Minireview

Control of biofilm formation and colonization in *Vibrio fischeri*: a role for partner switching?

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Summary

Bacteria employ a variety of mechanisms to promote and control colonization of their respective hosts, including restricting the expression of genes necessary for colonization to distinct situations (i.e. encounter with a prospective host). In the symbiosis between the marine bacterium *Vibrio fischeri* and its host squid, *Euprymna scolopes*, colonization proceeds via a transient biofilm formed by the bacterium. The production of this bacterial biofilm depends on a complex regulatory network that controls transcription of the symbiosis polysaccharide (*syp*) gene locus. In addition to this transcriptional control, biofilm formation is regulated by two proteins, SypA and SypE, which may function in an unusual regulatory mechanism known as partner switching. Best characterized in *Bacillus subtilis* and other Gram-positive bacteria, partner switching is a signalling mechanism that provides dynamic regulatory control over bacterial gene expression. The involvement of putative partner-switching components within *V. fischeri* suggests that tight regulatory control over biofilm formation may be important for the lifestyle of this organism.

Introduction

To achieve efficient colonization of their respective hosts, bacteria have evolved complex signalling networks to ensure the proper expression of the genes necessary to respond to the host environment. The result of these regulatory cascades is the induction of key cellular responses required for successful host colonization, such as biofilm formation. Biofilms, or surface-associated communities of cells encapsulated in a matrix, often play an integral role in the attachment of bacterial cells to host or environmental surfaces and in bacterial survival, both within and outside of a host.

The formation of a biofilm is a common strategy utilized among numerous *Vibrio* species, in which it is predicted to promote bacterial persistence in the environment and/or colonization of eukaryotic hosts (for a recent review see Yildiz and Visick, 2009). *Vibrio* species are Gram-negative bacteria, typically present in marine environments. Among the *Vibrios*, several species engage in pathogenic or symbiotic partnerships with specific eukaryotic hosts. This review focuses on the bacterium *Vibrio fischeri*, as recent work has shown a clear relevance of biofilm formation to the ability of this organism to establish symbiotic colonization of its eukaryotic host, the Hawaiian bobtail squid *Euprymna scolopes*. Biofilm formation by *V. fischeri* requires expression of a cluster of polysaccharide biosynthetic genes, termed the symbiosis polysaccharide (*syp*) locus, that is conserved among several *Vibrio* species (Yip et al., 2005; 2006). Importantly, the regulation of this polysaccharide locus involves an intricate network of regulatory proteins, which is predicted to restrict biofilm formation such that it occurs transiently in response to specific host-derived signals (Visick, 2009).

Biofilm formation also appears to be regulated by two largely uncharacterized regulatory proteins encoded within the *syp* cluster, SypA and SypE. Bioinformatic analyses of these regulatory proteins suggest they contain elements of a regulatory signalling mechanism, termed partner switching. A signalling mechanism most extensively characterized in Gram-positive bacteria, partner switching provides yet another layer of regulatory control over gene expression. In recent years, genome analyses have suggested that potential partner-switching components are present in a wide range of bacteria, including Gram-negative species (Mittenhuber, 2002; Mattoo et al., 2004). Here, following brief overviews of the *V. fischeri*–squid symbiosis and biofilm formation, we review the partner-switching mechanism, as understood within characterized models, then speculate on the role of
Vibrio fischeri and E. scolopes: symbiotic initiation depends on biofilm formation

The symbiotic relationship between the marine bacterium V. fischeri and its eukaryotic host, E. scolopes, provides an elegant model of symbiotic bacteria–host interaction (for recent reviews, see Nyholm and McFall-Ngai, 2004; Stabb, 2006; Visick and Ruby, 2006). Newly hatched juvenile squid are aposymbiotic and must acquire their bacterial symbionts from the surrounding seawater. Successful establishment of this symbiotic colonization involves a number of both host- and symbiont-derived responses (Nyholm and McFall-Ngai, 2004). Importantly for this review, exposure to environmental bacteria stimulates newly hatched squid to secrete mucus onto the surface of their symbiotic light organs (Nyholm et al., 2000; Nyholm and McFall-Ngai, 2004). Vibrio fischeri appears particularly adept at adhering to the mucus and forming a biofilm-like aggregate of cells that are poised to enter the light organ (Nyholm and McFall-Ngai, 2003). Subsequently, V. fischeri, but not other bacteria, productively enter and migrate to the crypts, where they establish colonization by multiplying to high cell density.

The formation of a biofilm aggregate outside the squid light organ is essential for efficient initiation of host colonization. Mutants defective in biofilm formation exhibit a significant defect in colonization, while a strain with an enhanced ability to form biofilms exhibits a significant colonization advantage (Yip et al., 2005; 2006). Formation of this biofilm requires the syp locus, consisting of 18 genes predicted to be involved in the synthesis and regulation of a polysaccharide biofilm matrix (Yip et al., 2005; 2006). syp mutants exhibit a significant defect in biofilm formation and host colonization (Yip et al., 2005).

Regulation of biofilm formation: a complex network of regulators

Biofilm formation appears to be under complex regulatory controls. At least four regulators encoded within the syp locus (SypA/E/F/G) and two regulators encoded elsewhere (RscS and VpsR) appear to regulate biofilms at the level of syp transcription or at an unknown level beyond syp activation (recently reviewed in Visick, 2009). Transcription of the syp locus is controlled by the response regulator SypG, which is predicted to be activated via phosphorylation from an upstream sensor kinase, RscS (Fig. 1) (Hussa et al., 2008). Overexpression of either rscS or sypG promotes substantial biofilm formation, and loss of either gene results in a severe colonization defect similar to syp mutants (Visick and Skoufos, 2001; Yip et al., 2006; Hussa et al., 2007). Two other regulators, the sensor kinase SypF and the response regulator VpsR (a predicted DNA-binding protein), also appear to regulate biofilm formation, but it remains unknown how these proteins contribute to the regulatory network (Darnell et al., 2008).

SypA and SypE also contribute to control of biofilm formation, but appear to exert their effects downstream of syp transcription (Hussa et al., 2008; A.R. Morris and K.L. Visick, unpubl. data). Current unpublished data indicate the SypA is required for biofilm formation by V. fischeri (S. Shibata, E.S. Yip and K.L. Visick, unpubl. data). Sequence analysis indicates that sypA codes for a single-domain protein with a predicted sulfate transporter and anti-sigma factor antagonist (STAS) domain (Fig. 2). This domain is conserved among anti-anti-sigma factors, which generally function as positive regulators (Aravind and Koonin, 2000).

In contrast to SypA, SypE appears to play a dual role in biofilm formation. SypE inhibits SypG-induced biofilm formation, but is required for full biofilm induction under conditions in which the sensor kinase, RscS, is overexpressed (Hussa et al., 2008). Sequence and domain analysis of SypE suggests a unique multi-domain protein. Due to the presence of a conserved receiver (REC) domain, SypE is a predicted response regulator. The activity of response regulators is generally controlled via the phosphorylation of a conserved aspartate residue within the REC domain (Stock et al., 2000). The
phosphorylation state of the REC domain activates an effector domain within the response regulator that results in a response, usually a change in gene transcription (Stock et al., 2000). Distinct from the typical response regulator, which contains the REC domain in the N-terminus, SypE contains a central REC domain (Fig. 3A). This central REC domain is flanked by two domains of putative opposing functions. The N-terminal domain of SypE exhibits weak sequence similarity to serine kinases of the GHKL (gyrase, Hsp90, histidine kinase and MutL) family, which includes SpoIIAB of Bacillus subtilis (Dutta and Inouye, 2000) (Fig. 3B). The C-terminus of the protein contains a putative serine/threonine phosphatase domain and exhibits strong sequence similarity to the PP2C family of serine phosphatases (Fig. 3C).

How these proteins contribute to the biofilm regulatory network remains an area of active research. Although the roles of SypA and SypE in the regulation of biofilm formation remain unknown, bioinformatics suggest that these proteins may constitute a novel partner-switching system.

The partner-switching system

First coined by Alper and colleagues (1994), the term ‘partner switch’ describes a network of interacting proteins whose interaction with cognate ‘partners’ depends upon a reversible phosphorylation event. Dependent upon which partner proteins interact, the outcome of this partner switching can either negatively or positively regulate a target protein, generally a transcription factor or enzyme. Specifically, the partner-switching mechanism depends upon key regulatory elements, including a serine kinase/anti-sigma factor, a serine phosphatase, an antagonist protein/anti-anti-sigma factor and a target protein (often a sigma factor) (Yang et al., 1996).

The partner-switching mechanism has been best characterized in the Gram-positive bacterium B. subtilis. In B. subtilis and other Gram-positive bacteria, partner-switching systems contribute to the signalling networks that control the activity of sigma factors, the subunit of RNA polymerase that provides promoter specificity. For example, B. subtilis uses partner switching to control the activity of the sigma factor, sigma B, which regulates the general stress response pathway (Dufour and Haldenwang, 1994). In this system, the anti-sigma factor RsbW negatively regulates the activity of sigma B by directly binding it and preventing interaction with the RNA polymerase core (Dufour and Haldenwang, 1994; Hughes and Mathee, 1998) (Fig. 4A). The binding of an anti-anti-sigma factor, RsbV, to RsbW relieves this negative regulation by sequestering the anti-sigma factor (Dufour and Haldenwang, 1994). This releases sigma B, which can now bind to the RNA polymerase core and promote transcription of sigma B-dependent genes. This results in the induction of proteins involved in protection against multiple bacterial stresses (Yang et al., 1996; Price et al., 2001).

Activity of the anti-anti-sigma factor RsbV is controlled via phosphorylation of a conserved serine residue within the STAS domain (Figs 2B and 4A). In the unphosphorylated state, RsbV can bind and inactivate the anti-sigma RsbW, thus freeing sigma B and upregulating sigma B-dependent genes. However, when this serine residue becomes phosphorylated, RsbV is rendered unable to bind RsbW, which permits RsbW to sequester sigma B.
and downregulate sigma B-dependent gene expression. The phosphorylation state of this serine residue is controlled by two sets of proteins, one of which is RsbW itself; in addition to its role in sequestering sigma B, RsbW also functions as a serine kinase. Dephosphorylation of this residue is carried out by the serine phosphatases, RsbU and RsbP, which are activated by environmental and energy stress signals respectively (Voelker et al., 1996). Thus, in this regulatory network, RsbW functions as a regulatory switch, as it reversibly interacts with its cognate sigma factor and anti-anti-sigma factor. Furthermore, the partner switch is regulated by reversible phosphorylation of RsbV.

Conservation of partner switching: a Gram-positive mechanism?

Since its characterization in *B. subtilis*, the partner-switching mechanism has been identified as a signalling component in a wide range of Gram-positive bacteria.
These include *Bacillus cereus* (van Schaik et al., 2005), *Bacillus anthracis* (Fouet et al., 2000), *Mycobacterium tuberculosis* (Beaucher et al., 2002), *Staphylococcus aureus* (Miyazaki et al., 1999) and *Listeria monocytogenes* (Chaturongakul and Boor, 2004; 2006). In these bacterial systems, partner-switching modules are utilized in a manner similar to that observed in *B. subtilis*: primarily, the regulation of sigma factor activity. However, the output of these modules varies among the individual bacteria. Partner-switching modules have been demonstrated to contribute to the regulation of the general stress response of *L. monocytogenes* and many other bacteria (Chaturongakul and Boor, 2004), biofilm formation in *Staphylococcus epidermidis* (Knobloch et al., 2004), and the expression of virulence-associated genes in *M. tuberculosis* (Beaucher et al., 2002; Manganelli et al., 2004). Additionally, bacteria may possess multiple partner-switching pathways regulating distinct sets of target proteins. For example, in addition to the RsbU/V/W module that regulates the general stress response, *B. subtilis* possesses a second set of partner-switching regulators, SpoIIAA/SpoIAB/SpoIIE. Similar to the sigma B regulatory pathway, these partner-switching proteins control the activity of a sigma, in this case sigma F, which regulates sporulation (Magnin et al., 1997). The partner-switching mechanism, while similar among diverse Gram-positives, has been adapted to respond to specific stimuli and regulate distinct cellular responses.

**Partner switching within the Gram-negatives?**

Analyses of diverse bacterial genomes suggest that partner-switching orthologues may exist in a wide range of eubacteria (Mittenhuber, 2002; Mattoo et al., 2004). Despite its predicted widespread distribution, partner switching has remained relatively uncharacterized within Gram-negative bacteria. Indeed, partner-switching systems have been characterized in only two Gram-negative bacteria, *Bordetella bronchiseptica* and *Chlamydia trachomatis* (Kozak et al., 2005; Hua et al., 2006). Furthermore, only in the case of *B. bronchiseptica* has a partner-switching module been experimentally demonstrated to regulate a physiological response. This respiratory pathogen utilizes a partner-switching module to control production of a type III secretion system (T3SS) (Mattoo et al., 2004). The T3SS consists of a needle-like secretory apparatus that directly transports virulence proteins into the cytoplasm of host cells. In *B. bronchiseptica*, the T3SS contributes to persistent colonization of the host trachea and the avoidance of the host immune response (Yuk et al., 2000; Mattoo et al., 2001). The production of the T3SS requires transcription of a gene cluster, the *bsc*
negative bacterium may represent a partner-switching module in the Gram-

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In vitro and in vivo analyses of the B. bronchiseptica proteins demonstrated that they constitute a regulatory network similar to their B. subtilis counterparts (Kozak et al., 2005). However, this partner-switching system appears to deviate from that of the B. subtilis RsbU/V/W paradigm. First, disruption of any component of the BtrU/V/W partner-switching module results in the loss of type III secretion (Mattoo et al., 2004), a result that is inconsistent with the B. subtilis model (Fig. 4A). Second, positive regulation of the T3SS requires both the formation of the BtrV/BtrW complex and its dissociation, via phosphorylation of BtrV by BtrW (Kozak et al., 2005). Finally, although the BtrU/V/W module regulates type III secretion, it does not appear to control transcription of the bsc locus (Kozak et al., 2005). Instead, Kozak and colleagues (2005) suggest that these partner-switching proteins may regulate the T3SS at the posttranscriptional level possibly by interacting with yet unknown regulatory proteins or playing a structural role in the secretory pathway. Thus, although there is conservation of the partner-switching components, the regulatory mechanism appears to vary from that of the Gram-positive paradigm.

Genome analysis of the obligate intracellular pathogen C. trachomatis identified several components of a putative partner-switching module (Hua et al., 2006). In vitro analysis of the candidate genes demonstrated that these proteins could interact. As with B. bronchiseptica, it appears that the C. trachomatis partner-switching system may vary from the B. subtilis paradigm, as in vitro binding assays failed to demonstrate an interaction with any of the three sigma factors encoded in the C. trachomatis genome (Hua et al., 2006). However, the lack of genetic tools and difficulty in culturing C. trachomatis have delayed analysis of this potential partner-switching module in vivo.

Together, these studies suggest that the partner-switching mechanism, previously observed only among the Gram-positives, also contributes to regulatory control in Gram-negative bacteria. It remains unknown how these Gram-negative partner-switching proteins regulate downstream targets. Furthermore, it remains unclear how widespread this regulatory mechanism is among Gram-negative bacteria.

SypA and SypE: a potential partner-switching module in V. fischeri?

Several lines of evidence suggest that SypA and SypE may represent a partner-switching module in the Gram-negative bacterium V. fischeri. First, the physical proximity of the sypA and sypE genes and their known roles in biofilm formation suggest a regulatory connection. Second, searches for V. fischeri proteins with sequence similarity to the RsbU/V/W of B. subtilis, and other orthologues, yielded only two candidate proteins, SypA and SypE. Third, these proteins not only contain the conserved domains, but also critical active-site residues within these domains.

Specifically, SypA not only contains the conserved STAS domain present among RsbV-like anti-anti-sigma factors, but also retains the conserved serine residue (S56) predicted to be the site of phosphorylation (Fig. 2B). Our preliminary data are consistent with a role for this residue in SypA function (A.R. Morris and K.L. Visick, unpublished). SypE contains two partner-switching domains: an N-terminal serine kinase (RsbW) and a C-terminal serine phosphatase (PP2C) domain. As shown in Fig. 3B, sequence alignment of the N-terminal domain of SypE with RsbW orthologues indicates that SypE contains the conserved N-, G1- and G2-boxes present in the Bergerat ATP-binding fold of serine kinases (Dutta and Inouye, 2000). Importantly, SypE possesses the invariant asparagine residue (N52) of the N-box, which is required for Mg2+ ion chelation and coordinates binding of ATP to the nucleotide pocket (Fig. 3B) (Dutta and Inouye, 2000). Sequence alignment also reveals the conserved G1-box aspartate and glycine residues (D81 and G83), predicted to participate in ATP binding and formation of the ATP lid of the nucleotide binding pocket respectively (Dutta and Inouye, 2000). The conservation of these key residues suggests that, despite poor overall sequence similarity, the N-terminus of SypE may function as an RsbW-like serine kinase. The C-terminus of SypE possesses strong sequence similarity to serine/threonine phosphatases of the PP2C family, including RsbU. As shown in Fig. 3C, the C-terminal domain of SypE also possesses the invariant aspartate residues (D306, D323, D443 and D495) that form part of the catalytic core and are predicted to coordinate binding of Mg2+/Mn2+ ions necessary for PP2C catalytic activity (Adler et al., 1997; Rantanen et al., 2007; Shi, 2009).

The antagonistic domains within SypE suggest that this protein may possess both negative and positive regulatory activity, depending on which domain is active (Fig. 4B). Studies of syp-dependent biofilms support this hypothesis: SypE inhibits SypG-induced biofilms, but is required for full expression of biofilms produced by over-expression of the sensor kinase, RscS (Hussa et al., 2008). Furthermore, recent genetic analyses indicate that the antagonistic domains of SypE may be responsible for these apparent dual regulatory activities (A.R. Morris and K.L. Visick, unpublished data).

Based on bioinformatics and experimental observations, we propose a model in which SypE and SypA

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constitute part of a partner-switching module (Fig. 4B). In partner-switching systems, RsbW-like serine kinases generally function as antagonists, and inhibit cellular responses via the binding to cognate partner proteins. Thus, potentially the N-terminal (RsbW) domain of SypE regulates biofilm formation by interacting with SypA or a yet unknown regulatory protein. These interactions are possibly dictated by the phosphorylation state of SypA, which may be controlled by the serine kinase and/or the serine phosphatase domains of SypE. The phosphorylation state of the central REC domain may regulate whether SypE functions as a serine kinase or a serine phosphatase. The outcome of this partner-switching network is either the negative or the positive regulation of biofilm formation. Work is currently in progress to assess whether these components indeed function as predicted by this model.

Conservation of SypA and SypE among Vibrio species

The syp locus is relatively conserved among several Vibrio species, including both pathogenic and symbiotic bacteria. Although biofilm formation has been investigated in diverse Vibrio species, the role of the syp cluster in species other than V. fischeri is not fully understood. Recently, Kim and colleagues (2009) demonstrated that the syp locus plays a role in biofilms formed by the pathogenic bacterium Vibrio vulnificus. Specifically, the syp cluster contributes to the production of exopolysaccharides and bacterial attachment to biotic and abiotic surfaces (Kim et al., 2009). Due to the conservation of the syp locus among Vibrio species, we performed a bioinformatic survey of syp-containing Vibrio genomes for SypA and SypE, specifically, or other potential partner-switching proteins. Interestingly, SypA is well conserved among the syp-containing Vibrio genomes, but SypE appears to be absent in several species. For example, V. vulnificus possesses a SypA orthologue, but lacks any clear SypE-like genes. Intriguingly, the genome of V. vulnificus contains RsbW- and RsbU-like proteins, VVA0582 and VVA1682, which encode a putative RsbW-like anti-sigma factor and an RsbU-like serine phosphatase respectively. The genomes of Vibrio parahaemolyticus and Allobacillus salmoneicida similarly lack SypE, but encode RsbW and RsbU-like proteins elsewhere. Thus, in several Vibrio species lacking SypE, other putative partner-switching components exist; whether or not they function as predicted or control the activity of SypA remains to be determined.

Concluding remarks

The symbiotic bacterium V. fischeri employs a variety of regulatory proteins to control biofilm formation and consequently host colonization. While much of the regulation occurs via canonical two-component signalling pathways that control syp transcription, it remains unknown how the syp-encoded regulators SypA and SypE control biofilm formation. Bioinformatics suggest the possibility that SypA and SypE participate in a partner-switching mechanism. However, the downstream target of this potential control mechanism remains unknown. In contrast to the well-established Gram-positive partner-switching paradigms, studies from other Gram-negative bacteria suggest that the downstream target may not necessarily be a sigma factor.

The potential integration of partner-switching components within the V. fischeri biofilm regulatory network would provide yet another layer of signal control. Together, these mechanisms may confine expression of colonization genes to defined conditions (i.e. interaction with juvenile host squid) and/or control the timing of the transient biofilm formation, thus permitting cells to leave the biofilm to enter the symbiotic organ. The presence of the syp locus within multiple Vibrio species exhibiting varied lifestyles (pathogens versus symbionts) suggests that syp-dependent biofilms may play a role in diverse responses, such as the colonization of respective hosts. The elucidation of the roles of SypA and SypE and the signals to which they respond in V. fischeri thus has the potential to provide a paradigm for understanding partner switching in Vibrios and other Gram-negatives.

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