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An intricate network of regulators controls biofilm formation and colonization by *Vibrio fischeri*

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Abstract

The initial encounter between a microbe and its host can dictate the success of the interaction, be it symbiosis or pathogenesis. This is the case, for example, in the symbiosis between the bacterium *Vibrio fischeri* and the squid *Euprymna scolopes*, which proceeds via a biofilm-like bacterial aggregation, followed by entry and growth. A key regulator, the sensor kinase RscS, is critical for symbiotic biofilm formation and colonization. When introduced into fish symbiont strains that naturally lack the *rscS* gene and cannot colonize squid, RscS permits colonization, thereby extending the host range of these bacteria. RscS controls biofilm formation by inducing transcription of the symbiosis polysaccharide (*syp*) gene locus. Transcription of *syp* also requires the σ^{54}-dependent activator SypG, which functions downstream of RscS. In addition to these regulators, SypE, a response regulator that lacks an apparent DNA binding domain, exerts both positive and negative control over biofilm formation. The putative sensor kinase SypF and the putative response regulator VpsR, both of which contribute to control of cellulose production, also influence biofilm formation. The wealth of regulators and the correlation between biofilm formation and colonization adds to the already considerable utility of the *V. fischeri-E. scolopes* model system.

Introduction

20 years after the model system was established, the symbiosis between the bacterium *Vibrio fischeri* and its symbiotic host, the squid *Euprymna scolopes*, continues to provide rich and novel insights into a variety of problems in bacteria-host interactions (reviewed by (McFall-Ngai, 2008; Ruby, 2008; Stabb *et al.*, 2000; Visick and Ruby, 2006)). Indeed, in the past year alone, researchers have uncovered numerous components of the bacteria-host interaction network, including the ability of the squid's symbiotic light organ to respond to light, colonization, and bacterially-released small molecules including autoinducers and a component of peptidoglycan, Trachael Cytotoxin (Chun *et al.*, 2008; Tong *et al.*, 2009; Troll *et al.*, 2009). These studies suggest an interaction of a complexity that rivals that of traditional mammalian models. Furthermore, host defense cells called haemocytes contribute to specificity by binding to and engulfing non-native or mutant bacteria preferentially over native symbionts, thus demonstrating the utility of this system as a model for innate immunity (Nyholm *et al.*, 2009). Understanding of the evolution and ecology of the interaction has advanced through studies of natural populations combined with experimental manipulations, which suggested that a limited number of bacteria (1–2) likely enter and populate each of the 6 internal crypts that comprise the light organ of the newly hatched and initially uncolonized squid; as a result, polyclonal but segregated populations can exist within a single squid (Wollenberg and Ruby, 2009). Finally, genomic analysis of representative symbiotic and non-symbiotic *V. fischeri* strains led to the recognition that a single regulatory gene, *rscS*, could be sufficient to alter host specificity, as it enabled a fish symbiont to colonize squid (Mandel *et al.*, 2009). The molecular details underlying the

influence of this regulator on colonization and specificity will be the main subjects of this review.

Symbiotic initiation and host specificity

The *V. fischeri-E. scolopes* symbiosis is highly specific. Juvenile squid hatch uncolonized but become colonized within hours following exposure to symbiosis competent bacteria. Despite the presence of numerous other bacteria in the seawater, including closely related bacteria such as *Vibrio parahaemolyticus*, only *V. fischeri* successfully colonizes the squid's symbiotic light organ (McFall-Ngai and Ruby, 1991). Furthermore, not all strains of *V. fischeri* are equally capable of colonizing, or are even symbiosis-competent (Nishiguchi *et al.*, 1998; Ruby and Lee, 1998). For example, *V. fischeri* strains isolated from symbiosis with the fish *Monocentris japonica* generally fail to colonize *E. scolopes* (Mandel *et al.*, 2009).

A number of factors contribute to this remarkable host specificity. Upon entering the light organ, *V. fischeri* must pass through mucus-filled ducts that contain outward-beating cilia (McFall-Ngai and Ruby, 1998; Visick and McFall-Ngai, 2000) and nitric oxide (Davidson *et al.*, 2004), an anti-bacterial defense. In addition, the crypts contain halide peroxidase, a host defense protein that catalyzes the production of hypochlorous acid, which is toxic to bacteria (Small and McFall-Ngai, 1999; Tomarev *et al.*, 1993; Weis *et al.*, 1996). Finally, macrophage-like haemocytes circulate within the crypts (Nyholm and McFall-Ngai, 1998). *V. fischeri* must survive each of these host-imposed stresses that presumably decrease the chances of colonization by other microbes.

Surprisingly, host specificity appears to begin even before the bacteria enter the ducts. Newly hatched juvenile squid exhibit a short permissive period in which non-symbionts (and even particles such as beads) can enter the light organ, followed by a non-permissive period during which nothing can enter (Nyholm *et al.*, 2002). Subsequently, mucus is secreted to the surface of the light organ (Nyholm *et al.*, 2000; Nyholm *et al.*, 2002). *V. fischeri* aggregates within this mucus, an event that is critical to colonization (Nyholm *et al.*, 2000; Yip *et al.*, 2006). For example, a mutant lacking the sensor kinase RscS fails to aggregate, a phenotype that correlates well with its severe defect in symbiotic initiation (Visick and Skoufos, 2001; Yip *et al.*, 2006). Studies of additional mutants have provided further support for the connections observed between aggregation and initiation of colonization (Millikan and Ruby, 2002; Whistler *et al.*, 2006).

Some non-symbionts, such as the closely related *V. parahaemolyticus*, appear to be capable of adhering to the light organ, but others, such as the Gram positive *Bacillus cereus*, are not, supporting the idea that specificity occurs at this stage of colonization (Nyholm *et al.*, 2000). Furthermore, a 1:1 mixture of *V. fischeri* and the non-symbiont *V. parahaemolyticus* results in an aggregate consisting of more than 80% *V. fischeri* cells (Nyholm and McFall-Ngai, 2003). These latter data suggest that specificity results, in part, from a superior ability of *V. fischeri* to interact with the surface of the squid=s light organ.

RscS and colonization

Initiation of symbiotic colonization requires a two-component sensor kinase gene that we designated *rscS*, for regulator of symbiotic colonization sensor (Visick and Skoufos, 2001). A subset of juvenile squid exposed to an *rscS* mutant remain uncolonized, while the rest become colonized only after a significant delay (Visick and Skoufos, 2001). Initial characterization failed to reveal defects in any known or suspected colonization traits, including motility and bioluminescence; however, subsequent studies (described below) uncovered an important role for RscS in inducing biofilm formation. The symbiotic defect

of the *rscS* mutant is likely to be due to its failure to aggregate on the surface of the light organ, an event that we hypothesize to correspond to biofilm formation (Yip *et al.*, 2006).

In the sequenced squid symbiont strain ES114, the *rscS* gene is located between *glpR* and *glpK*, genes involved in glycerol regulation and metabolism, respectively (Ruby *et al.*, 2005; Visick and Skoufos, 2001). When the genome of a second *V. fischeri* strain, the *M. japonica* isolate MJ11, became available, it was noted that *rscS* is absent from the *glp* locus (Mandel *et al.*, 2009). Because the fish symbiont is not proficient at squid colonization, it was proposed that lack of *rscS* could account for the colonization deficiency of this strain. Indeed, introduction of *rscS* on a multi-copy plasmid permits squid colonization by MJ11 (Mandel *et al.*, 2009). These data demonstrated that RscS alone is sufficient to extend the host range of *V. fischeri* from fish to squid. In further support of this notion, disruption of *rscS* in a variety of *V. fischeri* isolates from different geographic locations impairs symbiotic initiation (Mandel *et al.*, 2009). These results demonstrate that a single regulatory gene can alter the host range of an animal-associated microorganism and point to RscS and the genes it controls as key regulators of early steps in host colonization.

RscS and the *syp* locus

RscS is predicted to be a member of the histidine sensor kinase class of two-component regulators (Visick and Skoufos, 2001). Sensor kinases typically sense specific environmental signals and transduce that information by initiating a phosphorelay, resulting in the phosphorylation of their cognate response regulators and thus an output such as altered transcription of target genes (Gao and Stock, 2009). The gene for *rscS*, however, is not linked to a gene encoding a response regulator, and the 40 response regulators recognizably encoded in the *V. fischeri* genome made the hunt for a partner non-trivial (Hussa *et al.*, 2007). Furthermore, neither a partner nor a target could be readily identified based on similarity in phenotype, as the *rscS* mutant does not exhibit any phenotype in culture (Visick and Skoufos, 2001).

A break-through in understanding the role of RscS came with the discovery of an 18-gene symbiosis polysaccharide gene locus, *syp* (Yip *et al.*, 2005; Yip *et al.*, 2006). Mutants defective for specific *syp* genes exhibit phenotypes similar to those of the *rscS* mutant: defective for symbiotic initiation, but not for a variety of traits tested in culture (Yip *et al.*, 2005). The *syp* genes encode proteins with similarity to those involved in exopolysaccharide biosynthesis, including six putative glycosyltransferase genes. Of note, the *syp* locus also encodes two response regulators, potential partners for RscS. One of them, SypG, is a putative a σ^{54}-dependent enhancer binding protein, while the other, SypE, lacks any apparent DNA binding sequences.

A connection between RscS and *syp* was made when RscS was overproduced in a *syp* reporter strain. Because RscS is predicted to sense an environmental signal, potentially missing under standard laboratory conditions, a plasmid containing the *rscS* gene was mutagenized in an attempt to generate an allele with increased activity. This approach yielded a plasmid that substantially overproduces the RscS protein, resulting in increased transcription of the *syp* locus (Geszvain and Visick, 2008a; Yip *et al.*, 2006). It also induces biofilm formation: whereas control cells produce smooth colonies, *rscS*-overexpressing cells produce wrinkled colonies (Fig. 1). This wrinkled colony phenotype is similar to other biofilm-forming *Vibrio* species such as *Vibrio cholerae*, which produces rugose colonies under certain conditions (Yildiz and Visick, 2009). Furthermore, *rscS*-overexpressing cells produce a thick pellicle at the air-liquid interface of statically grown cultures, a phenomenon not seen with wild-type *V. fischeri* (Yip *et al.*, 2006); incredibly, these pellicles are so strong

that the cultures can be inverted without loss of the liquid medium (Fig. 1). These biofilm phenotypes depend upon a functional *syp* locus as well as RscS overproduction.

The physiological importance of these biofilm phenotypes was underscored by the finding that RscS-overproducing cells form substantially enhanced aggregates on the surface of the light organ — upwards of 20X bigger than controls. As with the *in vivo* biofilms, disruption of the *syp* locus eliminates or substantially reduces the size of the symbiotic aggregates induced by RscS. Importantly, this RscS-directed activity substantially promotes symbiotic colonization, as determined by competitive colonization experiments: squid inoculated with mixtures of vector-control and RscS-overexpressing wild-type cells become colonized exclusively by those that contain the RscS expression plasmid (Yip *et al.*, 2006)

In summary, a major function of RscS appears to be in controlling the *syp* locus, resulting in biofilm formation and symbiotic colonization. The ability of RscS to induce squid colonization by normally non-symbiotic strain MJ11 can likely be attributed to its ability to promote symbiotic biofilm formation, although this idea has not yet been directly tested. In support of it, however, are the findings that the *syp* locus is conserved in MJ11 and that RscS overexpression promotes biofilm formation by MJ11 (Mandel *et al.*, 2009).

RscS integrates positive and negative signals

RscS is predicted to be a hybrid sensor kinase. The C-terminus contains putative histidine kinase/ATPase (HisKA/HATPase), receiver (REC), and histidine phosphotransferase (Hpt) domains (Visick and Skoufos, 2001). This domain structure is similar to such well-characterized proteins as ArcB and BvgS that undergo two internal phosphorelay events prior to donation of the phosphoryl group to the cognate response regulator (Beier and Gross, 2008; Gao and Stock, 2009). Such additional domains involved in an intramolecular phosphorelay provide opportunities for multiple levels of control. The N-terminal region of RscS contains a large periplasmic domain flanked by two transmembrane helices, and a cytoplasmic PAS domain, all of which could contribute to sensory perception. The natural ligand(s) remains unknown, however.

Mutant alleles of *rscS* were constructed to study the roles of the various domains (Geszvain and Visick, 2008b). Perhaps not surprisingly, alterations of key residues in the HisKA and REC domains disrupt function, as measured by induction of *syp* transcription and biofilm formation. These data indicate that RscS functions as a kinase. In contrast, a substitution within the Hpt domain at a histidine predicted to be critical for phosphotransfer reduces but does not eliminate function. Perhaps the specific change facilitates phosphotransfer to the downstream response regulator from the conserved histidine within the HisKA domain, or perhaps the putative Hpt domain does not function as an Hpt. Additional work will be necessary to clarify the role of the predicted Hpt domain.

Substitutions of key residues in the PAS domain also abolish RscS activity. In RscS, the predicted location of the PAS domain is on the cytoplasmic face of the inner membrane, where it might be expected to respond to an internal cue. PAS domains in other proteins detect small molecules such as FAD or FMN, light, or oxygen (Taylor and Zhulin, 1999). Alterations to residues predicted to be involved in binding FAD lead to a greater disruption in RscS activity than do substitutions in those predicted to be critical for FMN binding, suggesting that the PAS domain of RscS might bind to a FAD cofactor (Geszvain and Visick, 2008b).

Signaling by FAD, if it occurs, might be influenced by membrane localization and/or by signaling through the periplasmic loop or transmembrane regions. Indeed, membrane localization of RscS seems important, as a deletion derivative containing only the

cytoplasmic portion of the protein (including PAS) exhibits reduced activity (Geszvain and Visick, 2008b). Surprisingly, however, deletions of the periplasmic loop result in increased activity, as do some substitutions within the first transmembrane helix, indicating that binding of an unidentified ligand to the periplasmic domain might inhibit, rather than activate, RscS activity. Together, these data reveal that RscS is a complex regulator, potentially integrating both inhibitory and stimulatory signals to initiate a phosphorelay that critically requires predicted phosphorylated residues in the HisKA and REC but not Hpt domains. An exciting future direction of this work will be to determine the nature of the signal(s) received by RscS.

RscS works upstream of SypG

The availability of RscS-induced phenotypes permitted a search for the response regulator(s) that functions downstream of RscS (Hussa *et al.*, 2008). The *rscS* over expression plasmid was introduced into each of 35 response regulator mutants (all but five of the 40 encoded by the *V. fischeri* genome), and the resulting phenotypes were evaluated. Most mutants exhibit biofilm phenotypes indistinguishable from those of the wild-type overexpression control. In several cases, some measures of biofilm formation (including glass attachment and pellicles) were impacted by disruption of specific response regulators, including *sypG*, *sypE*, *vpsR*, *flrC*, *arcA*, and *VF1401*. However, wrinkled colony formation is affected only by the disruption of either *sypG* or *sypE*, and induction of *syp* transcription by RscS depends only on *sypG*. The strong requirement for SypG in all RscS-induced phenotypes suggests that RscS works upstream of SypG (Hussa *et al.*, 2008).

Somewhat inconsistent with the above conclusion is the finding that overproduction of SypG alone does not induce the formation of wrinkled colonies or strong pellicles, although it does induce *syp* transcription. Furthermore, altering SypG by substituting glutamate for a conserved aspartate, a change that in many other response regulators produces a constitutively active protein (e.g., (Freeman and Bassler, 1999)), increases *syp* induction but does not promote biofilm formation (Hussa *et al.*, 2008). Although it is not always the case that the phenotypes of response regulators phenocopy those of their partner sensor kinases, these data raised the possibility that additional factors might be involved. Indeed, it was subsequently determined that deletion of the response regulator gene *sypE* permits the SypG overproduction strain to form wrinkled colonies and pellicles (Hussa *et al.*, 2008). This latter work thus established conditions under which overproduced SypG and overproduced RscS could induce similar phenotypes, supporting the idea that these proteins could work together. Furthermore, it appears that overproduction of RscS must somehow lead to inactivation of SypE. The role of SypE will be discussed further below.

SypG and σ^{54} regulate *syp* transcription

SypG is a putative σ^{54}-dependent response regulator, and binding sites for σ^{54}-containing RNA polymerase exist within the *syp* locus (Fig. 1). Specifically, σ^{54} sites exist within each of the four largest gaps between genes of the 18-gene *syp* locus (Yip *et al.*, 2005). Results from primer extension experiments designed to map the start sites of three of the genes (*sypA*, *sypI*, and *sypM*) are consistent with the putative σ^{54}-dependent promoters, and mutagenesis studies verify that transcription of at least *sypD* and *sypN* depends upon the gene for σ^{54}, *rpoN* (Yip *et al.*, 2005). Because σ^{54}-containing RNA polymerase requires a transcriptional activator to provide the energy for open complex formation (Buck *et al.*, 2000), it seems reasonable that the *syp* locus would require an activator such as SypG to bind and activate transcription. Indeed, bioinformatic analysis revealed the presence of a conserved 22 bp sequence upstream of each of the putative σ^{54} binding sites that we hypothesize to be the SypG binding site (Yip *et al.*, 2005). Our preliminary data support the

importance of the conserved sequences in *syp* transcription (Hussa and Visick, unpublished), but the precise role remains to be determined.

SypE, a novel response regulator

Although RscS functions upstream of SypG, the regulatory network is far from straightforward. Biofilm formation, while readily induced by overexpression of RscS, requires inactivation of *sypE* when SypG is overproduced. However, loss of SypE impairs biofilm formation induced by RscS. Thus, SypE appears to play both positive and negative roles in biofilm formation. Loss of *sypE* exerts only small effects on *syp* transcription, regardless of whether RscS or SypG is overproduced, suggesting that its impact is not at the level of *syp* transcription ((Hussa *et al.*, 2008) and Hussa and Visick, unpublished). These data indicate that SypE controls biofilm formation at another level, such as post-transcriptional control of the synthesis of the Syp polysaccharide or via control of the synthesis of another component or regulator of the biofilm matrix.

SypE is an unusual two-component response regulator in that its REC domain is located in the center of the protein (Yip *et al.*, 2005). In addition, instead of the typical DNA binding domain, SypE contains a putative serine kinase domain in its N-terminus and a putative serine phosphatase domain in its C-terminus. The potential opposing activities of these domains suggest a possible molecular basis for the apparent dual role of this protein in controlling biofilm formation. The phosphorylation state of the REC domain might then determine which activity is favored.

SypE thus is another complex regulator of biofilm formation in *V. fischeri*. Although the *syp* locus is conserved in many Vibrio species, *sypE* is generally lacking in these other *syp* loci (Yip *et al.*, 2005). These data suggest that *V. fischeri* uses SypE to fine-tune its control of biofilm formation, perhaps to coordinate Syp production with other factors necessary for squid colonization. However, SypE itself is not a critical colonization factor, as disruption of *sypE* does not prevent symbiotic initiation (Hussa *et al.*, 2007). Many intriguing questions remain to be answered about this unusual regulatory protein.

Roles for SypF and VpsR

In addition to the response regulators SypE and SypG, the *syp* cluster encodes a hybrid sensor kinase, SypF. Like RscS, SypF contains three domains predicted to be involved in a phosphorelay, HisKA/HATPase, REC, and Htp, as well as a putative periplasmic loop (Yip *et al.*, 2005). Overproduction of SypF exerts no discernible effect on biofilm formation. However, an increased activity allele, *sypF1*, was isolated that induces wrinkled colony formation, pellicle production, and increased adherence to a glass surface (Darnell *et al.*, 2008). SypF1 contains a serine to phenylalanine change at amino acid 247. S247 is located within the HisKA domain, three residues N-terminal to the conserved histidine predicted to be the site of phosphorylation; a change at this position could impact kinase activity. However, the involvement of these conserved residues in a phosphorelay has not yet been assessed biochemically.

Given the location of *sypF* between the response regulator genes *sypE* and *sypG*, it seemed likely that one or both would be necessary for SypF1-mediated induction of biofilm formation. However, the biofilm phenotypes are reduced, but not eliminated, in *sypE* and *sypG* mutants (Darnell *et al.*, 2008). Subsequent work revealed that the residual biofilm formation induced by SypF1 overexpression in *sypE* and *sypG* mutants depends on an unlinked gene encoding VpsR, a putative response regulator. Disruption of only *vpsR* also fails to eliminate biofilm formation, but a *vpsR sypG* double mutant mimics the uninduced

wild type. These data indicate that, at least under these conditions, SypF contributes to biofilm control through two distinct pathways.

VpsR exhibits sequence similarity to a protein with the same name in *V. cholerae* (Darnell *et al.*, 2008; Yildiz *et al.*, 2001). The *V. cholerae* protein is a major regulator of biofilm formation, through its control of the *vps* polysaccharide locus (Yildiz *et al.*, 2001). Although *V. fischeri* contains genes similar to those within one segment of the *V. cholerae vps* locus (termed *vps-II*), this locus does not appear to be responsible for the biofilms induced by SypF1 overexpression. Instead, *V. fischeri* (but not *V. cholerae*) contains a cellulose biosynthesis locus that is necessary for biofilm formation induced by SypF1 overproduction (Darnell *et al.*, 2008). Similarly, overproduction of VpsR also induces the formation of a cellulose-dependent biofilm. The roles of SypF, VpsR, and cellulose in biofilm formation and symbiotic colonization need to be clarified although, intriguingly, a mutant defective for *vpsR* exhibits a small defect in symbiotic initiation (Hussa *et al.*, 2007).

Concluding remarks

Wild type *V. fischeri* does not make impressive biofilms in culture. However, through genetic analysis and an appreciation of the natural symbiotic lifestyle of the organism, remarkable progress has been made to elucidate a complex regulatory network that controls biofilm formation (Fig. 1). The integrated signaling circuitry underlying biofilm development and symbiosis includes the hybrid sensor kinase RscS (a protein that is sufficient to broaden the host range of *V. fischeri* to include *E. scolopes*) and the downstream response regulator, SypG. Together, these proteins respond to as-yet-unknown squid or environmental signals and activate transcription of the symbiosis polysaccharide (*syp*) locus, which leads to biofilm-dependent initiation of colonization. An additional, novel response regulator, SypE, interacts with the RscS-SypG pathway and plays both positive and negative roles in control of biofilm formation. Adding to the complexity, production of the Syp polysaccharide might be coordinated with the biosynthesis of cellulose, through the putative sensor kinase SypF and the putative response regulator VpsR. A potential model that encompasses these findings is that multiple signals are received and integrated such that *V. fischeri* produces one type of biofilm (e.g., Syp-produced) at some stage(s) of its symbiotic or free-living life cycle and a distinct type (e.g., cellulose) at another stage(s).

While much remains to be understood, evidence to date suggests that some aspect of the *V. fischeri* interaction with its host activates biofilm formationBat least during the initial encounter, if not beyond. Negative control also appears to be importantBperhaps to fine-tune synthesis of biofilm components, to produce a distinct type of biofilm under different conditions, or even to turn biofilm formation off when it is not needed. This system is one of the few in which factors involved in biofilm formation in culture have been clearly correlated with biofilm formation in a natural model of bacteria-animal associations. This ability to correlate biofilm formation in culture and during the association of *V. fischeri* with *E. scolopes* thus adds to the already considerable versatility and utility of this remarkable model system for the study of both benign and pathogenic interactions between bacteria and eukaryotes.

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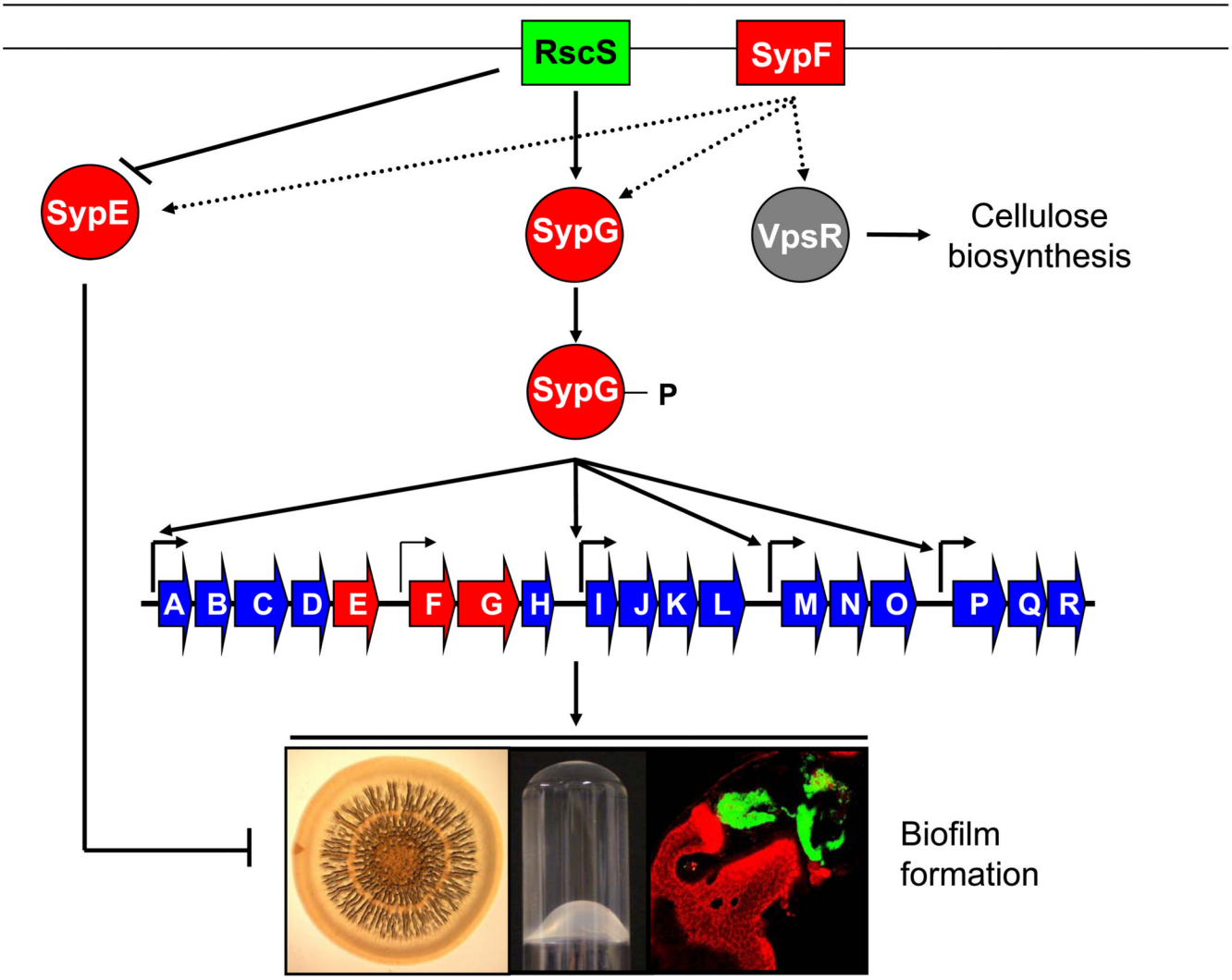

Figure 1. Model for control of biofilm formation in *V. fischeri*

Depicted here is a model based on current data regarding the potential roles of the *syp* regulators. The sensor kinase RscS acts upstream of SypG, presumably serving as a phosphodonor in response to some as-yet unidentified environmental signal, perhaps from the squid. Once phosphorylated, SypG is predicted to directly bind to sequences upstream of each of 4 *syp* operons to activate *syp* transcription by σ^{54}-containing RNA polymerase. The Syp proteins contribute to biofilm formation in culture, including the formation of wrinkled colonies and pellicles, as well as *in situ* biofilm formation and colonization. RscS overproduction also appears to inactivate SypE, which inhibits biofilm formation induced by overproduction of SypG at a level downstream from *syp* transcription; how RscS inactivates SypE is as yet unknown. Biofilm formation can be also induced by overexpression of a *sypF* allele with increased activity (SypF1) in a manner that depends in part on SypG and SypE, and in part on VpsR, which promotes cellulose biosynthesis.