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A lasting symbiosis: how *Vibrio fischeri* finds a squid partner and persists within its natural host

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Abstract

As our understanding of the human microbiome progresses, so does the need for natural experimental animal models that promote a mechanistic understanding of beneficial microorganism–host interactions. Years of research into the exclusive symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri* have permitted a detailed understanding of those bacterial genes underlying signal exchange and rhythmic activities that result in a persistent, beneficial association. as well as glimpses into the evolution of symbiotic competence. Migrating from the ambient seawater to regions deep inside the light-emitting organ of the squid, *V. fischeri* experiences, recognizes and adjusts to the changing environmental conditions. Here, we review key advances over the last 15 years that are deepening our understanding of these events.

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In this Review, Visick, Stabb and Ruby describe recent advances in understanding the squid–vibrio symbiosis from the symbiont's perspective.

[H1] Introduction

Since humans first realized they were affected by the presence of microscopic organisms within their bodies, most of our focus has been directed on those few microbial species that cause disease – who they are, what their impact is and how to eliminate them. Little

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The authors contributed equally to all aspects of the article.

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interest was shown in those bacteria found associated with a healthy body, and which were considered only ‘commensals’, an ecological term the meaning of which specifically connotes their lack of importance to the host. This viewpoint now seems as naïve as a belief in spontaneous generation. The importance of microorganisms to their hosts was well illustrated by the difficulties that pioneering scientists had to overcome to establish the first viable germ-free animals. Moreover, we now recognize that, when compared to pathogens, there are a much greater number and complexity of beneficial microorganisms associated with our bodies, as is the case for virtually all animals and plants. This rapidly expanding realization of the critical importance of the normal microbiota to organismal health has increased our desire to understand the mechanisms underlying the interactions that create and sustain microbial symbioses with eukaryotic partners. In response to such an intellectual frontier, scientists have always turned to model systems in which they could more easily address fundamental questions the answers to which can be broadly applied to many other organisms, including humans. Such ‘simple’ model organisms as *Escherichia coli, Caenorhabditis elegans*, *Drosophila melanogaster*, and others, were made ‘famous’ for their basic, and critical, contributions to the fields of biochemistry, genetics and development. Now, as the revolution in microbiome research expands, established models of symbiosis are leading the way, and new models are emerging and being developed[1].

One such well-studied animal–bacterial symbiosis is the light-organ association between the squid *Euprymna scolopes* and its bioluminescent bacterial partner, *Vibrio (Aliivibrio) fischeri*. This highly species-specific symbiosis begins when a newly hatched juvenile squid emerges into seawater containing planktonic cells of *V. fischeri*[2]. These cells aggregate on the surface of the juvenile’s nascent light organ (Fig. 1) and specifically induce host activities, including those that release a chemoattractant from the pores on the surface[3]. Within 1-3 hours[4], the bacteria detach from the aggregate, migrating through surface pores, down a duct, across an antechamber and into the epithelium-lined crypts[5] where a few founding cells will grow into a population of several hundred thousand. This dense population induces a visible bioluminescence, which the adult squid uses to avoid predation during their nocturnal activity[6]. Each morning at dawn, the host expels almost all of the symbiont population into the surrounding seawater[2,7]; the remaining bacteria repopulate the crypts and are ready to produce light by nightfall. Meanwhile, the vented bacteria cycle through the environment, perhaps in other as yet unknown niches, until they or their progeny colonize the light organs of other *E. scolopes* hatchlings.

The diurnal rhythmic venting activity of the squid continues as the host develops into maturity, and until its death, which in laboratory aquaria occurs after about 9 months. Throughout the initiation of, accommodation to and persistence in the symbiosis, the species specificity and activity of the bacterial population must be maintained, and the development and immunological response of the host needs to be regulated[8-10]. To achieve these intricate, intertwined goals, the two partner species have evolved a complex system of physiological and biochemical communication, drawing upon and modifying pre-existing adaptations and capabilities such as chitin-driven fermentation, bacterial chemotaxis to specific nutrients, and the host hemocyanin’s Bohr effect (see companion article by Nyholm and McFall-Ngai [11]), to perform new roles[3,13,14]. Identifying the molecular language of this communication has revealed not only adaptations unique to this association (for example, luminescence),

but also modes of signaling (for example, microbe-associated molecular patterns (MAMPs), circadian rhythms, and non-coding RNA)[9,15,16] that have later been, or are likely to be, found to occur between mammals and their normal microbiota[17,18].

Over a decade ago, a previous contribution to *Nature Reviews Microbiology*[19] focused on the impact that the application of bacterial genetic approaches was having on our understanding of the squid–vibrio association as well as several other emerging models of host–bacteria mutualism. In this Review, we update this analysis with new findings and conclusions about the squid–vibrio system from the symbiont's perspective. This contribution is preceded by several excellent reviews of the association (see, for example, Refs. [19-23]), and accompanies another contribution (see Nyholm & McFall-Ngai[11]) that describes recent advances in understanding the squid–vibrio symbiosis from the host's perspective.

[H1] Events at the light organ surface

Initial engagement between the symbiont and its host occurs when ambient seawater containing *V. fischeri* cells is drawn into the mantle cavity of a juvenile squid (Fig. 1), where the bacteria come into contact with host-derived mucus covering the surface of the light organ. There, the activities of two types of cilia ('long' and 'short') on the nascent organ combine to direct the flow of bacteria-sized particles in the seawater into stagnant zones above pores in the light organ surface[24] (see Nyholm & McFall-Ngai[11]). Within this zone, the bacteria attach to the cilia[25] and form a cohesive cluster known as an aggregate, or symbiotic biofilm[26], a critical colonization behavior (for example, Ref. [27]). The aggregates can be from 10s to 1000s of bacteria in number, depending on the strain of *V. fischeri* and its ambient concentration[28]. Intensive study[29,30] has shown that this biofilm, and efficient colonization, depends on an exopolysaccharide (Syp-PS) that is synthesized and exported by proteins encoded in the 18-gene *syp* locus[31,32]. Although most biofilm studies have focused on Syp-PS, *V. fischeri* produces at least one other polysaccharide, cellulose, that contributes to *in vitro* biofilm formation; however, its influence on the symbiosis is unclear[33–36]. In addition, other secreted components may contribute to the biofilm matrix, including Bmp proteins[37] and outer membrane vesicles (OMVs)[38].

Syp-PS production is controlled at the level of transcription by a network of two-component regulators (sensor kinases and response regulators). Sensor kinase proteins sense and relay (via phosphorylation) information about the environment to response regulator proteins that then control bacterial behavior. Because the signal or signals that modulate biofilm formation are generally unknown, for experimental studies biofilm formation is typically induced by overexpression of positive regulators or disruption of negative regulators. For example, overexpression of the sensor kinase RscS is sufficient to induce *V. fischeri* biofilm formation, contingent on the response regulator SypG, the direct activator of *syp* transcription (Fig. 2)[27,39-42]. RscS also depends on the presence of the sensor kinase SypF[43]. This regulatory scheme is atypical because sensor kinases generally signal directly to a downstream response regulator. Subsequently, it was determined that another sensor kinase, HahK, similarly functions through SypF[35]. Thus, SypF has an unusual role in integrating

sensory information gleaned by itself and other sensor kinases to control SypG and, thus, *syp* transcription and host colonization[35,43,44] (Fig. 2).

Syp-dependent biofilm formation and symbiotic colonization are inhibited by the response regulator BinK[45,46]. Loss of *binK* alone is sufficient to promote *syp* transcription and *syp*-dependent biofilm formation *in vitro*, but only when concentrations of Ca^{2+} typical in seawater are present[35]. This combination of factors (lack of BinK and addition of Ca^{2+}) permitted a more natural investigation of the regulation of biofilm formation (without artificial overexpression) and, indeed, facilitated the identification of HahK as a key biofilm regulator[35]. How BinK and Ca^{2+} control *syp* transcription remains unknown. Syp-PS production is also controlled post-transcriptionally, via the response regulator SypE. This response regulator lacks a DNA-binding domain, instead controlling the activity of its target, SypA, via serine phosphorylation/dephosphorylation (Fig. 2)[47,48]. Unphosphorylated SypA promotes biofilm formation, while phosphorylated SypA is inactive and unable to promote biofilm formation or colonization[48]. The chaperone protein DnaJ may also function in this pathway: while expressing normal levels of *syp* transcription, a *dnaJ* mutant fails to produce Syp-PS or biofilms[49]. Intriguingly, *syp* is conserved in most *Vibrio* species[32], and *sypA* from the pathogens *Vibrio vulnificus* and *Vibrio parahaemolyticus* could partially complement a *V. fischeri sypA* mutant[50]. Thus, the mechanisms uncovered in the squid symbiosis may have application to understanding biofilm formation and infection by human pathogens as well.

Historically, *V. fischeri* strain ES114 has been the principal strain used to study the squid–vibrio symbiosis, but new insights are being gleaned with additional isolates (Box 1). For example, the positive regulator *rscS*, which is required for colonization by ES114[51], is either absent or frame-shifted in some colonization-competent isolates, indicating that these strains likely have evolved other mechanisms to induce *syp* transcription and/or control post-transcriptional processes[52]. Notably, some isolates, termed 'dominant', readily out-compete ES114 for colonization[53]. For at least one, MB13B2, dominance seems to coincide with hyper-production of Syp-PS, as this strain produces substantially larger *syp*-dependent aggregates, and does so faster than ES114[28]. MB13B2 contains a frame-shifted *rscS* allele[52] and, thus, understanding how *syp* regulation is altered in different strains of *V. fischeri* will be key to determining the diversity of mechanisms by which colonization competence is achieved.

Despite hyper-aggregation exhibited by both MB13B2 (Ref. [28]) and *rscS*-overproducing ES114[27], these (and wild-type) strains ultimately disperse from their symbiotic biofilms to migrate into the light organ. Mechanisms of dispersal remain poorly understood, because it is rare to visualize this transient behavior in squid, it has not been observed in laboratory culture, and it is hard to differentiate mechanisms that promote biofilm formation from those that decrease dispersal, and vice versa. However, recent work has provided the first mechanistic insight by identifying a homolog of the *Pseudomonas* LapG protease[54] as responsible for cleaving a large adhesin from the *V. fischeri* cell surface, permitting dispersal[55]. The activity of LapG is controlled by the inner-membrane protein LapD. Loss of LapD results in cells with an increased ability to disperse, a phenomenon that seems to delay symbiotic initiation. Because the lack of LapD does not prevent colonization, it is

clear that other dispersal mechanisms must be in place. Additional insights may be gained from conditions or mutations that alter aggregate size (for example, Ref.[56]). For instance, it is possible that dispersal is facilitated by squid-produced nitric oxide (NO) found on the light organ surface[56,57]. Consistent with this possibility, addition of a NO synthesis inhibitor causes the formation of larger aggregates[57] and could restore aggregation to a mutant deficient for Hmp, which eliminates NO[58]. NO is recognized and bound by HnoX, which in turn inhibits *syp* activator HahK. This results in the down-regulation of Syp-PS production (Fig. 2), thereby potentially facilitating dispersal[59-60]. These observations suggest that other factors[49] or biosynthetic capabilities[61] are necessary to orchestrate either the production of or a timely exit from the symbiotic aggregate.

[H1] Beyond the light organ surface

[H2] Chemotaxis and motility.

Many horizontally transmitted symbionts are believed to use chemotaxis to find target tissues within their respective hosts[62]. For instance, the migration of *V. fischeri* cells from the aggregate to their ultimate destination in the deep crypts depends on bacterial motility and chemotaxis (for example,[26,63-67]). *V. fischeri* produces a tuft of sheathed flagella at one pole of the cell, and magnesium in the environment enhances that production[68,69]. Intriguingly, these flagella are largely lost from symbionts in the crypts, indicating that internal conditions signal the formation of non-motile cells[69]. A large-scale, unbiased search for factors important for motility yielded both genes previously established as required for flagella biosynthesis and a number of novel genes[63]. Of note, this work identified VF_1491, which seems to function in chemotaxis, and flagella structural components FlgO, FlgP, and FlgT. These latter proteins are now known to contribute to the scaffolding structure that permits the flagellar motor to produce the high torque necessary to propel migration through high-viscosity environments such as mucus[22,70]. Confirming previous smaller-scale studies, non-motile mutants were unable to colonize, likely because they could not reach the crypts, whereas mutants with diminished motility exhibited decreased efficiency of colonization[63]. In addition, mutants defective for chemotaxis regulators CheA and CheZ only colonized ~50% of animals when presented at a dose that enabled full colonization by the wild type, supporting earlier studies (for example,[71]) that suggested a role for chemotaxis in effective colonization.

Recent work describing the cilia-driven flow of fluids around the light organ surface revealed that these dynamics could cause an accelerated formation of chemical gradients that may permit *V. fischeri* to chemotax into the light organ[24]. However, defining chemotactic mechanisms in *V. fischeri* is particularly challenging because this organism encodes ~43 chemoreceptors[63,72]. Initial investigations into the roles of 19 of these chemoreceptors revealed the function of only one, VfcA, which recognizes serine as well as certain other amino acids[63]. Subsequently, VfcB and VfcB2 were shown to recognize short-chain fatty acids such as propionate and butyrate[73]. However, none of these chemoreceptors seem to be important during the early stages of symbiosis. By contrast, *V. fischeri* also chemotaxes toward *N*-acetylneuraminic acid and chitin oligosaccharides, two squid-produced molecules that could promote the recruitment of *V. fischeri*[71,74] (also, see Nyholm & McFall-

Ngai[11]). Indeed, efficient entry depends on the ability to recognize a gradient of chitin oligosaccharides: addition of the disaccharide *N, N´* diacetylchitobiose (chitobiose), but not the monosaccharide *N*-acetylglucosamine, disrupted squid colonization by preventing migration into the duct, while not preventing migration from the aggregate to the pore[74]. This behavior mimicked that of the colonization-defective *cheA* mutant. Similarly, efficient colonization could also be blocked by interfering with chitobiose production by the squid enzyme chitotriosidase, present in the ducts and pores[3]. Thus, it seems that early colonization comprises three stages: aggregate formation; migration to the pore; and movement from the pore into the duct in a manner dependent on the recognition of squid-generated chitin oligosaccharides. To date, the chitobiose chemoreceptor or chemoreceptors remain unknown; however, at least four of the genes encoding methyl-accepting chemotaxis proteins (MCPs) in the *V. fischeri* genome encode MCPs that recognize *N*-acetylated sugars like chitobiose[75].

Finally, in addition to promoting chemotaxis-based movement, flagellar rotation seems to indirectly trigger the host immune response. Rotation results in the release of lipopolysaccharide molecules from the flagellar sheath and/or outer membrane, which in turn triggers apoptotic cell death in epithelial cells present on the light organ surface[76]. This apoptosis is part of a program of tissue remodeling that removes colonization-promoting ciliated structures after successful entry by *V. fischeri* (see below and companion paper) Thus, the role of flagella in symbiosis seems to be multi-factorial[22].

[H2] Symbiotic bioluminescence and quorum-dependent regulation.

After colonizing its host, *V. fischeri* induces the *lux* operon, which is responsible for bioluminescence (Fig. 3). Once the animal grows large enough to cast a discernable shadow, this light emission is used by the squid to obscure its silhouette in a camouflaging ‘counterillumination’ behavior[6], and perhaps other behaviors as well[77]. Bioluminescence enables *V. fischeri* to persist in the host[78,79]; dark mutants persist in the host; mutants deficient in bioluminescence (dark mutants) initially colonize, but within two days their colonization levels begin to diminish relative to the wild type. This initial colonization attenuation seems restricted to the largest, early-developing crypts[80], perhaps because *lux* transcription is delayed in the smaller, later-developing crypts[81-83]. Although several possibilities have been proposed, the mechanism or mechanisms underlying the contribution of luminescence to colonization persistence remains unknown. Long-term colonization, either with or without a competing wild-type parent, drove a dark mutant to extinction[84], perhaps because the host either eliminates the dark cheaters, or restricts their ability to proliferate after the dawn expulsion. These results suggest that luminescence eventually is required in all crypts, and that the light emission of an overwhelming majority of symbionts cannot compensate for the colonization defect of a nearby dark mutant[84]. The symbiotic fitness advantage to light production is particularly noteworthy given that bioluminescence presents a metabolic fitness cost in culture[78], and dark lineages of *V. fischeri* have arisen in environments that lack hosts with light organs[85]. Symbiont bioluminescence not only affects the cellular and tissue morphology of the light organ, but also the host transcriptome[9], including two transcripts that encode homologs of cryptochromes, light detectors that may permit a response to symbiont-generated light[14,79,86-88].

Bioluminescence is regulated by an archetypic, widespread, transcriptional mechanism controlled by cell–cell signaling and driven by the accumulation of signaling molecules known as pheromones (autoinducers). Only at a high cell density, or a 'quorum', do these pheromones reach a sufficient concentration to induce the *lux* operon. At least three pheromones contribute to induction, although the two acyl-homoserine-lactone (AHL) based systems are the most influential. This interconnected regulatory network has been reviewed extensively[23,89,90] (Fig. 3). Briefly, the two AHL-based systems operate sequentially[91], with signaling by AinS–AinR triggered at moderate cell densities, and jump-starting the downstream LuxI–LuxR system. LuxR, in turn, provides negative-feedback repression of *ainSR*[92]. Consistent with a sentinel role for AinS–AinR, AinR is exquisitely sensitive to its cognate signal, at concentrations <100 pM[93]. This signaling also influences central metabolism[91,94,95] and, thus, exogenous addition of the pheromones, or chemical antagonists, affects not only the initiation of symbiosis, but also the subsequent bioluminescence and symbiotic stability[96].

Although a quorum is necessary to stimulate luminescence, it is not sufficient. Regulators of gene expression and positive feedback loops mean that both the environment and the recent history of system induction are also critical for the decision to bioluminesce. This context dependence is evident in *V. fischeri* strains isolated from squid, which are far more luminescent in the host than in culture, even at similar cell densities[97]. The combination of environmental regulation and positive feedback could enable *V. fischeri* to launch population-wide behaviors in response to an environmental cue experienced by only a sub-population[98,99]. *V. fischeri* cells in different crypts can communicate using their pheromones, potentially transmitting information about the conditions they experience, despite their physical separation[98]. Such environmentally responsive regulators that control luminescence include ArcA, CRP, Fur and PhoB, among others[98,100-103], and activating conditions and/or signals include oxidative redox, low iron, low phosphate, and the absence of catabolite-repressing sugars (such as glucose). Among these parameters, phosphate availability seems to vary between light organ microenvironments, although this unevenness alone cannot explain the heterogeneity of *lux* expression in the host[104]. Although some of these regulatory mechanisms are conserved, the *lux* locus and its regulation seems to have evolved rapidly among *V. fischeri* strains[105], which suggests distinct selective pressures in different associations.

[H1] Nutritional metabolism

The metabolism of *V. fischeri* cells in the symbiotic light organ environment has been studied extensively and reviewed elsewhere[23,106,107]. Bioinformatic analyses illustrate the metabolic diversity of *V. fischeri* (for example,[108]), including various catabolic, anabolic, scavenging and energy-generating pathways, and transcriptional profiling shows that *V. fischeri* actively uses much of this genomic potential in the symbiosis[109]. Not only is *V. fischeri* physiology in the light organ complex, but it varies temporally both on a diurnal cycle[110] and during maturation of the host[106,107,111,112] (see Nyholm & McFall-Ngai[11]).

Mutant analyses have provided key insights into the nutrients available to symbiotic cells. For example, a pioneering study examining the colonization proficiency of amino acid

auxotrophs indicated both that some amino acids were available to symbionts and that *de novo* amino acid biosynthesis contributed to symbiotic competence[7]. More recently, mutants with a disrupted tricarboxylic acid cycle (TCA) cycle that required glutamate were found to be proficient in colonization[113], suggesting this amino acid or a related compound like glutamine is available in the light organ. Similar mutant analyses have shown that the host provides symbionts with guanine[114] and δ-aminolevulinate[115], or related compounds, that can overcome these auxotrophies. A recent comprehensive and groundbreaking insertion sequencing (InSeq) study catalogued an expansive number of mutants and their relative abilities to grow in a rich medium or in the light organ, providing a wealth of evidence with respect to what symbionts do or do not have to produce *de novo* in the light organ[49]; this study will continue to provide insights that must await additional investigations.

Considerable interest has focused on finding the primary carbon source or sources underpinning symbiont growth, although many questions remain unanswered. *N*-acetylglucosamine (NAG), and to a lesser extent glucose, have been areas of focus, in part because NAG is a component of chitin, which is the most abundant polysaccharide in the marine environment. Studies of the metabolic regulators CRP[116] and NagC[111,117] suggest that neither glucose nor NAG is amply available to symbionts initiating colonization, and this interpretation seems corroborated by the poor colonization of a NAG auxotroph[115]. Interestingly, although the regulatory response of CRP to glucose is consistent with the activity of this regulator in *E. coli*, the influences of NAG and cellobiose on CRP activity are difficult to square with prevailing models, and may suggest selective pressure to de-couple responses to NAG and glucose[20,118]. Although NAG seems to have a minor role during initiation of the symbiosis, the host provisions symbionts with chitin, a source of NAG, later during symbiotic development and presumably for most of the duration of this mutualism[13] (see below).

Energy generation by the symbionts seems to involve a combination of respiratory and fermentative pathways[119,120]. Bioluminescence requires oxygen, and light output therefore indicates that the symbiont's metabolism is not strictly anaerobic; however, luciferase seems to be oxygen-limited at points in the symbiosis, despite its high affinity for this substrate[121]. These observations and others suggest low-oxygen physiology is important for *V. fischeri* symbionts[12,122]. The ArcA–ArcB two-component system, which mediates regulatory changes at the transition from respiration to fermentation, contributes to symbiotic competence[100], whereas the anaerobic regulator FNR does not[123]. *V. fischeri* possesses three terminal oxidases for aerobic respiration: CydAB, CcoNOQP and the unusual heme-independent NO-resistant AOX, each of which may contribute to symbiotic physiology, although the role of CydAB has been difficult to study owing to its importance in growth *ex vivo*[106,124]. Trimethylamine-*N*-oxide (TMAO) reductases are expressed in the symbiosis, but these anaerobic respiratory chains are not required during initiation of the symbiosis[125]. Other evidence suggests *V. fischeri* induces fermentative physiology in the light organ, acidifying the environment and notably producing acetate, which the host itself may use[13,110]. *V. fischeri* along with other members of this genus is capable of rapid generation times, which it achieves in the symbiosis[69], so production of fermentation acids could indicate a form of overflow metabolism that reflects selection for rapid energy

generation over efficient conversion of resources. Thus, the presence of fermentation products need not reflect a lack of electron acceptors to support respiration.

As with many pathogenic bacteria, in host tissues symbiotic *V. fischeri* cells induce scavenging pathways to obtain essential nutrients such as iron[126], phosphate[104] and sulfur[112]. For example, a low-iron response mediated by Fur is induced during colonization, and symbionts use siderophores[127,128] and scavenge heme[126] to obtain iron. Interestingly, sources of phosphate[104] and sulfur[112] are non-uniformly distributed across light organ microenvironments, illustrating the heterogenous biogeography of these symbiotic tissues. *V. fischeri* may obtain these nutrients at least in part from organic biomolecules like glycerophosphate[109,110] and cystine[7,112] during colonization.

[H1] Responses to the host

One of the key characteristics of bacterial symbioses is that, unlike when they are living outside the host, the surroundings of the microbial partner (that is, host tissues) reciprocally respond to the activities of the symbiont. Furthermore, that response changes depending on the age of the host, health, metabolic state and circadian clock. Because host engagement with the co-evolved mutualistic microbial species, and sometimes even strain, is critical for both partners[129,130], there must be effective communication between the partners during the initiation and throughout the persistence of the symbiosis. To achieve this goal in the squid–vibrio association, the host responds to a complex language of chemical signals and cues from *V. fischeri*[87] (see Nyholm & McFall-Ngai[11]), including OMVs[131,132], the composition of which is modified in response to low ambient pH[133]. Conversely, as in other symbioses[134], the squid shapes the microenvironments of its symbiont, which reacts by changing its physiology and behavior.

For example, during the initiation stage of colonization, aggregating *V. fischeri* cells encounter NO and acidic conditions produced within host mucus[3,57]. Although the levels of these stresses are insufficient to stop colonization, they do prime *V. fischeri* to induce resistance responses to withstand not only these conditions but also host-derived antimicrobial proteins[135,136] that the symbionts will encounter during their subsequent passage into the crypts[59,137,138]. In some cases, *V. fischeri* cells seem uniquely capable of responding to this priming[138], providing a possible mechanism that promotes symbiosis specificity. Importantly, transcriptional analysis of symbiont cells immediately after venting from the juvenile host provides evidence of the conditions they were experiencing in the crypts. Specifically, enzymes involved in resisting oxidative stress and antimicrobial peptides are highly upregulated in these cells[109], which suggests that the symbionts are under physiological stress[139]. Having exited the host, the symbionts transition to their planktonic environment by reducing their luminescence and increasing motility, as facilitated by a habitat-transition regulator, HbtR[75].

Each day after the morning venting, the remaining symbionts must repopulate the crypts, as well as produce bioluminescence that night. These two energy-intensive activities are supported by nutrients provided by the host. In the developing juvenile host, little is known about the identity of host-derived organic nutrients, although there is evidence from flux-

coupling analysis that glycerolipids (perhaps from host membranes) and amino acids and *N*-acetylneuraminic acid (perhaps from host mucus) are fermented[7,109]. In addition, the availability of inorganic nutrients[104,112], and the resulting nature of population growth[82], differ between crypt populations. In contrast to this biogeographic variation within juvenile light organs, the symbionts in adult light organs experience temporally distinct nutritional conditions depending on the time of day[110]. Specifically, during the night, the host supports and enhances bioluminescence by the provision of chitin oligonucleotides[12,13,110]. In laboratory studies, catabolism of NAG, the chitin monomer, leads to an increased production of fermentation products, a lowering of ambient pH and a decrease in respiratory oxygen consumption[120]. Such conditions in the crypt[10] would be expected to promote higher levels of luminescence production at night, when the squid needs this activity. Thus, the host controls its symbiont's bioluminescence by a temporally restricted nutrient availability. How the host constrains any growth in the bacterial population during this period of intense light emission remains an unresolved question.

[H1] Population biology and evolution

It is becoming increasingly clear that strain-level differences (Box 1) have an important role in establishing symbioses[19,129,130,140,141]. For instance, phenotypically distinct classes of symbiotic *V. fischeri* isolates exhibited different competitive behaviors during initial colonization of the squid[142], and genomic characterization of these strains revealed these classes were phylogenetically consistent, and defined a clade of strains with dominant ('D') behavior[53]. Extensive imaging of these D strains as they initiated light organ colonization showed that they often produced hyperaggregates[28], and migrated from their aggregates sooner, reaching the crypts more rapidly[4] than non-D strains. Interestingly, the D-strain clade also contains an additional 250 kb of DNA not found in any other *V. fischeri*[53] and these additional 194 ORFs are distributed in 41 loci ('islets') that are spread across the two chromosomes, unlike in a single locus as is typical for colonization traits on pathogenicity islands (for example,[143]). This pattern is similar to the scattering of unique loci of a divergent light organ symbiont from the Mediterranean sepiolid squid *Sepiola robusta*[144]. As of yet, none of these genes have been connected to the D-type behavior[145]. In fact, recent work indicates that strategies for achieving D-type behavior have evolved independently multiple times in *V. fischeri*[145].

Several studies have sought to understand the evolution of symbiosis competence in the highly specific squid–vibrio symbioses, using ecological[146], comparative genomic[145] or experimental evolution approaches[46,147]. The latter was designed to uncover specific mutations that increase the effectiveness of poorly colonizing strains of *V. fischeri*. In one study of two poor colonizers, mutations altering an extracellular polymeric substance (EPS) attenuator, BinK, previously identified in a transposon mutant screen[45], readily resulted in mutants with improved ability to initiate colonization[46]. Besides an increase in the synthesis of Syp-PS, the mutation caused additional altered phenotypes, including reduced pheromone-signal production and decreased luminescence output. Such pleiotropic mutations may produce fitness trade-offs for the bacterium in other environments[148], a hypothesis that has not been fully tested. In spite of this potential to increase the effectiveness of poorly colonizing strains, the squid–vibrio symbiosis remains notably

species-specific: to date, there have been no effective efforts to convert other *Vibrio* species into successful colonizers. Thus, with the exception of some strains of the closely related sister species, *Vibrio (Aliivibrio) logei*[149], *V. fischeri* remains the sole species found colonizing the *E. scolopes* light organ. Interestingly, like other *Vibrio* species, *V. fischeri* has a surprisingly low spontaneous mutation rate that is not uniformly represented around the chromosomes[150,151], and identical deletion events resulting in the loss of the entire *lux* operon[85] have apparently occurred across different lineages of this symbiont species. Thus, the population biology of *V. fischeri* is driven by both known and unknown evolutionary mechanisms.

A single light organ from a field-caught animal typically is colonized by a few of distinct strains[152], yet little is known about their distribution among the crypts owing to the complex biogeography of the mature light organ[153] and the difficulty of identifying different *V. fischeri* strains in a naturally colonized adult. An exciting advance in understanding the dynamic nature of symbiont-strain interactions has resulted from directly imaging the juvenile light organ after colonization by pairs of *V. fischeri* strains. Earlier work using isogenic strains indicated that, although individual crypts are most often colonized by single symbiont cells, ~20% typically develop as co-colonizations[142]. Colonization of the majority of crypts by only a single cell is believed to occur because of a physical 'bottleneck' along the migration pathway that constricts after the first *V. fischeri* cell enters[82]. Thus, it was a surprise when co-colonization experiments involving some strains resulted exclusively in singly colonized crypts[154], suggesting a competitive incompatibility between certain strains. Subsequent work revealed that some *V. fischeri* strains encode a second type-6 secretion system (T6SS2) that is lethal to strains that lack this system[155], the expression of which is regulated by environmental viscosity[156]. Carriage of the genes encoding the T6SS2, which includes functionally redundant structural proteins[157], apparently constitutes a trade-off for the bacterium, as such strains have not swept through the light organs of squid populations, and still constitute a minority of symbionts; however, when they co-occur in a crypt, these genes are expressed[158], and the strain prevails. Interestingly, only some of the dominant-behavior D strains[53] carry this lethal T6SS2, which is phylogenetically more widespread among *V. fischeri* strains[155], indicating that additional factors contribute to D-strain dominance. Although this discovery is still a new development in *V. fischeri* population dynamics, the announcement of the genome sequences of two T6SS-carrying strains[159] will facilitate the use of comparative genomics approaches to better understand the origin and function of the mechanisms underlying competitive interactions.

[H1] Concluding comments

Microbial symbionts can affect essentially all aspects of their host's behavior, life history and evolution and, in turn, adapt to their partner as a changing but predictable habitat. These processes are likely to occur across the extensive array of microbiomes that have become the subject of study in recent decades, including those of humans; however, the complexity of these associations make it difficult to examine individual interactions within a cacophonous consortium. By contrast, a naturally binary symbiosis like the squid–vibrio association has fewer voices in the conversation to decipher, permitting the discovery of novel, but shared, modes and components of a single partner's reciprocal signaling and response to

its host[9,14,15]. Once identified, these same components can often be recognized within the more complex, consortial symbioses, where they underlie mechanisms of health, resilience and homeostasis. Furthermore, new insights are anticipated from applying conceptual and technological advances to the study of this symbiosis (Box 2). As summarized in this review, the richness of the interaction between *V. fischeri* and its host is remarkable and continues to provide evidence of the extent to which the two partners have co-evolved, each increasing its fitness as a member of a functioning unit, relative to its success living as an individual organism.

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Glossary

Light organ:
Symbiotic bioluminescence organ inside the squid *E. scolopes* where *V. fischeri* resides

Chemoattractant:
Nutrient or other molecule to which a bacterium migrates, typically using flagella-driven motility

Mantle cavity:
The region inside *E. scolopes* where the light organ is located

Biofilm matrix:
The collection of secreted polysaccharides, proteins, and other substances, produced by and surrounding a group of bacteria, that functions to promote attachment and provide protection

Oxidative redox:
Chemical conditions that create an oxidative stress on bacteria

Auxotrophs:
Bacteria that lack the ability to synthesize one or more essential biomolecules from a limited nutrient source

Cellobiose:
disaccharide composed of glucose

Siderophores:
Iron-binding molecules secreted by and taken up by bacteria, thereby providing iron to the cell

Extracellular polymeric substance (EPS):

Polysaccharides, proteins, and other substances produced and secreted by a bacterium, many of which facilitate cell-cell and/or cell-surface attachment, and that contribute to the biofilm matrix

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BOX 1: Genomics and molecular genetics

The availability of the genome for *Vibrio fischeri* (strain ES114) in 2005 greatly accelerated reverse genetics studies of specific genes, typically chosen through comparisons with those important in pathogens[72]. The subsequent determination in 2008 of the genome for strain MJ11, a fish light-organ symbiont that fails to colonize squid, permitted a more symbiosis-focused comparison and revealed the absence of a single, key regulator in the fish symbiont[40]. More recently, the genomes of numerous other *V. fischeri* symbionts have been reported (see the figure) and contain, in some cases, over 250 kb of additional sequences not found in ES114 (Refs. [52,53,159]); the distinct phenotypes of these isolates are prompting investigations of factors that permit superior colonization. Whole-genome sequencing has also facilitated identification of point mutations, ushering in a new era of evolutionary biology. For example, experimental evolution experiments in which the non-colonizing MJ11 acquired the ability to colonize *E. scolopes* revealed the importance of a negative regulator in preventing colonization[46].

At the same time, other advances have facilitated labeling and genetic manipulation of *V. fischeri*. Study of pES213, a native *V. fischeri* plasmid, permitted the development of stable, pES213-based expression vectors and reporter plasmids[81,161]. The application of a hyperactive Tn*5* transposon enabled phenotype assessments via generating random mutants[162,163]. Another major genetic advance came with the finding that ES114 could be induced to take up DNA from the environment, and recombine it into the genome[164]. This ability to induce DNA uptake, combined with the use of new tools that facilitate mutant construction and complementation, has greatly accelerated the rate at which putative symbiosis factors can be investigated and confirmed through approaches such as ‘backcrossing’[165]. It has also facilitated deletion mapping of mutant derivatives[166]. Finally, the ability to interrogate the symbiosis has been advanced by other new approaches, including promoter probe[104] and InSeq[49] tools.

The geographical and biological sources of the isolates, as well as the number of strains sequenced, are indicated. Almost all the sequences are draft genomes [40,53,72,144,145,159,167,168]

* These sequences were deposited into GenBank without an associated publication.

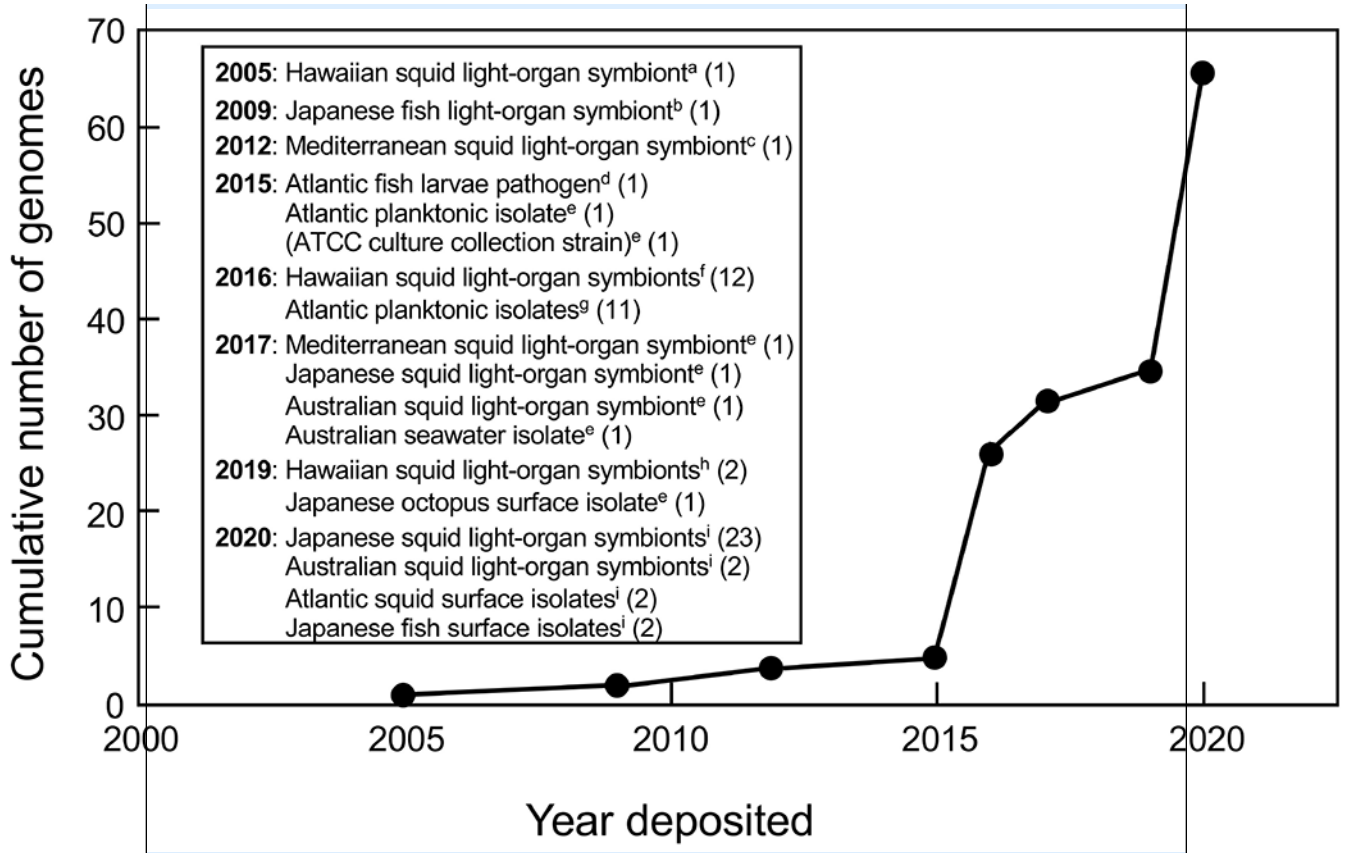

Fig. 4. The rise of *V. fischeri* genomic information.

Trajectory of the appearance of *V. fischeri* genome sequences over the last 15 years, which has seen a rapid increase since 2015. The geographical and biological sources of the isolates, as well as the number of strains sequenced, are indicated. Almost all the sequences are draft genomes. Associated publications are identified by their PMIDs: [a]15703294; [b]19182778; [c]22374964; [d]26044435; [e](none); [f]27128997; [g]27653556; [h]31331977; [i]32127462.

BOX 2: Research areas to watch

The next decade promises discoveries across a range of research topics, some examples of which are listed below.

- *Vibrio fischeri* isolate ES114 has been a unifying wild-type strain; however, recent studies have shown the power of comparative strain analyses[145]. *V. fischeri* populations naturally compete with one another, driving the evolution of distinct genotypes based on fitness tradeoffs that will be identified by strain comparisons and DNA sequencing technology.
- Future research will delve into issues of complex biogeography and diurnal variation, once simplified as ‘in the host’, using advances in microscopy such as hybridization chain-reaction fluorescence *in situ* hybridization (HCR-FISH)[75,169], transcriptional reporters and methods such as imaging mass spectrometry that enable the identification of molecules *in situ*.
- New insights into light organ environments enable researchers to better recapitulate relevant conditions *ex vivo*. No medium can reproduce all host conditions, but a systematic adjustment of media and physio–chemical growth conditions to better reflect light organ environments will give culture-based studies more ‘real-world’ relevance.
- Research on symbiotic *V. fischeri* will benefit from a greater use of computational and bioinformatic approaches. Across biology, mathematical analyses of complex datasets have enabled discoveries in a range of topics, from metabolism to regulatory circuits to the behavior of cells within populations. Research on the light organ symbiosis is generating molecular and imaging data ripe for such deeper analysis.
- Results from insertion sequencing (InSeq analysis)[49] remain a cornucopia of testable hypotheses, providing insight into the role of every *V. fischeri* gene with respect to its importance in culture and/or in the symbiosis. This seminal dataset will inform many future studies.
- An understanding of ‘global’ symbiont gene expression will benefit from the application of nanoString technology, which allows an accurate determination of the average expression levels for hundreds of genes in as few as 10^5 symbionts.

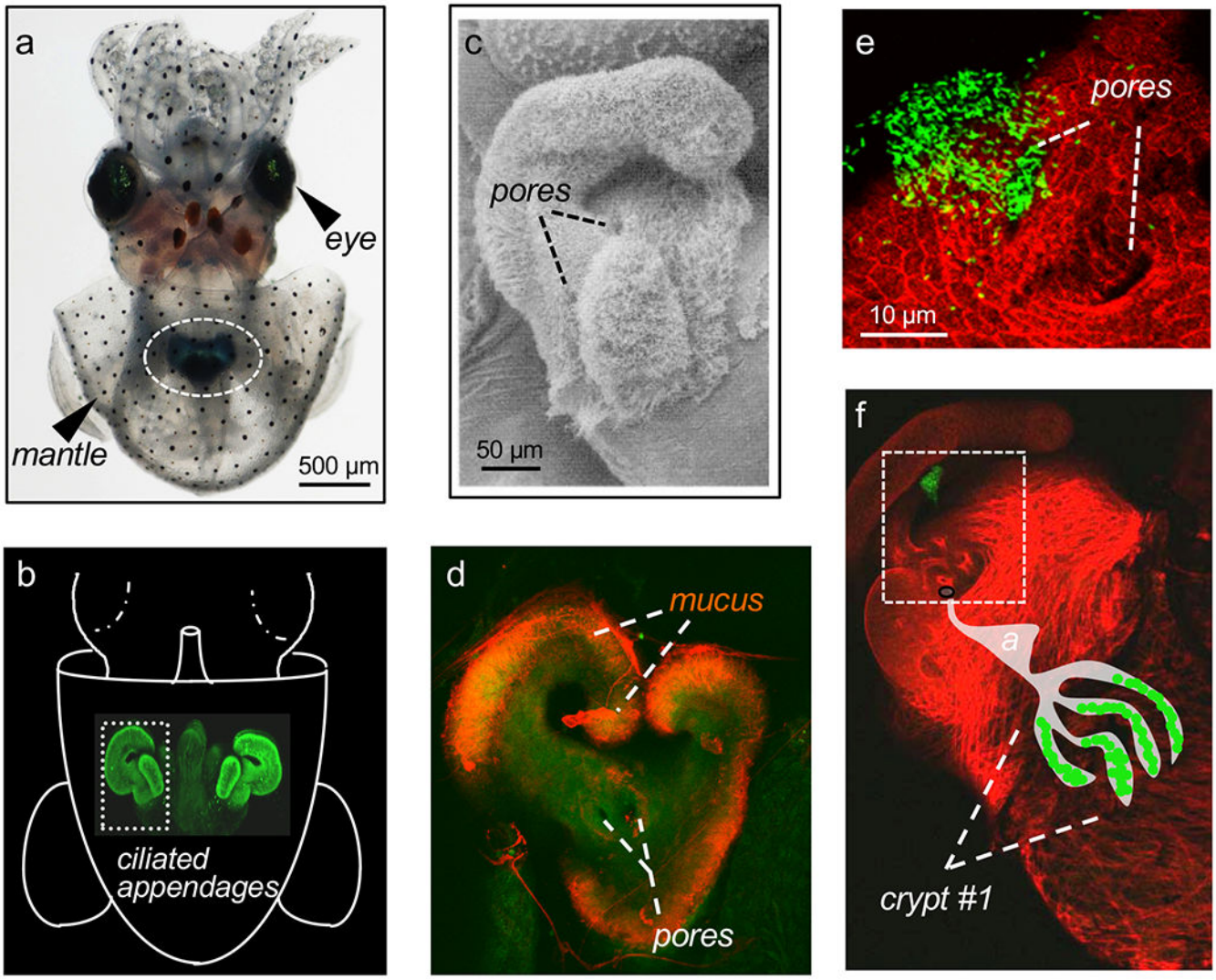

Fig. 1. The juvenile *E. scolopes* light organ.

a. The nascent light organ (circle) is located in the mantle cavity of a newly hatched squid. **b.** Two pairs of ciliated appendages emerge from the outer surface of the light organ, interacting with seawater drawn into the mantle cavity during respiration. **c.** Three pores are found at the base of each pair of appendages. **d.** Soon after hatching the appendages begin to produce mucus that covers the ciliated fields. **e.** Mucus production and the flow fields created by the cilia capture *V. fischeri* cells (here, labelled with GFP) present in the seawater, and direct them to a zone directly above the pores where they form aggregates. **f.** After a few hours, the aggregates chemotax to the three pores (square), and migrate into one of three interior ducts, each leading to an antechamber (*a*), through a bottleneck, and into a crypt. Once in the crypts, the *V. fischeri* cells proliferate and autoinduce luminescence. This interior pathway to crypt #1 is indicated as a cartoon.

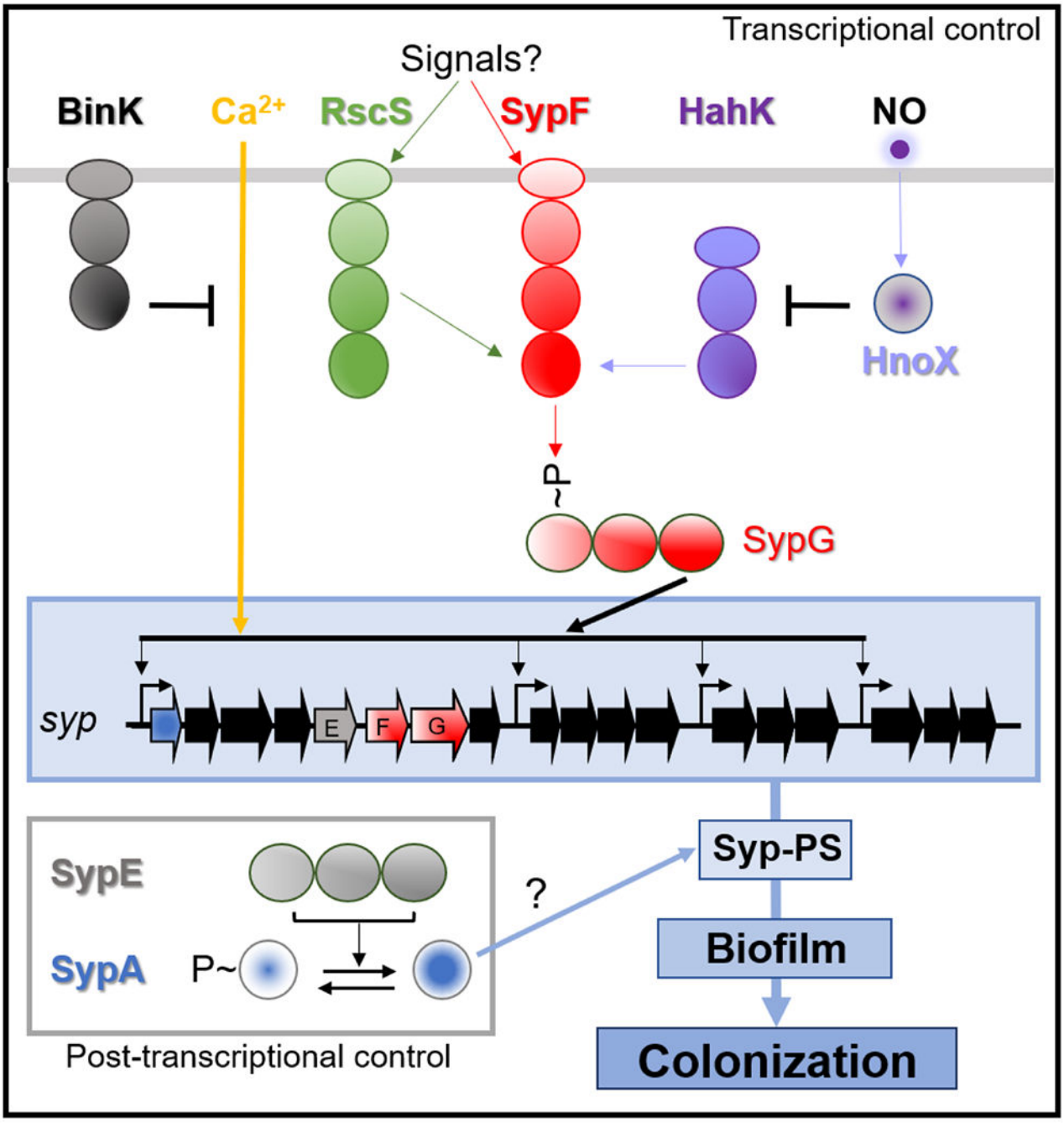

Fig. 2. Regulatory pathways controlling production of Syp-PS leading to biofilm formation and colonization.

As described in the text, the SKs RscS and HahK function upstream of the SK SypF, which in turn activates, via phosphorylation, the RR SypG. In turn, SypG activates transcription of the *syp* locus, resulting in production of the structural proteins necessary for Syp-PS production. Calcium is a positive signal inducing syp transcription that is inhibited by the SK BinK. Transcription is also inhibited by nitric oxide (NO) via HahK. Syp-PS production is further regulated post-transcriptionally by the action of the RR SypE, which

phosphorylates and dephosphorylates SypA. Unphosphorylated SypA promotes Syp-PS production through an unknown mechanism.

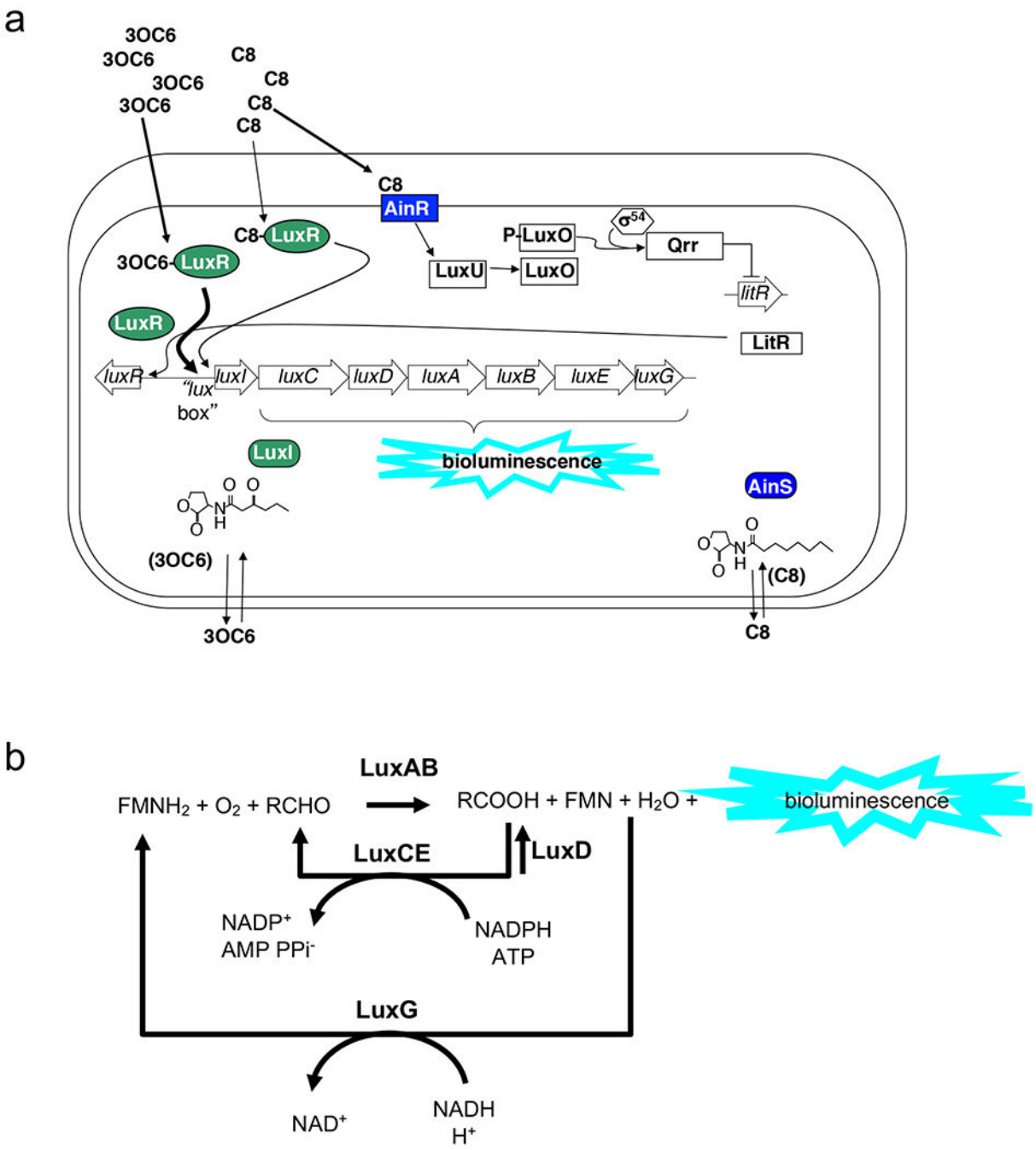

Fig. 3. Genetics and biochemistry of bioluminescence in *V. fischeri*.

a. Genetics and regulation: large arrows indicate the genes and their orientation at the *lux* locus. The bioluminescence reaction is driven by products of *luxCDABEG*, which are co-transcribed with *luxI*, and this "lux operon" is divergently transcribed from *luxR*. Two AHL signal molecules, *N*-3-oxohexanoyl homoserine lactone (3OC6), and *N*-octanoyl homoserine lactone (C8), produced by LuxI and AinS, respectively, are membrane permeable and can serve as cell-to-cell signals. When 3OC6 binds to LuxR, the activated regulator attaches to a sequence near the promoter of the *lux* operon called the "lux box", resulting in an activation

of transcription. While C8 is a weak activator of LuxR, it is strongly recognized by its cognate receptor, AinR, and works through a regulatory cascade to relieve the repression of LitR, a master regulator that controls transcription of several genes including LuxR.

b. Biochemistry: the LuxAB heterodimer forms the luciferase enzyme, which sequentially binds reduced flavin mononucleotide ($FMNH_2$), O_2, and an aliphatic aldehyde (RCHO), and converts these substrates to FMN, water, and the corresponding aliphatic acid (RCOOH), emitting a bluish light (~490 nm wavelength) in the process. LuxC, LuxD, LuxE, and LuxG are responsible for (re)generating the substrates for luciferase.