-1}$)] for 30 minutes at 28°C. In reactions without radiolabelled ATP, the same volume of 2 mM cold ATP was added. Samples were stopped with 5X sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 20% glycerol, 3% β-mercaptoethanol, 0.01%

bromophenol-blue), electrophoresed through a 10% SDS-polyacrylamide gel which was dried for 2 h and then exposed to film for 24–48 h. Phosphotransfer reactions: phospho-MBP-SypF or phospho-MBP-SypF* was obtained as described above. Equimolar concentrations of GST-SypE or MBP-SypG-REC were added and the reactions were incubated for 30 min. As a negative control, GST-SypE or MBP-SypG-REC was incubated in the same buffer conditions for 30 min but in the absence of a kinase. To assess levels of phosphorylated proteins, autoradiographs were generated as described above.

Colonization assay

V. fischeri strains were grown on agar plates overnight and then inoculated and grown to early log phase in liquid SWT media without shaking at 28°C. Aposymbiotic juvenile squid were collected shortly after hatching and placed in artificial sea water (ASW) (Instant Ocean, United Pet Group) that contained *V. fischeri* strains at a concentration of 1000 cells ml^{-1}. Colonization was allowed to proceed for 18 hours at which point individual *E. scolopes* were homogenized in 70% ASW. Serial dilutions of the homogenates were plated on SWT to determine the CFU of *V. fischeri* per squid. Limit of detection is 14 CFUs of *V. fischeri* per squid. 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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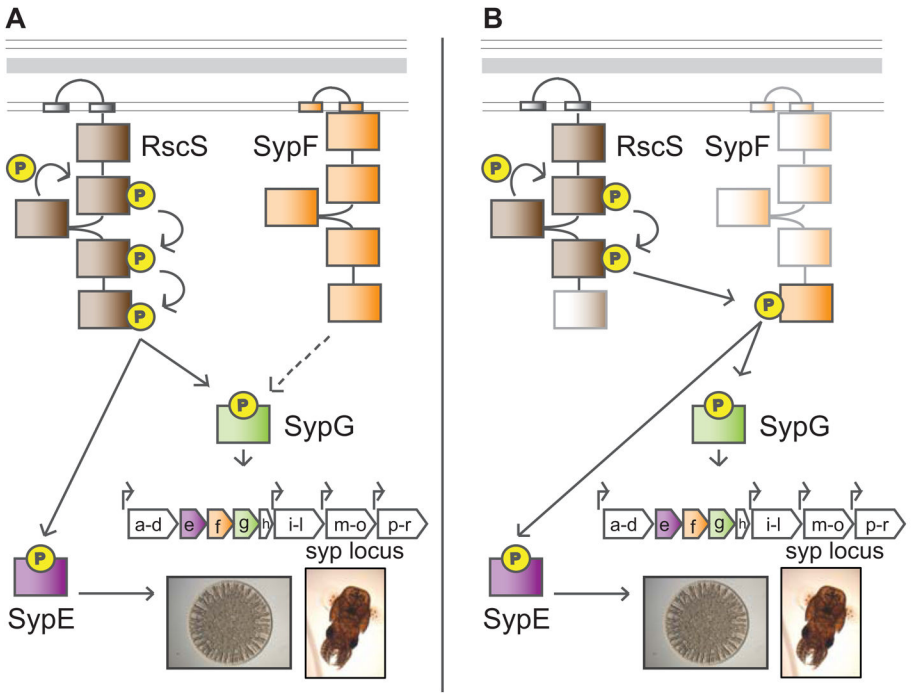

Figure 1. Syp biofilm regulation

Biofilm formation and host colonization by *V. fischeri* is controlled by a complex two-component signaling (TCS) pathway. (A) Previous model: the hybrid sensor kinase (SK), RscS, functions upstream of two response regulators (RR), SypE and SypG, to promote biofilm formation on agar plates (depicted as a wrinkled colony) and biofilm formation during colonization (represented by an image of a squid, the host for *V. fischeri*). Phospho-SypG functions as a transcription factor to activate the transcription of the *syp* locus at four promoters, and SypE inhibits biofilms at a level below *syp* transcription. When phosphorylated, SypE is no longer inhibitory. The *sypE* and *sypG* genes reside within the *syp* locus. Between *sypE* and *sypG* lies an additional hybrid SK gene, *sypF*, with an unclear role in biofilms. (B) Revised model: the C-terminal HPt domain of SypF functions between RscS and the two RRs, SypE and SypG, thus bypassing the requirement for the C-terminal domain of RscS. The faded colors indicate domains found to be non-essential for wrinkled colony formation and colonization.

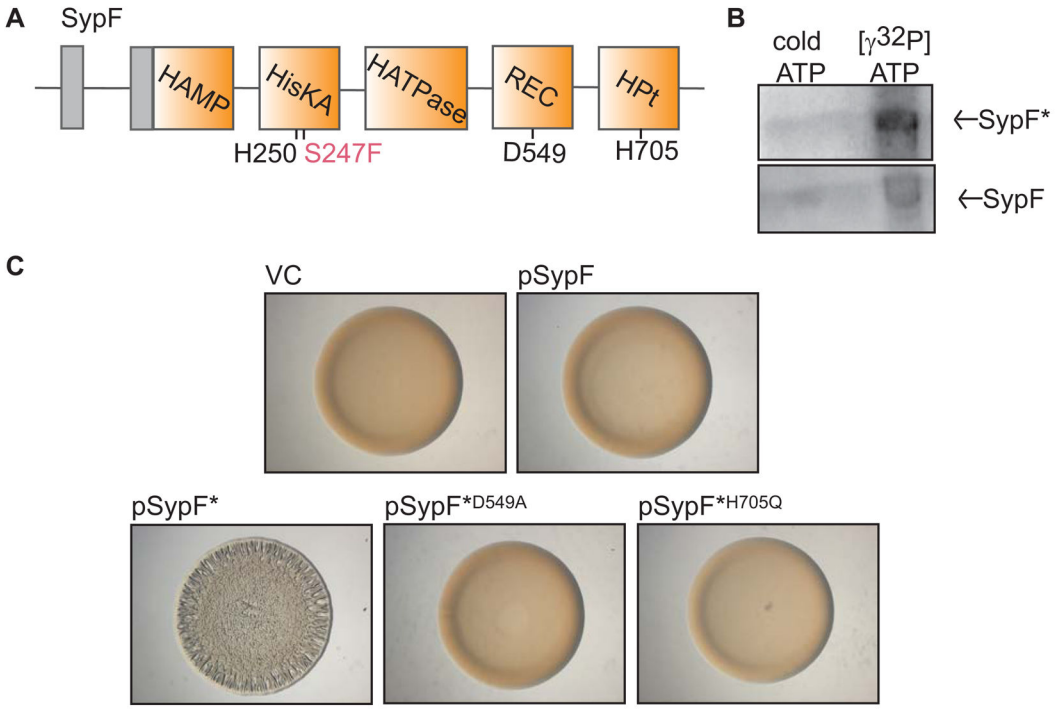

Figure 2. Function of SypF* as a SK

(A) Cartoon of the predicted functional domains within SypF, including HAMP, HisKA, HATPase_c, REC, and HPt domains (orange boxes) as well as transmembrane regions (gray boxes) flanking a putative periplasmic loop. Conserved putative sites of phosphorylation are indicated below in black type. SypF* contains two mutations. The critical mutation, S247F, is indicated in pink type. (B) Autoradiograph of purified MBP-SypF* (above) and wild-type MBP-SypF (below) after incubation with unlabeled ATP or [γ-^{32}P]-ATP. (C) Colony morphology of wild-type (WT) *V. fischeri* strain ES114 containing vector control (VC, pKV69) or various SypF and SypF* overproduction plasmids as follows: pSypF (pCLD54), pSypF* (pCLD29), pSypF*D549A (pANN61), and pSypF*H705Q (pANN62). Cultures of the indicated strains were spotted on agar plates and colony morphology was assessed after 24 h.

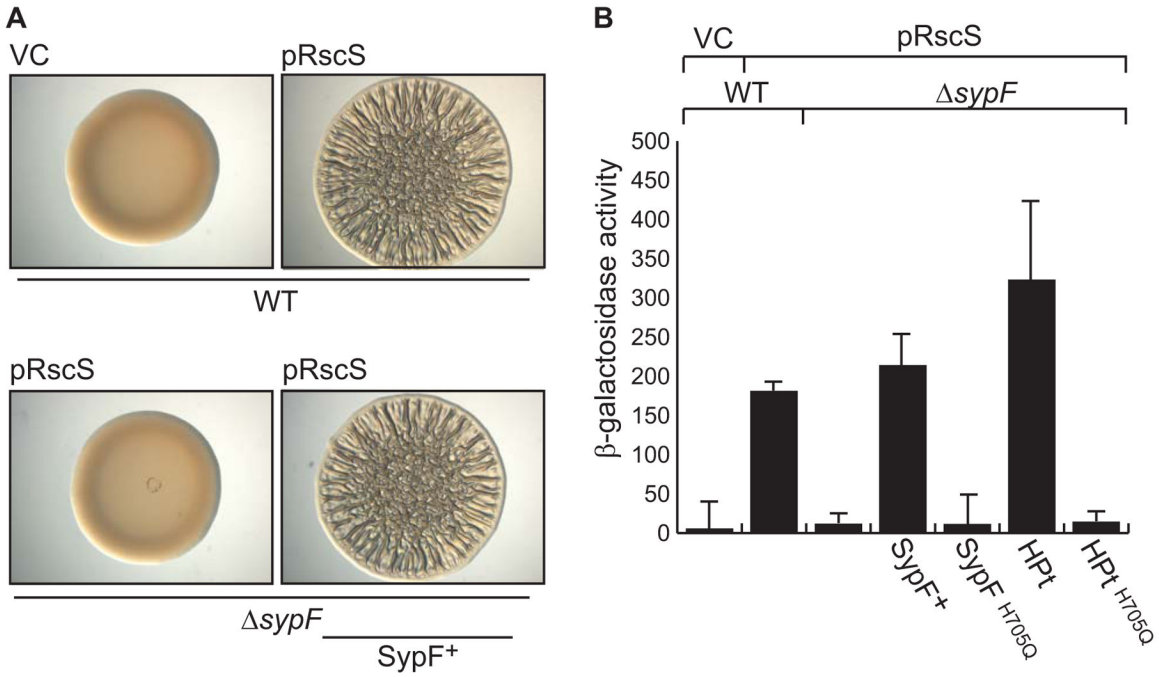

Figure 3. Role of SypF in RscS-induced biofilm formation and *syp* transcription

RscS-induced (A) biofilm formation and (B) *syp* transcription was assessed by overproducing RscS from a plasmid (pARM7). (A) Colony morphology of wild-type cells (WT, KV4389), a Δ*sypF* strain (KV6921), or the complemented Δ*sypF* strain (KV6659). These cells contained either vector control (VC, pKV282) or pRscS, as is indicated in the figure. Cultures of the indicated strains were spotted on an agar plate and colony morphology was assessed after 39 hours. (B) P_{sypA}-*lacZ* reporter activity in WT cells, the Δ*sypF* strain, and in Δ*sypF* strains producing the SypF proteins. The strains used for this experiment contained either VC or pRscS as indicated in the figure. The P_{sypA}-*lacZ* reporter base strains used are as follows: WT (KV7410); Δ*sypF* (KV7412), Δ*sypF sypF*$^{+}$ (KV7386), Δ*sypF sypF*^{H705Q} (KV7387), Δ*sypF sypF*-HPt (KV7377), and Δ*sypF sypF*-HPt^{H705Q} (KV7413). Error bars represent the standard deviation.

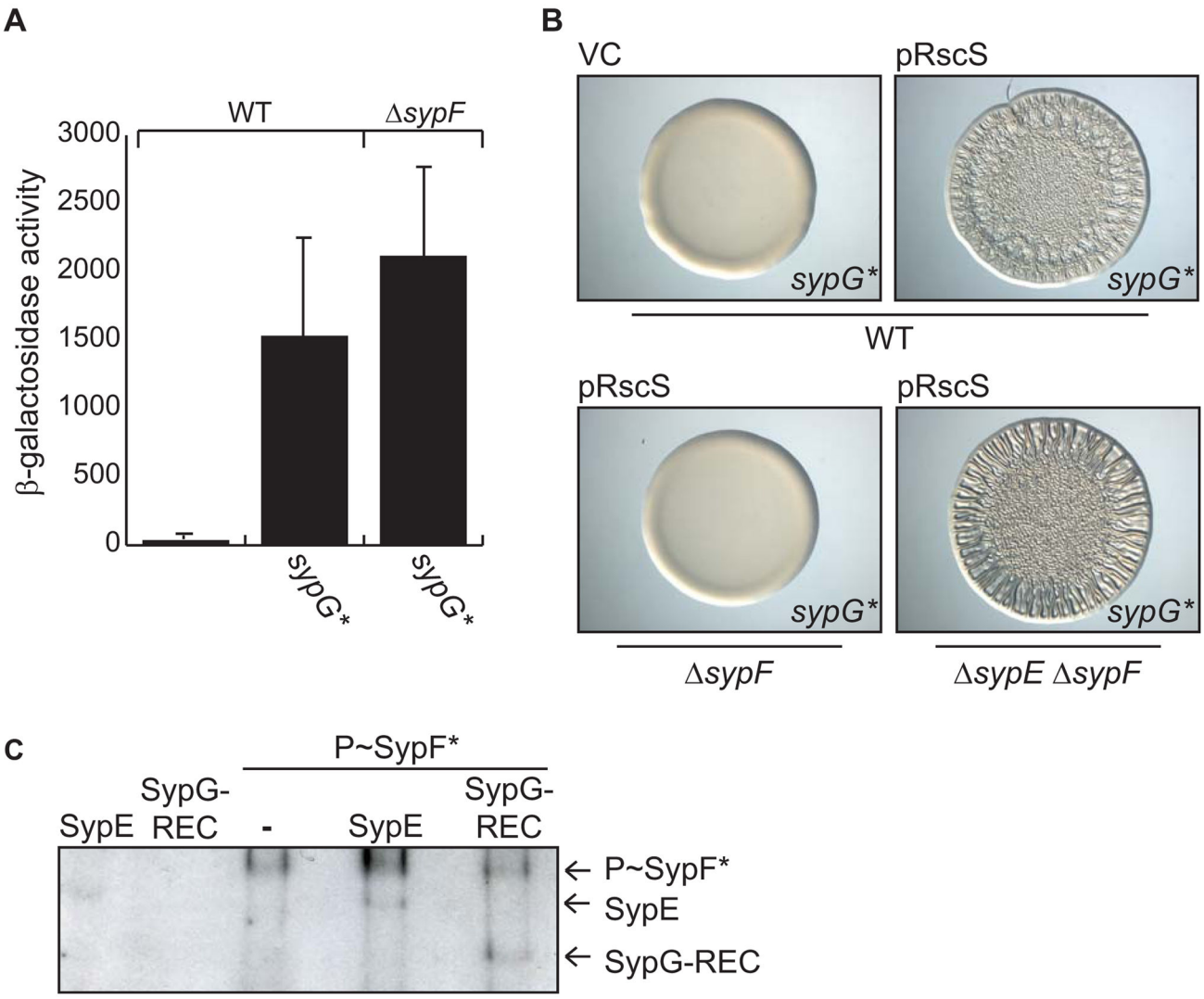

Figure 4. Determining where SypF functions in the Syp pathway

(A) SypG*-induced P_{sypA}-*lacZ* reporter activity in WT (KV7230) or Δ*sypF* (KV7231) strains. Error bars represent standard deviation. (B) Wrinkled colonies of WT *V. fischeri* strains producing SypG* (KV6527) with vector control (VC, pKV282) or pRscS (pARM7) (top two panels) and of pRscS, SypG*-producing Δ*sypF* (KV6526) and Δ*sypE* Δ*sypF* (KV6586) strains (bottom two panels). Cultures were spotted and colony morphology was assessed after 19 hours. (C) *in vitro* phosphotransfer assay. Left two lanes: GST-SypE or MBP-SypG-REC incubated with radiolabelled ATP. Right three lanes: phospho-SypF* incubated with or without GST-SypE or MBP-SypG-REC.

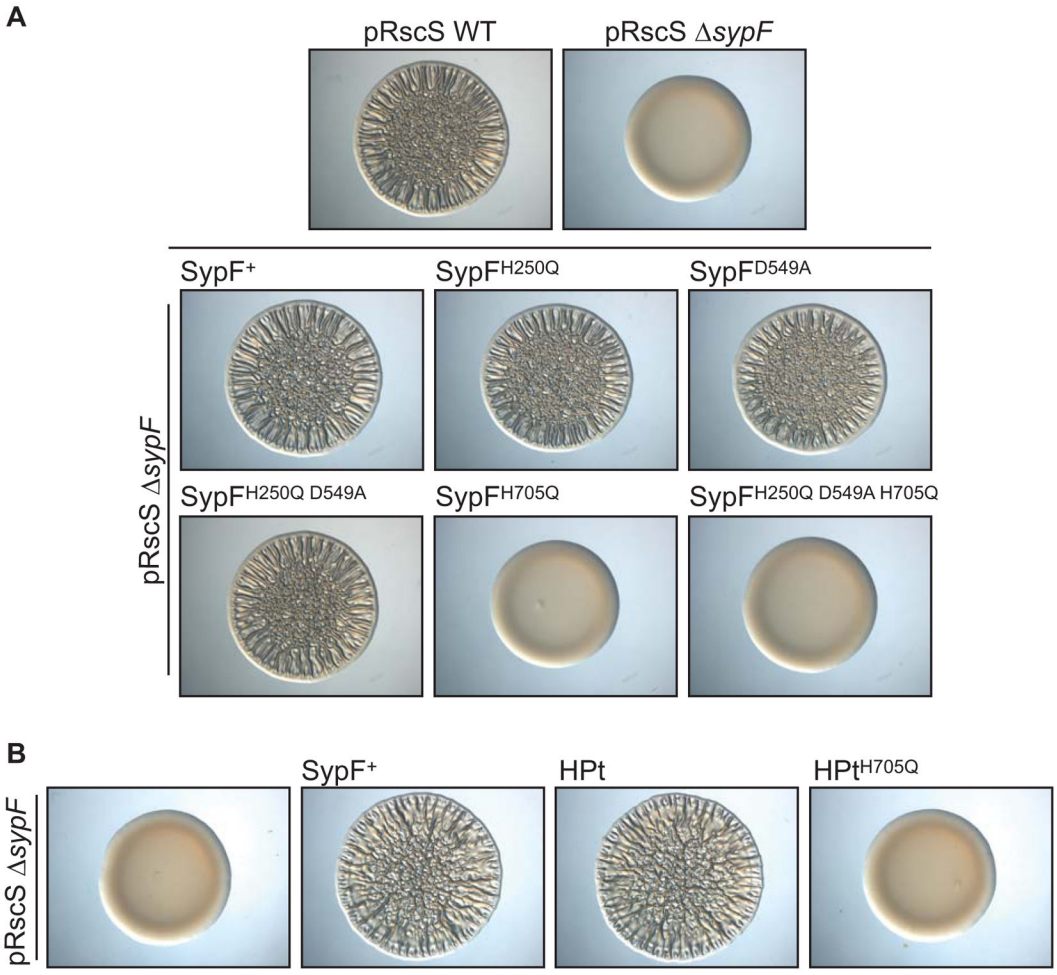

Figure 5. Function of the SK activity of wild-type SypF

(A) RscS-induced (pARM7) wrinkled colony formation in WT cells or *sypF* deletion strains with or without *sypF* alleles expressed in single copy. Strains used are as follows: WT (KV4389); Δ*sypF* (KV6921); Δ*sypF sypF*$^{+}$ (KV6659); Δ*sypF sypF*^{H250Q} (KV6896); Δ*sypF sypF*^{D549A} (KV6692); Δ*sypF sypF*^{H705Q} (KV7085); Δ*sypF sypF*$^{H250Q\ D549A}$ (KV7154); Δ*sypF sypF*$^{H250Q\ D549A\ H705Q}$ (KV7155). Strains were spotted on agar plates and colony morphology was assessed after 39 hours. (B) RscS-induced (pARM7) wrinkled colony phenotype of a Δ*sypF* strain (KV6291), or the Δ*sypF* strain containing full-length *sypF* (KV6659), *sypF*-HPt (KV7226) or *sypF*-HPt^{H705Q} (KV7485) after 39 hours.

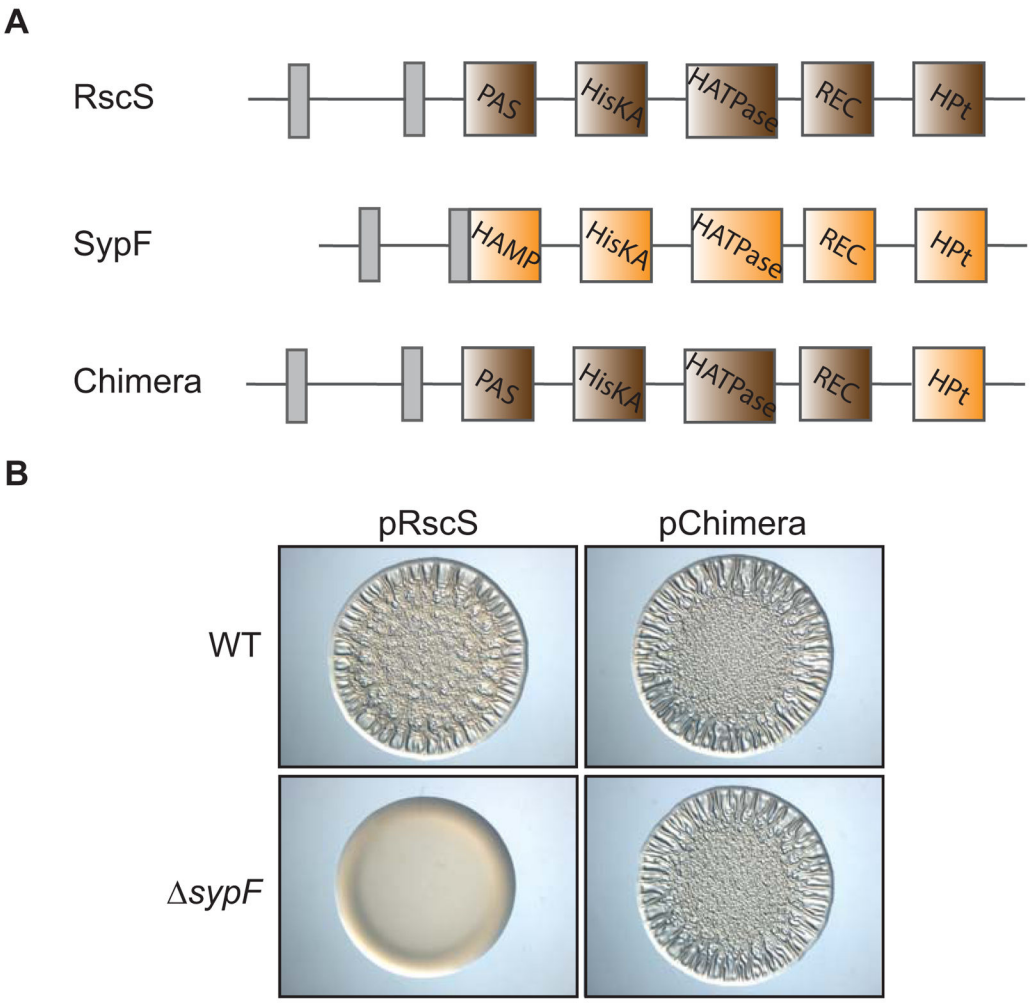

Figure 6. Interaction between RscS and SypF in the Syp biofilm pathway

(A) Cartoon image comparing the predicted functional domains of RscS (brown: PAS, HisKA, HATPase_c, REC, Hpt), SypF (orange: HAMP, HisKA, HATPase_c, REC, HPt), and an RscS-SypF chimera that contains the N-terminal regions of RscS and the HPt domain of SypF. Grey boxes indicate transmembrane regions that flank a putative periplasmic domain. (B) Wrinkled colony formation of WT (ES114) or *sypF* deletion (KV5367) cells overproducing RscS (pARM7) or the RscS-SypF chimera (pANN69). Indicated strains were spotted and grown for 22 hours.

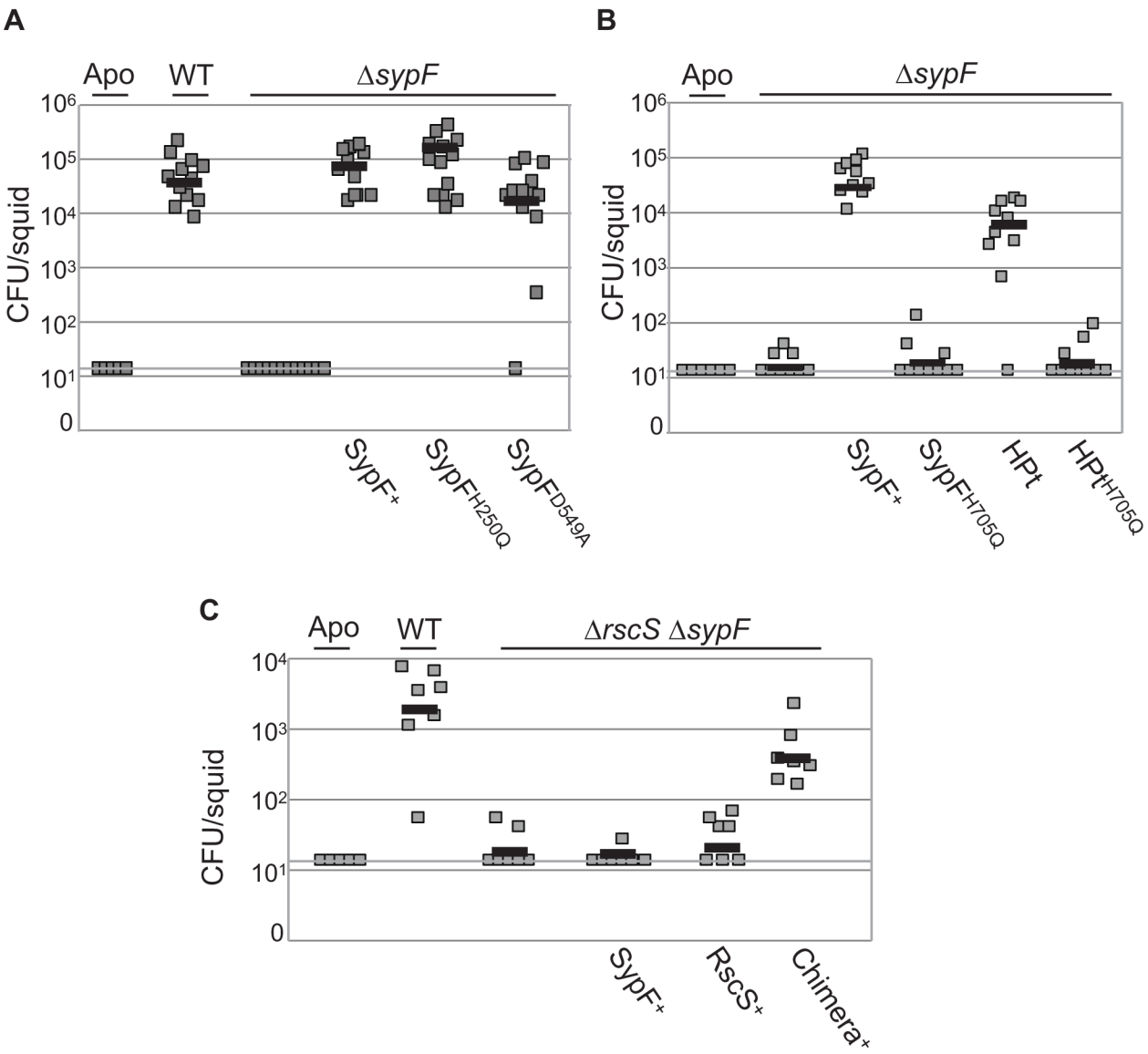

Figure 7. Mechanism by which SypF functions *in vivo*

(A, B, C) Indicated strains of *V. fischeri* were incubated with aposymbiotic (Apo) juvenile squid for 18 hours, and colonization of *E. scolopes* was determined by calculating colony-forming units (CFU) of *V.* fischeri in each squid. (Limit of detection, CFU = 14). (A, B) Colonization comparison between WT cells and *sypF* deletion strains expressing *sypF* alleles in single copy. Strains used are as follows: WT (KV4389), Δ*sypF* (KV6921); Δ*sypF* *sypF*$^{+}$ (KV6659); Δ*sypF* *sypF*^{H250Q} (KV6896); Δ*sypF* *sypF*^{D549A} (KV6692); Δ*sypF* *sypF*^{H705Q} (KV7085); Δ*sypF* *sypF*-HPt (KV7226), and Δ*sypF* *sypF*-HPt^{H705Q} (KV7485). Panels A and B represent independent experiments. (C) Colonization phenotype of WT cells (KV4389), a Δ*rscS* Δ*sypF* strain (KV7657), and the Δ*rscS* Δ*sypF* strain that produces SypF (KV7656), RscS (KV7654), or the chimera (KV7651).

Table 1

Strains and key plasmids used in this study

Strains		
Strain	***Relevant genotype***	***Source or Reference***
ES114	Wild-type *V. fischeri*	(Boettcher & Ruby, 1990)
KV3246	att*Tn7*::P_{sypA}-*lacZ*	(Morris & Visick, 2013)
KV4389	*att*Tn*7*::erm^R	(Morris *et al.*, 2011)
KV5367	Δ*sypF*	This study
KV6351	Δ*rscS* Δ*sypF*	This study
KV6526	Δ*sypF att*Tn*7*::*sypG**-FLAG	This study
KV6527	*att*Tn*7*::*sypG**-FLAG	This study
KV6586	Δ*sypE* Δ*sypF att*Tn*7*::*sypG**-FLAG	This study
KV6659	Δ*sypF att*Tn*7*::*sypF*-FLAG	This study
KV6692	Δ*sypF att*Tn*7*::$sypF^{D549A}$-FLAG	This study
KV6896	Δ*sypF att*Tn*7*::$sypF^{H250Q}$-FLAG	This study
KV6921	Δ*sypF att*Tn*7*::erm^R	This study
KV7085	Δ*sypF att*Tn*7*::$sypF^{H705Q}$-FLAG	This study
KV7230	*att*Tn*7*::*sypG**-FLAG P_{sypA}-*lacZ*	This study
KV7231	Δ*sypF att*Tn*7*::*sypG**-FLAG P_{sypA}-*lacZ*	This study
KV7154	Δ*sypF att*Tn*7*::$sypF^{H705Q\ D549A}$-FLAG	This study
KV7155	Δ*sypF att*Tn*7*::$sypF^{H705Q\ D549A\ H705Q}$-FLAG	This study
KV7226	Δ*sypF att*Tn*7*::*sypF*-HPt-FLAG	This study
KV7371	IG (*yeiR-glmS*)::P_{sypA}-*lacZ* [1]	This study
KV7372	Δ*sypF* IG (*yeiR-glmS*)::P_{sypA}-*lacZ* [1]	This study
KV7377	Δ*sypF* IG (*yeiR-glmS*)::P_{sypA}-*lacZ att*Tn*7*::*sypF*- HPt-FLAG[1]	This study
KV7386	ΔsypF IG (*yeiR-glmS*)::P_{sypA}-*lacZ att*Tn*7*::*sypF*- FLAG[1]	This study
KV7387	Δ*sypF* IG (*yeiR-glmS*)::P_{sypA}-*lacZ att*Tn*7*::$sypF^{H705Q}$-FLAG[1]	This study
KV7410	IG (*yeiR-glmS*)::P_{sypA}-*lacZ att*Tn*7*::erm^R [1]	This study
KV7412	Δ*sypF* IG (*yeiR-glmS*)::P_{sypA}-*lacZ att*Tn*7*::erm^R [1]	This study
KV7413	Δ*sypF* IG (*yeiR-glmS*)::P_{sypA}-*lacZ attTn7*::*sypF*- HPt^{H705Q}-FLAG [1]	This study
KV7485	Δ*sypF att*Tn*7*::*sypF*-HPt^{H705Q}-FLAG	This study
KV7651	Δ*sypF* Δ*rscS att*Tn*7*::*rscS-sypF* chimera	This study
KV7654	Δ*sypF* Δ*rscS att*Tn*7*::*rscS*	This study
KV7656	Δ*sypF* Δ*rscS att*Tn*7*::*sypF*	This study
KV7657	Δ*sypF* Δ*rscS att*Tn*7*::erm^R	This study

Key Plasmids		
Plasmid	**Description**	***Source or Reference***
pANN61	pKV69 + *sypF**^{D549A} generated using primer 1295	This study

Key Plasmids

Plasmid	Description	*Source or Reference*
pANN62	pKV69 + *sypF**^{H705Q} generated using primer 1569	This study
pANN69	pCLD29[2] + *rscS-sypF* chimera-FLAG generated using primers 1899, 1900, 1901, 1882	This study
pARM7	pKV282 + *rscS*	(Morris *et al.*, 2011)
pCLD29	pKV69 + *sypF**	(Darnell *et al.*, 2008)
pCLD54	pKV69 + *sypF*	(Darnell *et al.*, 2008)
pKV69	Vector; Cm^R, Tet^R	(Visick & Skoufos, 2001)
pKV282	Vector, Tet^R	(Morris *et al.*, 2011)

[1] IG (*yeiR-glmS*): intergenic (IG) region between the *yeiR* and *glmS* genes directly upstream of the Tn*7* site in the chromosome

[2] the original *sypF** sequence was removed from pCLD29 using restriction enzymes before the insertion of indicated DNA sequences