

## MINIREVIEW

# A Squid That Glows in the Night: Development of an Animal-Bacterial Mutualism

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### INTRODUCTION

The field of microbiology has traditionally shown considerable interest in the association of bacteria with higher plant and animal life. The fundamental question that has driven this interest is, How do certain bacterial species effectively utilize the tissues of other organisms as their preferred (and sometimes required) growth environment? This question has been successfully addressed over the last several decades through studies of a number of model systems that have uncovered the physiological, biochemical, and molecular adaptations that underlie specific bacterial-host associations. Remarkable progress has been achieved with two classes of symbioses: the pathogenic associations of bacterial species with animal and plant tissues and the cooperative associations between nitrogen-fixing bacteria and root nodule-forming plants.

Examination of the onset and development of a number of animal (primarily mammalian) and plant infections has produced striking examples of the extent to which an "arms race" exists between a pathogen and its host. The resulting association exhibits a complex pattern of adjustments between specific biochemical attacks by the bacterium and the more generalized compensating defenses of the host (47). While the specific patterns that characterize such diverse pathogens as *Vibrio cholerae* (12), *Bordetella pertussis* (68), and *Agrobacterium tumefaciens* (69) vary considerably, they all involve a programmed series of changes in virulence gene expression aimed at promoting the growth of the bacterium at the expense of the host (20).

In contrast, the root nodule symbiosis induced by species of *Rhizobium* and related genera is an example of an exquisitely cooperative interaction between the bacterium and its plant host (28). These mutualistic associations are highly sophisticated in their pattern of interspecies "cross-talk," with both partners employing specific regulatory and recognition signals to modulate their own, as well as their partner's, pattern of gene expression (10, 34). As a result, coordinated changes in the metabolism and morphology of both partners occur, culminating in the formation of novel, differentiated plant tissues whose sole function is to support and enhance the symbiotic activities of its nitrogen-fixing bacteria (36).

In comparison to current knowledge about either animal and plant pathogenic associations or plant mutualistic symbioses, our understanding of the development of cooperative associations between any animal species and its beneficial bacterial symbionts is as yet rudimentary. However, might we not anticipate that bacterial-animal mutualistic symbio-

ses will present as rich an opportunity to study the mechanisms underlying the development of such associations as the *Rhizobium*-plant symbiosis has? We review here the results of recently emerging studies of the mutualistic symbiosis between the luminous bacterium *Vibrio fischeri* and the sepiolid squid *Euprymna scolopes*. Although this association has been under study for only the last 4 years, its biological characteristics have revealed exciting information about the events leading to the initiation and development of the symbiosis (45).

### THE *V. FISCHERI*-*E. SCOLOPES* SYMBIOSIS

The recruitment of luminous bacteria into light organ symbioses has been an evolutionary strategy employed by at least 35 species of squids, belonging to two major cephalopod families, whose members are distributed throughout the coastal and deep waters of the world (54). One of these, *Euprymna scolopes*, is a small sepiolid squid (average adult length, 40 mm) that is indigenous to the Hawaiian archipelago, where it lives in the shallow sand flats that are associated with coral reefs (3). The squid buries itself in the sand during the day to escape predators but comes out to forage in the water column at night (62). *E. scolopes* is bioluminescent owing to the maintenance of luminous bacteria housed in a complex, bilobed light organ that lies within the center of the mantle cavity of the animal, where it is continually bathed with seawater as a result of normal ventilatory activity (Fig. 1A) (31). Light emitted by the bacterial symbionts is apparently used by the host squid in a strategy called counterillumination (40). An animal exhibiting this behavior camouflages itself from any predators below it by controlling the intensity of ventrally projected light, thereby matching the moonlight shining down, and eliminating any shadow it would otherwise cast.

The luminous bacterium *V. fischeri* is the specific light organ symbiont of *E. scolopes* (5). Upon emerging from the egg, each juvenile squid must obtain from the surrounding seawater an inoculum of symbiosis-competent *V. fischeri* (33, 43), which then colonizes the developing light organ and initiates the animal's ability to become bioluminescent (65). The squid attains sexual maturity within a few months and is believed to live for about a year in nature (44). During this entire period, the host nourishes and maintains within the light organ a pure culture of as many as  $10^8$  *V. fischeri* cells without either contamination from the environment or an opportunistic invasion by these bacteria of other host tissues.

Histological and ultrastructural analyses of the adult light organ have revealed that the symbionts are housed in a complex set of tissues that serve to support the bacterial

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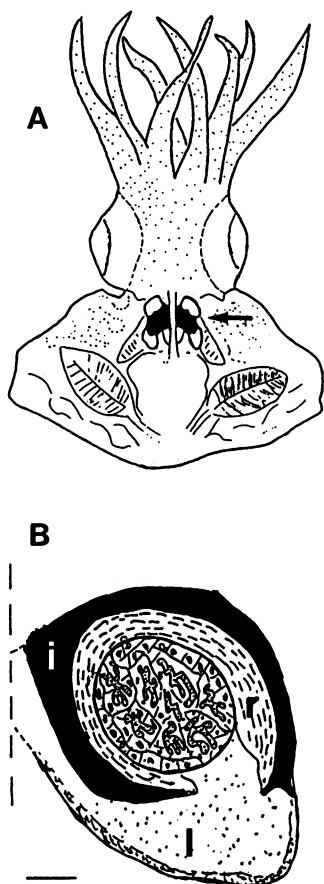


FIG. 1. (A) Schematic diagram of an adult specimen of *E. scolopes* (approximately 50 mm in length) viewed from the ventral surface. The mantle cavity has been dissected to expose the bilobed light organ within (indicated arrow). (B) Diagrammatic cross section through the right lobe of the light organ at the level of the arrow in panel A, revealing the ink sac (i), reflector (r), and lens (l). Cells of *V. fischeri* are contained in numerous lacunae in the central core tissue of the organ. The lens is located on the ventral, or downward-facing, portion of the organ. Bar = ca. 0.4 mm.

culture and control their bioluminescence (Fig. 1B) (43). Lacunae containing the bacteria are lined with a complex microvillous epithelium that provides a large area of surface contact between the bacteria and the squid tissue, presumably enhancing nutrient and regulatory exchange (43). A thick reflective tissue layer and a muscle-derived lens serve to direct and diffuse the light over the animal's ventral surface, while moveable diverticula of the squid's opaque ink sac dynamically control the intensity of light emission leaving the organ (43).

The luminescence is a product of the oxidation of FMNH<sub>2</sub> and tetradecanal by the enzyme bacterial luciferase (53). In *V. fischeri*, the structural genes responsible for the synthesis and activity of luciferase (the *lux* operon [for recent reviews, see references 15 and 46]) are under the transcriptional control of two regulatory genes: *luxI*, which codes for the synthesis of a membrane-permeable molecule called autoinducer (18, 30), and *luxR*, which codes for a DNA-binding protein that mediates the effect of autoinducer (46). The functional result of this regulatory circuit is to depress the expression of the *lux* operon under conditions of low *V. fischeri* population density (52). However, in the confines of

the light organ, the concentration of autoinducer is believed to increase, and transcription of the *lux* operon is induced, leading to an increase in the specific activity of light production of as much as 10,000-fold (5). At least some strains of *V. fischeri* have lowered specific activities of light emission when grown in the laboratory under certain conditions (in the presence of catabolite repressors, excess iron, or normal concentrations of oxygen) (for a recent review, see reference 15); however, no such responses were reported in a strain of *V. fischeri* isolated from *E. scolopes* (5).

#### INITIATION AND ESTABLISHMENT OF THE SYMBIOSIS

In the presence of a symbiosis-competent strain of *V. fischeri*, the nascent light organ of a newly hatched *E. scolopes* is infected within a few hours and becomes functionally bioluminescent in less than a day. As early as 3 days after initiation of the symbiosis, morphological and biochemical maturation of the organ has begun (45), culminating in a fully differentiated state within 2 weeks. The remainder of this minireview will concentrate on the first few days of the symbiosis, during which the association is initiated and established. Specifically, three classes of events in symbiotic development will be considered: (i) recognition and selection, (ii) growth and communication, and (iii) differentiation.

**Recognition and selection.** Light organ symbioses of at least 10 families of marine fishes and squids are species specific (for recent reviews, see references 41 and 53). For example, *E. scolopes* and its Japanese relative *Euprymna morsei* form symbiotic associations only with *V. fischeri* (6, 15, 58), even though the animals are found in waters containing an abundance of other luminous bacteria, including the loliginid squid symbiont *Photobacterium leiognathi* (22). Recently, by using newly hatched, uninfected juvenile *E. scolopes*, it has been demonstrated that the specificity is expressed during the initiation of the association (45). The light organs of animals maintained in seawater that does not contain symbiosis-competent *V. fischeri* do not become infected, either by any of the typical, indigenous marine bacterioplankton or by experimentally added symbiotic strains of other *Vibrio* or *Photobacterium* species. Such a finding demonstrates the remarkable ability of the exposed tissues of the nascent light organ to withstand nonspecific colonization, while encouraging a rapid infection and proliferation by an appropriate symbiont (see below).

Interestingly, the specificity for the *E. scolopes* symbiosis extends to only a subset of strains of *V. fischeri*. While strains of this species that have been recently isolated from the light organs of either *E. scolopes*, *E. morsei*, or two species of monocentrid fishes (*Monocentris japonicus* and *Cleidopus gloriamaris*) were symbiosis competent, several *V. fischeri* strains isolated directly from Southern California seawater were not (45). Thus, it appears that not all strains of *V. fischeri* carry or express the genetic determinants necessary for successful association with *E. scolopes*. The fact that strains recently isolated from *M. japonicus* light organs were able to infect the juvenile squids was particularly intriguing because *V. fischeri* MJ1 (61), a well-studied *M. japonicus* symbiont that has been transferred on laboratory medium for 15 years, was not symbiotically competent (60). This latter observation suggests that strain MJ1 may have lost its symbiotic competency over time, a phenomenon also reported with symbiotic *Rhizobium* strains after extended laboratory maintenance (8).

It is too early in the study of this symbiosis to say what the

nature of such apparent symbiotic determinants actually is; however, some possibilities are being explored. During a routine screening, it was observed that almost all strains of *V. fischeri* isolated from *E. scolopes* carried between 1 and 10 cryptic plasmids, while extrachromosomal DNA was only rarely present in symbiosis-incompetent strains (4). Because of the importance of plasmid-borne genes in symbiotic *Rhizobium* species (35), as well as the presence of virulence determinants on plasmids of pathogenic species of *Vibrio* and other genera (11, 27, 63), the potential role of such extrachromosomal elements in a successful symbiosis was examined. The results of these experiments suggested that plasmid-borne genes are not necessary for the effective initiation of a symbiotic association under laboratory conditions (7).

Mixed infections of a single light organ have shown that certain strains of symbiosis-competent *V. fischeri* exhibit competitive dominance over others (32). In these experiments, two strains (designated P and H) that were equally effective at colonizing the juvenile light organ by themselves were mixed at equal concentrations in water containing uninfected juvenile squids. While the initial light organ infections that developed contained a mixture of cells of the two strains at roughly a 1:1 ratio, within 4 days cells of strain P dominated by a ratio of at least 100:1. As found previously (7), the presence of plasmid DNA resulted in no significant advantage in such competition experiments; that is, infections initiated by mixed inocula containing plasmid- and non-plasmid-bearing *V. fischeri* resulted in no apparent dominance of the resulting symbiotic population by either strain (7).

Recent development and application of molecular genetic approaches to the study of *V. fischeri* (16, 25) as well as its host, *E. scolopes*, hold particular promise for rapid identification of the mechanisms underlying the physiological and biochemical processes that mediate the events through which the association is initiated and established. For example, nonmotile strains of *V. fischeri* ES114 have been produced by transposon mutagenesis and were no longer symbiosis competent (23) in a standard infection assay (45). Other phenotypes of presumptive importance to the symbiosis (e.g., the ability to acquire iron) can be obtained and studied by similar means. To identify symbiosis genes that have initially unknown functions but that encode proteins targeted for the cell surface, the application of *TnphoA* mutagenesis and selection is likely to help identify important recognition and specificity determinants (34).

**Growth and communication.** The newly hatched juvenile squid bears only a simple rudimentary light organ (45, 49). On each side of the bilaterally symmetrical organ are three empty saclike lacunae formed from invaginations of the epithelium. Each lacuna initially communicates with the seawater within the mantle cavity by a pore (45). These six openings are the entrances through which the lacunae become initially infected and colonized.

Studies of the kinetics of colonization of the juvenile squid light organ have revealed that the process has two distinct phases (58). For the first 9 to 12 h after the initial infection of the organ, the bacteria grow rapidly, with an apparent generation time of approximately 30 min, which is near the maximum growth rate of these bacteria at the experimental temperature (24°C). By 15 h postinfection, the rate of proliferation is significantly attenuated, resulting in a generation time of between 10 and 17 h. This diminished growth rate, in conjunction with a periodic expulsion of excess symbionts,

results in an established light organ population of about  $10^6$  cells that remains stable for a period of at least several days.

While it is clear that the host must be the source of nutrients for the symbiont population, little is known at this point about the characteristics of this nutritional communication between any light organ bacteria and their host; however, the ability of an auxotrophic mutant (e.g., requiring arginine) to colonize the juvenile squid suggests that the host tissue provides a diverse set of nutrients (24). Somewhat more can be hypothesized about the nature of interspecies regulatory communication, although up to now such communication has been largely inferred from studies of the bacteria in culture (15).

One of the best described systems of gene expression in *V. fischeri* is the *lux* operon (for a recent review, see reference 46). Of the several known regulatory components that are involved in the complex circuitry controlling luminescence, the most potent effector known is autoinducer. Although autoinducer functions within the cell as a transcriptional activator, it also functions extracellularly, allowing the cell to sense whether it is in an enclosed space such as the light organ lacunae (52). The result of this level of luminescence control is particularly dramatic in the symbionts of *E. scolopes*, which are unique among the known light organ symbionts: they are not visibly luminous when grown in laboratory culture because of an unusually low constitutive level of autoinducer synthesis (5). Gray and Greenberg (25) have cloned the *lux* operon from *V. fischeri* ES114, an *E. scolopes* symbiont, and shown that the underproduction of autoinducer by this strain is not due to a deficiency of substrates for autoinducer synthesis. Instead, underproduction of autoinducer by autoinducer synthetase, the putative product of the *luxI* gene (19), appears to be due to a limitation at the posttranscriptional level, perhaps resulting from either a decreased catalytic activity of LuxI in ES114 or an inefficient translation of ES114 *luxI* message (25). Support for the latter hypothesis comes from sequence comparisons of the regulatory region of *luxI* from strain ES114 with those of other strains of *V. fischeri*. Such comparisons have revealed an altered Shine-Dalgarno region in ES114 *luxI*, which may result in a diminished ribosome binding efficiency relative to other genes of the *lux* operon (26).

Recent estimates of the amount of autoinducer present in the light organ of an adult *Euprymna* specimen suggest an effective concentration of at least 30  $\mu$ M, a level well above that needed to maximally induce strain ES114 in culture (6). Although this strain constitutively produces autoinducer at extremely low levels, induction of luminescence occurs within 3 to 5 h of light organ colonization (58). This rapid response might result from the production of autoinducer by the host; thus, although there is no evidence for such a capability in the squid, light emission by juvenile squids infected with *luxI* mutants would be a highly suggestive finding.

Alternatively, if the autoinducer is produced solely by the bacteria, its concentration must increase very rapidly within the lacunae of a newly infected squid, which would suggest that while the lacunae walls allow the passage of nutrients and gases, they are an effective barrier to the rapid efflux of autoinducer. If this explanation is true, it follows that other forms of biochemical communication between the bacterium and the host tissue also may not be based simply upon diffusion kinetics. Other regulatory effectors, such as host-derived cyclic AMP (17), may also function in the communication between host and symbiont.

**Differentiation.** The development of the *V. fischeri*-squid

symbiosis, like that of the *Rhizobium*-legume association, appears to involve cellular differentiation by both the host and its symbionts.

(i) **Host development.** Studies of the changing morphology and biochemistry of the nascent light organ suggest that the presence of the symbiont may play an important role in development. The light organ of a newly hatched squid bears a pair of ciliated, microvillous epithelial projections on each side of the light organ (45). The tips of these structures are typically in close apposition, so that the projections form a ring of tissue, with the three pores that lead to the lacunae at the inside base of the ring (45). High-speed cinematography of the living animal has revealed that the ciliary beat created by this tissue draws seawater through the ring (42), thereby increasing the likelihood that a potential symbiont will encounter the pore openings. Within 48 to 72 h after the juvenile light organ becomes infected with symbiosis-competent bacteria, these epithelial projections begin to regress; however, if symbionts are not present, the structures persist for up to 8 days (45). The cinematographic and developmental data suggest both that these ciliated, microvillous structures are involved in the infection process and that the infection event signals the onset of the developmental program of the host organ. The fact that these structures are located several tissue layers away from the bacterial culture makes this entire process reminiscent of the induction of cortical cell division in the formation of the root nodule by interaction of bacterial symbionts with the surface of root hairs (10). Future experiments to determine the cellular basis of such morphological changes in the squid tissue and whether a specific bacterial signal is responsible are being designed.

In addition to these changes in the surface morphology of the light organ, the process of early development involves significant remodelling and elaboration of integral light organ tissues (44). Three-dimensional reconstructions of histological sections of the developing juvenile light organ have revealed that the size of the lacunae that house the bacteria increases by a factor of 3 or 4 within the first few days postinfection, a change in volume that does not occur in the absence of symbionts (49). Furthermore, within a week of infection, the three pores that are initially present on each lobe of the organ have coalesced into the single lateral pore that is characteristic of the adult condition.

At least three other host tissues that are involved in modifying the light produced by symbiotic bacteria (i.e., the reflector, ink sac, and lens) also undergo significant post-hatch elaboration in infected animals. Perhaps the most dramatic example is the development of the light organ lens (Fig. 1B). While there are no differentiated lens cells present on the uninfected juvenile light organ, within 2 weeks of infection, cells that exhibit the same ultrastructural characteristics as the cells forming the mature light organ lens have arisen on the organ's ventral surface (66). This process continues throughout host ontogeny, resulting in the formation of a thick pad of transparent lens tissue covering the entire ventral surface of the light organ.

Such anatomical and morphological changes are also reflected in the expression of genes and gene products that are characteristic of the developed light organ. For example, the differentiated lens cells do not express high levels of proteins specific to muscle, the tissue type from which these cells are derived (50). Instead, the major protein species is aldehyde dehydrogenase (ALDH), the same protein that is expressed in the eye lens of the squid as a crystallin (64); it appears, then, that ALDH also functions as a structural

protein in the light organ. Thus, the squid eye lens and light organ lens, which have the same functions of transmitting and focusing light, have each achieved this convergent function by amplifying the levels of ALDH (50). The induction of ALDH in the developing light organ apparently occurs within days because while newly hatched squids do not have differentiated lens cells, immunocytochemical analysis of incipient lens tissue reveals expression of ALDH as early as 10 days after infection (67).

Whereas two differentiation events that are apparent soon after infection have been correlated with the presence of symbiotic bacteria (45, 49), at this stage in our studies it is unclear whether the timing of any or all of the later developmental events described above is signaled by the onset of the symbiosis.

(ii) **Bacterial development.** It is a common phenomenon for bacteria to coordinately regulate the expression of a number of genes when they enter a symbiotic association (36, 47). As a result of these symbiotically signaled induction and repression events, the bacterium is generally regarded as having entered a specialized, differentiated state. In many cases, such as the bacteroid phase of *Rhizobium* spp. (37) or the reticulate bodies of chlamydia (51), morphological changes accompany the host-induced differentiation. Within 12 h of entering the lacunae of a juvenile squid, *V. fischeri* cells no longer display their characteristic polar tuft of sheathed flagella (58, 59); however, this flagella-deficient phenotype has not yet been physiologically induced in the laboratory (15, 57).

A hint to the possible mechanism by which this (and perhaps other) morphological symbiotic response is signaled in the squid symbiosis may lie in recent observations that the repression of flagellar formation that occurs in *Bordetella* and *Yersinia* spp. after entering the host is apparently coordinately linked with the induction of a number of virulence genes (1, 21, 48). Perhaps, as is often the case in the global regulation of virulence genes, a simple environmental cue, such as the decreased availability of iron or phosphate ions or the presence of a tissue surface, is the signal for coordinated changes in gene expression (47). Interestingly, such environment effectors control the expression of both flagellation and virulence genes in pathogenic *Vibrio* species (2, 38, 39).

Alternatively, the apparent differentiation of *V. fischeri* in the light organ may result from a more generalized symbiosis-induced stress response, as has been reported for other bacteria (9, 55). The rapid and sustained drop in apparent growth rate soon after the establishment of its association with *E. scolopes* suggests that symbiotic *V. fischeri* may also be experiencing nutrient limitation at just the time that flagella synthesis is terminated and maximal luminescence is induced (58). It is interesting to note that the induction of stress genes (e.g., *groEL*) is a response to starvation in some bacteria (29) and that maximal transcription of the *V. fischeri* *lux* operon (maintained in *Escherichia coli*) requires expression of the *groEL* gene (13). Taken together, these findings may explain why luminescence by *V. fischeri* and other light organ symbionts in the association is greater than can be achieved in laboratory culture (5, 14) and suggest a possible mechanism for host-controlled regulation of symbiont functions.

## CONCLUDING STATEMENT

The past decade has witnessed an exciting unveiling of many of the molecular mechanisms that characterize the

interactions between bacterial pathogens and their animal or plant hosts, as well as those between plants and their beneficial symbionts. This remarkable record of success encourages an increasing effort to understand another important class of associations, animal-bacterial mutualisms. Of particular interest are those issues that may well be specific to this class of associations (45), such as (i) the role of immunological adaptation in the stability of long-term host-bacterial mutualisms; (ii) the importance of symbiosis as a driving force in the normal developmental biology of many, if not all, animals; and (iii) the molecular basis for specificity and cooperative signaling between animal tissues and their bacterial inhabitants. The symbiosis between *V. fischeri* and *E. scolopes* can serve as an effective model system to approach such questions: it exhibits strain-level specificity, is accessible by using molecular genetic techniques, includes a rapid and complex program of developmental events in the host, and allows the comparison between axenic and symbiotic juvenile tissue differentiation.

In addition to serving as a model system for the study of mutualistic symbioses, certain characteristics of the *V. fischeri*-*E. scolopes* association may also be particularly relevant to studies aimed at understanding the derivation of pathogenic associations involving other *Vibrio* species. It is interesting to speculate that perhaps the numerous pathogenic conditions elicited in mammalian tissues by such organisms as *V. cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* may indeed have their evolutionary roots in certain adaptations that contribute to their nonpathogenic symbioses with aquatic invertebrate hosts known to be important in other stages of the bacterium's ecological cycle (56).

This minireview has described the early events in the initiation and establishment of the *V. fischeri*-*E. scolopes* symbiosis and summarizes what we now know about three steps in the process: the expression of specificity, the regulation of growth, and the appearance of programmed development. Although the stories behind these events are only just beginning to be elucidated, they clearly offer a rich opportunity to tease apart, by experimental manipulation and hypothesis testing, an understanding of the development of an animal-bacterial mutualism.

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