Two-Component Regulators in the *Vibrio fischeri-Euprymna scolopes* Symbiosis

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Abstract

The symbiotic relationship between the marine bioluminescent bacterium *Vibrio fischeri* and its host, the Hawaiian bobtail squid *Euprymna scolopes,* depends upon the ability of the two partners to sense and respond to each other. *V. fischeri* colonizes a specialized squid organ called the light organ in three general stages: initiation, accommodation, and persistence. To respond to the different environments encountered during these stages of colonization, *V. fischeri* utilizes specialized two-component signal transduction systems to regulate processes such as biofilm formation, motility and chemotaxis, and luminescence. In this chapter, we discuss in detail the two-component systems that regulate these processes and how they impact successful colonization of the squid host.

Introduction

Bacteria are ubiquitous in nature and can be found free-living or associated with a host. An important subset of associations of bacteria with hosts leads to disease and sometimes death. Another subset of associations involves beneficial, or symbiotic, bacteria–host interactions. Pathogenic and symbiotic relationships share certain host association characteristics, and thus studies of symbiosis can inform our thinking about pathogenic associations. One simple, yet elegant model system used to study bacteria–host interactions is the symbiotic relationship between the marine bioluminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes*. Some advantages of this model system include the following: (1) the symbiosis is highly specific: *V. fischeri* is the only bacterium capable of colonizing the squid in a specialized squid organ called the light organ (Fig. 18.1), and thus this one–to-one relationship can be probed in detail; (2) the symbiosis can be manipulated: the symbiosis initiates anew with each generation of squid and both partners can be maintained separately, and then placed together to evaluate bacteria–host interactions; thus, the process by which the two organisms recognize and respond to one another can be readily assessed; (3) colonization can be monitored non-invasively by measuring bioluminescence, a natural product of this symbiosis; only productive colonization results in high levels of bioluminescence; and (4) the stages of colonization (Fig. 18.2) parallel those that occur in pathogenic associations, permitting an understanding of the commonalities and differences between pathogenic and symbiotic associations (Ruby, 1996; Stabb, 2006). Such studies will promote our ability to generate more specific therapies that will target detrimental pathogenic bacteria over the beneficial symbiotic bacteria generally present in the host.

The *V. fischeri–E. scolopes* symbiosis

Studies on the symbiosis between *V. fischeri* and *E. scolopes* have provided a wealth of information about the process of colonization and the environments experienced by the bacteria during colonization (Ruby, 1996; Nyholm and McFall-Ngai, 2004; Stabb, 2006). These studies have identified three main stages of colonization: initiation, accommodation, and persistence (Fig. 18.2A) (Ruby, 1996). During the initiation stage,

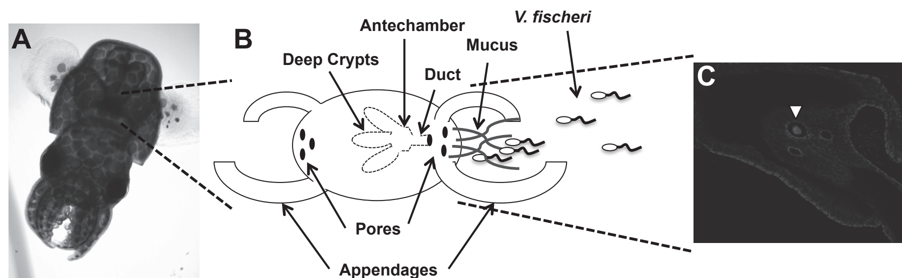

Figure 18.1 The light organ of juvenile *E. scolopes*. (A) Juvenile *E. scolopes*. The light organ can be seen as a dark mass in the centre of the body (mantle cavity) between the dotted lines. (B) A cartoon depiction of the light organ. This organ is bi-lobed, with a set of three pores located on each side at the base of two ciliated epithelial appendages. Each pore connects to a deep crypt space via a duct and antechamber (only one is represented for simplicity). *V. fischeri* cells are represented as white ovals with a curved line representing flagella. The bacteria aggregate outside of the light organ in the squid-secreted mucus (lines extending from the light organ surface). After aggregation, these cells migrate to the pores, through the ducts and the antechambers to the deep crypt spaces where they grow to high cell densities and bioluminesce. (C) A confocal microscopy image from (Yip *et al*., 2006), showing one side of the squid light organ with both appendages on the right side of picture (top and bottom). The three pores can be seen in the middle of the image, with an aggregate of *V. fischeri* located above one of the pores (indicated by the white triangle).

newly hatched juvenile squid (Fig. 18.1A) obtain their symbiotic partner from the surrounding environment. Seawater containing *V. fischeri* and other bacteria is continuously flushed through the body cavity and, as a result, across the light organ surface. The presence of bacteria and bacterial products induces the squid to secrete mucus to the light organ surface (Fig. 18.1B) (Nyholm *et al.*, 2000). Within about 3 hours after exposure of squid to *V. fischeri*, the bacteria form biofilm-like aggregates in the secreted mucus (Figs. 18.1C and 18.2B) (Nyholm *et al.*, 2000, 2002). The ability of *V. fischeri* to aggregate in the mucus is critical for colonization: mutants that cannot aggregate exhibit severe defects in colonizing the light organ (Yip *et al.*, 2006; Morris *et al.*, 2011). Within a few hours, *V. fischeri* cells transition from a biofilm-like state to a planktonic state and migrate towards the light organ pores (three on each side of the organ, six total) (Figs. 18.1B and 18.2B) (Nyholm *et al.*, 2000). This migration may be due to some chemotactic gradient associated with the pores. *V. fischeri* can sense and swim towards numerous attractants, including *N*-acetylneuraminic acid (a component of the mucus) and nucleosides (DeLoney-Marino *et al.*, 2003). Thus, these components, or some other attractant, may serve as a signal for entry into the light organ.

Once *V. fischeri* cells enter the light organ, they migrate through ducts and antechambers (Fig. 18.1B), both of which are non-permissive to colonization, to the deep crypt spaces where colonization and growth occur. The ducts and antechambers contain high concentrations of nitric oxide (NO) and other antimicrobial compounds, which *V. fischeri* is able to sense and respond to accordingly (Davidson *et al.*, 2004; Dunn *et al.*, 2010; Wang *et al.*, 2010a,b). Furthermore, the light organ is patrolled by macrophage-like immune cells, known as haemocytes (Nyholm and McFall-Ngai, 1998), yet *V. fischeri* is able to reside within the deep crypts. A recent study suggests that these haemocytes become 'educated' to the presence of *V. fischeri* within the light organ, and this apparent 'tolerance', mediated by both host and bacterial factors, prevents removal of the symbiont (Nyholm *et al.*, 2009).

Once in the deep crypt spaces, *V. fischeri* grows to high cell densities. This is the accommodation stage. The squid supplies the bacteria with nutrients, including amino acids in the form of small peptides, which permit rapid bacterial growth (Graf and Ruby, 1998). At high cell densities, the bacteria initiate production of bioluminescence (Fig. 18.2B), which the squid uses to avoid detection by predators: the light, which is directed downwards, disrupts the shadow that

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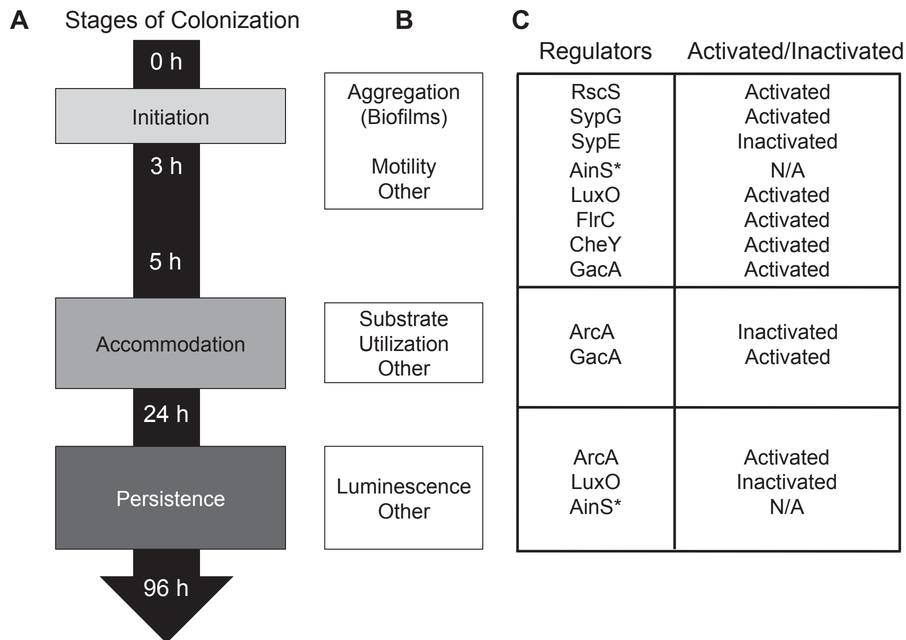

Figure 18.2 Model of the stages, processes, and predicted activities of a subset of regulators involved in colonization. (A) Time line and stages of colonization with the approximate times of and between each stage. At the initiation stage, the bacteria aggregate outside of the light organ and then migrate to the pores to enter and passage through the ducts and antechambers to reach the deep crypts (between hours 1–5 after hatching). At the accommodation stage, the bacteria reach the deep crypt spaces and grow to high cell densities and produce bioluminescence (between hours 5–24 after hatching). At the persistence stage, the bacteria must be maintained throughout the life of the squid, re-populating the light organ each day after the daily expulsion at dawn (between hours 24–96+ after hatching). (B) A brief list of the processes required for colonization that are discussed in this chapter. The approximate timing of these processes correspond to the time line and stages shown in panel A. (C) TCS regulators associated with the stages and processes required for colonization are listed along with their corresponding putative state of activation (activated or inactivated). For SypE, there is experimental evidence that strongly supports the hypothesis that this protein is inactivated at the indicated stage; for the other proteins, the putative state of activation is based on whether a mutant defective for the regulator exhibits a colonization defect at that stage. *AinS is not a TCS regulator, but it produces a signal proposed to be sensed by a TCS regulator.

would otherwise result from the down-welling moonlight shining on the animal as it forages for food in shallow bays at night (Jones and Nishiguchi, 2004). Motility, which is essential for entry, appears unnecessary at this stage of colonization, as most bacteria lack flagella in the deep crypts (Ruby and Asato, 1993).

The last stage of colonization is termed persistence. At dawn every morning, the squid expels approximately 95% of the bacteria into the seawater (Lee and Ruby, 1994; Boettcher *et al.*, 1996). The remaining *V. fischeri* cells then re-populate the light organ within a few hours. Thus, this partnership is dynamic, yet it is maintained for the life of the squid.

It is clear from this brief description of the initiation of the *V. fischeri*–squid symbiosis that the bacteria experience numerous different environments as they transit from seawater to symbiosis, adhering to the surface of the light organ, migrating through the ducts and antechambers and into the deep crypts, and growing rapidly once in the right location. Thus, it seems likely that *V. fischeri* recognizes and responds to specific signals in different environments to enter into a productive relationship with its squid host. A well-studied and important mechanism by which many bacteria, including *V. fischeri*, assess the environment is through two-component signal transduction systems (TCSs). Here, we will describe the known

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TCS systems encoded by *V. fischeri*, with a focus on those known to play a role in symbiosis.

Two-component signal transduction systems

Bacteria use TCSs to adapt to changes in their environment. A 'simple' TCS system is typically composed of a membrane-bound histidine sensor kinase (SK), which senses some environmental or cellular signal (e.g. membrane stress), and a response regulator (RR), which elicits the appropriate cellular response (e.g. transcriptional regulation of a gene or set of genes) (Fig. 18.3A). More specifically, when the SK senses a signal, it autophosphorylates on a conserved histidine residue, located within its transmitter domain

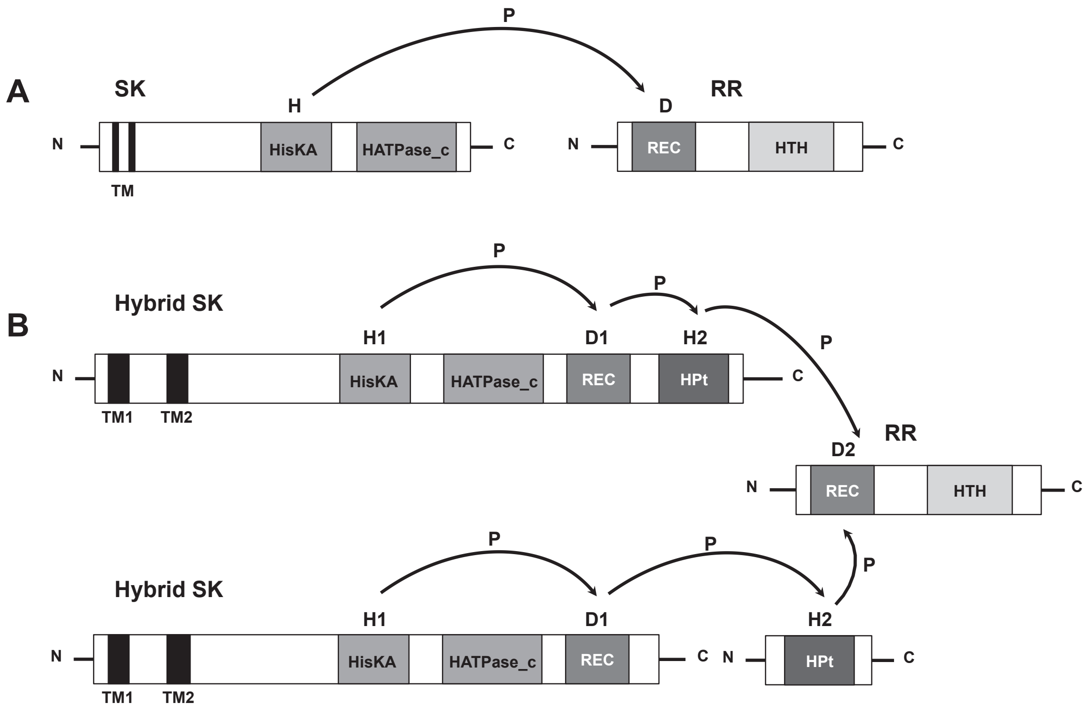

Figure 18.3 Two-component systems. (A) A simple TCS system composed of a sensor kinase (SK) and a response regulator (RR). A SK typically contains: (1) a periplasmic signal sensing loop, with at least two transmembrane spanning regions (depicted by the black boxes), and (2) HisKA and HATPase-c domains (light grey boxes with black lettering); the latter domain binds ATP and promotes phosphorylation of a conserved histidine residue located in the HisKA domain (depicted as an H above the HisKA domain) upon signal receipt. SKs can also contain cytoplasmically located signalling domains, such as PAS or HAMP domains (described in the text) (not shown). A RR typically contains: (1) a receiver (REC) domain (depicted as a darker grey box with white lettering), which catalyses the transfer of the phosphoryl group from the SK to a conserved aspartate residue (indicated by a D above the REC domain) within this domain, and (2) an attached effector domain, depicted here as a helix–turn–helix (HTH) DNA binding domain (very light grey box with black lettering); effector domains are not always DNA binding domains. (B) A more complex TCS system is known as a phosphorelay, and typically contains between two and four proteins and a total of four domains with residues that become phosphorylated. These domains, known as HisKA, REC, HPt (histidine phosphotransferase), and REC, contain H1, D1, H2 and D2, respectively. Two representative phosphorelays are depicted. At the top, a hybrid sensor kinase is depicted that contains three of the four domains involved in phosphotransfer to a RR, while at the bottom, a hybrid sensor kinase with two of the four domains is shown along with a separate phosphotransferase protein containing an HPt domain that serves as the phosphodonor to the RR. As with the simple TCS system, a signal stimulates the hybrid SK to autophosphorylate on H1. The phosphoryl group is transferred intramolecularly to D1 and, in either the same protein or a second protein, to H2. The phosphorelay is completed by donation of the phosphoryl group to D2 on the RR.

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(HisKA), and serves as a phospho-donor to its partner RR (Stock *et al.*, 2000; West and Stock, 2001). The RR then catalyses the transfer of the phosphoryl group to a conserved aspartate residue, located within its receiver (REC) domain (Fig. 18.3A) (Bourret *et al.*, 1990). RR phosphorylation is thought to stabilize the protein in a particular conformation, typically activating an attached effector domain (e.g. DNA binding domain), which promotes the necessary response (Stock *et al.*, 2000).

A more complex type of TCS system is known as a phosphorelay (Fig. 18.3B). A typical phosphorelay relies on multiple phosphotransfer events, between two or up to four separate proteins. One common type of phosphorelay involves a hybrid SK, which contains multiple residues involved in phospho-transfer, and a partner RR (Fig. 18.3B) (West and Stock, 2001). The hybrid SK usually contains a conserved histidine residue (H1) located within its transmitter domain (HisKA), a conserved aspartate residue (D2) located within a REC domain, and a second conserved histidine (H2) located within a histidine phosphotransferase (HPt) domain; alternatively, the HPt domain may be contained within a separate protein (Fig. 18.3B). The RR contains the second conserved aspartate residue (D2), located within its REC domain. Upon sensing a signal, the hybrid SK autophosphorylates on H1 and subsequently transfers the phosphoryl group intra-molecularly to D1 and then H2 (Fig. 18.3B) (West and Stock, 2001). The RR then catalyses the transfer of the phosphoryl group from H2 to D2 (Fig. 18.3B), which promotes RR activity (West and Stock, 2001).

Two-component regulators in *V. fischeri*

V. fischeri contains 40 genes that encode proteins with a predicted, conserved REC domain (Hussa *et al.*, 2007). The larger of *V. fischeri's* two chromosomes contains about half of the RR genes, with the remaining genes located on the smaller chromosome. These 40 RR proteins can be grouped into four classes: class one contains 15 non-DNA binding RRs, including CheY, a protein similar to the well-studied chemotaxis regulator in *E. coli* (Bourret *et al.*, 1990; Silversmith and Bourret, 1999); class two contains six RRs that are NarL-like owing to the presence of a classical helix–turn–helix (HTH) DNA binding domain (Gunsalus *et al.*, 1989; Baikalov *et al.*, 1998); class three contains 13 RRs that contain a winged helix–turn–helix (wHTH) domain, making them OmpR-like (Martinez-Hackert and Stock, 1997); and class four contains six NtrC-like RRs, which contain three domains, including an N-terminal REC domain, a centrally located σ^{54}-interaction domain, and a C-terminal DNA binding domain (Popham *et al.*, 1989; Weiss *et al.*, 1991). Of the 40 RR genes, 30 have been linked, by function or location, to a SK gene. A similar survey, however, has not yet been performed for SK genes.

Of the 40 RRs, 35 of them were disrupted and assessed for various phenotypes, including bioluminescence, motility, and colonization capability in the presence of wild-type *V. fischeri* (i.e. competitive colonization) (Hussa *et al.*, 2007). In addition to this global survey, a number of other studies have investigated the roles of specific two-component regulators in the *V. fischeri*–squid symbiosis. Most of these studies have focused on RRs (i.e. SypG, SypE, VpsR, FlrC, CheY, LuxO, ArcA, and GacA) (Figs. 18.2C and 18.4A), although a few have characterized hybrid SKs (i.e. RscS and SypF) (Figs. 18.2C and 18.4B). These studies have uncovered important roles of specific regulators in controlling biofilm formation (RscS, SypG, SypE, SypF, and VpsR), motility and chemotaxis (FlrC and CheY), and bioluminescence (LuxO and ArcA); one of these proteins (GacA) contributes to numerous phenotypes and thus is an important global regulator. The remainder of this review will focus on discussing the known roles of these regulators, both with respect to their control of the indicated phenotypes and to their importance in symbiosis.

Regulation of biofilm formation

Biofilms are populations of bacteria adherent to a surface and embedded in a matrix, typically self-produced and composed of polysaccharides, proteins, and DNA, although the exact composition varies depending on the microbe and its environment (Flemming and Wingender, 2010). Biofilms promote adherence of the bacteria to

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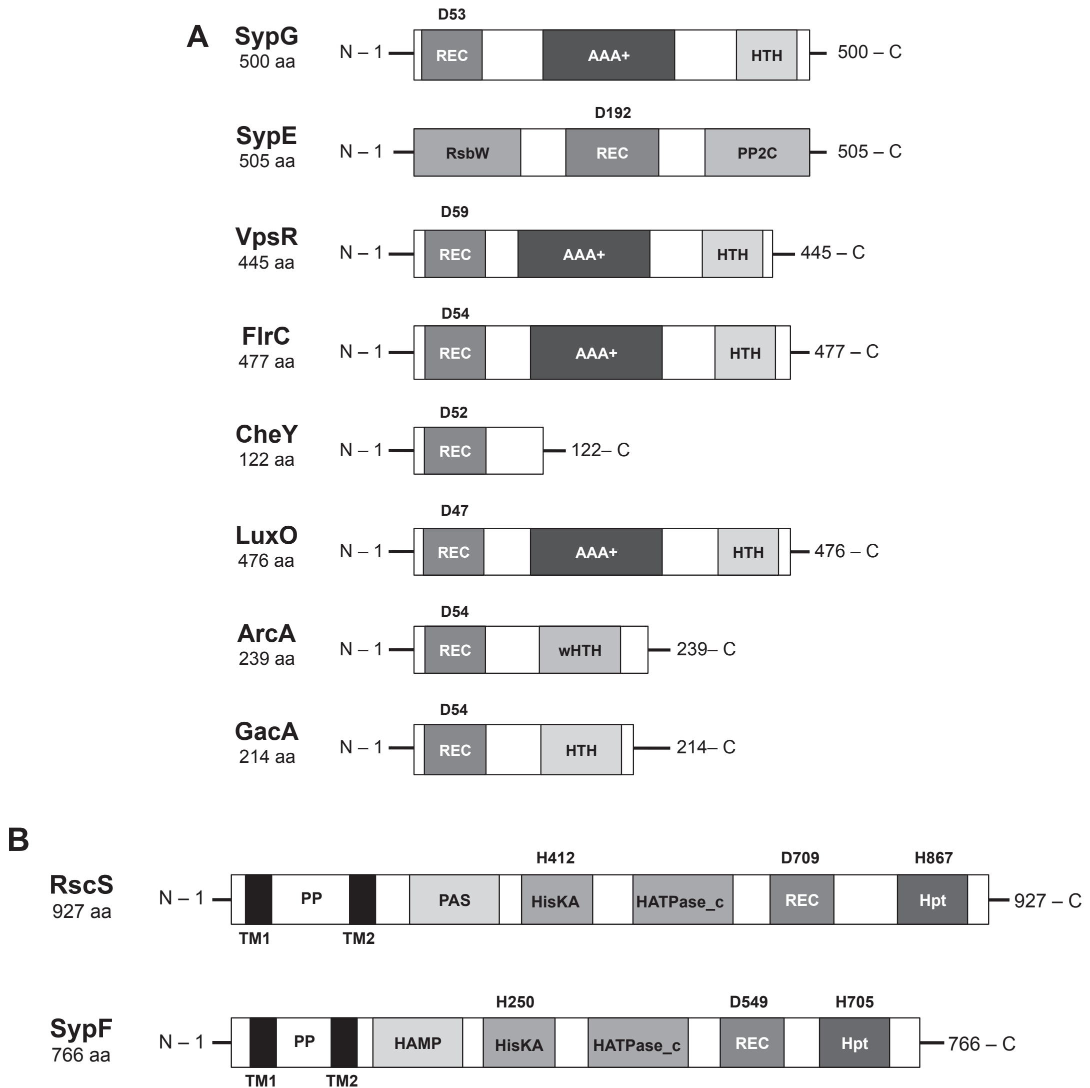

Figure 18.4 Domain structures of the RR and (hybrid) SK proteins described in detail in this chapter. (A) All of the RRs contain a REC domain (grey boxes with white lettering) with the predicted, conserved aspartate residue numbered and shown above this domain. All of these RRs, with the exception of SypE and CheY, contain a HTH or wHTH DNA binding domain (light grey boxes with black lettering). Four of these RRs (SypG, FlrC, LuxO, and VpsR) contain an AAA+ domain (dark grey box with white lettering), which is involved in interactions with RNA polymerase carrying the alternative sigma factor σ^{54}. SypE contains an N-terminal putative RsbW-like serine kinase domain and a C-terminal PP2C-like serine phosphatase domain, and CheY lacks these other domains. (B) Each SK contains a cytoplasmically located signal domain (either PAS or HAMP, described in the text; very light grey box with black lettering) and the HisKA and HATPase-c domains (light grey boxes with black lettering), with the predicted, conserved histidine numbered and shown above the HisKA domain. RscS and SypF are hybrid SKs and contain a REC (dark grey box with white lettering) and HPt (very dark grey box with white lettering) domain; the predicted, conserved aspartate and histidine residues are numbered and shown above these domains, respectively. The hybrid SKs each have two predicted transmembrane spanning regions (black boxes) flanking a periplasmic loop likely involved in the detection of an external signal.

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themselves and/or a surface. Furthermore, bacteria in biofilms exhibit an altered physiological state that permits increased resistance to antimicrobials such as antibiotics. As a result, biofilm formation is an important survival and colonization strategy used by bacteria. To date, few models have been described that allow the study of biofilm formation during host colonization; the *V. fischeri*–squid symbiosis is one of these. During the initiation stage of colonization, *V. fischeri* cells form a biofilm-like aggregate in mucus on the surface of the light organ (Fig. 18.1C), and subsequently disperse from the aggregate to enter the organ (Nyholm *et al.*, 2000). For *V. fischeri*, it seems likely that this aggregate is a form of biofilm, as its formation depends upon genes with known roles in biofilm formation in culture (Fig. 18.2B) (Yip *et al.*, 2006; Morris *et al.*, 2011). Whether or not the bacteria exist in a biofilm at a subsequent stage of colonization remains to be determined. Surprisingly, under traditional growth conditions in laboratory culture, *V. fischeri* fails to form any substantial amount of biofilm, although some adherence to the test tube surface can be detected (Yip *et al.*, 2005; Hussa *et al.*, 2008). Thus, it is possible that *V. fischeri* recognizes some host-specific signal(s) in its environment to promote aggregation on the surface of the light organ. Here, we describe the TCS regulators known to control biofilm formation in *V. fischeri*.

RscS

The gene *rscS* (regulator of symbiotic colonization – sensor) encodes a hybrid SK that was first identified in a screen for mutants unable to colonize juvenile *E. scolopes* (Fig. 18.4B) (Visick and Skoufos, 2001). The mutant defective for *rscS* exhibited a severe defect in initiating colonization: when exposed to the *rscS* mutant, most squid remained uncolonized. It was subsequently determined that this colonization defect resulted from the inability of the *rscS* mutant to aggregate on the surface of the light organ (Yip *et al.*, 2006). These experiments revealed for the first time a bacterial determinant necessary for aggregate formation, thus supporting the identification of this process as a stage critical to colonization. They also demonstrated that, rather than being passively taken up by the squid, *V. fischeri* plays an active role in promoting colonization. More recently, the importance of RscS was further emphasized when it was discovered that the gene that encodes this hybrid SK was missing in the fish symbiont MJ11, a strain of *V. fischeri* incapable of colonizing squid (Mandel *et al.*, 2009). When *rscS* was introduced into MJ11, the resulting strain became competent to colonize squid (Mandel *et al.*, 2009). Thus, this work determined that *rscS* – a single regulatory gene – was able to specify the interaction between a bacterium and its host. Together, these data demonstrated that RscS is a critical factor necessary for colonization, owing to its ability to promote aggregation.

RscS is now known to regulate the symbiosis polysaccharide (*syp*) locus, which is composed of 18 genes predicted to be involved in the regulation, production, and export of a polysaccharide necessary for biofilm formation (Fig. 18.5) (Yip *et al.*, 2005). Overexpression of *rscS* from either of two increased activity alleles, termed *rscS1* and *rscS2*, caused a marked increase in *syp* transcription (Yip *et al.*, 2006). Overexpression of these alleles also led to the production of distinctive *syp*-dependent phenotypes associated with biofilm formation (Fig. 18.6). These phenotypes included wrinkled colony formation on solid media, pellicle formation at the air–liquid interface of static cultures, cell aggregation in liquid cultures grown with shaking, increased attachment to glass surfaces, and increased hydrophobicity. Furthermore, examination of the wrinkled colonies formed by *rscS1*-containing cells using scanning and transmission electron microscopy revealed the presence of an extracellular matrix between the cells and at the colony surface. This polysaccharide matrix contained, in part, glucose and/or α-linked mannose sugars (Yip *et al.*, 2006).

These dramatic biofilm phenotypes begged the question, are they relevant to symbiosis? The answer was yes, as an *rscS1* overexpression strain showed a dramatic increase in the size of the symbiotic aggregate relative to the control (est. 50–200 μm vs. 10–20 μm in diameter) (Yip *et al.*, 2006). Furthermore, aggregate formation depended on *syp*: loss of *sypN* abolished the increase in aggregate formation induced by RscS overexpression. Finally, cells overexpressing *rscS1* substantially out-competed the vector-containing

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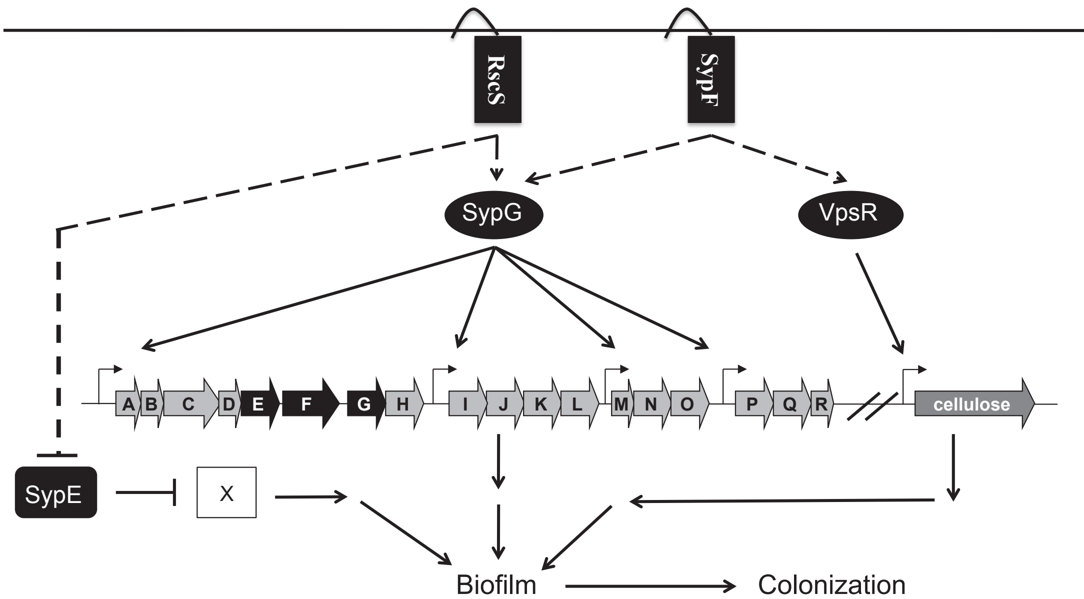

Figure 18.5 Model of *syp*-dependent biofilm formation. The hybrid SK RscS is predicted to activate SypG via phosphorylation, thus promoting transcription of each of four operons in the *syp* locus (from promoters indicated by the bent arrows). The *syp* genes encode proteins involved in the production and transport of a polysaccharide involved in biofilm formation. SypE is predicted to work downstream of *syp* transcription and inhibit biofilm formation in its unphosphorylated form. When RscS becomes active, the inhibitory activity of SypE is inactivated, promoting biofilm formation. SypF also appears to act upstream of *syp* transcription, as well as upstream of the RR VpsR, which is necessary for the production of cellulose, another component of biofilm formation. Biofilm formation in culture correlates with aggregation of *V. fischeri* within the squid secreted mucus, which promotes subsequent colonization events. The signals sensed by RscS and SypF are currently unknown. Furthermore, it is unknown whether cellulose promotes colonization.

control strain during mixed inoculation experiments, indicating that the increased ability to aggregate conferred an advantage to the cells during colonization (Yip *et al.*, 2006). Thus, these studies reveal a clear correlation between biofilm formation in culture and both symbiotic aggregation (i.e. biofilm formation) and colonization in an animal model.

What are *rscS1* and *rscS2*, and why do they have increased activity? These alleles were generated in a study that sought to determine the function of RscS. It was predicted that the signal sensed by RscS might exist only in the context of the symbiosis, and thus, a signal-insensitive mutant might be necessary to evaluate the role of RscS in culture. It was expected that the increased-activity alleles isolated, *rscS1* and *rscS2*, would contain changes to the coding sequence that made the RscS protein independent of the inducing signal. Instead, however, each increased activity allele contained a mutation in or near the putative ribosome binding site (RBS), while the *rscS1* allele also contained a silent mutation at codon Leu_{25} (Yip *et al.*, 2006; Geszvain and Visick, 2008a). Consistent with their locations, these mutations caused an increase in the level of the RscS protein, but did not impact the *rscS* transcript level (Geszvain and Visick, 2008a). Importantly, no RscS protein was detected when the wild-type allele was expressed from the chromosome or overexpressed from a plasmid lacking the mutations (Geszvain and Visick, 2008a), suggesting that this protein is normally expressed poorly or not at all under standard culturing conditions. Surprisingly, when the effects of the mutations in *rscS1* were separately assessed, the silent mutation at codon Leu_{25} appeared to exert a greater impact on RscS activity than the RBS-linked mutation. The silent

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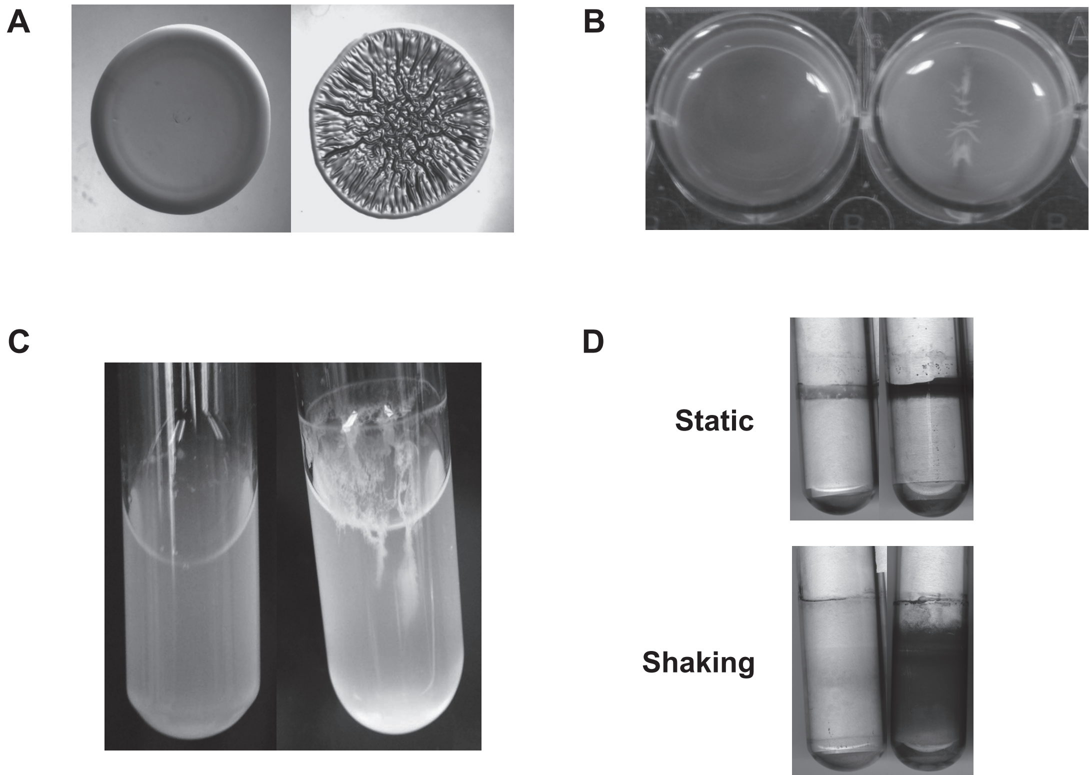

Figure 18.6 *V. fischeri* biofilm phenotypes. The left image in each section is of a non-biofilm forming *V. fischeri* strain, while the right image is of a biofilm-forming strain. (A) Wrinkled colony formation of spotted cultures on solid agar media. (B) Pellicle formation at the air–liquid interface of a static culture. The pellicle can be seen as a 'bunched' cell mass, generated to promote visualization by dragging a sterile pipette tip over the surface. (C) Bacterial aggregation of shaking cultures. 'Stringy' material, observed for the biofilm forming strain, consists of clumps of adherent cells. (D) Glass attachment of either statically grown cultures or shaking cultures, visualized following staining with crystal violet. Cells in static cultures attach at the air–liquid interface of the tube, while cells in shaking cultures can attach throughout the tube. These images are compiled from a variety of strains that produced or lacked biofilms.

mutation substitutes a rare Leu codon (used in 4% of Leu codons in *V. fischeri*) for a more common Leu codon (used in ~20% of Leu codons). This Leu codon occurs within a region ($L_{23}ML_{25}TRN_{28}$) that contains, with one exception, all rare codons (Geszvain and Visick, 2008a). Thus, there may be multiple levels at which the production of RscS protein is controlled. These studies concluded that the increase in protein production likely accounts for the increase in RscS activity and subsequent biofilm phenotypes when *rscS1* and *rscS2* are overexpressed. It will be of interest to determine the extent of regulatory control over RscS translation in *V. fischeri*. Furthermore, future studies should address whether simple overexpression is sufficient to overcome a need for a specific, potentially host-associated signal, or if the signal naturally exists in laboratory culture but depends upon sufficient protein production.

The identification of phenotypes for both RscS and the *syp* locus paved the way for understanding the specific roles of RscS and other biofilm regulators. RscS is an orphan hybrid SK (*rscS* is physically unlinked to a RR gene), and thus its partner(s) was initially unknown. However, the *syp* locus encodes two RRs, SypE and SypG (Fig. 18.5), and since RscS regulates the *syp* locus, it was possible that one of these RRs could serve as a partner for RscS. SypE is a unique RR that contains a centrally located REC domain, flanked by putative domains of opposing function (an N-terminal serine kinase domain and a C-terminal serine

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phosphatase domain; Figure 18.4A) (Hussa *et al.*, 2008; Morris and Visick, 2010); however, this protein lacks any sequences that resemble a DNA-binding motif. In contrast, SypG is a member of the well-studied NtrC family of RRs: it contains three predicted domains, an N-terminal REC domain, a central σ^{54} interaction domain, and a C-terminal DNA binding domain (Fig. 18.4A). Like RscS, SypG activates the *syp* locus (Yip *et al.*, 2005), making this RR a likely partner for RscS. In support of this idea, deletion of *sypG* disrupts all of the RscS-induced phenotypes, including *syp* transcription (Hussa *et al.*, 2008). Thus, it appears that RscS works upstream of SypG to induce *syp* transcription and thus biofilm formation (Fig. 18.5). The current knowledge of the roles of SypE and SypG in biofilm formation and colonization will be described later in this review.

Structurally, RscS resembles the hybrid SKs ArcB and BvgS (Visick and Skoufos, 2001). These proteins contain three conserved domains (HisKA, REC, and HPt) with residues predicted to be involved in phosphotransfer (H412, D709, and H867 in RscS) (Fig. 18.4B). Investigation of the roles of the conserved residues in RscS supported the function of RscS as a hybrid SK: mutations of H1 (H412Q), D1 (D709A), and H2 (H867Q) abolished (H1 and D1) or diminished (H2) induction of RscS-dependent biofilm phenotypes (Geszvain and Visick, 2008b). Since H2 was not essential for RscS-induced phenotypes, the signal transduction pathway may be complicated. For example, it is possible that the phosphorelay is branched, with RscS donating its phosphoryl group to another HPt domain-containing protein, or that phosphotransfer to the RR could occur directly from H1 to D2. Such events are not unprecedented [e.g. ArcA/B (Georgellis *et al.*, 1997)], but further studies are needed to fully dissect the dispensability of the Hpt domain for RscS function.

RscS also contains a large periplasmic (PP) loop and a PAS domain, both of which are implicated in signal recognition (Fig. 18.4B). The PP loop is ~200 amino acids in length and is flanked on either side by two transmembrane (TM) regions. The PAS domain is located in the cytoplasm after the second TM region (Fig. 18.4B). In other SKs, the PP loop is responsible for sensing an environmental signal, and transmitting that information through TM regions to the cytoplasmic signalling portion of the protein (Mascher *et al.*, 2006). This appears to be the case for RscS, as disruption of the PP loop or the first transmembrane domain promoted RscS activity (Geszvain and Visick, 2008b), indicating that these regions may serve to negatively regulate RscS function. In contrast, disruption of the cytoplasmically localized PAS domain abolished RscS function (Geszvain and Visick, 2008b), indicating that this domain may positively regulate RscS function. PAS domains can sense signals such as oxygen, redox potential, light, and small molecules such as ATP, and frequently require binding of a cofactor for signal transduction (Taylor and Zhulin, 1999). RscS may sense an FAD cofactor, since its PAS domain shares homology with the PAS domain of NifL from *Azotobacter vinelandii*, which senses an FAD cofactor (Key *et al.*, 2007). In support of this idea, conserved residues required for FAD binding are essential for RscS activity (Geszvain and Visick, 2008b). Taken together, it seems likely that RscS senses multiple signals using the PP loop and PAS domain, which regulate its function accordingly. Thus, determining what these signals are and understanding how RscS responds to them will provide a better understanding of how biofilm formation is regulated in the context of the squid host.

SypG

Like *rscS* mutants, *sypG* mutants exhibited severe defects in colonization as well (Hussa *et al.*, 2007). As noted above, SypG is a member of the NtrC family of RRs and contains three predicted domains: an N-terminal REC domain, a C-terminal DNA binding domain, and between those two domains, a σ^{54} interaction domain (Fig. 18.4B). This latter domain is predicted to provide the ATPase activity necessary for transcription: RNA polymerase containing σ^{54} binds to DNA and forms a closed complex, but cannot form the transcriptionally active open complex without the help of an activator protein to provide the energy (Fig. 18.7) (Buck *et al.*, 2000; Wigneshweraraj *et al.*, 2008). Intriguingly, the *syp* locus contains four promoters (Fig. 18.5) with σ^{54} recognition sequences (Barrios *et al.*, 1999; Yip *et al.*, 2005);

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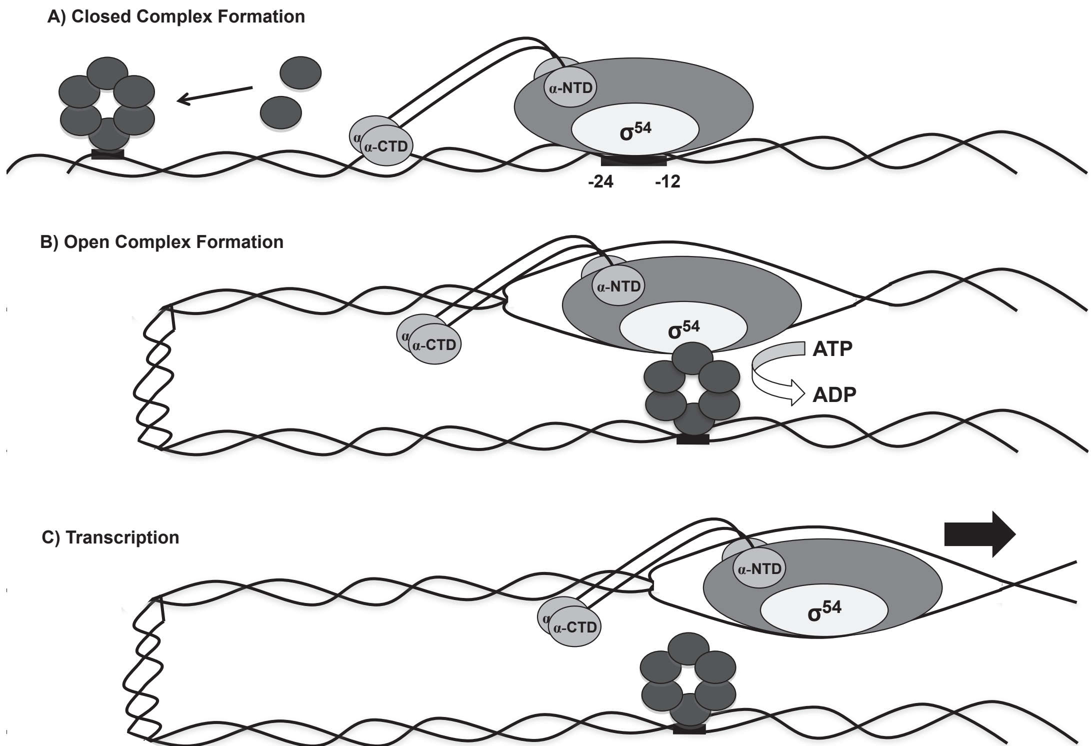

Figure 18.7 Model for σ^{54}-dependent transcriptional activation. (A) σ^{54}-containing RNA polymerase (shaded grey circles) binds to the promoter region (typically at conserved sequences centred at –12 and –24 relative to the start of transcription; large black box), promoting closed complex formation. Concurrently, activation of a σ^{54}-dependent activator protein (dark grey circles) typically promotes the formation of a hexamer, which binds to an enhancer sequence upstream of the promoter region. (B) Bending of the DNA enables interaction of the σ^{54}-dependent activator with σ^{54}. The σ^{54}-dependent activator then hydrolyses ATP to ADP, providing the energy for open complex formation. (C) With the energy provided by the σ^{54}-dependent activator protein and formation of the open complex, RNA polymerase is able to promote transcription (indicated by the black arrow). [The steps of transcription promoted by σ^{54}-containing RNA polymerase and an activator protein are adapted from Wigneshweraraj *et al*. (2008).]

three of the four promoters were confirmed by primer extension analyses (Yip *et al.*, 2005). Located upstream of each of the σ^{54} promoters is a conserved 22-bp sequence that could serve as a binding site for a σ^{54}-dependent activator (Fig. 18.7). Since *sypG* encodes such a protein, Yip *et al.* (2005) hypothesized that SypG could activate transcription of the *syp* locus. Although there was little *syp* transcription in wild-type cells, overexpression of *sypG* from a multicopy plasmid caused a 37- to 70-fold increase in *syp* transcription over that of the vector control strain. This SypG-induced transcription depended upon the presence of σ^{54}, as disruption of the *rpoN* gene, which encodes σ^{54}, abolished transcriptional activation of *syp* (Yip *et al.*, 2005). Together, these data suggest that SypG, in conjunction with σ^{54}, promotes transcription from the *syp* locus (Fig. 18.5). To date, however, it remains unknown whether SypG binds to the 22-bp sequences located upstream of the σ^{54}-dependent promoters within the *syp* locus.

Coincident with induction of *syp* transcription, overexpression of *sypG* induced biofilm formation (Yip *et al.*, 2005). Specifically, *sypG* overexpression induced a 3.5-fold increase in glass attachment (e.g. Figure 18.6D) when cells were grown statically. Furthermore, when grown under shaking conditions, cells that overexpressed *sypG* exhibited a >30-fold increase in glass attachment relative to the vector control strain. Biofilms formed under either condition (static or shaking)

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depended upon a functional copy of *rpoN* (Yip *et al.*, 2005). Together, these data demonstrated that SypG activates *syp* transcription, and thus biofilm formation, in a σ^{54}-dependent manner.

Although both RscS and SypG induce *syp* transcription and biofilm formation, and activity of the SK clearly depends on the RR, the specific biofilm phenotypes displayed by the two overexpression strains were dissimilar: overexpression of *rscS* resulted in the formation of wrinkled colonies and a strong pellicle (Yip *et al.*, 2006), while overexpression of *sypG* only led to weak pellicle formation (Hussa *et al.*, 2008). Two possibilities could account for these differences: (1) when overexpressed in the absence of its SK, SypG is not sufficiently activated to promote transcription of the genes required for strong biofilm phenotypes, or (2) RscS signals through a second RR to either inactivate a negative regulator or activate a positive regulator to promote biofilm formation. To assess the first possibility, a constitutively active version of SypG (SypG*) was generated, in which the conserved aspartate residue (D53) within the REC domain was changed to a glutamate (D53E); these types of mutations have been shown to promote RR activity in the absence of phosphorylation in other RRs [e.g. CheY, NtrC, and LuxO (Sanders *et al.*, 1989, 1992; Freeman and Bassler, 1999)]. Overexpression of *sypG** led to an increase in *syp* transcription, but could not induce wrinkled colony formation or enhance pellicle formation (Hussa *et al.*, 2008). Support for the second possibility came from an unexpected result: when *sypG* was overexpressed in a mutant lacking SypE, the second predicted RR encoded within the *syp* locus (Fig. 18.5), the cells formed wrinkled colonies and pellicles that were indistinguishable from those induced by RscS (Hussa *et al.*, 2008). Subsequent analyses, described in the SypE section below, supported the idea that RscS promotes inactivation of an inhibitory activity of SypE (Morris *et al.*, 2011). These results thus strengthen the hypothesis that RscS and SypG function together to promote *syp*-dependent biofilm formation (Fig. 18.5), but suggest that control over biofilm formation is complex and extends beyond transcriptional activation of the *syp* locus.

In summary, SypG plays a key role in controlling *syp* transcription and, thus, biofilm formation and colonization. At least one SK, RscS, appears to signal through SypG to control biofilm formation (Fig. 18.5). The function of another, SypF, which is encoded adjacent to the *sypG* gene, also appears to partially depend on SypG (discussed below in the SypF and VpsR section) (Fig. 18.5). Thus, from these studies it is clear that *V. fischeri* expresses numerous regulators that exert significant control over biofilm formation, indicating that it is important to *V. fischeri* to prevent biofilm formation when it is not needed—perhaps to restrict it to times when *V. fischeri* encounters its host.

SypE

The *syp*-encoded RR SypE is unusual. It contains a centrally located REC domain that is flanked by putative effector domains with apparently opposing enzymatic functions (Fig. 18.4A) (Morris and Visick, 2010). The N-terminal domain of SypE exhibits sequence similarity to SK-like serine kinases of the GHKL (Gyrase, Hsp90, HK, MutL) superfamily. These types of proteins contain a conserved asparagine residue, D52 in SypE (Morris and Visick, 2010), necessary for ATP binding (Dutta and Inouye, 2000). The C-terminal domain exhibits sequence similarity to the PP2C family of serine phosphatases, which contain invariant aspartate residues, D443 and D495 in SypE (Morris and Visick, 2010), involved in catalytic activity (Adler *et al.*, 1997; Jackson *et al.*, 2003). To date, no other RR protein with this unique domain structure has been characterized.

Consistent with the presence of two domains of opposing activity, SypE appears to exert both positive and negative control over biofilm formation. First, as described above, SypE appears to antagonize SypG-induced biofilm formation: *sypG* overexpression induced robust biofilms only when *sypE* has been disrupted (Hussa *et al.*, 2008), indicating that SypE functions as a negative regulator of biofilm formation. Second, SypE enhances biofilms produced by RscS overexpression: overexpression of *rscS* in a strain deleted for *sypE* resulted in wrinkled colony formation that was delayed relative to the *sypE*$^{+}$ control (Hussa *et al.*, 2008; Morris *et al.*, 2011). Taken together,

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these data suggest that SypE can function to both promote and inhibit biofilm formation.

How is this dual regulatory activity controlled? The answer to this question came from an elegant study that examined the function and interplay between the three domains of SypE and their impact on biofilm formation and host colonization. This study assessed the ability of a variety of SypE mutants to control biofilm formation induced by RscS (Morris *et al.*, 2011). It was found that the C-terminal putative serine phosphatase domain was responsible for the positive regulatory activity of SypE (i.e. promoting biofilm formation), while the N-terminal putative serine kinase domain was responsible for the negative regulatory activity of SypE (i.e. inhibiting biofilm formation). The negative and positive functions of the two domains depended upon conserved residues predicted to be necessary for serine kinase and serine phosphatase activities, respectively. Finally, mutation of the predicted site of phosphorylation in the REC domain (D192) to an alanine 'locked' SypE into an inhibitory state. This type of mutation can result in a protein that mimics the unphosphorylated state, since the residue can no longer become phosphorylated (Freeman and Bassler, 1999). Thus, it appears that D192 and, presumably, phosphorylation is necessary for switching the activity of SypE from negative to positive.

Which, if any, of these activities is important for colonization? A deletion of *sypE* exerted little effect on colonization, suggesting that the positive activity is not critical. However, expression of *sypE*^{D192A}, the REC domain mutant that is 'locked' into the inhibitory state, severely impaired colonization (Morris *et al.*, 2011). For example, in single strain colonization experiments, most squid remained uncolonized, while those that became colonized contained up to 1000-fold fewer bacteria than wild-type colonized squid. This colonization defect could be attributed to a defect at the aggregation stage: whereas RscS-overexpressing wild-type cells formed large aggregates above the light organ pores, RscS-overexpressing *sypE*^{D192A} cells were unable to aggregate (Morris *et al.*, 2011). These data, along with previous studies of RscS, demonstrate the importance of symbiotic aggregation in promoting colonization, as well as indicate that SypE naturally becomes inactivated during this stage of host colonization.

To date, no studies have confirmed phosphorylation of SypE, but it seems reasonable to expect that D192 becomes phosphorylated in response to some as-yet-unknown signal. The next question is, what SK works upstream of SypE? Morris *et al.* (2011) reasoned that RscS functions upstream of SypE (Fig. 18.5), because overexpression of RscS but not SypG seemed to turn off the inhibitory activity of SypE. Thus, RscS would have two activities: (1) activating SypG and (2) inactivating the negative regulatory activity of SypE. As a result of the latter activity, SypE would function as a negative regulator of colonization in an *rscS* mutant. If so, then deletion of *sypE* should suppress at least part of the colonization defect of an *rscS* mutant. Indeed, an *rscS sypE* double mutant colonized better than the single *rscS* mutant (Morris *et al.*, 2011), supporting the hypothesis that SypE functions as a negative regulator in the absence of RscS. Thus, during symbiotic colonization, SypE may serve to restrict colonization until RscS is activated (Fig. 18.5). Whether RscS directly or indirectly influences the phosphorylation state of SypE remains to be determined.

In summary, SypE is a positive and negative regulator of biofilm formation and, at least, a negative regulator of host colonization. Many questions remain unanswered, such as, at what level does SypE regulate biofilm formation and what is the target of SypE-mediated activity? For the first question, current data suggest that SypE works downstream of *syp* transcription (Hussa *et al.*, 2008), though the exact level remains to be determined. As for the target of SypE-mediated activity, one likely candidate is SypA, a putative anti-σ-factor antagonist with a conserved STAS (anti-σ-factor antagonist and sulfate transporter) domain; these types of proteins require a conserved serine residue (S56 on SypA) to regulate their activity (Morris and Visick, 2010). However, whether SypA functions to control biofilm formation, and whether it functions downstream of SypE remain unknown. As SypE is conserved in a number of distantly related bacteria, understanding its mechanism of action will be of benefit in a variety of fields.

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SypF and VpsR

The *syp* locus contains a gene, located between the two RR genes (*sypE* and *sypG*), that encodes a putative hybrid SK, SypF (Fig. 18.5). SypF contains three conserved domains (HisKA, REC, and HPt) and residues predicted to be involved in phosphotransfer (H250, D549, and H705, respectively) (Fig. 18.4B). Similar to RscS, SypF contains two additional putative signalling regions, a PP loop and a cytoplasmic domain (Fig. 18.4B). Whereas RscS contains a PAS domain, SypF contains a HAMP domain. Similar to PAS domains, HAMP domains are often involved in signal transduction. HAMP domains contain a highly conserved helix–turn–helix fold, a motif also common in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases; the fold but not the amino acid sequence is conserved, making it difficult to identify these domains (Kishii *et al.*, 2007). While the HAMP domain and periplasmic loop of SypF are likely involved in signal transduction, the signal(s) sensed by SypF are unknown.

Like RscS, SypG, and SypE, SypF also appears to serve as a regulator of biofilm formation in *V. fischeri*. Overexpression of a signal-independent allele of *sypF*, *sypF1*, resulted in a 10-fold increase in *syp* transcription and four distinct biofilm phenotypes: (1) cell aggregation (the clumping of cells in liquid culture under shaking growth conditions, not to be confused with symbiotic aggregation), (2) wrinkled colony formation, (3) pellicle formation, and (4) increased glass attachment (3-fold increase vs. the wild-type strain) (Fig. 18.6) (Darnell *et al.*, 2008). Sequence analysis of the *sypF1* allele showed that this allele contains two amino acid substitutions, one at residue 247 (S247F) and the other at residue 439 (V439I). Of these two mutations, the S247F substitution, located in the HisKA domain three residues N-terminal to the conserved histidine (H250), appeared to be more important for the increased activity of *sypF1* (Darnell *et al.*, 2008). Owing to the proximity of this residue to the conserved histidine, it is likely that this mutation impacts the predicted kinase activity of SypF.

Because SypF is a hybrid SK, it is predicted to exert its effect by acting through a downstream RR. In the chromosome *sypF* is adjacent to *sypG* (see the SypG section above; Figure 18.5), thus, it seemed reasonable to expect that SypF worked by activating SypG. Indeed, disruption of *sypG* eliminated the 10-fold induction of *syp* transcription caused by overexpression of *sypF1* (Darnell *et al.*, 2008). Furthermore, disruption of *sypG* also diminished SypF1-induced cell aggregation, a known *syp*-dependent biofilm phenotype. Surprisingly, however, the SypF1-induced wrinkled colony and pellicle formation phenotypes were only diminished, not eliminated, by loss of *sypG*. In addition, loss of *sypG* did not impact SypF1-induced glass attachment. These results suggested that some of the SypF1-induced phenotypes are SypG-dependent (*syp* transcription and cell aggregation), while other phenotypes are only partially dependent upon SypG (wrinkled colony and pellicle formation), and yet others were SypG-independent (glass attachment). Overall, while SypF appears to work upstream of SypG to regulate the *syp* locus (Fig. 18.5), it also appears to work through another regulator(s) to control biofilm formation.

What is the identity of the other regulator(s)? Of the 40 RRs encoded by *V. fischeri* (Hussa *et al.*, 2007), a prime candidate was VpsR, which is an NtrC-like σ^{54}-dependent activator (Figs. 18.4A and 18.7). In a related vibrio, *V. cholerae*, VpsR controls biofilm formation through activating expression of the *vps* polysaccharide locus (Yildiz *et al.*, 2001). In *V. fischeri*, a *vpsR* mutant produces colonies that are mucoid and opaque, distinct from the smooth and yellowish colonies formed by the wild-type strain (Darnell *et al.*, 2008), suggesting that VpsR might play a role in biofilm formation. Indeed, overexpression of *sypF1* in a *vpsR* mutant led to diminished wrinkled colony and pellicle formation, similar to when *sypG* was disrupted, as well as a total loss of glass attachment (which was unaffected by loss of *sypG*). However, disruption of *vpsR* did not impact cell aggregation (which was diminished by loss of *sypG*). Thus, wrinkled colony and pellicle formation appear to depend on both *syp* and *vpsR*, while *syp* transcription and cell aggregation (i.e. cell–cell interactions) are *sypG*-dependent phenotypes, and glass attachment (i.e. cell–surface interactions) is a *vpsR*-dependent phenotype. When *sypF1* was overexpressed in a double *sypG vpsR* mutant, all SypF1-induced

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biofilm phenotypes were lost (Darnell *et al.*, 2008), indicating that *syp* and *vpsR* together account for all of these phenotypes. Thus, SypF appears to modulate biofilm formation through regulation of the *syp* locus and an unknown, *vpsR*-dependent pathway (Fig. 18.5).

To begin to elucidate the role of VpsR in biofilm formation, a *vpsR* overexpression construct was generated. Overexpression of *vpsR* increased glass attachment under static growth conditions and allowed for weak pellicle formation, but did not induce wrinkled colony formation (Darnell *et al.*, 2008), suggesting that VpsR plays a role in biofilm formation that is distinct from *syp*. However, despite the role of the *V. cholerae* homologue in controlling the *vps* polysaccharide locus, VpsR-induced biofilm formation did not require the *vps*-like locus present in *V. fischeri* (Darnell *et al.*, 2008; Grau *et al.*, 2008; Yildiz and Visick, 2009). Instead, disruption of another polysaccharide locus, responsible for the production of cellulose, eliminated glass attachment and diminished pellicle formation. It also eliminated another phenotype associated with VpsR and SypF1: overexpression of either regulator caused an increase in the binding of the dye Congo Red, a phenotype which is associated with cellulose production (Teather and Wood, 1982). Together, these data suggest that SypF works upstream of VpsR to regulate cellulose biosynthesis and implicate cellulose as a player in *V. fischeri* biofilm formation and, specifically, cell–surface interactions (Fig. 18.5).

Intriguingly, there's another connection between *syp* and cellulose. Just downstream of the *syp* locus is a gene, *binA*, that encodes a c-di-GMP phosphodiesterase (PDE) involved in the degradation of c-di-GMP. In many bacteria, increased levels of c-di-GMP correspond to increased biofilm formation, in some cases owing to increased cellulose production (Ross *et al.*, 1987; Römling *et al.*, 2005). Consistent with that idea, disruption of *binA* resulted in increased c-di-GMP levels and increased cellulose-dependent biofilm formation (Bassis and Visick, 2010), findings that implicate BinA as a negative regulator of biofilm formation. Interestingly, transcription of *binA* increased upon overexpression of *sypG*, likely owing to read-through from the *syp* locus. However, BinA did not appear to impact *syp*-dependent biofilm formation (Bassis and Visick, 2010). Thus, BinA is both physically and functionally connected to two polysaccharide loci, potentially serving to down-regulate one when the other is up-regulated.

Of these regulators (SypF and VpsR), only VpsR has been investigated for a role in host colonization. When juvenile squids were exposed to a mixture of the *vpsR* mutant and wild-type *V. fischeri*, the resulting symbiotic animals contained a higher percentage of the wild-type strain (Hussa *et al.*, 2007), suggesting that the *vpsR* mutant exhibited a slight colonization defect. However, since this mutant exhibited multiple phenotypes (e.g. colony morphology and cellulose biosynthesis) (Hussa *et al.*, 2007; Darnell *et al.*, 2008), it is unclear whether the slight colonization defect resulted from a lack of one of these processes, from the loss of a combination of them, or from an as-yet-unknown factor. Further studies are needed to better assess the roles of SypF and VpsR in host colonization.

In conclusion, these studies (1) further demonstrate the complexity of biofilm formation in *V. fischeri*; (2) indicate that *vpsR* is conserved, but serves a distinct role in biofilm formation in *V. fischeri* relative to *V. cholerae*; (3) suggest that cell–surface interactions (glass attachment) depend upon cellulose, while cell–cell interaction (pellicles and cell aggregation) depend upon *syp*; and (4) suggest that the cellulose biosynthesis is regulated by multiple proteins, including those encoded in/near the *syp* locus (*sypF* and *binA*). Many unanswered questions remain, including the identity of the environmental signal(s) that stimulate the formation of *syp*- and cellulose-dependent biofilm formation and the role, if any, of cellulose in promoting host colonization.

Motility and chemotaxis

How does *V. fischeri* find its way to and into the squid? The answer to that is unknown, but one part of the puzzle is clearly motility and, likely, chemotaxis, the biased movement towards attractants and away from repellents. *V. fischeri* is lophotrichous (i.e. contains a tuft of polar flagella) and is predicted to encode about 40 different chemoreceptors that could direct its movement

(DeLoney-Marino *et al.*, 2003, Ruby *et al.*, 2005). Early studies demonstrated that this flagella-based motility is essential for colonization, as non-motile strains of *V. fischeri* were unable to colonize *E. scolopes* (Graf *et al.*, 1994; Millikan and Ruby, 2002, 2003, 2004; Wolfe *et al.*, 2004). The defect, however, was not at the aggregation stage, as flagella mutants formed normal aggregates (Nyholm *et al.*, 2000). Thus, some subsequent event, potentially entering and/or navigation through the ducts and antechambers, depends on motility and potentially, chemotaxis (Fig. 18.8A). Once *V. fischeri* cells have colonized the deep crypt spaces, the bacteria no longer contain flagella (Fig. 18.8A) (Ruby and Asato, 1993). Finally, upon release, the bacteria once again become flagellated (Fig. 18.8A). The signal(s) and regulators that control flagella expression in symbiosis remain unknown. Furthermore, whether the presence of flagella on colonized bacteria is detrimental or merely unnecessary remains to be determined. Therefore, an understanding of the regulatory mechanisms controlling the production of flagella is important to facilitate studies of mechanisms of control during symbiosis.

FlrC

In many bacteria, the production of flagella depends upon multiple regulators that control transcription at different levels within a transcriptional hierarchy (McCarter, 2006). For example, in *V. cholerae* there are four classes of flagellar genes: I, II, III, and IV (Fig. 18.8B) (reviewed by McCarter, 2001). Class I contains one gene, *flrA*, which encodes the master flagellar regulator (Fig. 18.8B) (Prouty *et al.*, 2001). FlrA is a σ^{54}-dependent transcriptional activator (Fig. 18.7) that promotes transcription of class II genes, which encode the SK/RR pair FlrB/FlrC, along with other genes involved in flagellar synthesis (Fig. 18.8B). FlrB, a cytoplasmic SK, serves as a phospho-donor to the RR FlrC (Correa *et al.*,

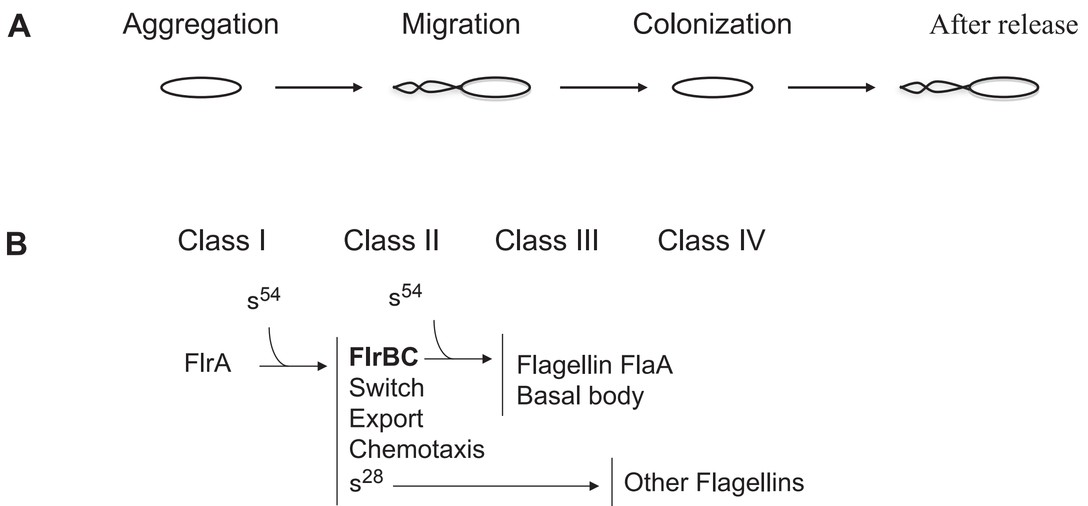

Figure 18.8 Control of *V. fischeri* motility. (A) *V. fischeri* adapts to its environment by the expression of flagella or lack thereof at various stages of colonization (flagella are represented by wavy lines coming off of the bacteria). Flagella are not necessary for aggregate formation, but are critical for some early stage in initiating symbiosis (migration). Once *V. fischeri* colonizes the squid, most cells lack flagella (colonization). Finally, upon release from the light organ, *V. fischeri* cells rapidly re-grow their flagella (after release). (B) Transcriptional hierarchy controlling flagella gene expression in *V. cholerae*, which serves as a model for *V. fischeri*. Class I includes the *flrA* gene (designated as FlrA to indicate its activity in controlling transcription of Class II genes), and the other three classes contain numerous genes involved in the production and rotation of flagella; these are listed simply as ring, switch, export, flagellin, and basal body (for a review, see McCarter, 2006). Of note is Class II, which includes the *flrB* and *flrC* TCS regulatory genes (designated as FlrBC to indicate their role in activating transcription of Class III genes). Both σ^{54} and σ^{28} contribute to regulation of flagellar genes. This figure was adapted from (Prouty *et al*., 2001; Correa *et al*., 2005).

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2000). Upon phosphorylation, FlrC promotes transcription of class III genes by RNA polymerase carrying the alternative sigma factor σ^{54}. The last class of genes, class IV, are transcribed by RNA polymerase carrying σ^{28}. Class III and IV genes encode the remaining components necessary to complete flagellar synthesis (Fig. 18.8B). Genes for many of the *V. cholerae* regulators are conserved in the squid symbiont, including *flrA* and *flrB*/*flrC* (Millikan and Ruby, 2003); thus, it seems likely that this type of hierarchical regulation also occurs in *V. fischeri*.

The FlrB and FlrC proteins of *V. fischeri* are 72% and 84% identical, respectively, to their homologues in *V. cholerae* (Millikan and Ruby, 2003). FlrB is predicted to contain an N-terminal PAS domain and a C-terminal HisKA domain. FlrC is predicted to contain an N-terminal REC domain, with a predicted, conserved site of phosphorylation (D54), a centrally located σ^{54}-interaction domain, and a C-terminal helix–turn–helix DNA binding domain (Fig. 18.4A) (Millikan and Ruby, 2003). Consistent with the predicted role of FlrC as a motility regulator, a *flrC* mutant of *V. fischeri* was non-motile (Hussa *et al.*, 2007). The mutant also exhibited a couple of other phenotypes: it consistently reached a higher optical density in culture than the wild-type strain and reached levels of luminescence that were slightly, but reproducibly, decreased (Hussa *et al.*, 2007). The reason(s) for these additional phenotypes remains unclear. FlrC also appears to play a role in biofilm formation: the *flrC* mutant exhibited a 2.3-fold decrease in glass attachment as compared with the wild-type strain, and exhibited diminished pellicle formation (Hussa *et al.*, 2008). Since biofilm formation was assessed under RscS-inducing conditions, these data suggest that motility and/or another *flrC*-controlled gene is necessary for *syp*-dependent biofilm formation.

Since motility is critical for *V. fischeri* to colonize *E. scolopes* (Graf *et al.*, 1994; Millikan and Ruby, 2002, 2003, 2004; Wolfe *et al.*, 2004), it is not surprising that the *flrC* mutant exhibited a severe colonization defect: juvenile squid inoculated with a 1:1 mixture of the *flrC* mutant and wild-type strains contained only the wild-type strain (Hussa *et al.*, 2007). Likely, this defect is due to the mutant's motility defect. However, because the *flrC* mutant exhibited additional phenotypes in the limited survey that was performed, it is possible that other FlrC-controlled traits could contribute to colonization proficiency. At this time, it is not possible to distinguish impacts on motility from the other effects of the *flrC* mutation.

Further studies of FlrC, as well as of its SK FlrB, will aid in our understanding of the role that motility, and its loss, play in symbiotic colonization. The signal sensed by FlrB to activate FlrC is unknown, although it is presumably present in normal laboratory conditions; potentially, this signal is absent in the deep crypts. It would be of interest to determine whether mutants that express a constitutively active version of FlrC (i.e. signal independent) would retain their flagella in the deep crypts. If not, then the control over flagellation likely occurs at a distinct level, such as at a higher level of transcriptional control (at FlrA or above) or, potentially, post-transcriptionally. Assessing the levels of flagellar transcripts from symbiotic cells would provide important insights into the level of control exerted over flagellation in symbiosis (Fig. 18.8). Analyses of how both bacteria and squid transcriptomes change during symbiosis are beginning to be performed, but the question of flagella control remains to be addressed (Jones and Nishiguchi, 2006; Wier *et al.*, 2010).

CheY

Chemotaxis is a random biased movement towards favourable substrates (e.g. nutrients) and away from unfavourable compounds (e.g. toxic molecules). In many bacteria, chemotaxis controls the direction of rotation of the flagella such that they alternate between counter-clockwise (CCW) and clockwise (CW) (Silversmith and Bourret, 1999). CCW rotation promotes swimming in a relatively linear path (i.e. 'smooth swimming'), while CW rotation generally disrupts the bundle of flagella such that they dissociate, causing the bacteria to tumble; tumbling permits re-orientation of the cell. In general, cells that are heading towards an attractant or away from a repellent suppress CW rotation and thus tumbling, and on average swim for extended amounts of time. As one might expect, the ability to switch the

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direction of flagellar rotation involves an intricate network of regulators. One such regulator, the RR CheY, appears to be critical in the ability to switch flagellar rotation from CCW to CW. Upon phosphorylation, CheY binds at the base of the flagellar motor to the 'switch' complex, increasing the likelihood that the flagella will switch from CCW to CW rotation (Bren and Eisenbach, 1998). In *E. coli*, *cheY* mutants exhibit a smooth swimming phenotype with relatively few 'tumbles', since the switch to CW rotation is absent (reviewed in Silversmith and Bourret, 1999). As a result, the cells are unable to re-orient to follow a gradient of attractant.

In *V. fischeri*, a *cheY* mutant also exhibits a 'smooth swimming' phenotype and is unable to migrate through soft agar (Hussa *et al.*, 2007). Furthermore, this mutant is defective during colonization: when juvenile squids were inoculated with a mixture of *cheY* mutant and wild-type strains, only the wild-type strain was recovered from the animals (Hussa *et al.*, 2007). Together, these data suggest that *V. fischeri* utilizes chemotaxis to promote migration into the light organ and/or to reach the deep crypt spaces (Fig. 18.2B and C). *V. fischeri* has been shown to swim towards *N*-acetylneuraminic acid (a component of the mucus) and nucleosides (DeLoney-Marino *et al.*, 2003), but whether either of these molecules promotes directed migration into the light organ crypts remains to be determined. The question of how *V. fischeri* finds its way into the light organ thus remains an exciting outstanding question to be explored.

Control of bioluminescence

The major product of the *V. fischeri*–squid symbiosis is bioluminescence, which the squid uses to avoid detection by predators (Jones and Nishiguchi, 2004). Indeed, the light organ is comprised tissues whose function it is to control light emission (McFall-Ngai and Montgomery, 1990; Montgomery and McFall-Ngai, 1992, 1993). For example, a reflector tissue directs the light downwards through a lens, which diffuses the light (Montgomery and McFall-Ngai, 1992; Crookes *et al.*, 2004). In addition, the wavelength of the light production is thought to be modified by passage through an accessory tissue to match that of the down-welling moonlight (McFall-Ngai and Montgomery, 1990). Furthermore, the light can be concealed by a muscle-controlled ink sac, a mechanism that permits the squid to adapt rapidly to prevent light emission when there's no moonlight, such as when the moon is obscured by clouds (McFall-Ngai and Montgomery, 1990). In addition to these mechanisms, the squid is thought to control light production by: (1) controlling the amount of oxygen delivered to the bacteria; luminescence production requires oxygen (Boettcher *et al.*, 1996); and (2) controlling the size and growth of the bacterial population through the daily expulsion of *V. fischeri* (reviewed in Stabb, 2006). Finally, in contrast to many bioluminescent bacteria, and even other strains of *V. fischeri*, the bacteria isolated from squid are non-visibly bioluminescent, although light production can be measured (Stabb *et al.*, 2008). In fact, the amount of light per cell (specific luminescence) is about 1000-fold higher in symbiosis than standard laboratory conditions (Boettcher and Ruby, 1990), suggesting that symbiotic conditions favour induction of bioluminescence. Given the role of luminescence and the evidence of the various levels of control over its production and emission, it is perhaps not surprising that the ability to bioluminese is important for *V. fischeri* to colonize its squid host: mutants defective for genes involved in the production and regulation of bioluminescence exhibit initiation and/or persistence defects during colonization (Stabb *et al.*, 2008). In this section, we describe the TCSs involved in bioluminescence production, and their roles in symbiosis.

Lux

Production of bioluminescence requires the *lux* operon, *luxCDABEG*. The *luxA* and *luxB* genes encode the two subunits of the enzyme luciferase, which utilizes long-chain aliphatic aldehyde, reduced flavomononucleotide ($FMNH_2$), and oxygen to produce light, aliphatic acid, oxidized flavomononucleotide (FMN) and water (Ziegler and Baldwin, 1981). The other *lux* genes encode an aliphatic acid reductase complex (LuxC, LuxD, and LuxE), involved in recycling aliphatic acid to aldehyde (Boylan *et al.*, 1989), and a protein that

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reduces FMN to $FMNH_2$ using NADH (LuxG) (Zenno and Saigo, 1994). These proteins together are sufficient to permit a non-bioluminescent organism, such as *E. coli*, to produce light (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984).

V. fischeri utilizes a complex network of regulators, including two-component regulators, to control light production in a cell density-dependent manner (i.e. quorum sensing): bioluminescence is repressed at low cell densities and induced at high cell densities. Cell densities are sensed through the use of autoinducers (AI), small molecules that are secreted from the cell and accumulate at high cell density. Specifically, two separate AI-based regulatory circuits control bioluminescence: the LuxR/LuxI (LuxR/I) circuit and the Lux phosphorelay (Fig. 18.9C).

LuxR is a transcription factor that becomes competent to activate transcription when bound to the AI *N*-3-oxo-hexanoyl homoserine lactone (3-O-C_6-HSL), synthesized by LuxI (Sitnikov *et al.*, 1995). During growth in culture, luminescence is initially low, as is the concentration of 3-O-C_6-HSL, but as the cell density increases, so does the concentration of this AI. This increased concentration of AI favours its interaction with LuxR, which becomes competent to induce *luxICDABEG* transcription, leading to the production of bioluminescence (Fig. 18.9C) (Engebrecht and Silverman, 1984; Meighen, 1991; Stevens *et al.*, 1994). As a result of this regulatory mechanism, *luxR* and *luxI* are required for luminescence: *luxR* and *luxI* mutants of visibly luminescent strains (e.g. the non-squid symbiont MJ1) are dark in culture (Dunlap and Kuo, 1992). In ES114, a non-visibly luminescent strain isolated from squid, *luxR* and *luxI* mutants exhibited only slight decreases in luminescence in culture (Visick *et al.*, 2000). However, during squid colonization, the *luxR* and *luxI* mutants of ES114 produced no detectable light at any time, indicating that these regulators are, in fact, critical for luminescence induction as predicted from the literature. Furthermore, the *luxR* and *luxI* mutants exhibited a colonization defect: they were unable to sustain the high colonization levels of the wild-type strain (Visick *et al.*, 2000). These data indicate that these regulators are necessary for *V. fischeri* to persist within the light organ. Similarly, mutants defective for the luciferase gene *luxA* (Visick *et al.*, 2000) or the entire *lux* operon (Bose *et al.*, 2008) exhibited a defect in persistence as well, suggesting that the colonization defects of *luxR* and *luxI* mutants can be attributed to a lack of bioluminescence. Taken together, these data indicate that both regulation of bioluminescence and bioluminescence itself are important for *V. fischeri* to colonize *E. scolopes*.

The second *lux* regulatory circuit in *V. fischeri* consists of the Lux phosphorelay, which also reports on cell density but does not rely on the AI produced by LuxI. The Lux phosphorelay is predicted to be similar to the well-studied pathway in *V. harveyi* (Ng and Bassler, 2009), which consists of three AI synthase proteins (LuxS, LuxM, and CqsA), which produce the signals detected by three hybrid SKs (LuxQ, in association with LuxP, LuxN, and CqsS, respectively), the HPt protein LuxU, and the RR LuxO (Fig. 18.9A,B). Briefly, at low cell densities when the AI concentrations are low, the hybrid SKs function as kinases, donating phosphoryl groups to LuxU, which serves as a phospho-donor to LuxO. Phosphorylated LuxO directs the transcription of five small RNAs (sRNAs) (*qrr1–5*), which bind to and destabilize the mRNA transcript of a transcription factor known as LuxR and which we will designate as $LuxR_{Vh}$; the *V. harveyi* protein is not homologous to LuxR from *V. fischeri*. $LuxR_{Vh}$ serves as the direct transcriptional activator of the *luxCDABEGH* operon. Thus, at low cell densities, little $LuxR_{Vh}$ is produced owing to transcript instability, and luminescence is not induced (Fig. 18.9A). As the cell density increases, AI signalling induces the hybrid SKs to switch from kinases to phosphatases, promoting the removal of the phosphoryl group from LuxO, through LuxU. Unphosphorylated LuxO is no longer active, leading to increased $LuxR_{Vh}$ and subsequent induction of luminescence (Fig. 18.9B).

The Lux phosphorelay in *V. fischeri* is similar to that of *V. harveyi*, and is predicted to function in largely the same way (Fig. 18.9C). *V. fischeri* contains homologues of LuxP, LuxQ, LuxU, LuxO, and LuxS, as well as LuxN and LuxM (called AinR and AinS, respectively). However, there are a few distinct differences between the Lux phosphorelays in *V. harveyi* and *V. fischeri*:

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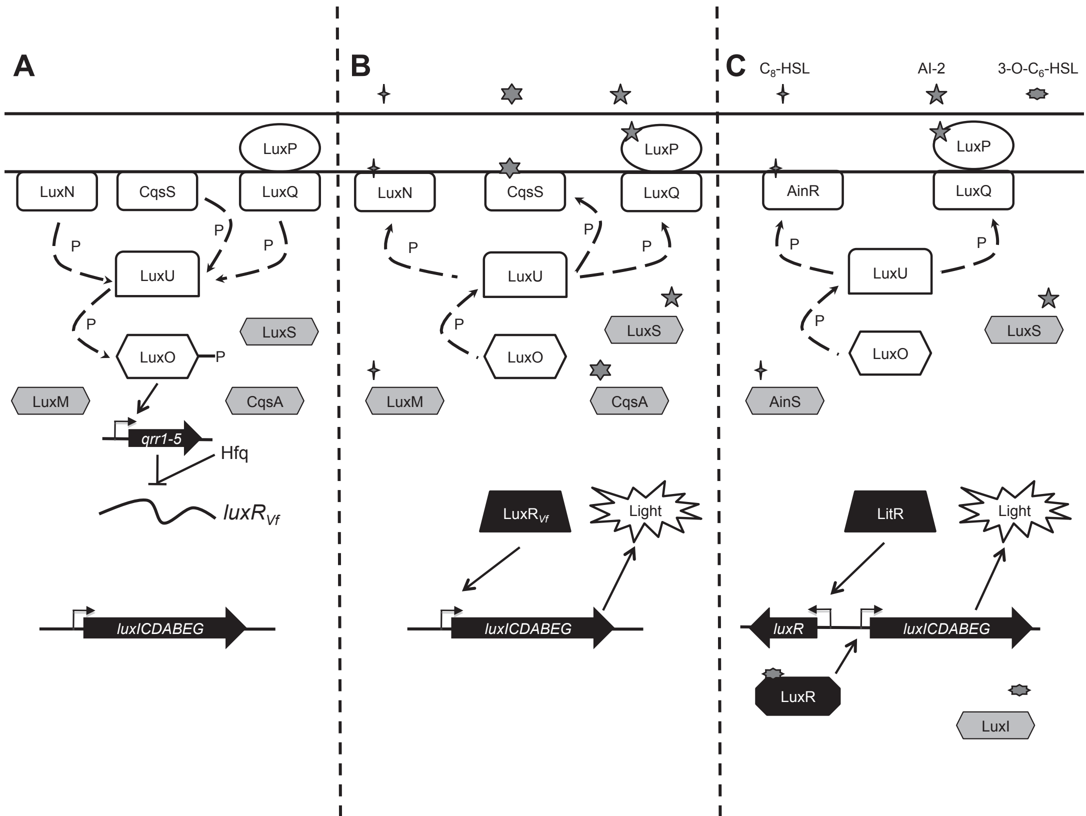

Figure 18.9 Models of bioluminescence regulation by Lux in *V. harveyi* and *V. fischeri*. (A) In *V. harveyi*, at low cell densities the AI concentration is low and the hybrid SKs (LuxN, CqsS, and LuxQ) act as kinases, serving as phospho-donors to the HPt protein LuxU, which serves as the phospho-donor to the RR LuxO. LuxO becomes active when phosphorylated and promotes the transcription of five sRNAs (*qrr1–5*), which, in conjunction with Hfq, bind to and destabilize the mRNA transcript of the transcriptional activator LuxR$_{Vh}$. Without LuxR$_{Vh}$, the *lux* operon is not transcribed and luminescence is not induced. (B) In *V. harveyi*, at high cell densities the AI concentration increases (represented by the diamond, sun, and star), and thus these molecules can interact with their respective hybrid SK and induce a conformational change that promotes phosphatase over kinase activity (in the case of LuxQ, AI-2 binds to LuxP, a periplasmic associated protein, which promotes the conformational change in LuxQ). As phosphatases, the hybrid SKs promote the removal of phosphoryl groups from LuxO, through LuxU. Unphosphorylated LuxO is no longer able to promote transcription of *qrr1–5*, in which case LuxR$_{Vh}$ is produced. LuxR$_{Vh}$ binds to the promoter region of the *lux* operon and promotes transcription, thus inducing luminescence. (C) In *V. fischeri*, Lux regulation at low cell densities and high cell densities is predicted to be similar to that of *V. harveyi*, except: (1) there is only one *qrr* sRNA, which, in conjunction with Hfq, binds to and destabilizes the mRNA transcript for the transcriptional activator protein LitR, which is similar to LuxR$_{Vh}$; (2) at high cell densities, LitR is produced and activates transcription of *luxR* in *V. fischeri*, which, when bound by its AI 3-O-C6-HSL promotes transcription of the *lux* operon in *V. fischeri*. *V. harveyi* does not contain this LuxR/I circuit, while *V. fischeri* does not contain CqsS or CqsA.

(1) LuxM and AinS do not produce the same AI (Cao and Meighen, 1989; Kuo *et al*., 1994); (2) *V. fischeri* does not encode CqsS or CqsA; (3) *V. harveyi* does not contain the LuxR/I circuit; and (4) LuxR$_{Vh}$ is homologous to LitR from *V. fischeri*, which is the transcriptional regulator for *luxR* from *V. fischeri*. Similar to *luxR*$_{Vh}$, *litR* is subject to LuxO-mediated regulation (Fig. 18.9C), which will be discussed further below.

At the current time, no studies have investigated the roles in symbiosis of the *V. fischeri* HPt protein LuxU or the hybrid SKs LuxQ and AinR,

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although one (Lyell *et al.*, 2010) examined the role of AinR in the control of bioluminescence in the squid symbiont. Furthermore, no genetic studies have verified that LuxQ and AinR work upstream of LuxU and LuxO in *V. fischeri*. Thus, for the remainder of this section, we will focus solely on the RR LuxO, the phenotypes it controls, and the input signals that control its activity. For a more comprehensive review of the Lux regulators in *V. fischeri* and their roles in bioluminescence and symbiosis, we recommend the review by Stabb *et al.* (2008).

LuxO-mediated regulation of bioluminescence

LuxO was initially identified as a regulator of bioluminescence in *V. harveyi* (Bassler *et al.*, 1994). LuxO homologues were subsequently identified in two *V. fischeri* strains: ES114, a squid symbiont, and MJ1, an organism that does not colonize squid, but in which luminescence has been extensively studied (Ruby and Nealson, 1976; Miyamoto *et al.*, 2000). In both strains, LuxO exerts negative control over bioluminescence: *V. fischeri luxO* mutants exhibited an increase in luminescence over the wild-type strains (Miyamoto *et al.*, 2000; Lupp *et al.*, 2003). These results are similar to what was observed in *V. harveyi*, and indicate that LuxO serves as an inhibitor of luminescence in both species.

The LuxO protein from MJ1 has been characterized in greater detail than that of ES114. The MJ1 protein shares 72% identity with LuxO from *V. harveyi*, and contains the conserved REC domain and putative site of phosphorylation, D47, in its N-terminus (Fig. 18.4A) (Miyamoto *et al.*, 2000). LuxO also contains a centrally located putative σ^{54}-interaction domain, with a predicted ATP-binding site, and a C-terminal putative DNA binding domain (Fig. 18.4A). These features are conserved in the ES114 protein, as the two *V. fischeri* proteins differ at only two residues (V/G114 and G/C177). Thus, like the *V. harveyi* protein, *V. fischeri* LuxO likely activates transcription at a σ^{54}-dependent promoter (Fig. 18.7) to control expression of genes involved in luminescence.

In support of that idea, Miyashiro *et al.* (2010) demonstrated that, as in other vibrios, LuxO promotes the transcription of an sRNA, designated *qrr1*. Unlike *V. harveyi*, which contains five *qrr* genes, and *V. cholerae*, which contains four *qrr* genes (Lenz *et al.*, 2004), *V. fischeri* contains only one *qrr1* gene (Miyashiro *et al.*, 2010). Based on the model generated in *V. harveyi* (Fig. 18.9A), Qrr1 from *V. fischeri* is predicted to bind to and destabilize the *litR* mRNA transcript, in conjunction with Hfq, resulting in little or no LitR protein expression. Thus, LitR would not be available to activate transcription of *luxR*, which is required to induce the *lux* operon and light production. The following data support this model: (1) in a *qrr1* mutant, *litR* transcript levels and luminescence are increased, similar to a *luxO* mutant (Miyamoto *et al.*, 2003; Miyashiro *et al.*, 2010), suggesting that Qrr1 negatively regulates luminescence through controlling *litR* transcript levels; (2) *qrr1* expression in wild-type cells was high at low cell density and decreased at high cell density (Miyashiro *et al.*, 2010), indicating quorum sensing control. Furthermore, *qrr1* expression remained low in the *luxO* mutant, indicating that LuxO activates *qrr1* expression at low cell density; (3) luminescence in a *luxO qrr1* double mutant was indistinguishable from either of the single mutants, indicating that these regulators function in the same pathway; (4) overexpression of *qrr1* in the *luxO qrr1* double mutant resulted in a decrease in *litR* transcript levels (Miyashiro *et al.*, 2010), suggesting that Qrr1 alone is sufficient to regulate *litR* mRNA transcript levels. Together, these data indicate that, like in *V. harveyi*, the *V. fischeri* LuxO protein is indirectly involved in regulating *litR* transcript levels through regulating the transcription of the sRNA Qrr1. Furthermore, these data demonstrate that LuxO connects the Lux phosphorelay to the LuxR/I circuit, since LitR is the direct transcriptional activator of *luxR* (Fig. 18.9C).

During squid colonization, a *luxO* mutant exhibits an initiation defect: when used to inoculate juvenile *E. scolopes*, the *luxO* mutant only reaches 37% of the colonization level of wild-type *V. fischeri* at 12 hours post-inoculation (Lupp and Ruby, 2005). This is an early time point, before the animals become fully colonized; thus, these data indicate that the *luxO* mutant has a defect in initiating colonization. However, at 72 hours post-inoculation, the colonization levels of the wild-type strain and the *luxO* mutant are

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comparable (Lupp and Ruby, 2005), suggesting that the *luxO* mutant is only defective at initiation, not persistence. This is perhaps not surprising, as at this late time point, luminescence is fully induced and LuxO should be inactivated. The *luxO* mutant is also defective for colonization when the wild-type strain is present: when juvenile squid were inoculated with a mixture of wild-type and *luxO* mutant strains, the *luxO* mutant exhibited a colonization disadvantage relative to the wild-type strain 24–48 hours post-inoculation (Lupp and Ruby, 2005; Hussa *et al.*, 2007; Miyashiro *et al.*, 2010). Whether the competition defect can be attributed to the initiation defect of the *luxO* mutant, or to the loss of control over a persistence factor not required during single strain colonization, remains to be determined.

What could account for the initiation defect of the *luxO* mutant? As mentioned above, bioluminescence is only required at later stages of colonization (i.e. persistence) (Visick *et al.*, 2000; Lupp and Ruby, 2005) and thus, light per se cannot account for the initiation defect of the *luxO* mutant. Array experiments revealed that mutations in *luxO* impacted expression of motility genes, and in support of this, *luxO* mutants exhibited decreased migration in motility assays (Lupp and Ruby, 2005; Hussa *et al.*, 2007). Previous studies have demonstrated that motility is essential for the initiation of colonization (e.g. Graf *et al.*, 1994), and thus the motility defect could be responsible for the initiation defect of the *luxO* mutant. Alternatively, other targets of LuxO activity identified in the array analysis, including regulatory proteins and genes involved in nutrient uptake and metabolism, could account for the initiation defect of the *luxO* mutant. Thus, the precise role of LuxO in initiation (Fig. 18.2C) remains unclear.

In conclusion, LuxO links the Lux phosphorelay to the LuxR/I circuit (Fig. 18.9C) by regulating transcription of a single sRNA, *qrr1*, that likely binds and destabilizes the *litR* mRNA transcript. Furthermore, LuxO is necessary for efficient colonization of the squid host, since a *luxO* mutant exhibits a defect in initiating colonization. Because its role in bioluminescence cannot account for the initiation defect, LuxO must control an additional gene(s) involved in this stage of colonization. Whether this additional gene(s) is controlled directly by LuxO or through downstream regulators (e.g. Qrr1 and/or LitR) remains unknown. Therefore, further studies of LuxO and LuxO-mediated activities are necessary to fully understand how LuxO impacts host colonization.

The autoinducer synthases – regulators of LuxO activity

In many two-component systems, the (hybrid) SK(s) have been well characterized, while the signal(s) sensed by these components are unknown or less well characterized. However, in *V. fischeri* there have been limited studies of the hybrid SKs (AinR and LuxQ), while multiple studies have examined the importance of the signal synthase proteins AinS and LuxS, which produce the AI signal molecules C_8-HSL and an uncharacterized molecule termed AI-2, respectively (Fig. 18.9C). In the following sections, we will discuss how AinS and LuxS regulate LuxO activity and thus luminescence, as well as what is known about how AinS and LuxS influence the symbiosis between *V. fischeri* and its squid host.

AinS

AinS synthesizes the AI, *N*-octanoyl-homoserine lactone (C_8-HSL), proposed to signal through the hybrid SK AinR (Fig. 18.9C) (Gilson *et al.*, 1995). C_8-HSL also appears to serve as a second AI that directly activates LuxR-mediated transcription of the *lux* operon (Kuo *et al.*, 1994, 1996), but we will not focus on this aspect of regulation here. AinS was initially identified and characterized as a luminescence regulator in MJ1 (Kuo *et al.*, 1994, 1996; Gilson *et al.*, 1995), although recent studies have focused on AinS from the squid symbiont ES114. Thus, we will focus our discussion on studies involving AinS from this squid symbiont.

In *V. harveyi*, LuxM (a homologue of AinS) synthesizes an AI sensed by the hybrid SK LuxN, which functions through LuxU to control the activities of LuxO (Fig. 18.9B) (Ng and Bassler, 2009). Thus, although the AinS produced signal is distinct from that produced by LuxM, it likely functions in a similar manner, signalling to the hybrid SK AinR to regulate LuxO activity in *V. fischeri* (Fig. 18.9C). Indeed, an *ainS* mutant of

ES114 produced no detectable light in culture (Lupp *et al*., 2003). Surprisingly, this mutant also exhibited a growth yield defect, reaching only 75% of the wild-type level (Lupp *et al*., 2003). This growth yield defect could be suppressed by the addition of exogenous C_8-HSL, indicating that the loss of the signal molecule was indeed responsible for the phenotype. Thus, it appears that, like LuxO, the AinS-produced signal regulates more than just luminescence. In support of this idea, an array analysis that compared transcripts from wild-type and *ainS* mutant cells revealed differences in the control of genes involved in metabolism and nutrient uptake (Lupp and Ruby, 2005). Subsequently, Studer *et al*. (2008) demonstrated that the *ainS* mutant was unable to utilize the acetate that is normally secreted and assimilated by *V. fischeri* cells. As a result, the mutant cultures became acidified, which caused the loss of cell viability. Thus, AinS activity appears to regulate multiple cellular functions, including luminescence and acetate utilization.

The *V. harveyi* paradigm predicts that the AinS-produced signal, C_8-HSL, should function to control LuxO activity. If this were true, then the phenotypes of the *ainS* mutant should depend on LuxO function. Indeed, this was the case: a double *ainS luxO* mutant exhibited luminescence and growth yield phenotypes indistinguishable from the *luxO* single mutant (i.e. high luminescence and normal growth) (Lupp *et al*., 2003). These results are consistent with the idea that AinS or, more specifically, C_8-HSL acts upstream of LuxO to control both luminescence and acetate utilization.

To understand how *ainS* itself was regulated, Lupp and Ruby (2004) evaluated levels of C_8-HSL and *ainS* transcription during growth of ES114. They found that over time the concentration of C_8-HSL increased over 2000-fold, concurrent with the increase in luminescence. Furthermore, *ainS* transcription was initially low, but increased exponentially until cells reached stationary phase, consistent with the increase in the amount of C_8-HSL produced during growth. These data suggested that *ainS* is autoregulated. The same pattern of *ainS* transcription occurred in *luxR* mutants, indicating that *ainS* was autoregulated independently of the LuxR/I circuit. However, autoregulation depended upon the Lux phosphorelay, as *ainS* transcription was decreased in an *ainS* mutant, but could be restored to wild-type levels by the addition of a mutation in *luxO* (Lupp and Ruby, 2004). Thus, the C_8-HSL signal not only controls luminescence and metabolism, but also its own production, in a manner that depends on LuxO.

Although the *ainS* mutant produced no light in culture (Lupp *et al*., 2003), similar to a *luxA* mutant (Visick *et al*., 2000), during colonization it produced detectable levels of light (between 10% and 40% of that observed for the wild-type strain) (Lupp *et al*., 2003). These data indicate that other factors contribute to luminescence control during symbiosis. This mutant also exhibited an initiation defect: at an early time (12 hours) following inoculation, the *ainS* mutant reached only 45% of the wild-type colonization level (Lupp and Ruby, 2005). This defect occurred after the aggregation stage, as this mutant was not defective in the timing of aggregate production or the size of the aggregate. Finally, the *ainS luxO* double mutant exhibited the same initiation defect as the *luxO* single mutant (37% and 36% colonization, respectively), which wasn't much different from the single *ainS* mutant (45% colonization). These results support the idea that AinS and LuxO function in the same pathway, and suggest that the initiation defect of the *ainS* mutant may be due to some LuxO-regulated process.

In addition to its initiation defect, the *ainS* mutant exhibited a persistence defect: at 24 hours post-inoculation, the levels of colonization by the mutant were 75% that of wild-type, and further decreased to 20% at 72 hours post-inoculation (Lupp and Ruby, 2004). However, this persistence defect was not due to the defect in acetate utilization: an *acs* mutant, which is similarly defective in acetate utilization, achieved the same level of colonization as the wild-type strain at 48 hours post-inoculation (Studer *et al*., 2008). Finally, disruption of *luxO* in the *ainS* mutant background restored colonization levels to that achieved by both the wild-type and single *luxO* mutant strains, indicating that the defect of the *ainS* mutant depends upon LuxO function (Lupp and Ruby, 2005).

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In summary, the AinS-produced signal (C_8-HSL) controls multiple phenotypes via its control over LuxO activity, and plays roles in symbiosis during at least two stages of colonization. Given the differences in the environments at these stages, it seems reasonable to expect that different C_8-HSL-controlled processes are important in initiation versus persistence. However, further studies of AinS are necessary to determine which genes under C_8-HSL control participate at these different times, and what other regulators might contribute to dictate the appropriate cellular response.

LuxS

In *V. harveyi*, LuxS produces AI-2, a furanosyl borate diester (Chen *et al.*, 2002). *V. fischeri* also encodes a LuxS protein, and it appears to produce a similar autoinducer molecule: supernatants from wild-type *V. fischeri*, but not a *luxS* mutant contained AI-2 activity, as measured by the ability to activate luminescence of a *V. harveyi luxS* mutant (Lupp and Ruby, 2004). Furthermore, AI-2 from *V. fischeri* appears to signal through LuxP/Q as it does in *V. harveyi* (Fig. 18.9): wild-type supernatant from *V. fischeri* was unable to induce luminescence in a *V. harveyi luxQ* mutant. To date, however, there have been no studies of LuxP or LuxQ in *V. fischeri*, nor confirmation of the structure of the autoinducer molecule.

In *V. fischeri*, a *luxS* mutant reaches about 70% of the luminescence achieved by the wild-type strain (Lupp and Ruby, 2004), suggesting that AI-2 contributes to luminescence regulation in *V. fischeri*, although not to the same extent as C_8-HSL. Consistent with its mild effect on luminescence, *luxS* transcription was low and unchanged throughout growth of the cells in culture, and AI-2 levels varied only 4-fold. These data indicate that *luxS* is constitutively expressed, rather than autoregulated like *ainS*. Finally, as predicted from the *V. harveyi* model, AI-2 from *V. fischeri* functions upstream of LuxO: the luminescence output of a double *luxS luxO* mutant resembled the *luxO* single mutant (i.e. increased luminescence) (Fig. 18.9B and C) (Lupp and Ruby, 2004). Together, these data suggest that LuxS plays a role, albeit a minor one, in controlling LuxO activity and thus luminescence regulation in culture.

Consistent with this relatively small role in luminescence regulation in culture, the *luxS* single mutant had no observable impact on either symbiotic bioluminescence or squid colonization (Lupp and Ruby, 2004). However, combining the *luxS* and *ainS* mutations resulted in a synergistic defect in colonization: the double mutant reached colonization levels that were 50–75% that of the *ainS* single mutant at both 24 and 48 hours post-inoculation (Lupp and Ruby, 2004). In contrast, this synergistic effect did not appear to occur during colonization initiation: at 12 hours post-inoculation, the *luxS ainS* mutant was not different from that of an *ainS* single mutant (both were approximately 45% that of wild-type), indicating that either AinS plays distinct roles during initiation and persistence, or that AI-2 (produced by LuxS) plays a stronger role during persistence than in initiation. In either case, LuxS and AinS appear to function in the same pathway to regulate luminescence and LuxO activity, since an *ainS luxS luxO* triple mutant did not exhibit a persistence defect, but resembled the *luxO* single mutant.

In summary, the Lux phosphorelay plays multiple roles during the symbiosis between *V. fischeri* and *E. scolopes* (Fig. 18.2B and C). The *V. harveyi lux* phosphorelay provides a good model for understanding luminescence regulation in *V. fischeri*, but it is clear that the pathways of these organisms differ in a number of ways, including the number of *qrr* genes and the existence of the LuxR/I circuitry in the symbiont (Fig. 18.9). Many questions remain. For example, because luminescence itself cannot account for the various symbiotic defects of regulatory *lux* mutants, what are the downstream targets that are responsible? Do the hybrid SKs LuxQ and AinR function similarly to their *V. harveyi* homologues, and if so, what is the strength of each input in controlling luminescence? Does the Lux pathway in *V. fischeri* regulate biofilm formation or other colonization factors as it does in *V. harveyi* and *V. cholerae* (Lilley and Bassler, 2000; Miller *et al.*, 2002; Zhu *et al.*, 2002; Vance *et al.*, 2003)? The answers to these questions and many others will aid in our understanding of how bacteria use quorum sensing during host interactions.

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ArcA

Lux is not the only pathway that controls bioluminescence in *V. fischeri*. The ArcA/ArcB TCS system is involved in repressing bioluminescence, as well as regulating a variety of other cellular functions. In *E. coli*, the ArcA/ArcB two-component system is thought to respond to the redox state of the cell (Gennis and Stewart, 1996; Georgellis *et al.*, 2001). Under reducing conditions, the hybrid SK ArcB initiates a phosphorelay, autophosphorylating on a conserved histidine residue and subsequently serving as a phospho-donor to activate the RR ArcA (Georgellis *et al.*, 1997). Phosphorylated ArcA then serves to either positively or negatively regulate transcription from a variety of genes (Lynch and Lin, 1996; Salmon *et al.*, 2005).

In *V. fischeri*, *arcB* encodes a hybrid SK that is 57% identical to ArcB from *E. coli* and contains the conserved PAS, HisKA, and REC domains, as well as conserved residues involved in redox sensing (C180 and C241) and phosphotransfer (H292, D576, and H717) in *E. coli* (Georgellis *et al.*, 1997; Malpica *et al.*, 2004). *arcA* encodes a RR that is 84% identical to ArcA from *E. coli* and contains the putative, conserved N-terminal REC domain, with the predicted, conserved site of phosphorylation (D54) and a C-terminal wHTH DNA binding domain (Fig. 18.4A) (Bose *et al.*, 2007). To determine whether the putative ArcA from *V. fischeri* had the same activity as the *E. coli* protein, Bose *et al.* (2007) introduced the *arcA* allele from *V. fischeri* into an *E. coli arcA* mutant; this *E. coli* mutant exhibits sensitivity to redox dyes, such as toluidine blue, due to changes in the electron flow in this mutant (Ruiz *et al.*, 2006). The *V. fischeri arcA* allele was able to restore toluidine blue resistance, demonstrating that ArcA from *V. fischeri* is functionally conserved (Bose *et al.*, 2007).

To further investigate how the role of ArcA in *V. fischeri* compares to *E. coli*, Bose *et al.* (2007) generated an *arcA* mutant and evaluated transcription of a putative ArcA-controlled gene. Specifically, control over the succinate dehydrogenase (*sdh*) gene, which is repressed by ArcA in *E. coli* (Ravcheev *et al.*, 2007), was assessed using a reporter construct with the *sdh* promoter fused to *lacZ*. Transcription from this reporter was increased in the *V. fischeri arcA* mutant (Bose *et al.*, 2007). Thus, it appears that *V. fischeri* ArcA functions like the *E. coli* protein.

The *V. fischeri arcA* mutant exhibited additional phenotypes. For example, it exhibited a slightly slower growth rate (Bose *et al.*, 2007, Hussa *et al.*, 2007) and a decrease in motility (Hussa *et al.*, 2007). This mutant also exhibited a substantial (approximately 500-fold) increase in bioluminescence (Bose *et al.*, 2007). This increased luminescence could be restored to wild-type levels when the mutant was complemented with the wild-type *arcA* allele, but not with an *arcA* allele in which the putative, conserved site of phosphorylation (D54) was changed to a glutamate (Bose *et al.*, 2007); in other RRs, this mutation results in a protein that is active but not phosphorylated (see, for example, Sanders *et al.*, 1989, 1992; Freeman and Bassler, 1999). These results suggest that ArcA represses luminescence, and requires the putative, conserved site of phosphorylation to do so.

One mechanism by which ArcA could control luminescence is to serve as a direct repressor by binding at the *lux* operon. In support of this hypothesis, the intergenic region between *luxR* and *luxI* contains a putative ArcA binding site that matches the core consensus sequence (5′-ATGTTAA-3′) recognized by *E. coli* ArcA (Liu and De Wulf, 2004; Bose *et al.*, 2007). More importantly, purified, phosphorylated ArcA from *E. coli* was able to bind to this promoter region in *in vitro* experiments (Bose *et al.*, 2007). Thus, ArcA serves as a direct repressor of luminescence in *V. fischeri*.

Given the known function of ArcA/ArcB in *E. coli*, it seemed probable that this system responds to the redox state of the cell in *V. fischeri* as well. Previous studies had demonstrated that activation of ArcA/ArcB from *E. coli* could be achieved by the addition of the reducing agent dithiothreitol (DTT) (Malpica *et al.*, 2004); thus, the same could be true for ArcA/ArcB from *V. fischeri*. Indeed, the addition of DTT to wild-type *V. fischeri* led to a 22-fold reduction in luminescence, but had no effect on light production by the *arcA* mutant (Bose *et al.*, 2007). Thus, the ArcA/ArcB two-component system in *V. fischeri* appears to function as a redox-responsive system, similar to the homologous system in *E. coli*.

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Since ArcA affects luminescence and certain metabolic processes in *V. fischeri*, it seemed likely that ArcA might also play a role in symbiosis. Indeed, during mixed inoculation experiments using the *arcA* mutant and the wild-type strain, the wild-type strain outcompeted the mutant by 4-fold at 48 hours post-inoculation (Bose *et al.*, 2007), although this defect was not observed at 24 hours post-inoculation (Hussa *et al.*, 2007). These results suggest that ArcA may be important later in host colonization (Fig. 18.2C); however, it is also possible that a 4-fold effect would not have been identified in the screen by Hussa *et al.* (2008). Surprisingly, the *arcA* mutant did not exhibit a colonization defect in single strain assays: there was no difference in the start of luminescence induction or number of bacteria per squid as compared with the wild-type strain (Bose *et al.*, 2007). These data suggest that ArcA might be inactivated during colonization (Fig. 18.2C). In support of this idea is the fact that the wild-type strain exhibits low luminescence in culture, but increased luminescence in the squid, while the *arcA* mutant achieves greater levels of luminescence than the wild-type strain in culture but equal levels in the squid (Bose *et al.*, 2007). Thus, inactivation of ArcA may be important during colonization, although it would be necessary to examine the impact of constitutively active alleles of *arcA* and/or *arcB* during colonization to support this idea.

In summary, ArcA is a RR that exerts a strong impact on luminescence control in *V. fischeri*. It is likely that ArcA-mediated repression of luminescence in culture can account for the observation that the squid symbiont ES114 exhibits a 1000-fold decrease in specific luminescence in culture relative to that achieved during symbiosis (Boettcher and Ruby, 1990). It will be interesting to understand what other functions, if any, are controlled by ArcA that may contribute to colonization.

Global regulation and host development

During symbiotic colonization, *V. fischeri* promotes a series of morphogenic changes in the squid light organ (Fig. 18.10). For example, mucus secretion, which facilitates aggregate formation on the surface of the light organ, ceases in animals colonized by *V. fischeri* (Fig. 18.10B and C) (Nyholm *et al.*, 2002). In addition, the epithelial cells within the appendages on the surface of the light organ (Fig. 18.1B <Should this be Fig. 18.10 rather than 18.1? >) undergo apoptosis (Foster and McFall-Ngai, 1998). Subsequently, the appendages undergo regression and are lost over a 4-day period (Fig. 18.10) (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995). These changes are a normal part of light organ maturation and considered hallmarks of successful colonization; likely, most if not all of these changes contribute to the specificity of the association and prevent subsequent 'super-infection' by additional *V. fischeri* cells. These

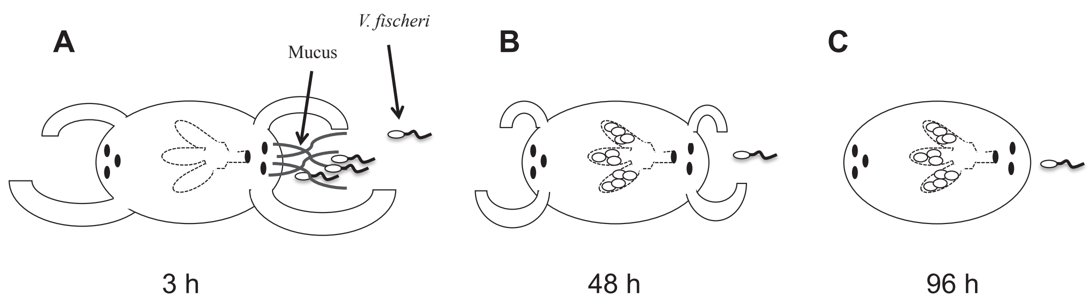

Figure 18.10 Host development induced by *V. fischeri*. (A) Shortly after hatching, *E. scolopes* juveniles secrete mucus, which facilitates aggregation by *V. fischeri*, from ciliated epithelial appendages present on the surface of the light organ. (B) Within 48 h, colonizing *V. fischeri* cells have signalled for mucus secretion to cease, and for the apoptosis and regression of the surface appendages. (C) Within 96 h, the appendages have fully regressed. The combinations of these developmental events restricts subsequent colonization by additional *V. fischeri* cells.

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developmental events are triggered by bacterial cell wall components released from colonizing bacteria, in particular LPS and a component of peptidoglycan (Foster *et al.*, 2000; Koropatnick *et al.*, 2004). Although these signals are known, much remains to be learned about the bacterial factors controlling host development. In this section, we focus on one RR, GacA, that appears to play a role in a variety of host developmental events.

GacA

The GacS/GacA two-component system (BarA/UvrY in *E. coli*) is used by a variety of bacteria to mediate bacteria–host interactions such as colonization, production of host- and tissue-specific effectors, and adaptation to the host environment (Heeb and Haas, 2001). This system has been well studied in bacteria involved in pathogenic associations (e.g. *Salmonella* spp., *V. cholerae*, and *Erwinia carotovora*), as well as in bacteria involved in benign (mutualistic) associations (e.g. *Pseudomonas* spp.–plant interactions) (Heeb and Haas, 2001). In *V. cholerae*, for example, the RR GacA, termed VarA, is involved in mediating pathogenesis (Wong *et al.*, 1998). The *V. fischeri* GacA protein is 85% identical to VarA, and, like other GacA proteins, contains a putative N-terminal REC domain with the predicted, conserved site of phosphorylation (D54) and a putative C-terminal helix–turn–helix DNA binding domain (Fig. 18.4A) (Whistler and Ruby, 2003).

The construction and analysis of a *gacA* mutant of *V. fischeri* revealed a significant role for this regulator in controlling a variety of cellular functions as well as symbiosis. For example, the *gacA* mutant exhibited altered colony morphology (smaller, less yellow, and translucent) when grown on nutrient agar and achieved a lower growth yield when cultured in a variety of complex liquid media (Whistler and Ruby, 2003). Furthermore, the *gacA* mutant was unable to grow on minimal media containing simple sugars as carbon sources (Whistler and Ruby, 2003). These results suggest that GacA regulates processes involved in growth-substrate utilization (Fig. 18.2B). The *gacA* mutant also exhibited decreased siderophore activity, and altered motility and chemotaxis (i.e. appeared to deplete serine more rapidly from the media than the wild-type strain) (Whistler and Ruby, 2003).

GacA also appears to regulate luminescence: the mutant produced no detectable light in culture, although this defect does not appear to be due to changes in the production or concentration of the AI signals (Whistler and Ruby, 2003). These data suggest that GacA may regulate luminescence via another mechanism, although this mechanism has yet to be determined. One possible explanation for how GacA regulates luminescence in *V. fischeri* comes from data in *V. cholerae*. VarA (the GacA homologue) controls the production of sRNAs that inhibit CsrA, a protein involved in post-transcriptional regulation of processes as diverse as biofilm formation and carbon metabolism (Romeo *et al.*, 1993; Romeo, 1998). CsrA also controls luminescence, apparently by affecting the activity of the RR LuxO (Lenz *et al.*, 2005). Thus, a similar mechanism may occur in *V. fischeri*, which contains a homologue of CsrA, as well as the inhibitor sRNAs (Kulkarni *et al.*, 2006).

During colonization, the *gacA* mutant exhibited an initiation defect: the mutant colonized only 51% of squid, and required longer incubation times (14–18 hours) and a higher inoculum to colonize 100% of squid (Whistler and Ruby, 2003). This defect was likely due, at least in part, to the inability of the mutant to aggregate outside of the light organ (Whistler *et al.*, 2007). Furthermore, animals that became colonized by the *gacA* mutant contained much lower bacterial loads than wild-type-colonized animals (approximately 1.5×10^3 vs. 1.2×10^5 bacteria per squid, respectively); thus, GacA appears critical for the accommodation stage as well (Fig. 18.2B and C) (Whistler and Ruby, 2003). Finally, GacA does not appear to be necessary for persistence, despite playing a role in regulating cellular functions important for persistence, such as luminescence and siderophore production (Graf and Ruby, 2000; Visick *et al.*, 2000): squid colonized by the *gacA* mutant maintained consistent (low) colonization levels up to 48 hours (Whistler and Ruby, 2003). Thus, GacA is an important regulator for both initiating symbiosis and achieving normal population densities within the light organ.

During the initial colonization experiments, an intriguing observation was made: some squid

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colonized by the *gacA* mutant were luminescent, while others were not (Whistler and Ruby, 2003). Because mutation of *gacA* impacted luminescence in culture, this observation warranted further investigation. Whistler and Ruby (2003) determined that (1) the luminescent squid contained higher population densities than non-luminescent squid, and (2) bacterial cells collected from *gacA*- and wild-type-colonized squid exhibited no difference in specific luminescence per cell (Whistler and Ruby, 2003). Together, these data indicated that as long as sufficient cell densities were reached, the cell density-dependent induction of luminescence could occur, permitting the squid to become luminescent. Thus, in contrast to what was observed in culture, GacA does not appear to be necessary for regulation of luminescence within the host.

Because the *gacA* mutant exhibited severe defects in colonization, Whistler and Ruby (2007) wondered whether the *gacA* mutant was defective in promoting morphogenesis of the light organ. Indeed, this mutant was unable to induce normal levels of apoptosis (Whistler *et al.*, 2007). This phenotype could be due to differences in the LPS profile of the *gacA* mutant: the LPS profile of the mutant, while resembling that of the wild-type strain, also contained lower molecular weight species not produced by the wild-type strain (Whistler *et al.*, 2007). Whether the defect in apoptosis is a result of the altered LPS profile of the mutant (and thus, potentially, the production of the wrong type of LPS, which could alter recognition between the bacterium and the host) or the decreased production of LPS (due to the lower colonization levels of the *gacA* mutant) or another explanation remains to be determined.

Squid colonized with the *gacA* mutant not only failed to undergo apoptosis but also continued to secrete mucus and permit bacterial aggregation at the light organ pores 96 hours post hatching, long after wild-type-colonized animals ceased mucus secretion (Whistler *et al.*, 2007). These data suggested that a *gacA*-mutant-colonized host might be susceptible to a secondary colonizer. Indeed, animals colonized with the *gacA* mutant were displaced from the light organ upon inoculation with the wild-type strain 60 hours after hatching and primary colonization (Whistler *et al.*, 2007). These results confirmed previous studies that suggested that cessation of mucus shedding limits subsequent colonization by other bacteria once a productive association is established (Fig. 18.10B and C) (Nyholm *et al.*, 2002). Furthermore, they suggested the possibility that the host can recognize defective symbionts, and respond by promoting colonization by other potential partners.

In conclusion, GacA appears to be a global regulator of a variety of processes in *V. fischeri*. Although it is difficult to pinpoint the cause of the numerous symbiotic defects that occur upon the loss of *gacA*, these studies have none-the-less increased our understanding of the role the bacteria play in influencing host development. In particular, the *gacA* studies have provided insights into how colonized animals limit subsequent colonization by additional *V. fischeri* bacteria. The targeted disruption of members of the GacA regulon in the future will permit a dissection of the various phenotypes impacted by GacA and their importance in symbiosis.

Concluding remarks

V. fischeri encodes numerous TCS regulators, and a significant subset of them play key roles in the ability of this organism to form a productive association with its symbiotic partner, the squid *E. scolopes*. *V. fischeri* clearly experiences different environments as it transitions from seawater to the site of colonization (Figs. 18.1 and 18.2), and likely it detects different signals in each location. For the regulators involved in quorum sensing, the signals are known and are self-produced, but depend on appropriate conditions for their production (i.e. growth to high cell density). For some other TCS regulators, such as ArcA/ArcB, the signal (redox state) is suspected but as yet unverified. Finally, for a number of other regulators, such as RscS and Syp, the signals recognized are as-yet unknown. Thus, an important area of investigation lies in understanding the specific environments experienced at the stage of colonization that requires the activity of a particular TCS system.

Detailed studies of TCS regulators have revealed their involvement at each of the known

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stages of symbiosis: initiation, accommodation, and persistence (Fig. 18.2). For example, RscS, SypG, and SypE are involved in biofilm formation and aggregation at the initiation stage, and FlrC and CheY are necessary for motility (and likely chemotaxis) somewhere between the initiation and accommodation stages (Fig 18.2B and C). In contrast, LuxO is important at both the initiation and persistence stages, likely regulating non-luminescence processes during the initiation stage and luminescence and/or other factors during persistence (Fig. 18.2B and C). A better understanding of the regulons of each regulator, both in the presence and absence of its signal (if known), will provide testable hypotheses for determining which downstream targets are critical at specific stages of symbiosis.

Of the many TCS regulators encoded by *V. fischeri*, the most important subclass for symbiosis appears to be the σ^{54}-dependent RRs. Only six of the 40 RRs are in this class. However, four of these, LuxO, SypG, FlrC, and VpsR, have been shown to play important roles in symbiosis. NtrC (VF_0095), an additional member of that class, also appears to play a role in symbiosis, based on competition assays reported in the global survey of RRs (Hussa *et al.*, 2007); this regulator has yet to be characterized further. Given the importance of these regulators in symbiosis, it is perhaps not surprising that an *rpoN* mutant, defective for production of σ^{54}, is also defective for symbiotic colonization (Wolfe *et al.*, 2004). This type of regulation lends itself to tighter control: RNA polymerase carrying σ^{54} is unable to promote transcription in the absence of an activator protein, which provides the energy (via ATPase activity) necessary for transcription initiation (i.e. open complex formation) (Fig. 18.7); the result is a decrease in basal levels of transcription (Buck *et al.*, 2000). Additionally, these activator proteins typically require a signal to promote ATPase activity: in the absence of a signal (e.g. phosphorylation in the case of a RR) ATPase activity is off, while the presence of a signal would promote ATPase activity. Given this information, one could consider σ^{54}-dependent promoters either 'on' or 'off', depending on the presence of environmental conditions and signals that dictate whether the regulatory proteins are activated. Thus, the use of so many σ^{54}-dependent RRs during symbiotic colonization may be an important mechanism by which certain processes are turned 'on' or 'off' at various times during colonization.

One observation made from this review is that inactivation of certain TCS regulators may be a common mechanism during symbiosis. For example, at least three regulators (SypE, LuxO, and ArcA) appear to be inactivated during colonization (Fig. 18.2C). This observation begs the question, how many regulators must become inactivated to promote symbiosis? The traditional approach of using gene disruptions to evaluate function may not be sufficient to determine whether a given TCS component plays a role in symbiosis, as disruption is equivalent to inactivation. For example, deletion of the RR *sypE* did not lead to noticeable defects in colonization, but the generation of a constitutively active (constitutively inhibitory) mutant led to a severe colonization defect (Morris *et al.*, 2011). Thus, it may become necessary to make point mutations in conserved residues to evaluate the roles of both activation and inactivation of a given regulator to fully test the necessity of a particular regulator in symbiotic colonization. Such point mutation studies, while difficult to accomplish, are becoming more feasible with the availability of the genome sequence and new approaches in mutant construction. Undoubtedly, such studies would provide invaluable insights into the regulatory mechanisms in place during colonization. The foundation is laid with a wealth of knowledge regarding traits and regulators critical to this symbiotic association. Future work should begin to fill in the details and provide a better understanding, in general, of bacteria–host interactions.

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