The Biology of Vibrios
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Chapter 14

The *Vibrio fischeri–Euprymna scolopes* Light Organ Symbiosis

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INTRODUCTION

The light organ symbiosis between the bioluminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* has received increasing interest from researchers representing a range of disciplines, including microbiology, zoology, oceanography, immunology, and genetics. Most of the scientists who study this symbiosis are lured to it by a fascination for these two remarkable organisms and their intriguing partnership. This allure is further reflected in the numerous reviews, popularized accounts, and even poetry dedicated to this symbiosis. Such "gee whiz" appeal has engendered great enthusiasm and scientific curiosity, which are powerful motivators, but, ultimately, progress in the field has stemmed from a rare combination of characteristics. Specifically, like all good model systems, this symbiosis is representative of important widespread phenomena while also possessing a unique combination of features that make it experimentally tractable.

WHAT DOES THIS MODEL SYSTEM MODEL?

All animals have associated microorganisms that can contribute to the host's health and normal development, and this native microbiota has recently been recognized as an important underrepresented research focus (Darveau et al., 2003). An improved understanding of the factors underlying these natural symbioses will help face such problems as the (re)emergence of diseases with environmental hosts, the effective application of probiotics, overreactions to commensal bacteria by the immune system, and the negative effects of broad-spectrum antibiotics.

The *V. fischeri–E. scolopes* symbiosis serves to model many animal–bacteria interactions. Like innumerable bacteria–animal interactions, this symbiosis is composed of an extracellular gram-negative bacterium colonizing the microvillous surface of a polarized epithelium. *V. fischeri* also persistently infects the host without causing disease, which is typical of most animal–bacteria associations. Furthermore, like the microbiota of many animals, *V. fischeri* triggers developmental changes in the host. Therefore, insight into the *V. fischeri–E. scolopes* symbiosis may shed light on other host-associated microbiota, including our own.

V. fischeri is also representative of a bacterial family that encompasses important host-associated bacteria, the *Vibrionaceae*. Although often benign colonists of marine animals, some species, such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are also opportunistic or emerging pathogens, and the spread of these pathogens through natural aquatic hosts poses a human health threat. Other species, e.g., *Vibrio harveyi*, *Vibrio anguillarum*, and *Vibrio campbellii*, can cause disease in farmed marine animals. Thus, the *V. fischeri–E. scolopes* symbiosis may also serve as a model for vibrio-host interactions that are important for disease epidemiology and aquaculture.

EXPERIMENTAL TRACTABILITY

As described above, the *V. fischeri–E. scolopes* symbiosis has many features typical of important host-bacteria interactions. However, what sets this interaction apart is that a unique set of features renders it experimentally tractable, allowing approaches that would be difficult to conduct or interpret in other systems.

One requisite feature for tractability is that both partners can be maintained in the laboratory. Typi-

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cal of many vibrios, *V. fischeri* is readily cultured. *E. scolopes* requires more husbandry, notably, a need for live prey. Nonetheless, adults, which are generally <5 cm long, can be kept in small aquaria, and inducing the animals to mate requires only that a male and a female are placed in the same tank. A typical female will lay 500 to 1,000 eggs during a 2- to 6-month reproductive life in the laboratory (Fig. 1). *E. scolopes* can even be raised from hatchlings to reproductive adults in captivity (Hanlon et al., 1997), although this requires live prey of varying sizes. In practice, most researchers replenish their adult breeding stock with wild-caught animals. The animals are common in shallow sandy reef areas less than a meter deep, so catching them does not require expensive equipment.

Ideally, maintaining the symbiotic partners separately is a first step toward experimentally reconstituting the symbiosis. Unfortunately, many bacterial symbionts are transmitted through the host's eggs, making it difficult or impossible to generate symbiont-free animals. However, *V. fischeri* is not present in *E. scolopes* eggs, and hatchlings must acquire *V. fischeri* by horizontal transmission from the environment. This allows controlled establishment of the symbiosis in a laboratory setting. Moreover, wild *V. fischeri* populations and the kinetics of natural infections are known, so experimental infections can be performed to mimic natural parameters.

An unusual and useful characteristic of this symbiosis is that colonization results in bacterial bioluminescence. As described below, this bioluminescence is apparently used as an antipredatory mechanism. Thus, in contrast to many (e.g., nutritional) symbioses, lab-reared *E. scolopes* are not compromised if they lack *V. fischeri* symbionts. Furthermore, in hatchling squid, bioluminescence intensity is correlated with the degree of bacterial colonization, allowing researchers to monitor the progress of infection noninvasively and nondestructively. As in many other systems, the animals can also be anesthetized, homogenized, and plated to determine the number of symbiont CFU. Luminescence data provide details of infection kinetics and help indicate key time points for plating.

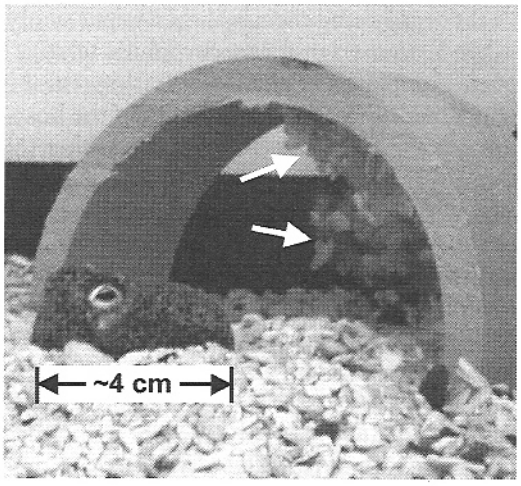


Figure 1. *Euprymna scolopes*. An adult female Hawaiian bobtail squid sits on coralline sand near eggs (indicated by white arrows) that she laid on a PVC half-pipe and on the side of her tank. While housed in a 50-liter tank at the University of Georgia, this female produced 1,616 juveniles over a 4.5-month span.

Another unusual feature that makes this system tractable is that only *V. fischeri* colonizes the host's light organ. Many beneficial bacteria, notably, gut symbionts in animals, live in mixed consortia, and the complexity of these communities makes it difficult to reconstitute a natural system or to tease apart the respective role(s) of different symbionts. The specificity of the *V. fischeri–E. scolopes* symbiosis not only obviates these problems, but it also presents a fascinating platform from which to study how hosts can distinguish "good" bacteria from "bad."

Another unusual property of *E. scolopes* is an anatomy that renders symbiotic tissues readily accessible. The light organ is essentially external and can be viewed simply by pulling back the mantle and funnel. Moreover, the light organ tissue is transparent, so fluorescence-based microscopy can be used to view bacterial colonization inside an intact organ. The light organ is also accessible to experimentally added solutes. Pores connect the outside environment with the site of infection in the light organ, so reagents can be added to cure an infection or monitor biochemical processes at the site of infection. Finally, the light organ can be readily plucked away from other tissues. This sort of accessibility stands in contrast to systems that require delicate surgeries to reach symbiotic tissues of interest.

Other features of the *V. fischeri–E. scolopes* symbiosis are less unique but also contribute to its utility as a model system. *V. fischeri* is genetically manipulable, and the introduction and mutation of genes are now common practice. These approaches are being augmented by the genome sequence of *V. fischeri* (Ruby et al., 2005). Similar progress is being made in nascent genomic and proteomic initiatives in the host (Doino and McFall-Ngai, 2000; Kimbell and McFall-Ngai, 2004). Finally, while the lack of a backbone is hardly a unique trait of *E. scolopes*, there are both ethical and regulatory advantages to studying an invertebrate host.

EVOLUTION AND BASIC BIOLOGY

The first recorded observations of sepiolid squid with bioluminescent bacterial symbionts date to the early 20th century. These squid, now classified in the genera *Sepiola* and *Euprymna*, are found in the Mediterranean Sea and the Pacific Ocean. Although the original reports are hard to find today, most of the seminal works are reviewed in the wonderfully complete book *Bioluminescence* (Harvey, 1952). In each species, a two-lobed organ was found closely associated with the ink gland, in the mantle cavity, near the animal's ventral surface. Of particular note, in 1928 Kishitani made detailed drawings of sections through a *Euprymna* light organ, revealing epithelia-lined pockets filled with bioluminescent bacterial symbionts. Kishitani also described pores connecting these pockets to the light organ surface, and reported that the light organs had reflective tissue on the dorsal surface and a lens on the ventral surface.

Selective Advantages

The light organ architecture suggests that it functions in the camouflaging behavior known as counterillumination (Fig. 2), wherein marine animals emit light downward, matching the light from above and obscuring their silhouette from predators beneath them (Harper and Case, 1999). This is consistent with the light organ morphology of adult *E. scolopes*, wherein reflector and lens tissue direct bioluminescence downward and the ink sac can be used as a shutter to control the amount of light emitted. Furthermore, the amount of light emitted by adult *E. scolopes* is correlated with the intensity of down-welling light

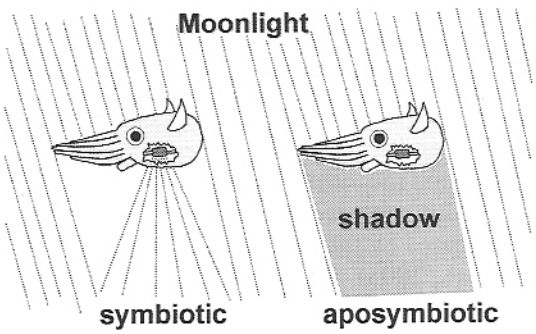


Figure 2. Proposed counterillumination behavior of *E. scolopes*. Based on light organ architecture, it is thought that an adult *E. scolopes* with bioluminescent symbiotic *V. fischeri* can obscure its silhouette with a controllable, ventrally directed luminescence, whereas an aposymbiotic (lacking symbionts) squid would cast a shadow.

(Jones and Nishiguchi, 2004). Camouflaging also seems to be a general strategy for *E. scolopes*, which routinely coats itself with sand (Shears, 1988), changes color to hide, and emits distracting squid-sized ink blobs when threatened (Anderson and Mather, 1996). Conversely, reports of animals emitting brief flashes of light (Moynihan, 1982) or even a persistent bright glow (A. Wier, personal communication) suggest that luminescence may have functions other than camouflaging. More detailed behavioral studies will help determine the benefit(s) of the symbiosis to the host.

The selective advantage of the symbiosis to *V. fischeri* is clear. Each morning, the nocturnal squid bury themselves with sand, and the light of dawn triggers the expulsion of most symbionts into the environment (Boettcher et al., 1996). The remaining *V. fischeri* regrow throughout the day, through two to four generations, so that the light organ is repopulated by the time the animals resume their nocturnal behaviors. As a result of this diurnal "venting," *V. fischeri* populations are relatively large in habitats occupied by *E. scolopes* (Lee and Ruby, 1994b). Further ecological studies and strain typing (Lee and Ruby, 1994a) leave little doubt that in near-shore Hawaiian waters the ability to colonize *E. scolopes* is a strong selective force on *V. fischeri*.

Coevolution

The *V. fischeri*–squid symbiosis appears to be an ancient association in which the host and bacteria have fine-tuned the specifics of their partnership. Many species in the *Sepiola* and *Euprymna* genera have light organs, and their bioluminescent symbionts are either *V. fischeri* or its psychrophilic relative *Vibrio logei* (Fidopiastis et al., 1998; Nishiguchi, 2000). In general, the molecular phylogenies of the hosts and their respective symbionts parallel each other (Nishiguchi et al., 1998; Nishiguchi, 2002), consistent with a model whereby a light organ symbiosis arose in an ancient squid, which coevolved with its symbionts as it speciated. Both early histological and recent molecular studies suggest that this original light organ may have arisen from another tissue colonized by bacteria, the accessory nidamental gland (Harvey, 1952; Nishiguchi et al., 2004).

This model of a coevolved association also fits the observation that *V. fischeri* isolates from the light organs of monocentrid fishes constitute a distinct lineage from the squid-associated *V. fischeri* (Lee, 1994). Coevolution is further intimated by apparent functional coadaptations between hosts and their respective symbionts. For example, *V. fischeri* isolated from the light organs of the fishes *Monocentris japonica*

and *Cleidopus gloramaris* can colonize *E. scolopes*, but the populations attained by these foreign strains in the squid light organ are 10- to 100-fold lower than those achieved by native *V. fischeri* isolates (Ruby and Lee, 1998). Similarly, when *V. fischeri* strains compete for host colonization in mixed inocula, the native strain, or the strain closest to the native lineage, tends to dominate (Lee and Ruby, 1994a,b; Nishiguchi et al., 1998; Nishiguchi, 2002).

Taken together, these data indicate that each host has a closely evolved relationship with its respective (native) *V. fischeri* symbionts. This has at least two practical implications for current research. First, it underscores the importance of using native *V. fischeri* in symbiotic studies. Thus, *V. fischeri* MJ1, a type strain used in many pioneering studies of bioluminescence and isolated from the light organ of *M. japonica* (Ruby and Nealson, 1976; Engebrecht et al., 1983), is not an appropriate strain for studies of the *V. fischeri–E. scolopes* symbiosis. Second, although it has become increasingly clear that some bacteria have shifted host range or tissue tropism rapidly through the acquisition of discrete "islands" of genes, this does not seem to be the case for the *V. fischeri* light organ symbionts. Rather, the *V. fischeri*–squid symbiosis probably predates the speciation of *E. scolopes*, and it seems likely that the composition and regulation of many genes will have adapted to maximize the benefits of this partnership.

Basics of the *V. fischeri–E. scolopes* Association

E. scolopes lives in shallow, sandy reef flats in the Hawaiian archipelago. These solitary predators feed mainly on shrimp and polychaetes as they grow from hatchlings just a few millimeters long to thumb-sized adults (Moynihan, 1982; Shears, 1988). The adults may live for several months but probably not more than a year (Singley, 1983; Hanlon et al., 1997). Females lay clutches of tens or hundreds of eggs, and, as mentioned above, each new generation of hatchlings must acquire *V. fischeri* from the surrounding environment (Wei and Young, 1989).

Most studies of this symbiosis focus on the establishment of *V. fischeri* in the light organ through the first 2 to 4 days after hatching. Hatchlings derive energy from a yolk sac; however, after 4 to 5 days, the animals have apparently depleted these reserves and begin to show signs of stress. Interestingly, such stressed animals can clear out established *V. fischeri* symbionts. Researchers generally terminate experiments before this point, although it is possible to extend studies by feeding the juveniles (Hanlon et al., 1997; Claes and Dunlap, 2000).

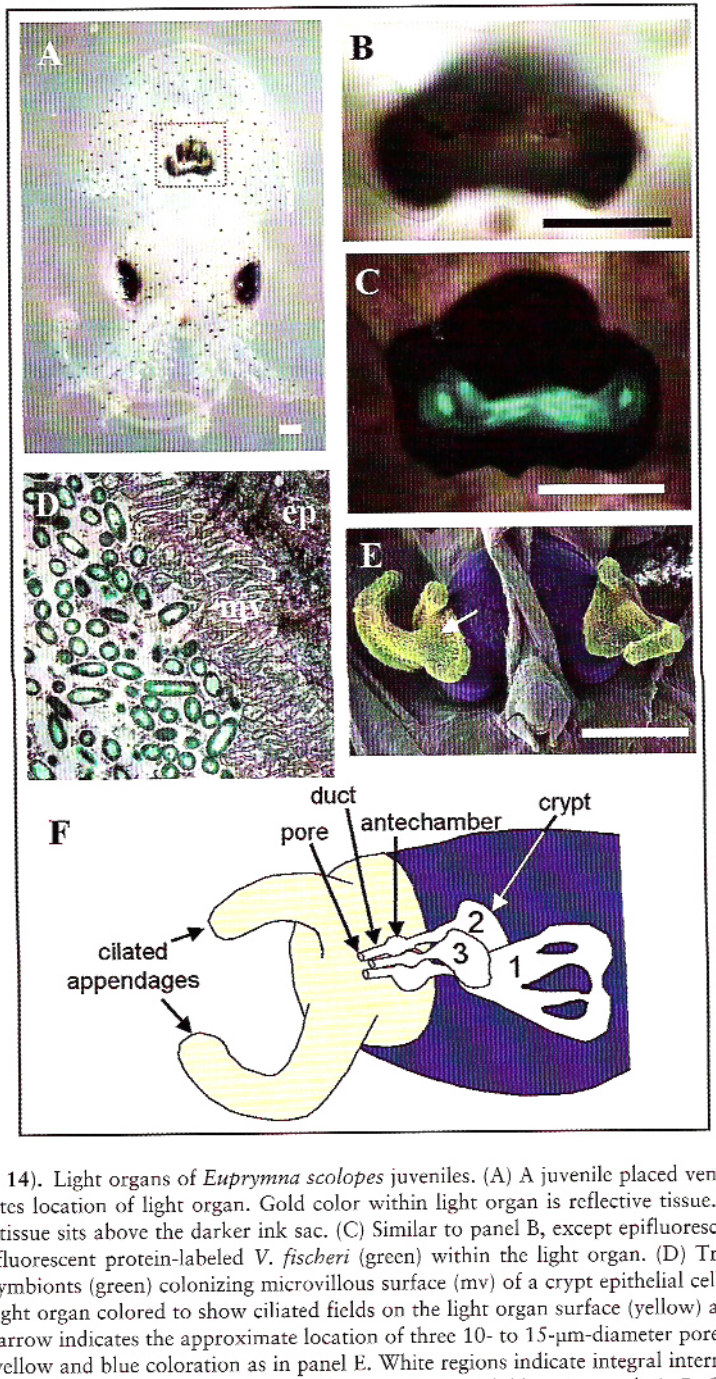
The light organs of juvenile *E. scolopes* are shown in Color Plate 6. The light organ is loosely covered by the mantle on the animal's ventral surface (Color Plate 6A), and it consists of relatively transparent tissue and a reflector closely associated with the ink sac (Color Plate 6A,B). Symbionts labeled with green fluorescent protein can be visualized inside this transparent tissue (Color Plate 6C), and electron microscopy reveals their extracellular association with epithelial microvilli (Color Plate 6D). In hatchlings, each side of the light organ has a field of ciliated cells on its surface, and each ciliated field includes two appendages that often approximate a discontinuous ring (Color Plate 6E). At the base of these ciliated appendages are three pores, each 10 to 15 μm in diameter, that lead through ciliated ducts into distinct "crypts" lined by microvillous polarized epithelial cells (Color Plate 6F). These crypts span a 10-fold range in size from crypt 1, the largest, to crypt 3, the smallest and also developmentally delayed relative to crypts 1 and 2 (Montgomery and McFall-Ngai, 1994). The crypts are topologically complex, encompassing enlarged "antechambers" between the duct and the largest space as well as deep veins extending medially from crypt 1 (Visick and McFall-Ngai, 2000; Millikan and Ruby, 2004). These extensions of crypt 1 have been termed "medial diverticula" (Visick and McFall-Ngai, 2000), although it is uncertain whether these are actually dead-end diverticular projections or an interconnecting network (as depicted in Color Plate 6F). Regardless, this medial region of crypt 1 is densely packed with bacteria once animals become colonized (Color Plate 6C).

EARLY EVENTS IN SYMBIONT ESTABLISHMENT

In the waters inhabited by *E. scolopes*, aposymbiotic hatchlings become infected very quickly (Wei and Young, 1989), and even the smallest animals that have been caught in the wild and examined are already luminescent (Ruby and Asato, 1993). When fresh hatchlings were placed in near-shore Hawaiian seawater, the onset of luminescence was detectable within 8 h (Ruby and McFall-Ngai, 1991). Initial colonization by small numbers of bacteria, which have not yet begun to generate light, occurs even earlier (Ruby and Asato, 1993).

Obstacles To Overcome

The speed and efficiency of symbiont establishment are astounding considering the obstacles to infection. *V. fischeri* is present in Hawaiian waters at densities of only 100 to 1,000 cells/ml (Lee and Ruby,



Color Plate 6 (Chapter 14). Light organs of *Euprymna scolopes* juveniles. (A) A juvenile placed ventral side up and lit from above. Red box indicates location of light organ. Gold color within light organ is reflective tissue. (B) Backlit light organ. Translucent symbiotic tissue sits above the darker ink sac. (C) Similar to panel B, except epifluorescence microscopy reveals the location of green fluorescent protein-labeled *V. fischeri* (green) within the light organ. (D) Transmission electron microscopy of bacterial symbionts (green) colonizing microvillous surface (mv) of a crypt epithelial cell (ep). (E) Scanning electron microscopy of a light organ colored to show ciliated fields on the light organ surface (yellow) and other light organ tissues (blue). The white arrow indicates the approximate location of three 10- to 15-μm-diameter pores. (F) Cartoon depiction of a light organ, with yellow and blue coloration as in panel E. White regions indicate integral internal symbiotic tissues, described in the text, with numbers labeling crypts 1, 2, and 3, respectively. Solid bars in panels A, B, C, and E are ~250 μm.

1995), representing <0.1% of the total bacterial population. The light organ is located in a ~1.3-μl mantle cavity, which one can estimate contains between zero and two *V. fischeri* cells at any given time, and the squid flush the mantle water every half second (Nyholm et al., 2000). Numerically, the odds seem stacked against infection occurring at all.

The prospects for infection appear even bleaker when taking into account the physiological state of *V. fischeri*. The population of 100 to 1,000 *V. fischeri* cells/ml noted above was determined by quantitative hybridization to environmental DNA; however, the *V. fischeri* cells that form colonies on solid media represent only about 1% of this total (i.e., 1 to 10 CFU/ml) (Lee and Ruby, 1992, 1995). The nonculturable majority appear able to initiate the symbiosis, because dilutions that contained <1 CFU/ml but 10 to 100 unculturable cells were still infective (Lee and Ruby, 1995). Thus, these cells resemble certain other viable but nonculturable (VBNC) vibrios. Little is known about the VBNC state of *V. fischeri*, but based on studies of VBNC *V. cholerae* cells (Kondo et al., 1994), VBNC *V. fischeri* may be relatively dormant and nonmotile.

Thus, one could conclude that between zero and two semidormant *V. fischeri* cells are in the mantle cavity of a hatchling squid at any time, and that these cells have about a second to revive, induce motility, and swim into one of the six small pores on the surface of the light organ before being pushed back out into the surrounding water. Yet juveniles are efficiently infected within hours of hatching! This riddle was solved in an elegant series of microscopic experiments using fluorescent labels and stains to observe the bacteria, abiotic bacteria-sized particles, and host components through the early infection process (Nyholm et al., 2000, 2002; Nyholm and McFall-Ngai, 2003). The results of these observations are described in stages below.

Stages in Symbiont Establishment

Stage 0: hatching

Events leading to symbiont infection begin shortly after *E. scolopes* juveniles emerge from the egg. This early time frame is categorized here as "stage 0," to connote processes that occur before contact with the *V. fischeri* that will ultimately establish an infection. Within 10 to 20 s of hatching, the light organ cilia begin beating, increasing the flow of water across the light organ surface (Nyholm et al., 2000, 2002). For the next hour, the light organ enters a mysterious "permissive" phase, during which particles ~1 μm in diameter or smaller are drawn into the crypts (Nyholm et al., 2002). Such particles can include small bacterial cells, even nonmotile cells. However, any cells that are brought into the crypts, including *V. fischeri*, are cleared out again and apparently do not establish an infection. For just a few particles to appear inside the crypts, they must be added to water at a high density (millions per milliliter), which is similar to total bacterial populations in seawater but roughly 1,000-fold higher than native *V. fischeri* populations. Furthermore, the absence of *V. fischeri* during the permissive phase does not seem to attenuate the eventual infection process. Taken together, these data indicate that the light organ somehow samples the environment upon hatching; however, the significance of this permissiveness and whether uptake of *V. fischeri* is relevant remain unclear.

An hour after hatching, the animals cease their permissive, nonspecific uptake of external particles. At about the same time, but in an independent event, epithelial cells in the ciliated epithelial appendages begin shedding mucus (Nyholm et al., 2002). Both sialomucin and neutral mucin are released from these epithelial cells, with the former dominating. This mucus secretion is stimulated by peptidoglycan from either gram-negative or gram-positive bacteria, and entry into the crypts is not required for perception of this signal. With the cilia still beating, mucus is moved onto the light organ surface, and seawater is continuously drawn across this mucus matrix.

Stage 1: aggregation

Water carried into the mantle cavity by ventilation and stirred by the ciliated epithelial appendages brings environmental bacteria into contact with the mucus on the light organ surface. Through this process, initial contact is made between the host and its eventual symbiont(s). Bacteria aggregate in the mucus, forming defined balls of cells visible by fluorescence microscopy (Nyholm et al., 2000). At natural densities of *V. fischeri*, these aggregates may be composed of <20 cells (Millikan and Ruby, 2002). Much larger and easily observable aggregates form when denser inocula are used.

Although other gram-negative bacteria will also aggregate on the light organ surface (Nyholm et al., 2000), *V. fischeri* outcompetes them (Nyholm and McFall-Ngai, 2003). The mechanism(s) that favors *V. fischeri* in the aggregate remains to be determined; however, the presence of nitric oxide (NO) in the aggregates and the observation that inhibitors of NO synthesis enable a nonsymbiotic bacterium to form unusually large aggregates suggest a role for this antimicrobial agent in enriching *V. fischeri* (Davidson et al., 2004). Cell surface functions associated with

attachment or motility may also contribute to formation of robust aggregates by *V. fischeri* (Millikan and Ruby, 2002), although motility is not required for aggregate formation.

Thus, in the aggregation stage, the squid overcome the challenge of obtaining symbionts in a dilute environment by concentrating bacteria on the light organ surface. An enrichment process in the aggregates further helps overcome the fact that *V. fischeri* represents a small minority of the total bacteria present.

Stage 2: migration

In the next stage of infection, bacteria that have sat essentially motionless in aggregates move toward the light organ pores. Usually, this migration occurs 4 to 6 h after hatching (Nyholm et al., 2000). Based on this timing, it appears most bacteria will have been in an aggregate for a few hours before migrating, allowing ample time for the bacteria to sense their new location and change gene expression accordingly. This could explain how, in nature, semidormant VBNC cells are able to revive and establish an infection.

It is not known what triggers the symbionts to proceed from the aggregation stage to the migration stage. It is tempting to speculate that gene expression in aggregates is controlled via "quorum-sensing" density-dependent regulation, and that a "quorum" of bacteria must be reached in an aggregate before migration proceeds. However, the quorum-sensing LuxIR regulon does not appear to be activated even in large aggregates (Nyholm and McFall-Ngai, 2003).

Migration from the aggregates to the pores requires flagellar motility (Nyholm et al., 2000). Moreover, fast-swimming hyperflagellated mutants move prematurely and do not aggregate or migrate normally (Millikan and Ruby, 2002). Thus, both motility and its coordinated regulation are important in this process. Chemotaxis may play a role in directing migration toward the pores, and *V. fischeri* has the unusual ability to sense and swim toward nucleosides and *N*-acetylneuraminic acid, the latter of which is a component of squid mucus (DeLoney-Marino et al., 2003). If one of these compounds, or some other attractant, is more abundant near the pores, this could help the bacteria determine the correct direction in which to migrate.

However, even if *V. fischeri* responds to an attractant from the host, this does not determine symbiont specificity. Other *Vibrio* species will, like *V. fischeri*, migrate through strands of mucus toward the light organ pores (Nyholm et al., 2000). However, once bacteria proceed through a pore into a duct, the *V. fischeri* cells appear uniquely well adapted. Nonsymbiotic bacteria that proceed beyond the ducts are extremely rare, even when they are present at very high concentrations. The mechanism(s) for this specificity is not known but may be mediated in part by the antimicrobial activity of NO (Davidson et al., 2004).

Stage 3: early colonization

Several *V. fischeri* cells may aggregate and migrate, but relatively few actually establish an infection (McCann et al., 2003). Other *V. fischeri* cells may fall victim to the presumed antimicrobial forces preventing infection by unwanted bacteria. When hatchlings were exposed to three isogenic *V. fischeri* strains, even at the relatively high inoculum dose of 5,000 CFU/ml, most animals were colonized by only one strain 1 day later. Although additional strains may continue to make the journey to the light organ crypts over time, initial colonization triggers a series of events (described below) that minimize the chances of such superinfection.

THE LIGHT ORGAN ENVIRONMENT

Once *V. fischeri* has successfully initiated colonization, it must not only persist but must actually grow through three to four generations each day following the diurnal expulsion of cells. Thus, the crypt environment must be hospitable enough to support bacterial growth. On the other hand, the host must prevent spread of this infection to other tissues and must restrict its hospitality to *V. fischeri*, preventing infection by other bacteria. Accordingly, there is great interest in understanding the environment the bacteria are exposed to in the light organ.

Neatly defining the light organ environment is probably impossible, given its heterogeneous and ever-changing nature. For example, bacteria proximal to the ducts or in antechambers may experience conditions different from those of bacteria deeper in the crypts. Moreover, as the squids grow from juveniles to adults, the light organ itself undergoes many changes (Montgomery and McFall-Ngai, 1995; Foster and McFall-Ngai, 1998; Lamarcq and McFall-Ngai, 1998; Claes and Dunlap, 2000; Doino and McFall-Ngai, 2000; Kimbell and McFall-Ngai, 2004). There is also a diurnal rhythm of changes in the light organ environment, illustrated by a relatively disorganized epithelial layer deep in the crypts immediately after diurnal venting, followed by progressive restructuring of the epithelial boundary (M. McFall-Ngai, personal communication). Taken together, these various considerations make the question "what is the light organ environment?" a complex one to answer in a detailed way. Nonetheless, many studies

have suggested how the light organ environment both supports and limits bacterial growth.

Support of *V. fischeri* Growth

A light organ crypt must be nutrient-rich, considering the rapid growth of *V. fischeri* in this environment. Unless unnaturally large inocula are used, colonization starts from a few cells (McCann et al., 2003), but populations reach $>10^5$ cells within hours (Ruby and Asato, 1993). In fact, early in infection, *V. fischeri* may achieve generation times of 20 to 30 min, rivaling the maximum growth rate typically observed in culture (Ruby and Asato, 1993). However, the specific nutrients supporting this growth are not entirely defined.

Numerous free amino acids and peptides are available in the crypts of adult squids (Graf and Ruby, 1998). Glycine is especially abundant in crypt peptides relative to its use in *V. fischeri*, and a glycine auxotroph was unaffected in colonization proficiency (Graf and Ruby, 1998). Arginine, proline, methionine, leucine, cysteine, threonine, serine, and lysine are also available to the symbionts; however, de novo synthesis of these (and possibly other) amino acids is required for full colonization (Graf and Ruby, 1998).

The availability and use of metabolites other than amino acids have gone largely untested, but there is good evidence that other nutrient sources are available. For example, the epithelial cells lining the crypts are glycosylated with mannose (McFall-Ngai et al., 1998). Also, sialomucin is secreted into the light organ crypts, and this could serve as a source of both protein and carbohydrate (Nyholm et al., 2002). Furthermore, dead host cells may be sloughed off the epithelium (Nyholm and McFall-Ngai, 1998), potentially spilling many diverse nutrients to the bacteria. *V. fischeri* grows on a variety of carbon sources, including both common compounds like chitin and unusual growth substrates like cyclic AMP (Dunlap and Callahan, 1993), and the possible roles of these nutrient sources merit further investigation.

How *V. fischeri* generates energy is also unknown. Because bioluminescence requires oxygen, we know that the light organ is not anaerobic. It is possible that aerobic respiration is functional, although its importance is far from certain. Oxygen levels may be relatively low (Boettcher et al., 1996); however, *V. fischeri* apparently possesses cytochrome *bd*- and cytochrome cbb_3-type oxidases, both of which have high affinity for O_2 in other organisms. Indeed, the latter is related to rhizobial FixN, which has very high O_2 affinity (K_m = 7 nM) and is required for symbiotic bacteroid development and N_2 fixation in legume hosts. In light of this, it is tempting to speculate that *V. fischeri* is well adapted for microaerobic respiration. *V. fischeri* may also generate energy via anaerobic respiratory pathways, which are induced in the light organ (Proctor and Gunsalus, 2000). Of particular interest, *V. fischeri* can use trimethylamine-*N*-oxide as a terminal electron acceptor, and trimethylamine-*N*-oxide levels are high (1 to 100 mM) in the tissues of many cephalopods (Yancey et al., 1982; Lin and Hurng, 1985, 1989).

Control of *V. fischeri* Populations

The squid must also keep *V. fischeri* in check and prevent growth of other bacteria. Interestingly, this seems to be accomplished largely by barriers and antimicrobial mechanisms similar to those used by animals to restrict pathogens. For example, tight junctions between epithelial cells help form a barrier, and macrophage-like cells monitor the crypts (Nyholm and McFall-Ngai, 1998). Two additional conserved antimicrobial responses elaborated by *E. scolopes* are production of reactive oxygen species (ROS) and antimicrobial peptides (Nyholm and McFall-Ngai, 2004). The squid possess a halide peroxidase, which uses H_2O_2 as a substrate to generate the antimicrobial ROS hypochlorous acid in the light organ (Weis et al., 1996; Small and McFall-Ngai, 1999), and the host may produce the H_2O_2 substrate via a respiratory burst. Consistent with the model that symbionts face extracellular H_2O_2, *V. fischeri* possesses a highly active periplasmic catalase that contributes to colonization competitiveness (Visick and Ruby, 1998). The hosts also secrete NO synthase to produce antimicrobial NO (Davidson et al., 2004).

Restricting *V. fischeri* infection may also be accomplished in part by limiting the availability of key nutrients. For example, iron availability limits symbiont growth in the light organ, much as it does in other animal–bacteria associations (Graf and Ruby, 2000). Furthermore, because *V. fischeri* motility requires Mg^{2+} (O'Shea et al., 2005), the host might prevent spread of the symbiont to other tissues by limiting Mg^{2+} availability. The squid may also control *V. fischeri* growth, while enhancing the bioluminescence of the symbiont, by maintaining high osmolarity (Stabb et al., 2004). The ionic content and composition of the light organ are not known, but based on ionic control in related cephalopods, these hypotheses are not unreasonable (Robertson, 1965; Yancey et al., 1982).

COLONIZATION FACTORS

Discovering the factors that enable *V. fischeri* to colonize *E. scolopes* has been a major research focus, with many studies using mutational approaches to de-

lineate the genes, and thereby the processes, required to colonize the host. As reviewed elsewhere (Ruby, 1996), mutants that are symbiotically attenuated can have a range of specific phenotypes. Some mutants are attenuated in initiating colonization but are normal thereafter, whereas others are just the opposite. Still other mutants may appear to be essentially wild type unless they are forced to compete in a mixed inoculum. Most mutants characterized to date can be categorized as defective in motility, regulation, nutrition, or surface molecules.

Motility Mutants

Nonmotile mutants unable to swim are the only class of mutants that are absolutely unable to colonize the host (Graf et al., 1994). Motility mutants do form aggregates in the mucus on the light organ surface (Nyholm et al., 2000), but they cannot make the journey from the aggregates through the pores and ducts into the light organ crypts. *V. fischeri* may also elaborate surface-mediated "twitching" motility, but this cannot compensate for a defect in swimming.

Regulation of motility may be critical during the infection process and may be tied to the regulation of other colonization factors. Analyses of spontaneous "hyperswimmer" mutants revealed that having too many flagella may interfere with attachment and aggregation (Millikan and Ruby, 2002). Certain hyperswimmer mutants also displayed pleiotropic phenotypes related to colonization, suggesting that motility may be coregulated with other colonization factors. A subsequent study found that the master regulator of motility, FlrA, also regulates several functions unrelated to motility but potentially tied to colonization.

Motility is down-regulated in the light organ (Ruby and Asato, 1993), raising the question of whether this system is expendable once the crypts are colonized. Contrary to this hypothesis, mutants lacking the flagellin subunit FlaA are preferentially expelled from the light organ during the daily venting process, suggesting a role for flagella in competitive retention and possibly even attachment to the light organ epithelium (Millikan and Ruby, 2004). Furthermore, the flagellar basal body might act as a secretion apparatus for extracellular colonization factors (Young et al., 1999). Future work with conditional motility mutants or microinjection of motility mutants into the crypts should yield insight into what role swimming plays in an established infection.

Regulatory Mutants

Certain regulatory mutants are also impaired in colonizing *E. scolopes*. Unlike amotile mutants, regulatory mutants are still able to colonize the host to some degree; however, their colonization deficiency is generally more severe than that displayed by the other mutant classes. The implication is that certain key regulators control the expression of multiple genes that contribute to symbiotic competence.

Perhaps the most intriguing regulator is RscS, which appears to be the sensor part of a classic two-component regulatory system (Visick and Skoufos, 2001). The name RscS derives from "*r*egulator of *s*ymbiotic *c*olonization-sensor," and *rscS* mutants are severely impaired in initiating infection. *rscS* mutants show no obvious phenotypic deviation from its parent in culture, suggesting that RscS recognizes a host-specific signal and prompts a regulatory response involving symbiosis-specific genes. RscS has a conserved signal transduction domain, but its putative sensor domain is novel, and the signal it recognizes is unknown.

The lack of an obvious phenotype to score in culture has made characterization of Rsc-mediated regulation difficult. Furthermore, *rscS* is not genetically linked to a response regulator, so the putative regulatory RscR component has remained elusive. However, overexpression of RscS elicits changes in expression of a gene cluster critical for symbiotic competence, and this cluster includes a response regulator (K. Visick, personal communication). This exciting development promises to unlock the important symbiotic functions downstream of RscS. A major challenge then will be identifying the environmental cue sensed by RscS.

Mutations in other regulatory systems also affect symbiotic competence, and some but not all of the resultant phenotypes relevant to symbiosis can be identified in culture. As mentioned above, FlrA regulates motility, which is required for symbiosis, but FlrA also regulates other factors that may contribute to colonization. Regulation by FlrA depends on the RNA polymerase sigma factor σ^{54}, and mutants lacking σ^{54} are decreased in motility and therefore do not infect the host (Wolfe et al., 2004). However, σ^{54} also modulates other phenotypes (e.g., biofilm formation) which may be relevant to the symbiosis. Similarly, the two-component global regulator GacS/GacA affects expression of motility, but the symbiotic phenotypes of this mutant suggest that it regulates additional symbiotic factors as well (Whistler and Ruby, 2003; C. A. Whistler, T. A. Koropatnick, M. McFall-Ngai, and E. G. Ruby, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. N-303, 2004).

Mutant analyses also indicate that the regulators LuxR, AinS, and, to a lesser extent, LuxS can contribute to symbiotic competence (Visick et al., 2000; Lupp et al., 2003; Lupp and Ruby, 2004). Each of

these regulates bioluminescence, a known colonization factor described in greater detail below; however, the symbiotic attenuation of *luxR*, *ainS*, and *luxS* mutants may not be fully explained by this phenotype. For example, LuxR also stimulates expression of QsrP, a colonization factor that is periplasmic but otherwise uncharacterized (Callahan and Dunlap, 2000). Another regulator of bioluminescence in culture is LitR; surprisingly, a *litR* mutant actually outcompetes the parent strain for squid colonization in mixed inocula (Fidopiastis et al., 2002). LitR has little or no effect on luminescence levels in the symbiosis, and the enhanced competitiveness of a *litR* mutant is likely due to another property of this pleiotropic mutant.

Thus, several regulators may each control multiple genes involved in the symbiosis. The relatively severe colonization attenuation of these regulatory mutants facilitated the identification of their symbiotic importance, but the challenge ahead lies in delineating how effects on these regulons add up to their net symbiotic phenotype. Defining the suites of genes regulated by σ^{54}, FlrA, GacA, LuxR, AinS, LitR, and LuxS, combined with mutational analyses of those genes, will help tease apart the contributions of various colonization factors in these regulons.

Mutants Affected in Surface and Attachment Functions

Mutant analyses have also indicated that certain cell surface attributes are symbiotically important, although none of these features are essential, and the mutants examined tend to have relatively minor colonization defects. For example, *pilA* encodes a putative type IV-A pilin protein; *pilA* mutants only show a defect in competition with the parent, and even this competition deficiency is quite moderate (Stabb and Ruby, 2003). Similarly, transposon insertions in the *had* locus, which also encodes a putative type IV pilin apparatus, were identified on the basis of a hemagglutination deficiency and were impaired in squid colonization (Feliciano, 2000). Of the two *had* mutants examined, one colonized to about 40% of wild-type levels, and the other displayed diminished symbiotic proficiency only when competed against the parent.

In addition to functioning in attachment, the cell surface acts as a barrier, and colonization defects have been observed for mutants altered in barrier functions. For example, a *pgm* mutant has shorter lipopolysaccharide (LPS) and increased sensitivity to detergents and cationic agents and does not colonize as well as its parent (DeLoney et al., 2002). Similarly, an *ompU* mutant lacks an outer membrane protein that may act as a porin, has increased sensitivity to detergents and the antimicrobial peptide protamine sulfate, and displays a slight decrease in colonization efficiency at low inoculum doses (Aeckersberg et al., 2001).

These data suggest that surface molecules associated with permeability and attachment may play a role in the symbiosis. However, multiple and somewhat redundant adhesins may be involved in attachment, because colonization phenotypes of strains with single-gene mutations have been relatively subtle so far. Genomic analyses, discussed below, appear to support the notion of semiredundant attachment mechanisms, especially pili (Ruby et al., 2005).

Nutritional Mutants

V. fischeri mutants with specific nutritional requirements are also attenuated in colonization of *E. scolopes*. Although some scientists are reluctant to label metabolic pathways "colonization factors," analyses of these mutants have unquestionably helped elucidate the nutritional environment of the light organ crypts and the requirements for symbiosis. Most notably, mutant analyses have revealed that siderophore-mediated iron acquisition and the ability to synthesize certain amino acids contribute to light organ colonization proficiency (Graf and Ruby, 1998, 2000).

THE IMPORTANCE OF BIOLUMINESCENCE

One of the most intriguing colonization factors is bioluminescence. Long considered an energetic drag on cultured cells, luminescence is critical for full colonization of the *E. scolopes* light organ by *V. fischeri* (Visick et al., 2000). Much is known about the genetics and biochemistry of bioluminescence, and several hypotheses have been proposed to explain why it may be useful to the bacteria during colonization of a host. These hypotheses and approaches for testing them have been reviewed elsewhere (Stabb, 2005) and will be described below.

In bacteria, light is generated by a heterodimeric luciferase composed of the *luxA* and *luxB* gene products. LuxAB sequentially binds reduced riboflavin 5′-phosphate ($FMNH_2$), O_2, and an aliphatic aldehyde, which it converts to flavin mononucleotide (FMN), water, and an aliphatic acid, generating light in the process (Hastings and Nealson, 1977; Tu and Mager, 1995). The *luxC*, *luxD*, and *luxE* genes flank *luxA* and *luxB* and are responsible for (re)generating the aldehyde substrate (Boylan et al., 1989; Meighen, 1994). Regeneration of $FMNH_2$ and the aldehyde substrate relies on reduction by NAD(P)H, and, in the

latter case, ATP hydrolysis. *luxG*, located downstream from *luxE*, encodes a protein that shuttles reducing power from NAD(P)H to FMN.

Despite this detailed knowledge, the selective advantage(s) of bioluminescence to bacteria remains a controversial topic (Nealson and Hastings, 1979; Timmins et al., 2001). The apparent costs of bioluminescence have especially motivated curiosity in its utility. These costs include sizable biosynthetic input (LuxAB can make up 5% of the protein in bright cells [Hastings et al., 1965]), ATP hydrolysis, and the consumption of reducing power and oxygen, which could otherwise theoretically be used to generate energy from aerobic respiration. Although an advantage for the host (e.g., antipredation) could confer a fitness advantage on the bacteria, thereby rationalizing such energetic input, this cannot explain why dark *lux* mutants are decreased in fitness in the absence of a selective pressure for the host (Visick et al., 2000).

Several hypotheses have been proposed to explain how bioluminescence aids bacteria directly. Two of these propose that light itself is the key. One hypothesis is that the squid detect luminescence in the light organ and impose sanctions on dark infections. This would allow the host to ensure that it received bioluminescence in exchange for the nutrients it provides the symbionts, much as legumes limit rhizobial symbionts that do not fix nitrogen (Kiers et al., 2003). Another hypothesis states that bioluminescence stimulates DNA repair mediated by photolyase (Czyz et al., 2003). Experimental data indicate that this hypothesis is possibly correct (Czyz et al., 2000), although it seems unlikely that the conditions used mimic the light organ environment.

Other hypotheses posit that the important function of bioluminescence is to burn oxygen. In consuming oxygen, the symbionts may create a hypoxic stress for the nearby squid epithelium, thereby attenuating ROS production or simply generating an environment that the facultative symbionts are better suited to live in than are the obligately aerobic host epithelial cells (Visick et al., 2000). Bioluminescence could also depress intracellular oxygen concentration in the bacteria, either to increase resistance to oxidative stress in general (Timmins et al., 2001) or to protect specific oxygen-sensitive enzymes. The high affinity of luciferase for oxygen is consistent with its having a role in decreasing either ambient or intracellular oxygen levels.

Another hypothesis is that the relevant reactant is reducing power, which is supplied indirectly from NADH. Luciferase could help recycle NAD^+ cofactor when it becomes limiting as its reduced form, NADH, builds up (Bourgois et al., 2001). In this model, luminescence acts as a valve to release excess reductant and becomes important for symbiotic bacteria when biomass production, another electron sink, becomes limited by spatial constraints in the light organ.

At present, none of these hypotheses can be ruled out. Indeed, it is possible that more than one is correct. For example, the host may recognize light production and impose a sanction of ROS on dark infections, while bioluminescence may also prevent or minimize the effects of ROS by lowering the ambient oxygen. Conversely, the models proposing that bioluminescence acts as an electron sink or as an antioxidant may be mutually exclusive, as they imply a reducing or oxidizing environment, respectively. Predictions can be drawn from each of the above hypotheses, and experimental analyses are under way to test them (Stabb, 2005).

INTERSPECIES RECOGNITION AND SIGNALING

Only *V. fischeri* colonizes the *E. scolopes* light organ, and this infection triggers specific developmental responses in the host. If kept in *V. fischeri*-free seawater, *E. scolopes* can be raised to adulthood without the light organ's becoming infected or developing bioluminescence (Hanlon et al., 1997). In most respects, such "aposymbiotic" animals mature normally (Claes and Dunlap, 2000); however, several distinct developmental changes do not occur without *V. fischeri*.

Specific developmental events triggered by *V. fischeri* symbionts include (i) regression of the ciliated fields accompanied by increased trafficking of hemocytes to the ciliated appendages and apoptotic cell death (Montgomery and McFall-Ngai, 1994; Foster and McFall-Ngai, 1998; Koropatnick et al., 2004), (ii) cessation of mucus shedding by the ciliated fields (Nyholm et al., 2002), (iii) constriction of the ducts (Kimbell and McFall-Ngai, 2004), (iv) downregulation of NO synthase (Davidson et al., 2004), (v) swelling of the crypt epithelial cells (Montgomery and McFall-Ngai, 1994), (vi) proliferation of the epithelial microvilli (Lamarcq and McFall-Ngai, 1998), (vii) increased mucus secretion in the crypts (Nyholm et al., 2002), and (viii) many molecular changes that have yet to be linked to distinct processes (Doino and McFall-Ngai, 2000; Kimbell and McFall-Ngai, 2003). Nyholm and McFall-Ngai (2004) reviewed these symbiont-triggered events and their timing.

Many of these developmental events are easily rationalized. Once the squid are colonized by *V. fischeri*, continued harvesting of bacteria from the environment is unnecessary and may encourage undesirable infections, so removal of the mucus-shedding

bacteria-collecting ciliated fields and constriction of the ducts are understandable. Meanwhile, inside the crypts, cell swelling and microvillar proliferation may help increase the surface area of contact between symbiont and host, allowing more efficient exchange of metabolites between the partners.

Some of the events triggered by *V. fischeri* are reversible by curing *E. scolopes* of its symbionts with antibiotics (Lamarcq and McFall-Ngai 1998; Nyholm et al., 2002). Although there are many explanations for this observation, one possibility is that some developmental changes are linked to an ongoing metabolic exchange between the animal and its symbionts. Interestingly, the swelling of the crypt epithelial cells is both reversible upon curing the symbionts and requires that the *V. fischeri* cells are bioluminescent (Visick et al., 2000). This swelling is consistent with the epithelium's experiencing hypoxic stress and could be tied to ongoing oxygen consumption by bioluminescence. Cell swelling could also represent a developmental response of the animal to light or to metabolic by-products related to the physiology of bioluminescence.

Other developmental programs cannot be stopped once they are set in motion. This includes the regression of the ciliated epithelial fields, which becomes irreversible 12 h after hatchlings are exposed to *V. fischeri* (Doino and McFall-Ngai, 1995). The regression process is triggered remotely, in that morphological changes occur several cell layers away from the bacterial infection. LPS from the bacteria triggers apoptosis in the ciliated field but not regression of this structure (Foster et al., 2000). A peptidoglycan monomer (PGM) can stimulate regression and acts synergistically with LPS to elicit a response closely resembling that induced by symbiotic infection (Koropatnick et al., 2004). Although LPS and PGM purified from other bacteria can also stimulate these responses in *E. scolopes*, it is likely that the squid only experience high levels of these two signaling molecules in infected light organs.

The observation that LPS and PGM stimulate developmental processes in a mutualistic animal–bacteria association is notable for at least two reasons. First, these molecules are also recognized by the innate immune systems of animals and often trigger antimicrobial responses. This suggests conserved mechanisms for mutualist and pathogen detection by animals, with host responses being context dependent. Second, the particular PGM molecule shed by *V. fischeri* is identical to "cytotoxins" of pathogens *Bordetella pertussis* and *Neisseria gonorrhoeae* (Koropatnick et al., 2004), revealing an interesting and unanticipated similarity between mutualistic and pathogenic bacteria–animal associations (Table 1).

INSIGHTS FROM GENOMICS

Rapid advances in genomics are transforming research on the *V. fischeri–E. scolopes* symbiosis. Given the relative genetic simplicity and manipulability of *V. fischeri*, it is not surprising that genomics is further along in the bacterial symbiont; however, genomic approaches are being applied to both partners and promise to revolutionize investigations of this symbiosis.

The *V. fischeri* Genome

The complete annotated genome of *V. fischeri* strain ES114 was recently published (Ruby et al., 2005). ES114 was picked from among various *E. scolopes* isolates in part because it contains a large (45.8-kb) plasmid, pES100, which represents a group of plasmids often found in *E. scolopes* symbionts. ES114 also lacks small multicopy plasmids, which would be overrepresented in DNA pools, thereby confounding sequencing efforts. Many *E. scolopes* symbionts contain small plasmids, however, so a representative of this group, pES213, was recently sequenced and characterized to complement the genome project (Dunn et al., 2005).

V. fischeri ES114 became the fourth *Vibrio* species to have its genome published, joining pathogens *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*. As the lone mutualistic *Vibrio* species sequenced,

Table 1. Varied roles for peptidoglycan monomer

Bacterium	Host	Result of infection	Activity of shed *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine
Bordetella pertussis	Human	Whooping cough	Causes death in ciliated airway epithelial cells
Neisseria gonorrhoeae	Human	Gonorrhea	Causes death in ciliated fallopian tube epithelial cells
Vibrio fischeri	Hawaiian bobtail squid	Bioluminescent light organ mutualism	Triggers regression of ciliated epithelial fields on light organ surface

these data are enriching studies of genome evolution. Interestingly, each of these *Vibrio* species has two chromosomes, a relatively large chromosome harboring many conserved "housekeeping" genes, and a second, smaller chromosome that is more variable between species. The smaller "chromosome 2" may therefore carry more lifestyle-specific genes and could be rich in genes that function in the symbiosis. Also, *V. fischeri* DNA is more A+T rich than the other *Vibrio* species, which parallels a trend of high A+T content in other bacteria that are especially dependent on associations with an animal host (Rocha and Danchin, 2002).

It would be difficult to overstate the importance of the *V. fischeri* genome database to current and future research. The sequence, which encompasses 4.28 Mbp and 3,802 putative open reading frames, has provided intriguing insight into old questions as well as unanticipated fodder for future research directions. For example, as described above, the subtle symbiotic phenotypes of putative attachment (e.g., pilus) mutants intimated possible functional redundancy. The appearance of no fewer than eight putative type IV pilus loci along with other putative adhesins supports that conjecture. Furthermore, the unanticipated discovery of a homolog to toxin-coregulated pili of *V. cholerae* provides an intriguing new research target (Ruby et al., 2005).

In this and other examples, genomic data provide a rapid way to identify and mutate specific genes to test hypotheses about what is important in the symbiosis. Although targeted mutational approaches were possible before genomics, they involved uncertain and lengthy cloning strategies, negative cloning results were uninterpretable, and possible redundancy was difficult to address. Genomics has streamlined the process of target identification and allows far more rapid cloning and mutant generation, and the resulting data can be interpreted in a broad context.

Analysis of the *V. fischeri* genome has also opened up a qualitatively new approach by allowing a broad assessment of regulatory and metabolic pathways. For example, as described above, RscS, the sensor component of a putative two-component regulatory system, was identified as an important regulator of colonization factors but was not genetically linked to a response regulator (Visick and Skoufos, 2001). A genomic search for putative response regulators unpaired with a sensor revealed several potential targets. This sort of approach would not have been possible without the genome sequence. Genomic analyses have also made it possible to view how the biochemistry of bioluminescence integrates into the overall physiology of *V. fischeri*, which should help define its role in symbiosis (see above and Stabb, 2005).

Microarray-based approaches will also shed light on the symbiosis, for example, by defining the suites of genes controlled by regulators that are important in the symbiosis (e.g., RscS/RscR). Ideally, microarrays will also elucidate the changes in global gene expression associated with entering the symbiosis. One hurdle to this approach is that obtaining sufficient mRNA from the bacterial symbionts currently requires the use of adult animals, which are not necessarily infected by strain ES114. This approach could also miss important regulatory events specific to establishing the symbiosis. If these do present a problem for interpreting microarray results, this should be overcome by technologies that require less input mRNA or by improved methods for large-scale recovery of symbionts from juvenile squid infected with ES114.

Comparative genomics will also be a valuable research tool. Comparison of *V. fischeri* with other sequenced *Vibrio* species already sheds light on the evolution of *V. fischeri* as a symbiont. In the future, sequencing of other *V. fischeri* strains, both those that infect squid efficiently and those that do not, will further define what enables a strain to partner with *E. scolopes*.

E. scolopes Genomics

Proteomic and subtractive cDNA experiments have revealed that the animal undergoes many molecular changes during the initiation of the symbiosis (Doino and McFall-Ngai, 2000; Kimbell and McFall-Ngai, 2003), and this is now being augmented by genomics. Fourteen thousand expressed *E. scolopes* genes have been sequenced and placed on a microarray (Nyholm and McFall-Ngai, 2004), and differences in gene expression in aposymbiotic animals and infected animals are being examined. These data will funnel into existing technologies for quantitating transcript abundance using real-time PCR and visualizing expression patterns in situ with fluorescent probes (Kimbell and McFall-Ngai, 2004). Mutational approaches are not yet possible with *E. scolopes*, but RNA interference technology may ultimately allow researchers to test the symbiotic importance of specific genes by disrupting their expression. The expressed sequence tag database is also being used to identify gene products that may be involved in LPS- and peptidoglycan-mediated signaling, including homologs of the receptors and signal transducers known to function in the innate immune systems of animals. In this way, genomic approaches in *E. scolopes* are helping connect the gross morphological changes associated with the symbiosis to changes in gene expression and underlying molecular mechanisms.

NEW PERSPECTIVES

Studies of the *V. fischeri–E. scolopes* symbiosis have yielded fresh insights into host-animal interactions. The remarkable specificity of this symbiosis has helped dispel the notion that invertebrate immune systems are "primitive" or indiscriminate, as these squid clearly possess sophisticated mechanisms for recognizing and controlling their microbiota. Another new insight has been the revelation that the same molecules that function in pathogenesis and pathogen detection can also function as signaling molecules in an animal–bacteria mutualism.

The *V. fischeri–E. scolopes* symbiosis is also yielding new perspectives as an archetype *Vibrio*-host interaction. Many *Vibrio* species associate with animals, but often the evolutionary and ecological relevance of the laboratory models studied are uncertain (Klose, 2000). The *V. fischeri–E. scolopes* symbiosis ties experimental tractability in the laboratory to an animal model that is relevant to the life history of the bacteria in the real world. Accordingly, studies of regulation, signaling, adherence, evolution, and physiology in the *V. fischeri–E. scolopes* system promise to shed new light on many associations between marine *Vibrio* species and animal hosts.

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