Discovery of calcium as a biofilm-promoting signal for *Vibrio fischeri* reveals new phenotypes and underlying regulatory complexity

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Running Title: Calcium-induced *V. fischeri* biofilms

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Abstract

*Vibrio fischeri* uses biofilm formation to promote symbiotic colonization of its squid host, *Euprymna scolopes*. Control over biofilm formation is exerted at the level of transcription of the symbiosis polysaccharide (*syp*) locus by a complex set of two-component regulators. Biofilm formation can be induced by overproduction of the sensor kinase RscS, which requires the activities of the hybrid sensor kinase SypF and the response regulator SypG, and is negatively regulated by the sensor kinase BinK. Here, we identify calcium as a signal that promotes biofilm formation by biofilm-competent strains under conditions in which biofilms are not typically observed (growth with shaking). This was true for RscS overproducing cells as well as for strains in which only the negative regulator *binK* was deleted. These latter results provided, for the first time, an opportunity to induce and evaluate biofilm formation without regulator overexpression. Using these conditions, we determined that calcium induces both *syp*-dependent and bacterial cellulose synthesis (*bcs*)-dependent biofilms at the level of transcription of these loci. The calcium-induced biofilms were dependent on SypF, but SypF’s Hpt domain was sufficient for biofilm formation. These data suggested the involvement of another sensor kinase(s), and led to the discovery that both RscS and a previously uncharacterized sensor kinase, HahK, functioned in this pathway. Together, the data presented here reveal both a new signal and a biofilm phenotype produced by *V. fischeri* cells, the coordinate production of two polysaccharides involved in distinct biofilm behaviors, and a new regulator that contributes to control over these processes.

Importance

Biofilms, or communities of surface-attached microorganisms adherent via a matrix that typically includes polysaccharides, are highly resistant to environmental stresses, and are thus problematic in the clinic and important to study. *Vibrio fischeri* forms biofilms to colonize its symbiotic host, making this organism useful for studying biofilms. Biofilm formation depends on the *syp* polysaccharide locus and its regulators. Here, we identify a signal, calcium, that induces both SYP-PS and cellulose-dependent biofilms. We also identify a new *syp* regulator, the sensor kinase HahK, and discover a mutant phenotype...
for the sensor kinase RscS. This work thus reveals a specific biofilm-inducing signal that coordinately
controls two polysaccharides, identifies a new regulator, and clarifies the regulatory control over biofilm
formation by V. fischeri.

Introduction

Biofilms are communities of microorganisms attached to surfaces and/or each other, and are
formed by bacteria in response to specific environmental signals (1-4). These signals can range from
small molecules to physical surface detection, and induce the production of biofilm matrices that contain
a complex array of molecular components. Notably, polysaccharides are prominent matrix components
that promote cell-cell and cell-surface attachment, and contribute to protection from environmental
stressors such as antibiotics and host defense molecules (5-7).

Calcium is one small-molecule signal that controls biofilm formation in multiple bacterial
species. Calcium affects biofilm formation through diverse mechanisms, either negatively (e.g., in
Staphylococcus aureus (8) and Vibrio cholerae (9)) or positively (e.g., in Xylella fastidiosa (10);
Rhizobium leguminosarum (11), Pseudomonas aeruginosa (12), and Vibrio vulnificus (13-15)). We
recently demonstrated that salts, including calcium chloride, modestly impact biofilm formation by Vibrio
fischeri (16). Specifically, calcium accelerates wrinkled colony formation, an indicator of biofilm
formation (1).

For V. fischeri, biofilm formation is critical for colonization initiation of its symbiotic host, the
Hawaiian bobtail squid, Euprymna scolopes (reviewed in (17-19)). Two polysaccharide loci, the
symbiosis polysaccharide (syp) locus and bacterial cellulose synthase (bcs) locus, are associated with
biofilm formation (19-22) (Fig. 1 and Supplemental Fig. S1). The syp locus is an 18 gene locus that
encodes glycosyltransferases and other proteins predicted to be involved in synthesis, modification, and
export of SYP polysaccharide (SYP-PS) (23, 24). The syp genes are necessary for the production of SYP-
PS, which promotes cell-cell interactions, while bcs encodes enzymes necessary for cellulose biosynthesis
and appears to promote cell-surface interactions. syp-dependent biofilm formation by V. fischeri is well
characterized; mutation of specific syp genes disrupts biofilm formation in culture as well as symbiotic biofilm formation (22, 23, 25-27).

Four two-component regulators control syp-dependent biofilm formation by V. fischeri (Fig. 1). Three regulators are encoded within the syp locus: SypG, a response regulator that serves as the direct transcriptional activator of syp; SypF, a sensor kinase that works upstream of SypG to control SYP-PS production; and SypE (not shown), a second response regulator that controls SYP-PS production at a level below syp transcription (21, 24, 26-29). The fourth regulator is a sensor kinase encoded by an unlinked gene, RscS. The two sensor kinases, SypF and RscS, are both hybrid kinases with similar domain architecture, containing putative sensory and conserved domains predicted to be involved in autophosphorylation (HATPase/HisKA) and subsequent phosphorelay (REC and Hpt domains) (30, 31). A role for RscS in biofilm formation in culture has been observed only in the context of overexpression: overexpression of RscS is sufficient to induce SYP-PS production and biofilm formation, as seen by the production of cohesive wrinkled colonies on solid media, the formation of pellicles in static liquid media, and enhanced symbiotic biofilms (22). These RscS-induced biofilms require SypF (26). Biofilm formation can be restored through complementation with the Hpt domain of SypF alone. As the Hpt domain of RscS is not essential for its activity (32), distinct domains within the two proteins, RscS and SypF, appear to work together to drive the signal transduction necessary for syp transcription and biofilm formation.

Recently, the involvement of a third sensor kinase, BinK, was reported (33, 34). BinK inhibits the production of syp-dependent biofilms induced by RscS overexpression, and loss of BinK enhances symbiotic biofilm formation and colonization (Fig. 1). The inhibitory effect of BinK occurs, at least in part, at the level of syp transcription, as disruption of binK increased expression of a syp reporter fusion. The mechanism of how BinK interfaces with other Syp regulatory proteins and exerts its effect on syp transcription remains unknown.

Here, we report the discovery that calcium supplementation induced the production of biofilms. These calcium-induced phenotypes were dependent on both the syp and bcs loci, indicating coordinate
production of these two polysaccharides. Moreover, we determined that a single mutation, disrupting the negative regulator \textit{binK}, was sufficient for \textit{V. fischeri} to produce biofilms in response to calcium. This finding is significant because it permitted assessment of biofilm regulation in culture in the absence of overexpression of positive biofilm regulators. As a result, we uncovered the involvement of a new \textit{syp} regulator, HahK, and identified, for the first time, a mutant phenotype in culture for the known \textit{syp} regulator RscS.

**Results**

**Calcium induces biofilm formation.** Previous work indicated that calcium accelerates wrinkled colony formation by \textit{V. fischeri} (16). To further explore the importance of calcium in biofilm formation, we assayed a number of strains under a variety of growth conditions in which calcium was added to the rich growth medium LBS. In many cases, the impact of calcium was modest. For example, calcium addition to plates promoted subtle changes in wrinkled colony formation by strain KV7655, which contains a second chromosomal copy of the gene for the positive biofilm regulator RscS (\textit{rscS}++) (Table 1), and, as seen previously (16), some slight colony architecture by wild-type strain ES114, relative to the absence of calcium (Fig. 2A). In other cases, however, the impact was striking: the same \textit{rscS}++ strain (KV7655) produced robust pellicles in static liquid culture only in the presence of calcium (Fig. 2B; note cohesive biofilm indicated by arrow). Furthermore, we found that calcium could induce biofilm phenotypes under conditions that are not typically permissive for biofilm formation, namely shaking liquid (LBS) cultures. While ES114 grows as a fully turbid culture in the presence of calcium under these conditions, the \textit{rscS}++ strain exhibited two distinct biofilm phenotypes: a ring around the test tube surface above the top of the liquid (in the “splash zone”), and a cohesive cellular clump at the bottom of the tube (Fig. 2C). These biofilm phenotypes were specific to calcium, and not induced by supplementation with other cations (Fig 2D). Calcium also induced clump and ring formation by other biofilm-competent strains, including strains overexpressing \textit{rscS} from a multi-copy plasmid, or overexpressing positive regulator \textit{sypG} in the absence of the negative regulator \textit{sypE} (Supplemental Fig S2, Table 2). For these plasmid-containing biofilm-competent strains, the ring and clump phenotypes were less robust than those seen for \textit{rscS}++.
due to the necessary addition of antibiotics for plasmid maintenance. Together, these data indicate that calcium is a strong inducer of biofilms, as it specifically triggers *V. fischeri* to form biofilms under classically non-permissive conditions (*i.e.*, shaking liquid cultures). These calcium-induced shaking liquid phenotypes also provide a novel phenotype to study regulatory pathways in *V. fischeri* biofilm formation.

**BinK inhibits calcium-induced biofilm formation.** Another strain that we examined was a strain deleted for the negative regulator, *binK*. The report that identified BinK had examined its role in the context of *rscS* overexpression. It showed that disruption of *binK* accelerated the onset of wrinkled colony formation when *rscS* was overexpressed, and that *binK* overexpression inhibited RscS-induced wrinkled colonies, resulting in smooth colonies (33). Given that both *binK* and calcium affect wrinkling of biofilm-competent strains, we hypothesized that loss of BinK might enhance calcium-dependent biofilm formation. We further hypothesized that the loss of this negative regulator alone might be sufficient to permit biofilm formation in the presence of calcium. We thus evaluated the biofilm phenotypes of a Δ*binK* mutant using our three assays, the formation of wrinkled colonies, pellicles, and rings/clumps. In the absence of calcium, the *binK* mutant did not produce any visible biofilms (Fig. 3A-C). However, when calcium was added, the *binK* mutant formed robust biofilms under all three conditions (Fig. 3A-C). As with the *rscS* ++ strain, the ring/clump formation was specific to calcium (Fig. 3D). These data reveal BinK as a strong negative regulator that alone is sufficient to suppress calcium-dependent biofilm formation in *V. fischeri*. Additionally, this simple combination of genetic (*binK* disruption) and environmental (calcium supplementation) conditions is sufficient to overcome the need for overexpression of positive regulators to induce *in vitro* biofilm formation.

**Calcium-induced rings and clumps form separately.** Because the rings and clumps produced in culture in response to calcium appeared as distinct phenotypes, we visually evaluated their development over time using the *binK* mutant. We found that ring formation occurred as early as 2-4 h after inoculation of a single colony into broth containing calcium (Supplemental Fig. S3), while clumping occurred later (around 11 h in the experiment shown in Fig. 4). The two biofilms progressed over time, with the rings often developing “tendrils” that merged with/attached to the cellular clumps. The distinct timing and
position of these biofilms suggested that discrete processes may be involved in their growth and maturation.

**Calcium-induced biofilms are syp and bcs dependent.** Since RscS and BinK both control syp transcription and syp-dependent wrinkled colony formation (33), we hypothesized that disruption of syp would abolish calcium-induced liquid biofilms. Deletion of most of the 18 syp genes eliminate wrinkled colony formation and pellicle production (23), so a representative gene, sypK, was chosen to assess the role of syp in the shaking biofilm phenotypes. Deletion of sypK abolished production of the cohesive cellular clump, but not ring formation, by the binK mutant (Fig. 5A). We quantified this effect by staining the biofilm material with crystal violet (Fig. 5A, middle), then solubilizing and measuring the stain (Fig. 5A, bottom). The amount of biofilm produced by the binK sypK double mutant was significantly less than that produced by the binK mutant alone. We thus conclude that cell clumping requires an intact syp locus.

Since disruption of syp had no impact on ring formation, we hypothesized that another polysaccharide locus, such as the cellulose locus (20), may be responsible for ring production. To test this hypothesis, we asked if deletion of bcsA, which encodes a subunit of cellulose synthase, abolished ring formation. A binK bcsA double mutant failed to form rings, indicating that ring formation requires an intact cellulose locus. This double mutant retained the ability to produce cohesive cellular clumps, and produced substantially less polysaccharide than the single binK mutant alone (Fig. 5A).

These data suggested that both SYP and cellulose polysaccharides contribute to the biofilm phenotypes observed under these conditions. Indeed, disrupting both syp and bcs (ΔbinK ΔsypK ΔbcsA) prevented production of both rings and clumps by the binK mutant (Fig. 5A). In fact, the phenotype of the triple mutant was similar to cultures grown in the absence of calcium (Fig. 5A). Each of the two phenotypes could be restored, separately, to the triple mutant by complementation with the appropriate syp or bcs gene (Supplemental Fig. S4A & B). In addition, we observed similar biofilm defects when we assayed syp and bcs mutants in an RscS overexpressing strain (Supplemental Fig. S4C). Thus, SYP-PS and cellulose are both required for liquid biofilm formation, and disruption of binK largely phenocopies overexpression of RscS under these conditions.
Calcium induces two distinct polysaccharide biofilms in liquid culture, but for wrinkled colonies, only SYP-PS is known to be important as disruption of syp results in smooth colonies in the context of RscS overexpression (23, 24). We therefore investigated whether both SYP-PS and cellulose were important for calcium-induced wrinkled colony formation. A binK sypK double mutant failed to form wrinkles or cohesive colonies in the presence of calcium, suggesting that SYP-PS is necessary for colony architecture and cohesion (Fig. 5B). Conversely, a binK bscA double mutant formed colonies phenotypically indistinguishable from a binK mutant in the presence of calcium, while the triple binK sypK bcsA mutant was smooth (Fig. 5B). Thus, robust wrinkling and cohesive colonies require only SYP-PS, and not cellulose.

**Calcium impacts transcription of syp and bcs.** Given that calcium induces liquid biofilm phenotypes that depend on two distinct polysaccharides, we hypothesized that this effect may occur at the level of transcription of the bcs and syp polysaccharide loci. Transcriptional reporters for the promoter regions of bcsQ and sypA revealed a significant increase in transcription of both promoters in the presence of calcium (Fig. 6A & B). This calcium-dependent increase was more substantial at both promoters in a binK mutant, especially at the sypA promoter (Fig. 6A & B). The effect of binK disruption on syp and bcs transcription is consistent with recent reports (33, 34). These data suggest that (1) calcium promotes biofilm formation, at least in part, by inducing transcription of bcs and syp loci and (2) BinK inhibits the effect of calcium on transcription of both loci.

**Calcium-dependent cell clumping depends on sypF and sypG.** The identification of new phenotypes and conditions that induce biofilm formation in the absence of overexpression of regulators provided an opportunity to reassess the roles of known syp regulators. We thus asked if SypF, SypG, and/or RscS were required for calcium-dependent biofilm formation (Fig. 1). We generated double deletion mutants and assessed cell clumping in shaking cultures and wrinkled colony formation on plates. All of the mutants retained the ability to form rings, but the binK sypF and binK sypG mutants produced turbid instead of clumped cultures. Visual observation of these cultures and subsequently of the crystal violet stained tubes confirmed that the double mutants formed substantially less biofilm than a single binK
The mutant (Fig. 7A). The binK sypF and binK sypG mutants generated smooth, non-cohesive colonies, compared to the fully wrinkled and cohesive binK mutant (Fig 7B). These results indicate the importance of these regulators in wrinkling and cell clumping, but not ring formation. In contrast, the phenotype of a binK rscS mutant was indistinguishable from that of the binK single mutant (Fig. 7A & B). Therefore, despite RscS’s clear positive contribution to biofilm formation (Fig. 2, Supplemental Fig. 2) (22), it does not seem to be required for biofilm formation in the absence of BinK; similarly, binK disruption is not required when RscS is overexpressed (Fig. 2). These data indicate that the calcium-dependent cell clumping and wrinkled colony formation that occurs under these conditions in the absence of binK requires sypF and sypG, but not rscS.

The Hpt domain of SypF is sufficient for calcium-dependent cell clumping. When RscS is overexpressed, only the Hpt domain of SypF is necessary for biofilm formation (Fig. 1) (26). Since SypF, but not RscS, is necessary for biofilms in a binK mutant (Fig. 7), we wondered whether full length SypF was required, or if only a specific domain would be sufficient for calcium-induced, syp-dependent cell clumping. We thus introduced, into the double binK sypF mutant, various sypF alleles that encode proteins with mutations in residues predicted to be involved in the phosphorelay, H250Q, D549A, and H705Q, as well as expressing the Hpt domain alone (Fig. 1). Consistent with our previous work (26), expression of wild-type SypF, SypF-H250Q, and SypF- D549A each restored cell clumping to the binK sypF mutant (Fig. 8). Expression of the Hpt domain alone was similarly able to restore clumping, while the Hpt domain with a H705Q mutation resulted in a significant and complete loss of cell clumping (Fig. 8). These data indicate that a phosphorylatable Hpt domain is the only domain of SypF necessary for BinK-inhibited, calcium-dependent cell clumping.

The sensor kinase HahK promotes cell clumping and colony wrinkling. As autophosphorylation activity of SypF is not required for calcium-dependent cell clumping (Fig. 8), the Hpt domain of SypF must become phosphorylated by another mechanism. We considered the involvement of another sensor kinase. Specifically, we looked for genes in the V. fischeri genome that encoded a sensor kinase with the right domain structure (poised to donate a phosphoryl group to the Hpt domain of SypF via a REC
domain) and were unlinked to genes for putative DNA-binding response regulators. Because biofilm formation is an important colonization determinant, we prioritized those sensor kinases that appeared important for symbiotic colonization (33). As a result, we focused our attention on four possible uncharacterized regulators, \textit{VF\_2379}, \textit{VF\_1296}, and \textit{VF\_1053}, and \textit{VF\_A0072}. Of these, only deletion of \textit{VF\_A0072} had any effect on calcium-induced biofilm formation by the \textit{binK} mutant, although the effect was subtle, with only a delay but not loss of biofilm formation (Supplemental Fig. S5). \textit{VF\_A0072} is a cytoplasmic sensor kinase with HTPase, HisKA, and REC domains (Fig. 1). Although uncharacterized, it has previously been named \textit{hahK} (HnoX associated histidine kinase) due to its location within an operon downstream of the gene for HnoX, a nitric oxide sensor (35, 36). For simplicity and consistency, we will refer to \textit{VF\_A0072} as HahK.

We hypothesized that, when SypF is intact, it is capable of promoting calcium-induced biofilm formation independent of \textit{hahK}, and that the role of HahK, if any, would be more apparent when only the Hpt domain of SypF was present. Therefore, we generated a strain deleted for \textit{binK}, \textit{sypF}, and \textit{hahK}, then introduced \textit{sypF-Hpt} into the chromosome. Biofilm formation by this strain was assessed using the cell clumping and wrinkled colony assays. While the control strain (\textit{binK sypF-Hpt}) was competent to produce cell clumps in response to calcium, the equivalent strain that lacked HahK formed significantly less biofilm, and very small clumps (Fig. 9A). In contrast, when full-length \textit{sypF} was restored to the \textit{binK sypF hahK} mutant, an intermediate phenotype was observed, as the cells clumped but overall biofilm formation was significantly reduced (Fig. 9A). These phenotypes were mirrored on plates as the \textit{\Delta binK sypF-Hpt} mutant was cohesive and wrinkled, while the mutant lacking HahK had only minimal wrinkling, and slight cohesiveness (Fig. 9B). Similar to the liquid phenotype, the wrinkled colony assay showed an intermediate phenotype for the HahK mutant in the context of a full-length SypF: this strain had slight architecture and retained cohesiveness (Fig. 9B). The triple mutant expressing SypF-Hpt was complemented by a plasmid overexpressing \textit{hahK} (Supplemental Fig. S6). Together, these data indicate...
that HahK is an active member of this pathway, potentially by acting through the Hpt domain of SypF (Fig. 1).

**RscS contributes to calcium-dependent biofilms.** Loss of *hahK* severely diminishes, but does not fully abolish polysaccharide production (Fig. 9), so we hypothesized that a third sensor kinase may be working through SypF-Hpt to promote SYP-PS. RscS was considered as a candidate for this sensor kinase, as it has previously been shown to work through the Hpt domain of SypF (26). To test this possibility, we first constructed an *rscS* mutation in the *binK sypF-Hpt* mutant background, and assessed its ability to form calcium-induced biofilms. In liquid culture, these mutants were virtually indistinguishable from the control strain, similar to what we observed previously in the context of a *binK* mutation alone (Fig. 10A & Fig. 6). Additionally, wrinkled colony formation of the *rscS* mutant strain was only slightly delayed compared to the control strain, with the control strain showing increased architecture at 30 h (Fig. 10B). If SypF, HahK, and RscS all work through the Hpt domain of SypF, then the presence of HahK in these strains may be obscuring the contribution of RscS. To test this hypothesis, we constructed a strain with mutations in both *rscS* and *hahK* (in the background of a *binK sypF-Hpt* strain), and assessed calcium-dependent biofilm formation. Biofilm formation was significantly decreased in these strains compared to the control, with cell clumping completely abrogated and ring formation substantially diminished (Fig. 10A). On solid agar, colonies were completely smooth, with no detectable cohesiveness when disrupted (Fig. 10B). The loss of both *rscS* and *hahK* could be complemented by a plasmid expressing either RscS or HahK (Supplemental Fig. S7). These data support a role for RscS in calcium-dependent cell clumping and wrinkled colony formation that was previously obscured by multiple sensor kinase inputs. This marks the first mutant phenotype in culture for *rscS* since its discovery, and highlights the complexity and redundancy of regulators in the control of *V. fischeri* biofilm formation.

**Discussion**

Wild-type *V. fischeri* naturally forms a biofilm during colonization of its symbiotic squid host, yet it forms biofilms poorly under standard laboratory conditions (22, 37). Substantial biofilm development has only been detected previously when positive regulators, such as RscS or SypG, are overexpressed.
These overexpression conditions have been extremely fruitful in identifying the contributions made by positive and negative factors, including specific proteins encoded by the *syp* locus (e.g., (22, 23, 27, 38)) and BinK (33). However, the use of overexpression conditions can limit the scope of our understanding by bypassing natural regulatory processes. Here, we report new conditions that obviate the need for overexpression of positive regulators to promote *in vitro* biofilm formation by *V. fischeri*. These new conditions have permitted a deeper understanding of biofilm regulation and have facilitated the identification of a new regulator in the control over biofilm by *V. fischeri*.

Specifically, we have identified calcium as a major regulator of biofilm formation. This requirement had not been apparent in previous work that depended on the overexpression of positive regulators of biofilm formation such as RscS, as these strains readily form wrinkled colonies and pellicles in the absence of calcium. Although recent work had hinted at a role for calcium in these phenotypes, the impact of calcium was modest, presumably because biofilms were already quite robust (16). In contrast, RscS-overexpressing cells do not form biofilms when cells are grown in liquid cultures with shaking. Thus, it was with some surprise that we observed that calcium supplementation induced the biofilm formation by RscS-overexpressing cells grown with shaking. Indeed, two distinct biofilm behaviors were noted, attachment to the surface at the air/liquid interface of shaking cultures (“rings”), and the production of a cohesive cellular clump (“clumps”). Because RscS-overexpressing cells do not normally form biofilms under these conditions in the absence of calcium, we conclude that calcium overcomes the regulatory processes that prevent biofilm formation by RscS-overexpressing cells under these conditions.

Calcium did not, however, permit SYP-PS dependent biofilm formation by wild-type cells, indicating that multiple levels of control are in place. One such regulator turned out to be the negative regulator BinK, as calcium also induced the same phenotypes by a mutant defective only for BinK. In culture, the role of BinK as a negative regulator of biofilm formation had been previously established in the context of RscS overexpression; like calcium supplementation, disruption of *binK* only modestly increased wrinkled colony formation (33). Indeed, in the absence of calcium supplementation (or RscS overexpression), the *binK* mutant does not form biofilms. The addition of calcium, however, promoted all
three biofilm phenotypes: wrinkled colony formation, pellicle production, and production of cohesive cellular clumps and rings. Together, these data further establish calcium as a powerful inducer of biofilm formation and reveal that a single regulator, BinK, is sufficient to prevent wild-type *V. fischeri* from responding to calcium to form biofilms.

Cohesive wrinkled colonies and pellicles are both dependent on SYP-PS (mutating *syp* genes fully disrupts both phenotypes). In contrast, disruption of SYP-PS production did not fully eliminate biofilms formed in calcium-supplemented shaking liquid cultures. Instead, only clumps, but not rings, were disrupted by mutation of *syp*. This result provided new insight into these biofilms, permitting the identification of cellulose as a contributing factor responsible for ring formation. Understanding the specific contributions of the two polysaccharides will be an important future direction.

The discovery of conditions that promoted biofilm formation in the absence of overexpression of positive regulators permitted a re-evaluation of the roles of known regulatory factors. Previous work using *rscS* overexpression indicated that RscS functioned upstream of the sensor kinase SypF (requiring only the Hpt domain of this protein) and the response regulator SypG. Similarly, SypF and SypG were required in the absence of BinK, suggesting that this pathway functions as previously determined using overexpression. However, the loss of RscS in a *binK* mutant did not significantly impact biofilm formation, even when only the Hpt domain of SypF was present. This finding indicated the involvement of another sensor kinase, and led to the discovery that a previously uncharacterized regulator, HahK, also functions in biofilm formation. However, loss of HahK severely diminished, but did not eliminate, biofilm formation, suggesting the involvement of yet another sensor kinase; indeed, the remaining biofilm phenotypes were lost when *rscS* was also disrupted. These results are significant, as they (1) reveal HahK as a new biofilm regulator and (2) identify, for the first time since it was identified in 2001 (39), a mutant phenotype in culture for *rscS*. We conclude that the activity of RscS is masked by redundancy with the activities of HahK and, potentially, SypF. The identification of conditions under which a phenotype for RscS can be observed in culture will permit additional studies designed to understand the signals and
factors that control activity of RscS. Similarly, understanding the control over HahK activity, potentially via the nitric oxide sensor HnoX encoded upstream (35, 36), is an important future direction.

Together, these findings reveal an increased complexity of the regulatory pathway controlling *syp*-dependent biofilm formation, with the involvement of four sensor kinases and two response regulators (Fig. 1). In other microbes, similarly complex pathways exist, e.g., *Vibrio lux* (40, 41), *E. coli* Rcs (42, 43), *Pseudomonas* Roc (44) and Gac/RetS/Lad (45-49). For example, in *P. aeruginosa*, four sensor kinases feed into a pathway that controls, among other things, biofilm formation. The central regulator, the hybrid sensor kinase GacS, autophosphorylates and donates phosphoryl groups to the response regulator GacA, which controls the downstream events. In addition, the hybrid sensor kinase LadS feeds into the pathway by donating a phosphoryl group to the Hpt domain of GacS. Another hybrid sensor kinase, RetS, forms heterodimers that inhibit the activities of GacS and a fourth sensor kinase, PA1611. We envision that analogous events are happening with the Syp regulators. SypF is known to donate phosphoryl groups to SypG and SypE (26), and yet its Hpt domain alone is sufficient for both biofilm formation in culture and symbiotic colonization, a result that validates our conclusions that other sensor kinases, presumably RscS and HahK, feed in to activate SypF.

A lingering question is, how does calcium induce biofilm formation by *V. fischeri*? The answer to this question is unknown, although some specific mechanisms can be ruled out. For example, *V. fischeri* lacks the CarRS two-component system that, in *V. cholerae*, is induced in response to calcium and regulates transcription of the *Vibrio* polysaccharide locus *vps*. *V. fischeri* also lacks the *Vibrio vulnificus* calcium binding matrix protein CabA that promotes biofilm formation in the latter organism (15). Further afield, *V. fischeri* also lacks the *Pseudomonas* sensor kinase LadS, which controls biofilm formation in response to calcium (50). Finally, it is unlikely that any of the known biofilm regulators function as a calcium sensor responsible for inducing biofilm formation: deletion of *sypF*, *rscS*, or *hahK* alone fails to prevent calcium-induced biofilm phenotypes. While SypF comes closest as a candidate for a calcium sensor, as the *sypF* mutant produces only cellulose-dependent biofilms in response to calcium, cell clumping is restored by just the Hpt domain of SypF, indicating that the sensory part of SypF is not
necessary for this response. Similarly, while deletion of binK promotes biofilm formation, biofilms only form when calcium is added, a result that indicates the involvement of another regulator. Thus, calcium may not be recognized by a two-component sensor in V. fischeri, and/or the response to calcium may be multi-factorial. Future work will be directed at understanding how V. fischeri recognizes and responds to calcium.

In summary, this work has substantially advanced our understanding of the signals, pathways, and regulators that control biofilm formation by V. fischeri. It has established calcium as an important signal controlling the production of two different but interacting biofilms at the level of transcription. It has revealed conditions that promote biofilm formation in the absence of overexpressed regulators, permitting the discovery of a new regulator, HahK, that feeds into the control of biofilm formation, and the identification of a mutant phenotype for rscS. These conditions, and the knowledge gained here using them, will permit a mechanistic investigation of the signals and pathways involved in promoting biofilm formation in response to calcium.

Materials and Methods

Strains and Media. V. fischeri strains, plasmids, and primers used in this study are listed in Tables 1, 2, and Supplemental Table S1, respectively. All strains used in this study were derived from strain ES114, a bacterial isolate from Euprymna scolopes (51, 52). V. fischeri strains were grown in the complex medium LBS (53, 54). To induce biofilm formation, calcium chloride was added to a final concentration of 10 mM (or other concentrations as indicated). Derivatives of V. fischeri were generated via conjugation, as previously described (55), or by natural transformation (56, 57). A variety of E. coli host strains, including GT115 (Invivogen, San Diego, CA, USA), CC118 λ pir (58), TAM1 or TAM1 λ pir (Active Motif, Carlsbad, CA, USA), DH5α (59) or DH5α λ pir (60), Top10 F’ (Invitrogen, now Thermofisher), S17-1 λ pir (61) and π3813 (62), were used for the purposes of cloning, plasmid maintenance, and conjugation. E. coli strains were grown in LB (63). Solid media were made using agar to a final concentration of 1.5%. The following antibiotics were added to growth media as necessary, at the
indicated final concentrations: chloramphenicol (Cm) at 1 μg ml⁻¹ (V. fischeri) or 12.5 μg ml⁻¹ (E. coli); erythromycin (Em) at 2.5 μg ml⁻¹ (V. fischeri); Tetracycline (Tc) at 5 μg ml⁻¹ (V. fischeri) or 15 μg ml⁻¹ (E. coli); ampicillin (Ap) at 100 μg ml⁻¹ (E. coli); kanamycin (100 μg ml⁻¹ (V. fischeri) or 50 μg ml⁻¹ (E. coli); trimethoprim at 10 μg ml⁻¹. Along with any necessary antibiotics, thymidine was added to a final concentration of 0.3 mM for E. coli strain π3813.

Molecular techniques and strain construction. All plasmids were constructed using standard molecular biology techniques, with restriction and modification enzymes obtained from Thermofisher (Pittsburgh, PA, USA). EMD Millipore Novagen KOD high fidelity polymerase was used for PCR SOEing (Splicing by Overlap Extension) (64) reactions, and Promega Taq was used to confirm gene deletion/insertion events. In some cases where PCR was used to generate DNA fragments, PCR cloning vector pJET1.2 (Fisher Scientific, Pittsburgh, PA, USA) was used as an intermediate vector prior to cloning into the final vector. Unmarked deletions of rscS and binK were generated using pKV456 and pLL2, respectively, using an arabinose-inducible ccdB toxin approach as previously described (62, 65). For deletions of other genes, including hahK (VF_A0072), VF1296, VF1053, and VF2379, a PCR SOEing approach was used. Briefly, sequences (~500 bp) upstream and downstream of each gene were amplified by PCR. In addition, either an antibiotic resistance gene, along with flanking FRT sequences, was similarly amplified. The PCR primers used to generate the three DNA fragments (upstream sequence, antibiotic resistance marker, downstream sequence) contained overlapping sequences that facilitated a SOEing reaction. Natural transformation was used to introduce the final spliced PCR product into tfoX-overexpressing V. fischeri strains (usually ES114), and the antibiotic resistance marker was used to select for the recombinant that contained the desired insertion/deletion mutation. Because natural transformation is more efficient using chromosomal DNA (56), chromosomal DNA was isolated from the recombinant strains using either the DNeasy Blood & Tissue Kit (Qiagen) or the Quick-DNA Miniprep Plus kit (Zymo Research) and used to introduce the desired mutation into additional strains. Insertion at the Tn7 site of the chromosome was performed via tetraparental mating (66) between the V. fischeri recipient and three E. coli strains, carrying the conjugal plasmid pEVS104 (67), the Tn7 transposase plasmid pUX-BF13 (68), and the pEVS107
derivative of interest, respectively. In some cases, sequences at or adjacent to the Tn7 site, or at other sites in the chromosome were introduced into *V. fischeri* strains via natural transformation and selection for the appropriate antibiotic resistance cassette. For example, the PsypA-lacZ reporter used here was positioned adjacent to the Tn7 site. Either the empty Tn7 cassette or the Tn7 cassette containing one of several specific sypF alleles was subsequently introduced at the Tn7 site of the PsypA-lacZ strain. Chromosomal DNA from the resulting strains was used to introduce the cassette and associated reporter into additional strains, such as those deleted for hahK, by selection for the EmR cassette. In some cases, the antibiotic resistance cassette was removed from *V. fischeri* deletion/insertion mutants using pKV496, which encodes Flp recombinase; this enzyme acts on FRT sequences to delete the intervening sequences, as has been shown previously (69).

**Calcium-induced biofilm assay.** To assess calcium-induced biofilm formation under shaking liquid conditions, LBS broth containing 10 mM calcium chloride was inoculated with single colonies of *V. fischeri* strains and grown overnight at 24°C with shaking. For these shaking liquid culture experiments, 13 x 100 mm test tubes were used with a culture volume of 2 ml of LBS broth. Pictures are representative of at least 3 independent experiments. Photos were captured with either a Canon EOS Rebel T3i, Nikon D60, or an iPhone 5 camera.

**Crystal violet staining assay.** Strains were grown in 2 ml LBS broth overnight, with 10 mM calcium chloride at 24°C as indicated. 200 μl of a 1% crystal violet solution was added for 30 min. Tubes were washed with deionized H2O, and liquid removed via aspiration. Tubes were destained with ethanol, and the OD600 was measured using a Synergy H1 microplate reader (BioTek). The data were compiled from at least three independent samples. Statistical analysis was performed using a one-way ANOVA.

**Wrinkled Colony assay.** *V. fischeri* strains were grown overnight at 28°C in LBS with antibiotics when necessary for plasmid maintenance. The overnight cultures were subcultured 1:100, grown until mid-log phase, and diluted to an OD600 of 0.2. 10 μl aliquots were spotted onto LBS agar, supplemented with antibiotics or calcium chloride as indicated. Spots were imaged at the indicated times, using consistent magnification with a Zeiss Stemi 2000-C dissecting microscope. At the final time point, the resulting
colonies were disturbed with a toothpick to assess cohesiveness as a measure of SYP-PS production (38). Photos are representative of at least three independent experiments.

**Pellicle assay.** *V. fischeri* strains were grown overnight at 28°C in LBS media. The overnight cultures were diluted to an OD$_{600}$ of 0.2 in 2ml of LBS media supplemented with calcium chloride as indicated. Pellicles were incubated statically at 24°C, and imaged at indicated times, using consistent magnification, with a Zeiss Stemi 2000-C dissecting microscope. Pellicles were disturbed with a toothpick at the final time point to assess cohesiveness. Photos are representative of at least three independent experiments.

**β-galactosidase assay.** Strains carrying a *lacZ* reporter fusion to the *sypA* promoter or to the *bcsQ* promoter were grown in triplicate at 24°C in LBS medium containing 10 mM calcium chloride. Strains were subcultured into 20 ml of fresh media in 125 ml baffled flasks, and the OD$_{600}$ was measured and samples (1 ml) were collected after 22 h of growth. Cells were resuspended in Z-buffer and lysed with chloroform. The β-galactosidase activity of each sample was assayed as described (70) and measured using a Synergy H1 microplate reader (BioTek). The assay was performed at least three independent times. Statistical analysis was performed using a two-tailed T-test.

**Acknowledgements.** We’re grateful for insight gleaned from preliminary data of Anne Marsden and Valerie Ray. We thank Christine Bassis, Cindy Darnell, and Allison Norsworthy for strain construction, and Jon Visick and members of the lab for thoughtful discussions and review of the manuscript. This work was supported by NIH grant R01 GM114288 awarded to K.L.V.

**Table 1. Strains used in this study**

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<th>Strain</th>
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<th>Derivation</th>
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<td>This study</td>
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<td>KV8069</td>
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<td>KV8078</td>
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<td>KV8297</td>
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<td>NT of KV6576 (73) with PCR DNA generated from primers 2057, 2103, 2089, 2090, 2062, and 2104</td>
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</table>

1 Abbreviations: FLAG, FLAG epitope-tagged; IG (yeiR-glmS), Intergenic between yeiR and glmS (adjacent to the Tn7 site); FRT, the EmR or CmR cassette was resolved using Flp recombinase, leaving a single FRT sequence.

2 Derivation of strains constructed in this study; NT, Natural transformation of a pLostfoX or pLostfoX-Kan-carrying version of the indicated strain with the indicated chromosomal (c) DNA or with a PCR SOE product generated using the indicated primers and, as templates, ES114 and either an EmR or CmR cassette.

Table 2. Plasmids used in this study
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<table>
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<td>pANN20</td>
<td>pEVS107 + sypF-FLAG</td>
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<td>pANN21</td>
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<td>pEVS107 + sypF-H705Q-FLAG</td>
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<td>pCMA26</td>
<td>pEVS107 containing PbcsQ-lacZ reporter from pCLD51</td>
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<td>pCP20</td>
<td>Encodes flp recombinase</td>
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<td>Vector for delivery of DNA into the Tn7 site, KnR, EmR</td>
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<td>pKPQ22</td>
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<td>Suicide vector, CmR</td>
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<td>pKV456</td>
<td>pKV363 + sequences flanking rscS</td>
<td>(26)</td>
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<td>pLL2</td>
<td>pKV363 + sequences flanking binK, generated with primers 1268, 1269, 1270, and 1271, to generate binK deletion</td>
<td>This study</td>
</tr>
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</table>
LostfoX Vector for tfoX expression for natural transformation, Cm\(^R\) (56)

pLostfoX-Kan Vector for tfoX expression for natural transformation, Kn\(^R\) (57)

pTMO82 Vector containing promoterless lacZ gene, Kn\(^R\), Ap\(^R\) (25)

pUX-BF13 Delivery plasmid for Tn7 transposase (68)

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References cited


Figure Legends

Figure 1. Model for the regulatory control over syp-dependent biofilm formation by *V. fischeri*.

Previous work with plasmid-based overexpression of regulators revealed that the hybrid sensor kinase RscS induces biofilm formation in a manner that depends on the *syp* locus and the *syp* regulators SypF and SypG. The activity of RscS requires the indicated conserved residues (H412 and D709) in RscS as well as the conserved histidine (H705) within the last (Hpt) domain of SypF, but not the conserved histidine (H250) or aspartate (D549) in the HisKA and REC domains of SypF (26, 32). SypF donates phosphoryl groups to both the response regulator SypG, the direct activator of the *syp* locus, and to the response regulator SypE (not shown), which controls *syp*-dependent biofilm formation at a level below *syp* transcription. BinK functions as a negative regulator of *syp*-dependent biofilm formation, at least in part due to the inhibition of *syp* transcription (33). This study confirms the position of RscS in the pathway and identifies HahK as another important sensor kinase whose activity feeds in through the Hpt domain of SypF.

Figure 2. Calcium induces biofilm formation. Biofilm formation was assessed for wild-type *V. fischeri* (ES114) and *rscS*++ (KV7655). (A) Wrinkled colony formation was assessed by a time course on LBS agar plates lacking or containing 10 mM CaCl₂ as indicated. Colonies were disrupted at the final time point to evaluate SYP-PS production. (B) Pellicle formation was assessed at 72 h after static incubation in LBS either lacking or containing 10 mM CaCl₂ as indicated. Pellicles were disrupted to determine cohesiveness. (C) ES114 and *rscS* were grown in LBS media with shaking either lacking or containing 10 mM CaCl₂. (D) ES114 and *rscS* were grown in LBS media alone or supplemented with 10 mM CaCl₂, KCl, NaCl, or MgSO₄ as indicated.

Figure 3. Calcium induces biofilm formation. Biofilm formation was assessed for *V. fischeri* Δ*binK* (KV7860). (A) Wrinkled colony formation was assessed by a time course on LBS agar plates lacking or containing 10 mM of CaCl₂ as indicated. Colonies were disrupted at the final time point to evaluate SYP-
Pellicle formation was assessed at 72 h after static incubation in LBS either lacking or containing 10 mM CaCl₂ as indicated. Pellicles were disrupted to determine cohesiveness. (C) ΔbinK was grown in LBS media with shaking either lacking or containing 10 mM CaCl₂. (D) ΔbinK was grown in LBS media with shaking either lacking or containing 10 mM CaCl₂. (D) ES114 and rscS⁺ were grown in LBS media alone or supplemented with 10 mM CaCl₂, KCl, NaCl, or MgSO₄ as indicated.

Figure 4. Calcium-induced rings and clumps form separately. Biofilm phenotypes of ΔbinK (KV7860) supplemented with 10 mM CaCl₂ were evaluated over time using multiple cultures grown from single colonies. The independent cultures behaved similarly. Representative images from different tubes were captured at the following times post-inoculation: 8.5 h, 9 h, 10 h, 11 h, 12 h, 13 h, 15 h, and 16 h.

Figure 5. Calcium-induced biofilms are syp and bcs dependent. The contribution of specific polysaccharides to calcium-induced V. fischeri biofilms was evaluated using strains ΔbinK (KV7860), ΔbinK ΔsypK (KV7906), ΔbinK ΔbcsA (KV7908), and ΔbinK ΔsypK ΔbcsA (KV7914). (A) (Top) Strains were grown shaking in LBS media either lacking or containing with 10 mM CaCl₂ as indicated, and imaged 16 h post inoculation. (Middle) Tubes were stained with crystal violet and imaged. (Bottom) Crystal violet was quantified, and a one-way ANOVA was performed (p=0.01, 0.01, 0.1 (n.s.), and 0.01 respectively). (B) Wrinkled colony formation was assessed by a time course on LBS agar plates lacking or containing 10 mM CaCl₂ as indicated. Colonies were disrupted at the final time point to evaluate SYP-PS production.

Figure 6. Calcium induces syp and bcs transcription. Transcription of the bcs and syp genes was assessed using a promoterless lacZ reporter gene fused to the promoter regions of bcsQ (A) and sypA (B). V. fischeri cells were grown at 24°C with shaking in 20 ml of LBS supplemented, as indicated, with 10 mM CaCl₂. (A) The effect of calcium on bcsQ transcription was monitored using strains PbcςQ-lacZ (KV8078) (p=0.02) and ΔbinK PbcςQ-lacZ (KV8076) (p=0.0025). (B) The effect of calcium on sypA
transcription was monitored using strains P_{psyp}\text{-}lacZ (KV8079) (p=0.03) and ΔbinK P_{psyp}\text{-}lacZ (KV8077) (p=0.004).

Figure 7. Calcium-dependent cell clumping depends on sypF and sypG. The contribution of SypF, SypG, and RscS to calcium-induced *V. fischeri* biofilms was evaluated in strains ΔbinK (KV7860), ΔbinK ΔsypF (KV7862), ΔbinK ΔsypG (KV7933), and ΔbinK ΔrscS (KV7861). (A) (Top) Strains were grown shaking in LBS media supplemented with 10 mM CaCl$_2$, and imaged 16 h post inoculation. (Middle) Tubes were stained with crystal violet and imaged. (Bottom) Crystal violet was quantified, and a one-way ANOVA was performed (compared to KV7860, p=0.09, 0.07, 0.5 respectively). (B) Wrinkled colony formation was assessed by incubation for 72 h on LBS agar plates containing 10 mM CaCl$_2$. Colonies were disrupted to evaluate SYP-PS production.

Figure 8. The Hpt domain of SypF is required for calcium-induced clumps. The requirement for specific SypF residues and domains in calcium-induced *V. fischeri* biofilm formation was evaluated. (Top) Strains were grown shaking in LBS media containing 10 mM CaCl$_2$, and imaged 16 h post inoculation. (Middle) Tubes were stained with crystal violet and imaged. (Bottom) Crystal violet was quantified, and a one-way ANOVA was performed (p=ns, ns, 0.004, ns, and 0.004 respectively). Strains from left to right: ΔbinK (KV7860), ΔbinK ΔsypF (KV7862), ΔbinK ΔsypF ΔsypF$^{+}$ (KV7878), ΔbinK ΔsypF sypF-H250Q (KV7875), ΔbinK ΔsypF sypF-D549A (KV7879), ΔbinK ΔsypF sypF-H705Q (KV7873), ΔbinK ΔsypF sypF-HPT (KV7877), ΔbinK ΔsypF sypF-HPT-H705Q (KV7871).

Figure 9. The sensor kinase HahK promotes cell clumping and colony wrinkling. The contribution of hahK to calcium-induced *V. fischeri* biofilms was evaluated in strains ΔbinK ΔsypF sypF-HPT (KV7877), ΔbinK ΔsypF ΔhahK sypF-HPT (KV8323), and ΔbinK ΔsypF ΔhahK sypF$^{+}$ (KV8324). (A) (Top) The strains were grown shaking in LBS media containing 10 mM CaCl$_2$, and imaged 16 h post inoculation. (Middle) Tubes were stained with crystal violet and imaged. (Bottom) Crystal violet was quantified, and a
one-way ANOVA was performed ($p=0.002, 0.03$, respectively). (B) Wrinkled colony formation was assessed by incubation for 72 h on LBS agar plates containing 10 mM of CaCl$_2$. Colonies were disrupted to evaluate SYP-PS.

**Figure 10. RscS contributes to calcium-dependent biofilms.** The contributions of RscS and HahK to calcium-induced *V. fischeri* biofilms were evaluated using strains $\Delta$binK $\Delta$sypF sypF-HPT (KV7877), $\Delta$binK $\Delta$sypF $\text{Tn}10$ sypF-HPT (KV7949), and $\Delta$binK $\Delta$sypF $\text{Tn}10$ $\Delta$hahK sypF-HPT (KV8325). (Top) Strains were grown shaking in LBS media containing 10 mM CaCl$_2$, and imaged 16 h post inoculation. (Middle) Tubes were stained with crystal violet and imaged. (Bottom) Crystal violet was quantified, and a one-way ANOVA was performed ($p=0.01$ and 0.0009 respectively). (B) Wrinkled colony formation was assessed by incubation for 72 h on LBS agar plates supplemented with 10 mM CaCl$_2$. Colonies were disrupted to evaluate SYP-PS production.