Sepiolids and vibrios: When first they meet
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Sepiolids and Vibrios: When First They Meet

Reciprocal interactions between host and symbiont lead to the creation of a complex light-emitting organ

Margaret J. McFall-Ngai and Edward G. Ruby

Microbiologists have long recognized that one of the principal ecological niches of bacteria is the cell surface of animal tissues. However, although the majority of these associations are either commensal or, quite often, beneficial, most studies of animal-associated microbes have focused on those rare bacterial species that cause disease. Similarly, zoologists usually ignore the normal associations that animals have with their microbiota and instead consider symbioses with bacteria as rare, highly derived conditions. These biases have arisen not only from the perspectives that these two fields of biology have developed historically, but also because an understanding of even the most common types of animal–bacterial symbioses (i.e., skin or enteric tract associations) is inherently inaccessible. For none of these ubiquitous associations has the consortium of microbial species even been fully identified, and among those taxa that have been identified, a substantial subset remains unculturable under laboratory conditions (Savage 1977, 1986).

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This symbiosis offers unique opportunities to understand the processes underlying the colonization of animal epithelia by benign bacteria

The symbiosis between the Hawaiian bobtail squid, *Euprymna scolopes*, and its luminous bacterial partner, *Vibrio fischeri*, offers a number of unique opportunities for studying the processes underlying the colonization of animal epithelia by benign bacteria (McFall-Ngai and Ruby 1991, Ruby 1996). First, the association comprises only two partners, a condition that makes the dynamics of the interaction more easily deciphered. Second, the host is relatively accessible and abundant in nature—neither expensive equipment nor substantial effort are required for its collection. Third, both partners of this symbiosis can be easily and independently raised in the laboratory.

In the early 1970s, developmental biologists used *E. scolopes* as a model species for cephalopod embryology (Arnold et al. 1972, Singley 1983) and, in so doing, showed that this squid could be maintained and reared in seawater aquaria. Similarly, for many years microbiologists have studied both the diverse ecology and the molecular biology of the easily cultured species *V. fischeri* (Nealson and Hastings 1991), primarily because of its presence in coastal seawater and its remarkable ability to emit blue-green light. The bioluminescence of *V. fischeri* not only makes it an easily recognizable component of bacterioplanktonic communities, but also led to the use of the genes associated with its light production (i.e., the *lux* genes) as molecular reporters of gene expression in other organisms (Stewart et al. 1996). Thus, *V. fischeri* has become one of the most extensively and broadly studied marine bacterial species.

These characteristics of the association between *E. scolopes* and *V. fischeri* have made it an attractive subject in which to examine the initiation of symbioses between animals and their bacterial partners. In 1989 Sarah Wei, a graduate student at the University of Hawaii, and her advisor, Richard Young, published the first paper describing the initiation of this symbiosis (Wei and Young 1989). They showed that newly hatched juvenile squids become bioluminescent within hours if they are placed in natural Hawaiian seawater; however, the animals do not become luminous when maintained in seawater that has been filtered to remove bacteria. This study provided the first evidence that the onset of the luminescence capacity in *E. scolopes* requires the initiation of a symbiosis between each new genera-

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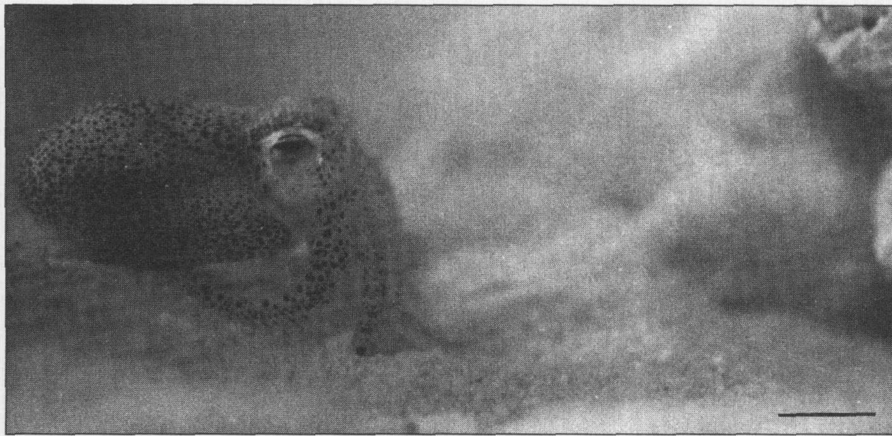

Figure 1. An adult *Euprymna scolopes* rests on the sandy substrate associated with a coral reef. This species and others within the squid family Sepiolidae typically bury in the sand during the day and come out to forage in the water column over the reef during the night. Bar = 10 mm.

tion of host and luminous bacteria present in the ambient seawater environment. It also set the stage for the development of this squid–luminous bacteria symbiosis as an experimental model for studying the role of recognition and specificity in the establishment and maintenance of symbiosis, and the reciprocal influence of each partner on the other's developmental biology during entry into the symbiotic state (McFall-Ngai and Ruby 1991).

In 1988, with the help and guidance of both Richard Young and David Karl, also of the University of Hawaii, we began to collect specimens of *E. scolopes* and to examine their symbiotic bacteria. Over the ensuing nine years, first at the University of Southern California and now at the University of Hawaii, we have begun to understand the basic nature of this symbiosis. This effort has benefited heuristically from dramatic progress in two areas of research: bacteria-induced diseases of animals, whose underlying mechanisms may well be homologous with those in cooperative relationships (Salyers and Whitt 1994), and root nodule symbioses between leguminous plants and nitrogen-fixing bacteria, which are analogous cooperative associations (van Rhijn and Vanderleyden 1995). For example, regulatory genes and toxins associated with virulence in pathogenic species of *Vibrio* can be found in *V. fischeri* (Reich and Schoolnik 1994, Reich et al. 1997). In addition, the complex, reciprocally induced developmental biology of the *Euprymna* light organ symbiosis has many parallels with that of root nodule formation. Both the squid and the legume exhibit a presymbiotic propensity for bacterial colonization, as well as morphological structures that reflect the inoculation, maturation, and persistence stages of the association's development (Hirsch 1992, Montgomery and McFall-Ngai 1993, 1994). In the plant symbioses, dozens of genes in the host and in the bacterial symbiont mediate the temporal program of nodulation (van Rhijn and Vanderleyden 1995). Within the squid–luminous bacteria symbiosis, we are just beginning to discover that a conversation of similar complexity occurs between the two partners. To date, most of our studies have focused on describing the initiation of this relationship (Ruby 1996, McFall-Ngai 1998). Thus, following a general introduction to the symbiosis, we describe what we have learned about the first 12 hours of the interaction between the juvenile *E. scolopes* and symbiotically competent *V. fischeri.*

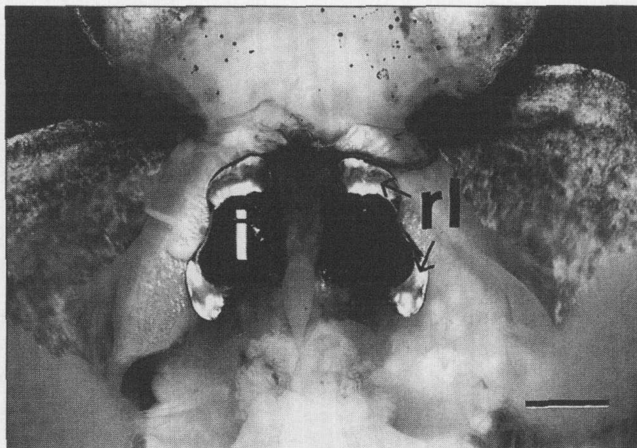

Figure 2. A ventral dissection of an adult *Euprymna scolopes* reveals the bilobed light organ. The core of the organ (not visible in the dissection) contains the crypt epithelial cells of the host and the bacterial symbionts that are supported by these cells. This symbiotic core is surrounded by accessory tissues that modify the bacterially produced luminescence. These accessory tissues include diverticula of the ink sac (i), which serve to control intensity of the light emission, and a reflector overlain by a lens (rl; indicated by arrows), which directs and diffuses light over the ventral surface of the animal. Bar = 5 mm.

The mature association

E. scolopes is one of several sepiolid squid species that have bacterial light-emitting organs (Figure 1; Nesis 1987). This small (the average adult mantle is 25 mm long) cephalopod is indigenous to the Hawaiian archipelago, where it lives in the shallow sand flats associated with coral reefs (Berry 1912). *E. scolopes* buries in the sand during the day and emerges at night to forage in the water column as a predator on invertebrates and small fishes (Moynihan 1983). In the center of its mantle cavity is a bilobed light-emitting organ (Figure 2) that consists of several differentiated tissues, including a core of epithelial tissue that supports a luminous bacterial culture and accessory tissues, such as a reflector and lens, which serve to modify the bacterially produced light so that it can be used by the host. Although specific behavioral uses of luminescence by the squid host have not been observed in nature, the morphology and position of the light organ suggest that it participates in a camouflaging strategy called counter-illumination

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(McFall-Ngai and Montgomery 1990, Montgomery and McFall-Ngai 1992). In this type of luminescence behavior, the animal emits a ventral glow that is of the same intensity and color, and is projected in the same direction, as downwelling moonlight and starlight (McFall-Ngai 1990). As a result, predators looking up from below cannot perceive the silhouette of the animal's body.

In Hawaii, *E. scolopes* reproduces over the entire year and readily mates and lays eggs under laboratory conditions (Singley 1983). During mating, the male bobtail squid passes a spermatophore into the female's mantle cavity using its sex-specific hectocotylized arm. When the female is ready to lay a clutch of eggs, she breaks open the spermatophore and fertilizes her eggs (Singley 1983). In captivity, females generally lay their eggs on hard substrates, such as coral rubble (Figure 3) or plastic piping, and they camouflage the egg mass by coating it with a layer of sand. Unlike many other cephalopods, such as the octopods, *E. scolopes* exhibits no parental care, leaving the eggs of the clutch to develop on their own. Also, unlike many other squid and octopus species, the female bobtail squid does not die after egg-laying. When a female in a laboratory aquarium is mated regularly (i.e., once each week), she will lay a dozen or more clutches, averaging approximately 200 eggs each, over a period of a few months. Maintaining a population of a dozen females has thus provided our laboratory with between 60,000 and 100,000 juveniles per year.

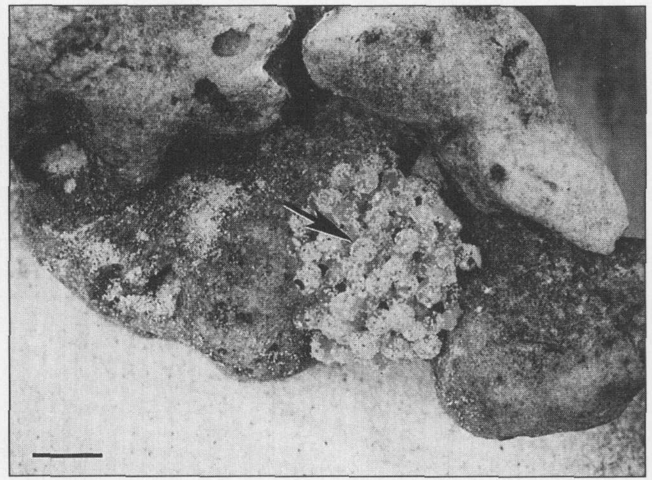

Figure 3. Clutches of eggs that have been attached by female *Euprymna scolopes* to pieces of dead coral. Typically, a female lays the eggs of a clutch one at a time over several hours during the night. She covers the eggs with sand before leaving them to develop on their own. The clutch shown contains approximately 150 eggs; the arrow indicates a single egg of the group. Bar = 10 mm.

The presymbiotic partners

The embryonic period of *E. scolopes* varies from 18 to 26 days, depending on the incubation temperature. During this time, the squid embryo develops specific tissues that will prepare the nascent light organ to be efficiently infected by *V. fischeri* cells that are present in the ambient seawater (Montgomery and McFall-Ngai 1993). About midway through embryogenesis, a thickening of the

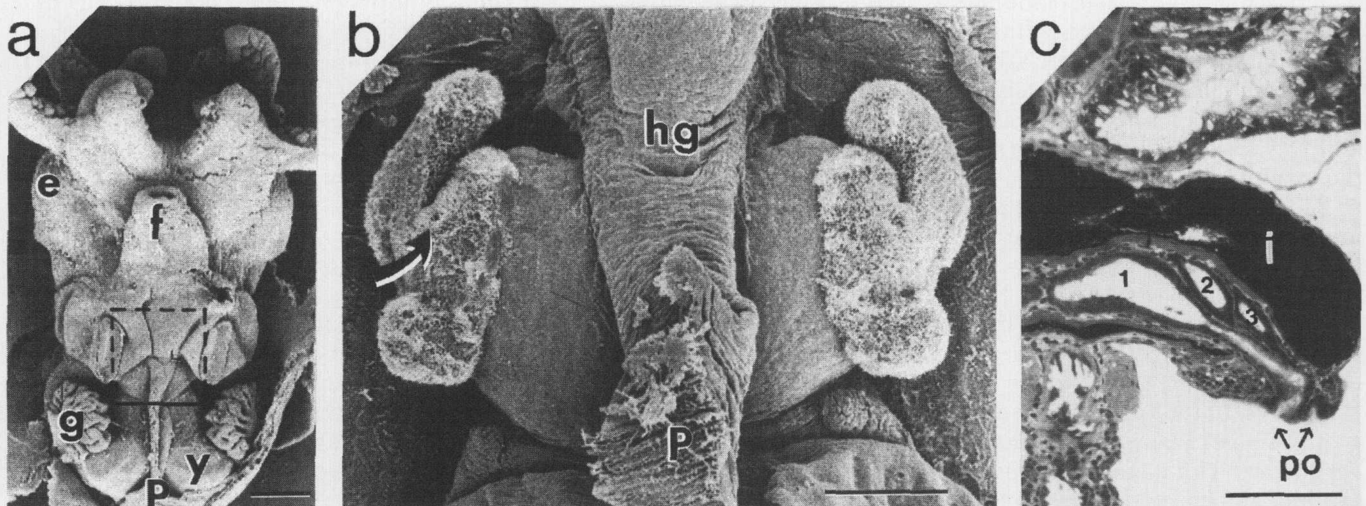

Figure 4. The light organ of a juvenile *Euprymna scolopes* at the time of hatching has a distinct anatomy and morphology that is very different from that of the mature organ of an adult animal. (**a**) A scanning electron micrograph (SEM) of a ventral dissection of a hatchling squid reveals that the light organ is situated beneath the funnel; the light organ is found in the area that is highlighted by the box. During ventilation, the squid brings water into the mantle cavity by expanding the mantle; the water is then expelled through the funnel. Thus, the nascent light organ of the juvenile squid is exposed to the *Vibrio fischeri*–rich water that is flowing through the mantle cavity. e, eye; f, funnel; g, gill; P, posterior; y, yolk sac. Bar = 200 μm. (**b**) In this SEM of a ventral dissection of a juvenile squid, the funnel has been removed to reveal the light organ, which lies under the hindgut (hg). The most conspicuous features on the juvenile organ are the ciliated microvillar fields on the lateral faces of the organ. Each field consists of an anterior and a posterior appendage and a more medial field adnate to the organ surface. At the base of each pair of appendages are three pores (one is visible here and is marked by an arrow). P, posterior. Bar = 100 μm. (**c**) A histological cross-section of one-half of the juvenile light organ shows three epithelial cell–lined crypts (labeled 1, 2, and 3); the opposite side of the organ is a mirror image of this anatomical arrangement. Reconstructions of serial sections through the organ reveal that the six crypts do not interconnect. In this micrograph, the pores (po) for crypts 1 and 2 can be observed (arrows). i, ink sac. Bar = 100 μm.

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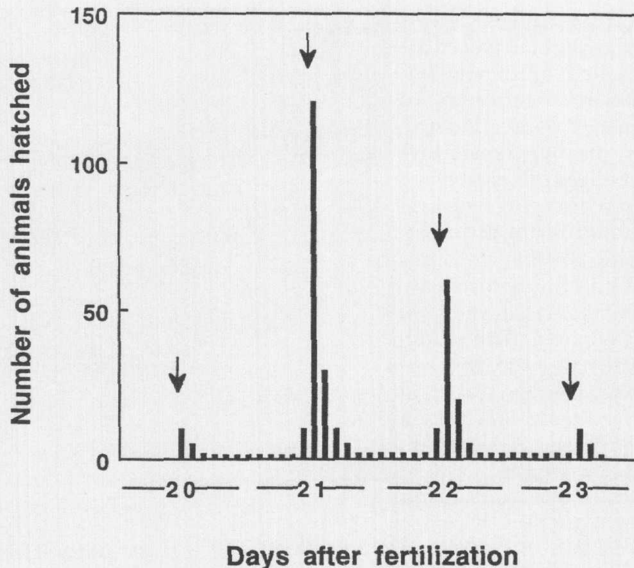

Figure 5. Juvenile *Euprymna scolopes* from a single clutch of eggs emerge over several days. Animals hatch in large numbers within minutes of sunset (arrows) over a period of several days. The clutches vary not only in the numbers of eggs, but also in the number of days following fertilization that the first hatching occurs and in the number of days over which the clutch continues to emerge. On the evening of the first day of hatching, which for the clutch illustrated occurred 20 days postfertilization, only a few animals will typically emerge. The majority of the animals hatch after sunset of the second and third days. Over the subsequent days, the number of juveniles hatching dwindles until all animals in the clutch have emerged. Most animals emerge during a period of one to two hours following sunset; only an occasional animal emerges during the rest of each night. Hatching can be experimentally delayed by retaining the clutch in the light.

mesenchyme associated with the hindgut–ink sac complex heralds the beginning of light organ development. Two tissues essential to the infection process are then elaborated: a superficial ciliated microvillus field that will serve to potentiate the bacterial inoculation, and a series of epithelia-lined crypts that will provide the eventual colonization site of the symbionts (Figure 4).

Because light inhibits egg hatching, all of the juveniles that are prepared to emerge on a given day hatch as a burst soon after sunset (Figure 5). Typically, it takes three to five such bursts for all the juveniles in a clutch to emerge. Such behavior is fortuitous for biologists studying this symbiosis because it allows the synchronization of such procedures as the inoculation of juvenile animals with bacterial symbionts. The newly hatched squid average 1.7 mm in mantle length (Arnold et al. 1972, Singley 1983), and they are easily maintained individually in small glass vials of seawater. The appearance and level of luminescence of sets of these animals can be monitored either by hand in a manual luminometer or continuously in an automated liquid scintillation counter programmed to operate as a photometer (Boettcher et al. 1996).

In nature, the squid hatches into water laden with *V. fischeri*. The cells of this bacterial species have been isolated from coastal marine environments in many parts of the world, where they inhabit seawater and sediments, as well as from the enteric tracts, diseased tissues, and surfaces of marine animals (Nealson and Hastings 1991). Their abundance in seawater, generally a few cells or less per milliliter, has been estimated by counting *V. fischeri* colony-forming units (CFUs) in aliquots spread on nutrient-agar medium. In the vicinity of populations of *E. scolopes*, ambient seawater and sediments have elevated levels of *V. fischeri*, probably due to a diel venting behavior by which the squid expel from 90% to 95% of their symbiont population into the surrounding seawater each morning (Lee and Ruby 1994b). Recent studies of *V. fischeri* in Hawaiian coastal waters (Lee and Ruby 1995) have indicated that only approximately 1% of the viable cells are capable of forming colonies on typical isolation medium; the remaining cells are in a dormant state that has been reported for other species of *Vibrio* as well (Oliver and Bockian 1995). Interestingly, these dormant cells, although temporarily unable to grow on laboratory media, remain symbiotically competent and can efficiently initiate colonization of the juvenile squid light organ (Lee and Ruby 1995). Thus, because *V. fischeri* cells in this dormant state are numerically dominant, they may well be responsible for most of the symbiotic colonization of *E. scolopes* in the natural environment.

Initiation of the symbiosis

A major goal of our laboratories is to discover the events that unfold when a newly hatched *E. scolopes* juvenile is maintained under different ambient conditions. Microscopic analyses have confirmed earlier studies suggesting that the juvenile is free of symbiotic cells when it emerges from the egg: If the animal is placed in natural seawater that is devoid of symbiotically competent *V. fischeri* cells, then its nascent light organ remains free of bacterial colonization (McFall-Ngai and Ruby 1991). This result is particularly remarkable because it proves that the crypts of the nascent light organ resist nonspecific colonization, even when they are exposed to seawater containing millions of other bacterial cells of a variety of species. By contrast, when placed in natural Hawaiian coastal seawater or in aquarium seawater that has either contained adult *E. scolopes* that have expelled symbiont cells or been supplemented with an inoculum of cultured *V. fischeri*, the juvenile squid become colonized within a few hours (Ruby and Asato 1993).

The colonization process begins when ambient seawater passes across the nascent light organ during the normal ventilatory processes of the host—that is, the mantle cavity is first expanded, drawing seawater into the cavity, then collapsed, flushing it out past the light organ and through the funnel. The ciliated microvillar surfaces of the organ appear to potentiate its inoculation with any *V. fischeri* that are present in the passing seawater. High-speed cinematography (Margaret J. McFall-Ngai, University of Hawaii, and Richard Emlet, Oregon Institute of Marine Biology, Coos Bay, OR, unpublished data) of the hatchling organ has revealed that a pair of anterior and posterior appendages on the

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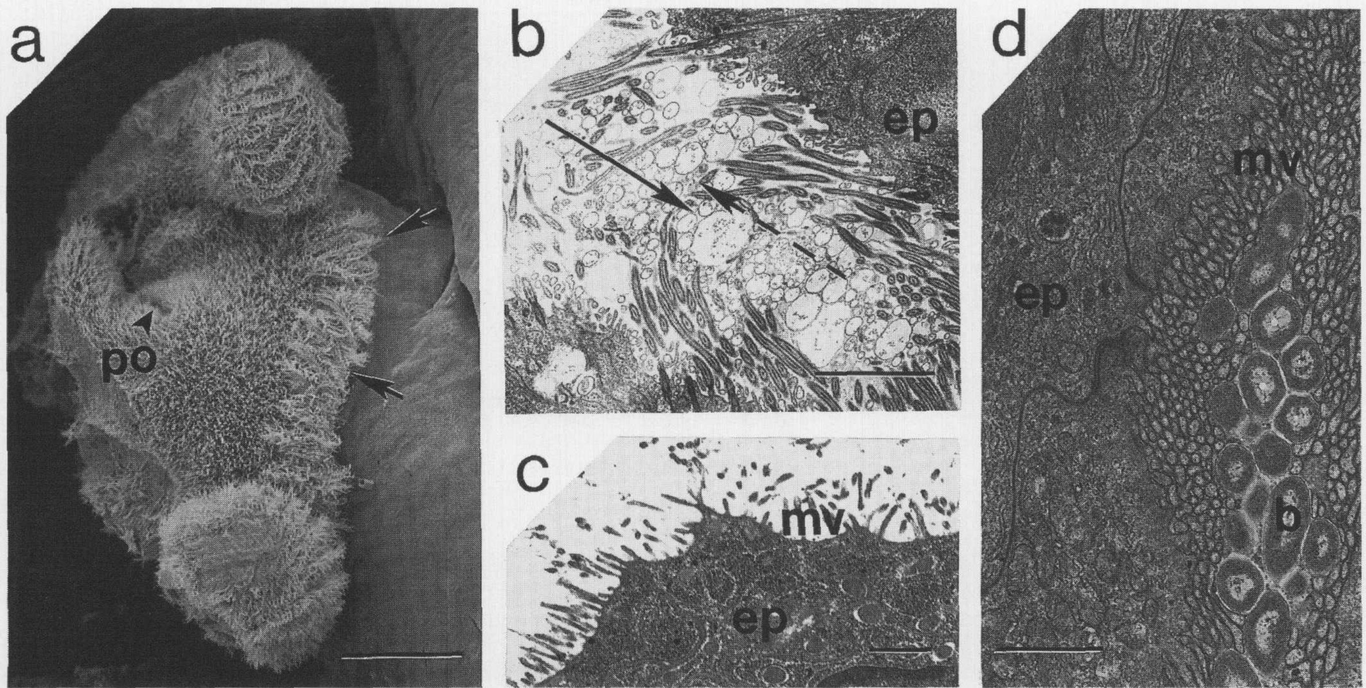

Figure 6. Bacterial symbionts travel a specific pathway on their passage from the mantle cavity to the brush border of the crypts. (**a**) This scanning electron micrograph shows the surface of a juvenile light organ in which the ciliary beat has been arrested midstroke by the application of the tissue fixative. The result of this treatment suggests that the effective stroke of the ciliary rows (arrows) is toward the pores (po; arrowhead) on the light organ surface. Bar = 100 μm. (**b**) The duct that connects the mantle cavity with the crypts is lined by cilia. The ultrastructure of the rootlets of these cilia indicates that they are beating material outward toward the pores (dashed arrow)—that is, the opposite direction from which the bacterial symbionts are entering the crypt spaces (solid arrow). ep, epithelial cell. Bar = 1 μm. (**c**) On entering the crypts, the bacteria associate with the microvilli (mv) of the brush border. Bar = 1 μm. (**d**) A transmission electron micrograph of bacteria (b) associated with the brush border of the adult light organ shows that the microvilli elaborate dramatically during the maturation of the light organ. Bar = 1 μm.

lateral face of each of the ciliated fields (Montgomery and McFall-Ngai 1995) form a ring by the apposition of their tips. Seawater is entrained through this ring by cilia on the appendages, bringing any suspended bacteria into the vicinity of three pores at the base of each pair of appendages. Treating the juvenile light organ with agents that arrest ciliary beat in midstroke has shown that the effective stroke of the superficial cilia appears to be in the plane of these pores (Figure 6a).

The pores lead into a ciliated duct; transmission electron microscopy of the cilia lining this duct, and of their associated rootlets, has indicated that the effective stroke of the ciliary beat is toward the pores (Figure 6b). The crypts of the juvenile organ expand out medially from the base of the ducts, creating dead-end spaces that will be populated by the bacterial symbionts. Once in the crypt spaces, the bacteria interact with the brush border of a polarized, columnar epithelium that is sparsely covered with microvilli (Figure 6c). Studies of the adult light organ indicate that the brush border becomes increasing elaborate during development (Figure 6d).

The presence of bacteria during the first few hours following inoculation causes significant changes in the host light organ (McFall-Ngai 1994). These changes can be divided into those associated with the superficial ciliated microvillus field (i.e., remote from the growing bacterial culture), and those of the crypt epithelia with which the bacteria associate directly (Montgomery and McFall-Ngai 1995). The bacteria trigger the loss of the superficial field through massive cell death over the first four days following inoculation. Although the bacteria must enter the light organ and interact with the crypt cells to initiate this program of cell death, bacteria need only be present for between 8 and 12 hours to initiate the four-day program of cell death resulting in morphogenesis of the organ (Doino and McFall-Ngai 1995). This minimum temporal window was determined by a series of experiments in which the light organ was cured of its symbiont population by treatment with antibiotics at different times after initiation of the association. Thus, the bacteria appear to deliver a transient, irreversible signal that results in the regression of these superficial structures through cell death.

V. fischeri symbionts also cause dramatic changes in the appearance of the cells of the crypts with which they associate directly, including a swelling of these cells that results in a fourfold increase in their volume (Montgomery and McFall-Ngai 1994). Similarly, we have recently found that the density of the microvilli along the crypt brush border increases markedly in response to colonization by bacterial cells (Lamarcq and McFall-Ngai 1998).Thus, the host cells respond to the presence of symbionts along their surfaces in ways not unlike those reported for the colonized epithelium of the enteric tract (Levine et al. 1983). We are currently determining whether these changes in light organ crypt cell mor-

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phology are irreversible or whether removing the bacteria by antibiotic treatment will return the cells to their presymbiotic state.

Further evidence that movement through the pores and into the crypts is essential for colonization has come from genetic studies of symbiotic competence in *V. fischeri* (Ruby 1996). Mutant strains of wild-type symbiotic *V. fischeri* have been constructed that are incapable of initiating a symbiotic infection of the *E. scolopes* light organ. Among these strains, the best characterized are those that are defective in either the production of the flagellar apparatus or its rotation (Graf et al. 1994). In both cases, regardless of the locus of the genetic lesion, the loss of motility was accompanied by an inability to colonize the nascent light organ.

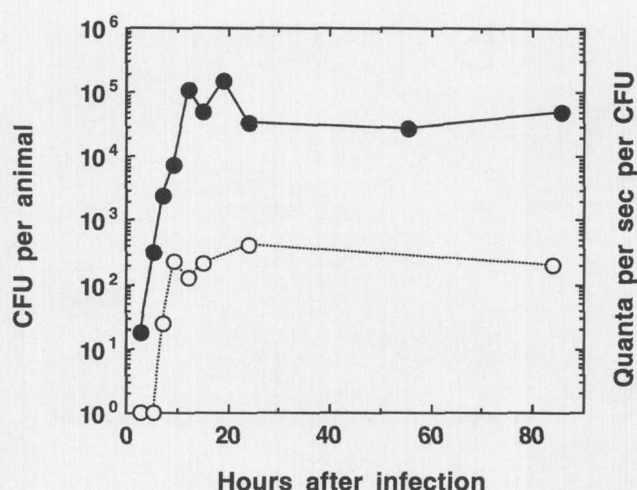

Figure 7. Growth and luminescence of *Vibrio fischeri* cells increase dramatically during the initiation of symbiosis. The light organs of newly hatched juveniles of *Euprymna scolopes* were inoculated by exposing them for three hours to seawater containing symbiotically competent *V. fischeri* cells and then placing them in symbiont-free seawater. At various intervals, the number of bacteria (i.e., colony-forming units, or CFU) present in the light organ of each animal was determined by plating homogenates of the organ on nutrient-agar medium. In addition, the amount of luminescence produced by each light organ homogenate was estimated photometrically. Averages of values obtained for the extent of bacterial colonization of the animals (solid circles) and for specific activity of bacterial luminescence (open circles) were calculated for each time point. Adapted from Ruby and Asato (1993).

Within three hours after newly hatched squids are exposed to symbiosis-competent *V. fischeri* cells, bacteria begin to proliferate in the light organ crypts (Ruby and Asato 1993). The nutrients available for bacterial growth in the crypt environment are of sufficient quality and supply to support a generation time of less than 30 minutes (Figure 7), a value that is near the maximum rate for *V. fischeri* (Boettcher and Ruby 1990). Within 10 to 15 hours, the symbiont population reaches a level of between 10^5 and 10^6 cells, after which it stabilizes. The mechanism setting this level is as yet unknown, but the host may turn out to largely control the final level. Experiments with newly released symbiont cells have suggested that their growth may be oxygen limited, through host control of oxygen delivery to the light organ, during certain periods of the symbiotic interaction (Boettcher et al. 1996).

As already mentioned, each morning over 90% of the symbiont population is expelled from the light organ crypts, and by the onset of nightfall the remaining cells have proliferated back to their characteristic maximum number. The expelled material can be collected and analyzed; thus, the composition of the biochemical environment (e.g., potential nutrients) surrounding the bacteria can be determined. The nature of the nutrients provided to the symbionts by the host has been a matter of considerable speculation (Nealson 1979, Dunlap et al. 1992); however, recent work with mutants of *V. fischeri* has begun to address this fundamental aspect of the symbiosis. Transposon mutagenesis was used to produce a collection of auxotrophs, each of which absolutely requires the presence of one of nine different amino acids for growth (Graf and Ruby in press). These strains are all capable of initiating colonization of the squid light organ; therefore, the host must supply the required amino acids to the symbionts. However, some of the strains are able to reach population levels of only 1–10% of that of the wildtype strain (i.e., 10^4–10^5 cells), suggesting that some of these amino acids are provided in limiting amounts. We are currently investigating the form in which the host provides these amino acids.

During the first 12–15 hours after the association is initiated, at least two significant changes occur in the symbiont cells (Ruby and Asato 1993). First, bacterial luminescence is rapidly induced, as revealed by an increase in the amount of light emitted per cell (Figure 7). This induction is due, at least in part, to the accumulation of the bacterially produced quorum-sensing molecule *N*-(3-oxohexanoyl) homoserine lactone (Boettcher and Ruby 1995). As the symbiont population reaches its maximum density, this continuously excreted molecule builds up in the crypt to levels that lead to a dramatic induction of the *V. fischeri lux* genes and to a resultant 100- to 1000-fold increase in luminescence specific activity (Figure 7). It is this increased light emission that provides the host squid with its source of bioluminescence.

Concomitant with reaching their maximum population density, at approximately 12 hours post-infection, the symbiont cells cease to synthesize a flagellar filament (Ruby and Asato 1993). This observation suggests that the presence of flagella must be of no importance once the bacteria have gained entrance to the crypts. The signal that suppresses the elaboration of this complex structure during the development of the symbiotic association is unknown; however, studies of *V. fischeri* grown in culture suggest that when its average generation time exceeds five hours, flagellar synthesis is suspended (Ruby 1996).

Recognition and specificity during initiation

The observation that the light organ remains sterile in the absence of symbiotically competent *V. fischeri* suggests that specific recognition occurs between the surfaces of the crypt epithelia and the symbionts, that the crypt environment is accessible and hospitable only to *V. fischeri* cells,

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or that a combination of these regulatory processes is operating. Studies of root nodule symbioses have indicated that recognition and specificity are controlled at a variety of levels by a complex pattern of reciprocal signaling between the partners of the symbiosis (van Rhijn and Vanderleyden 1995). Similarly, in the squid–luminous bacteria symbiosis, it is unlikely that either the host or the bacterial symbiont produces a single omnipotent "key determinant" that is sufficient to guarantee the establishment of a stable association. Instead, an array of features appears to be required, any one of which is insufficient to render a potential symbiont capable of establishing a symbiotic relationship with the squid (Ruby 1996). For example, although motility is necessary for infection, it is not sufficient to allow other motile bacteria to infect the light organ crypts.

To date, we have identified a number of indicators of, and processes modulating, the specific recognition between *E. scolopes* and *V. fischeri*. There is increasing evidence that a protein–glycan interaction, a feature common to many associations between bacterial and animal cells (Ofek and Doyle 1994), is an essential part of the recognition process between the squid and its symbiont (McFall-Ngai in press). First, the bacterial symbionts have mannose-recognizing proteins, called adhesins, on their cell surfaces, and the most common glycan on the crypt epithelial surfaces is mannose. Second, when an analog of mannose is added to the surrounding seawater concurrently with the introduction of bacteria, infection of juvenile squid is blocked. Analogs of other sugars (e.g., galactose, fucose, and glucose), by contrast, do not block infection. In addition, evidence is accumulating that additional surface molecules are also involved in the process of recognition (Hensey and McFall-Ngai 1992).

Beyond the presence of positive factors that apparently act to potentiate colonization by *V. fischeri* cells, the light organ crypt environment also may actually be inhospitable to potential colonizers other than *V. fischeri*. For instance, the crypts contain high levels of a peroxidase that is closely related to myeloperoxidase (Weis et al. 1996), an antimicrobial protein present in vertebrate neutrophils. This protein acts in vitro as a halide peroxidase, using hydrogen peroxide to oxidize chloride ions and yielding the highly toxic oxidant hypochlorous acid. The specific activity of this peroxidase is lower in symbiotic hosts than in uncolonized animals, indicating that the presence of the bacterial symbiont somehow modulates the expression of the host peroxidase gene, the production and secretion of the enzyme, or the functional activity of the enzyme. We are currently investigating which of these levels of control operates in the intact symbiosis and how these controls are mediated. The bacteria are resistant to oxidative stress while in the light organs (Boettcher 1994), perhaps because they produce a peroxidase (e.g., catalase) that competes with the halide peroxidase for its hydrogen peroxide substrate. Examination of the colonization competence of catalase-deficient mutant strains of *V. fischeri* has begun to determine the importance of this enzyme to the symbionts (Visick and Ruby 1997).

Different strains of *V. fischeri* appear to exhibit different degrees of specificity in host recognition. The evidence for this conclusion comes from the use of two comparative approaches. First, although *V. fischeri* strains that have been isolated from a number of different environmental sources can colonize *E. scolopes* juveniles (McFall-Ngai and Ruby 1991), strains that originated from the light organ of this host ("native strains") had a competitive advantage over other *V. fischeri* isolates in co-infection experiments (Lee and Ruby 1994a). The nature of this advantage is, as yet, unknown, but it becomes apparent within 24 hours after initiation of the symbiotic colonization. This competitive dominance provides a further indication that specific recognition events occur between the host and its symbionts and that native strains are more successful in this dialogue.

Given the host specificity of *V. fisheri* strains for their original *E. scolopes* hosts, one might predict that *V. fischeri* strains that are isolated from *E. scolopes* might be better adapted to colonizing this species than strains isolated from one of the many other species of sepiolid squids (Mangold and von Boletzky 1988). Indeed, we have recently obtained such evidence (Michele Nishiguchi, Edward G. Ruby, and Margaret J. McFall-Ngai, unpublished data). We isolated symbionts from nine species of sepiolids, including four species of the genus *Euprymna* from the Indo-West Pacific and five species of the genus *Sepiola* from the Mediterranean. We then performed cross-inoculation studies to determine how well non-native strains could infect the *E. scolopes* in the presence of native strains. Although all strains were capable of infecting the light organ of *E. scolopes* when presented alone, the native strain was always dominant over non-native strains in these competitions. In addition, when only non-native strains were presented, the strain from the squid host most closely related to *E. scolopes* won the competition. The competition was not won immediately but was resolved over the first couple of days after inoculation.

We are presently beginning experiments to determine the mechanisms underlying the competitive dominance of a given *V. fischeri* strain in these colonization experiments. In one set of experiments, we are labeling one of the co-infecting strains with green fluorescent protein, an easily visualized marker (Webb et al. 1995). This approach should help to define the specific distribution of the competing strains within the crypts, which could give an indication of the specific processes that mediate strain dominance. For example, a finding that native strains localize to the periphery of the crypts (i.e., along the brush border) would suggest that native strains may be better able to interact with receptors on the crypt microvillar border. Such studies will not only speak to the mechanisms of competitive dominance but also address the question of whether host species and their symbionts have coevolved through the divergence of specificity determinants (Triplett and Sadowsky 1992).

Direction of future studies

Development of the symbiotic association between a newly hatched *E.*

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scolopes and its specific bacterial partner *V. fischeri* follows a program of events that leads to the creation of a complex light-emitting organ. Both the host and the symbiont participate in the successful completion of this program, which can be initiated only by partners that recognize and respond to each other through specific signals. We have only begun to determine the nature of the biochemical phrases used in this symbiotic dialogue, but already it is clear that the process is a dynamic one. It expresses itself in a dramatic remodeling of host tissue, a process that provides both important developmental landmarks and clues to the mechanisms underlying this process. Similarly, the ability to identify *V. fischeri* genes that are specifically induced by entry into the symbiotic state, and whose mutation leads to a loss of symbiotic competence, has opened new possibilities for discovering the bases of the interaction. Together, these two approaches are already providing us with a unique opportunity to understand one of the many ways in which host species and their symbionts have learned to live together.

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