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CONSEQUENCES OF EVOLVING WITH BACTERIAL SYMBIONTS: Insights from the Squid-Vibrio Associations

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■ **Abstract** The squid-vibrio light-organ symbioses, which have been under investigation for just over 10 years, offer the opportunity to decipher aspects of the dynamics of stable associations between animals and bacteria. The two best-studied partners, the Hawaiian sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri*, engage in the most common type of animal-bacterial association, i.e., between extracellular, gram-negative bacteria and animal epithelia. Similar to most such symbioses, the squid-vibrio relationship begins anew each generation when the-host animal acquires the symbiont from the surrounding environment. To establish a specific association, mechanisms have evolved to ensure recognition between the host and symbiont and the exclusion of other potential partnerships. Once the association has been established, the bacteria induce significant morphological changes in the host that result in a transition of the light organ from a form associated with initiation of the symbiosis to one characteristic of the mature, functional relationship.

INTRODUCTION

Metazoans evolved in environments rich in bacteria and, as a part of this process, formed alliances with specific subsets of these microbes that persist from generation to generation. The partnerships that resulted allowed animals to take advantage of the unique and diverse metabolic capabilities of their prokaryotic partners. Thus, animals can be accurately viewed as complex communities comprised of a principal multicellular, eukaryotic cell type and an array of microbial species. It is likely that when species arise, a specific microbiota arises with them (37); and, similarly, when an animal host becomes extinct, it is likely that some subset of the microbial community with which they associate will also not persist (73).

Despite the ubiquity of animal-microbial associations, rarely have these communities been ecologically modeled, nor has the impact of these associations been extensively studied by evolutionary biologists (37). The lack of knowledge is likely

due to the complexity of these associations, which impose a sort of "Russian doll" nature to the system. A less complex biological model, while not comprehensive in scope, can provide insight into the mechanisms by which multicellular, eukaryotic organisms initiate and maintain stable associations with microbes. To date, very few such models have proven amenable to in-depth study of these questions; either they are rare or their host and symbiont are not culturable independently of one another (40). The notable exception to this trend has been in the study of the plant-bacterial associations between legumes and root-nodulating, nitrogen-fixing rhizobia, which have been the principal model for prokaryotic-eukaryotic symbioses (54, 76). Over 100 years of research with these associations, at all levels of analysis, from ecology to molecular biology, have provided the biological community with a window into the complexity of the interactions between bacteria and multicellular organisms.

With the emergence of new model systems (29, 41, 66) and the application of molecular, genetic and biochemical approaches, this frontier area of research has shown promise over the past few years (40). One model that is part of this nascent effort is the association between sepiolid squids and particular species of marine vibrios (41, 44, 65). In these relationships, the bacteria form a persistent, extracellular relationship with host epithelial tissue, the most common type of association between prokaryotes and animals. The two-partner nature of these symbioses offers opportunities for the study of animal-bacterial symbioses similar to those that have been available in plant-microbial symbioses, and it offers the opportunity to determine the similarities and differences between animal and plant associations with microbes. In addition, the study of how symbioses may interface with features unique to animals, such as cell-mediated immunity and animal developmental pathways, are possible in this system. The aim of this review is to provide an overview of progress of the study of the squid-vibrio association to date.

THE PARTNERS

Bobtail Squids

The sepiolid, or bobtail, squids are small (mantle length in sexually mature adults usually averaging between 1 and 8 cm) mollusks whose taxonomic position within the coleoid cephalopods is presently controversial (10, 11). Most systematists who study the relationships of cephalopods consider the sepiids, or cuttlefish, the sister group of this family. The family Sepiolidae contains 14 genera and between 50 and 60 species in 3 subfamilies: the Sepiolinae, the Rossiinae, and the Heteroteuthinae (53, 79). They are broadly distributed from tropical to boreal habitats. Whereas the species in the Heteroteuthinae are principally deep pelagic, the species in the Sepiolinae and Rossiinae are benthic, distributed from the shallow subtidal to deeper areas of the shelf and bathyl benthic regions of the ocean.

Over half of the 14 sepiolid genera are characterized by an internal, light-emitting organ associated with the ink sac (27, 53, 61). All members of the subfamily

Heteroteuthinae have light organs, although the luminescence is, in some cases, autogenic rather than bacterial in origin (14). Some of the genera within the Sepiolinae and Rossiinae have bacterial light organs, but the taxonomic distribution within these subfamilies suggests that either this character arose independently several times or was lost several times (10).

Within the Sepiolinae, two genera have members that are readily accessible for the study of bacterial light-organ symbioses: the Indo-Pacific genus *Euprymna* and the Atlantic/Mediterranean genus *Sepiola* (44). Presently, the array of species within these two genera is being studied as a mechanism by which to understand the evolution of animal-bacterial symbioses (see below). However, the principal focus for studies of the dynamics of the symbiosis itself have focused on the Hawaiian sepiolid species *Euprymna scolopes* (41).

Euprymna scolopes is endemic to the Hawaiian archipelago (6, 70), with populations occurring from Midway to Maui; no reports of this species have been madefrom the southernmost, and youngest, island of the archipelago, Hawaii. Some evidence exists for the divergence of subspecies within *E. scolopes*; i.e., morphological and molecular sequence data suggest reproductively isolated populations exist on either side of the island of Oahu (Kimbell, MJ McFall-Ngai and Roderick, unpublished results).

Luminous Bacterial Symbionts

Most luminous bacteria are facultative anaerobes belonging to the gamma-proteobacteria group of gram-negative bacteria. Four culturable species form specific associations with light organs of marine fishes and squids: *Vibrio fischeri*, *V. logei*, *Photobacterium phosphoreum*, and *P. leiognathi* (19, 26, 45). Populations of these four species of luminous bacteria can be found in a wide variety of habitats; in addition to being the microbes in fish and squid light organs, they occur as components of the enteric microbiota of marine animals, are opportunistic saprophytes and pathogens, and are relatively common members of the bacterioplankton. These planktonic populations of the luminous bacteria provide the inoculum for the light organs of juvenile squids and fishes that have light organs. Squid species are known to harbor three of these species, *V. fischeri* and *V. logei* in the sepiolid squids with light organs (19) and *P. leiognathi* in the loliginid squid species with light organs (21).

Until recently, these light-organ associations were thought to be exclusive, i.e., one particular luminous bacterial species always found in association with a specific squid or fish host. This pattern does hold true for all *Euprymna* species studied thus far; i.e., all identified luminous bacterial isolates from *Euprymna* spp. light organs have been *V. fischeri*, although the light organs have not been sampled at all times of year. However, studies of the Mediterranean sepiolids indicate that the light organs of an individual squid can harbor mixed cultures of *V. fischeri* and *V. logei* (19). *Vibrio logei* is more psychrophilic (i.e., it grows optimally at relatively low temperatures) (4) and may be a better competitor in the light organs

in colder months or in deeper populations. To determine the dynamics of these two symbionts in the host *Sepiola* spp., a more detailed study must be undertaken.

THE HOST LIFE CYCLE AND THE MAINTENANCE OF BREEDING COLONIES UNDER LABORATORY CONDITIONS

The life cycles of the sepiolid squids have been studied extensively, most likely because their life histories can be examined under laboratory conditions (3, 25). Unlike many cephalopods, which are semelparous, i.e., having a single reproductive effort (mating once, depositing their eggs, and then dying), the sepiolids are iteroparous (22). For example, the adult females of the Hawaiian sepiolid *E. scolopes* may mate numerous times and, over their life time, a given female will typically lay several clutches on hard substrates (44). Each clutch contains 50–400 eggs, each of which is about 2 mm in diameter. No direct parental care occurs during embryogenesis; immediately following the laying of a clutch, the female covers the clutch with sand and leaves the embryos to develop and hatch on their own.

Under laboratory conditions, we have found that female *E. scolopes* will not lay eggs unless they are frequently mated, although the females may be carrying many spermatophores from previous matings (MJ McFall-Ngai, personal observation). If the animals are fed to satiation and mated once per week, a breeding colony of between 10 to 12 females and 3 to 4 males produces 60,000–100,000 eggs per year. The rate of hatching success approaches 100% if the eggs are kept in clean, well-aerated seawater (44).

Following an embryonic period that varies from 17 to 25 days, the length of which depends on the environmental conditions under which the embryos develop (temperature, aeration, etc), the juvenile *E. scolopes* (averaging 1.7 mm in mantle length) hatch with internal yolk reserves that furnish nutrition over the next few days. These newly hatched juveniles have a gross morphology that is very similar to that of the adult, i.e., like most cephalopod species, *E. scolopes* does not have larval stages in its life history. Individuals of this species reach sexual maturity as early as 60 days posthatch (25), and their entire life span is thought to be about one year (70), although only fragmentary data are available to support this assumption.

The life histories of *Sepiola* spp. have also been studied extensively, and the details of their life cycle differ in several aspects from that of *E. scolopes* (10, 22). Although the adults are similar in size and gross morphology to *E. scolopes*, *Sepiola* spp. lay smaller clutches (between 1 and 200 eggs) of much larger eggs, each 4–7 mm in diameter (22). The embryonic period of *Sepiola* species is two to three times that of *E. scolopes*, and when the juveniles hatch, they are significantly larger, averaging 4–6 mm in mantle length (MJ McFall-Ngai, personal observation). These differences in the developmental program between *E. scolopes* and *Sepiola* spp. have proven valuable in advancing an understanding of the dynamics of squid-vibrio associations, particularly the role of symbionts in development of light organs (see below).

THE INITIATION OF THE SYMBIOSIS

Embryonic Development of the Host Organ

During embryogenesis the host squid develops an incipient light organ with a morphology that allows it to initiate a symbiosis with *V. fischeri* immediately upon hatching (39, 44, 48, 51). About half-way through embryogenesis, a lateral thickening in the hindgut-ink sac complex represents the first stages of organ development. Over the last half of embryogenesis, pairs of crypts, three on either side, invaginate from the surface epithelium in sequence. On each side one begins to invaginate early in this period of development and continues to grow throughout embryogenesis, the second pair begins several days later, and the third pair forms a few days before the juvenile host hatches. Thus, at hatching, the host has three pairs of epithelia-lined crypts of different sizes, each individual crypt connected by a pore to the surface of the organ. These crypts will house the symbiotic bacterial population after inoculation of the organ by *V. fischeri* from the environment.

Also during embryogenesis, the superficial epithelium of the developing organ, which is not directly connected with the crypt spaces, forms a complex ciliated, microvillous field (44, 48). This field spreads medially over the lateral surfaces of the organ,where it consists of three regions: an anterior appendage and a posterior appendage, which have the three crypt pores of that side at their base, and a basal field, which surrounds the pores and spreads medially. The appendages consist of a single layer of epithelium, separated from a blood sinus by a basement membrane. The medial extension of the ciliated field on each side of the developing organ terminates in a row of prominent, elongate cilia. High-speed cinematography (RB Emlet, MJ McFall-Ngai, unpublished data) has suggested that the arms form a ring-like arrangement that entrains water toward the vicinity of the pores leading to the crypt spaces. In addition, manipulation of the ciliated field with pharmacological agents has indicated that the cilia beat toward the pores (44). Studies with fluorescently labeled bacteria demonstrate that they do not adhere to this ciliated, microvillous surface but instead are entrained by the water currents into the vicinity of the pores (MJ McFall-Ngai, personal observation).

The crypt spaces are connected to the superficial pores by long cilia-lined ducts (Figure 1) (42, 48). Goblet cells embedded among the epithelial cells lining these ducts appear to release mucopolysaccharides into the duct spaces (MJ McFall-Ngai, personal observation.). The orientation of rootlets of the cilia, as viewed by TEM, suggests that they beat materials from the duct, and perhaps the crypt spaces, out through the pores (44). This ciliary activity may provide a gradient of a chemoattractant sensed by potential symbionts. However, while it is uncertain whether chemoattraction is involved in colonization of light organs, studies of the bacteria suggest that the anatomical arrangement of the duct presents a physical impediment to colonization. *Vibrio fischeri* mutants that are defective in motility, because of either a lack of flagella or an inoperative flagellar motor, are not able to enter the crypt spaces even when they are present at very high densities in the surrounding water (24). These data suggest that the bacteria must be capable of

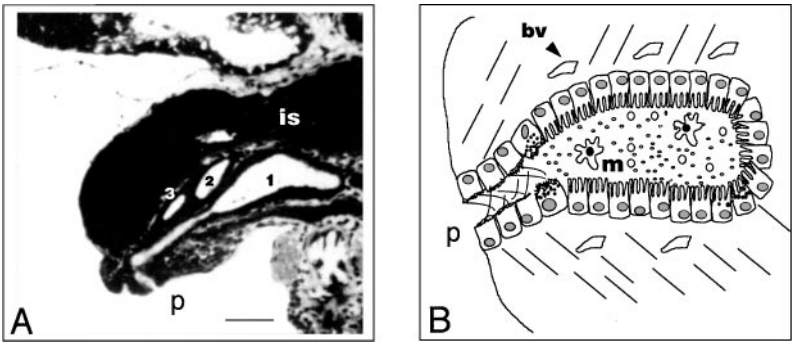

Figure 1 Cross section of the juvenile light organ of *Euprymna scolopes. A.* A histological section through the organ of a newly hatched animal. Three pores on each side of the light organ provide the site of entry into the light organ for the symbiotic bacteria. The bacteria enter the pores, travel along ducts, and enter into three separate crypt spaces [1, 2, 3]. The pore (p), the entire duct, and the largest portion of crypt 1 can be seen in this section. *is*, ink sac; bar = 50 μm. B. An illustration depicting the cells of the light organ with which the bacteria interact. Upon entering the pore, *V. fischeri* cells first encounter the ciliated duct cells and then move into the crypt spaces where they proliferate. In the crypt, they are surrounded by the microvillous epithelium that lines the crypt space. In addition, they also interact with macrophage-like cells that are likely to be delivered to the light organ through the blood vessels (*bv*) in the connective tissue matrix surrounding the crypts.

swimming to negotiate a physical barrier presented either by the mucus or by a cilia-generated current in the duct, or both. The bacteria also produce a mucinase (EG Ruby, personal communication), which may function to degrade the mucus in the duct. It has yet to be determined whether mutants defective in mucinase production are unable to infect the light organ.

The Bacterial Symbionts During the Colonization Process

Wei & Young (80) were the first to determine that the nascent light organ of juvenile *E. scolopes* was not capable of producing luminescence if the newly hatched animal was maintained in seawater from which the bacteria had been removed by filtration. Thus, it was concluded that in nature the animals obtain their initial inoculum of *V. fischeri* cells from the seawater into which they hatch. In later work under laboratory conditions, it was found that the addition of a few hundred to thousand *V. fischeri* cells per milliliter of filtered seawater is required to elicit a successful colonization of the *E. scolopes* light organ. However, methods traditionally applied to the enumeration of *V. fischeri* cells occurring naturally in the seawater [i.e., counting the number of colony-forming units (CFUs) that arise on a nutrient agar plate onto which seawater is spread] initially suggested that there existed a population density of fewer than 10 *V. fischeri* cells per milliliter in the

seawater where *E. scolopes* hatchlings are typically found, and in which they become rapidly colonized. Thus, either the bacteria in natural seawater samples are far more infective than those grown under laboratory conditions, or the numbers of CFUs obtained for natural seawater populations of *V. fischeri* underestimated the actual number. Using a probe specific for *V. fischeri*—a DNA sequence complementary to the *luxA* gene of this species—Lee & Ruby (32) showed that *V. fischeri* is present at a concentration of approximately 100 to 1000 cells per milliliter of seawater in areas where adult *E. scolopes* occur. More than 99% of these cells, while infective, are in a "nonculturable" condition (35). A number of species of marine bacteria have been demonstrated to enter this cryptic state (13, 60), the nature of which has not been characterized. However, the conditions of the juvenile light organ are capable of initiating growth of *V. fischeri* in this state, so the light-organ environment causes these cryptic bacteria to return to a culturable state. The specific conditions of the light organ that promote the growth of these bacteria have not been determined.

Seawater samples taken at increasing distances from locations with known populations of adult *E. scolopes* contained increasingly fewer *V. fischeri* and were increasingly less capable of promoting colonization light organs (34). Laboratory studies of the interactions between the light-organ populations of *V. fischeri* and their populations in the surrounding seawater indicated that the host seeds the water with symbionts by a diel venting of the light-organ population into the environment. Specifically, at dawn each day, the host expels between 90% and 95% of its resident *V. fischeri* cells into the surrounding seawater through the lateral pores on the light organ (9, 23, 34). This venting appears to result in the high population densities of *V. fischeri* cells that are detected in habitats with large numbers of adult animals. While this daily expulsion behavior may serve a purpose in the maintenance of a healthy symbiosis, an additional, ecological result is an increased likelihood that subsequent generations of squids will be successfully inoculated.

Recognition and Specificity

In experiments in which juvenile *E. scolopes* are placed in seawater containing *V. fischeri* cells at a concentration typical of their natural environment, the animals become colonized with the symbiont within a few hours (43, 67). The colonization process has been monitored in the laboratory in two ways: one, by following the onset and increase in luminescence over time; and two, by the enumeration of bacteria in the light organ as a function of time. Studies of the infection process (33, 67) have shown that the initial growth rate of the bacteria results in a doubling of the cell number every 20 min. However, after about 12 h, the growth rate slows to an average doubling time of 5.5 h.

Although the squid will readily be infected with *V. fischeri* upon hatching, when *V. fischeri* is either low in number or absent, under either natural or laboratory conditions, the light organ remains uncolonized even when the numbers of nonspecific environmental bacteria are high (43). These data suggest *V. fischeri* is not simply a

competitive dominant in the light organ, but rather that there is a host-imposed positive selection for *V. fischeri*. Experiments in which nonspecific, green-fluorescent protein (GFP)–labeled *Vibrio* spp.are exposed to juvenile squid have revealed that these bacteria enter the light-organ crypt spaces but do not grow and persist there (SV Nyholm, EV Stabb, personal communication). Further, having entered the host's crypt, these nonspecific bacteria are no longer culturable, indicating that they have lost viability. These data suggest that one aspect of specificity in the squid-vibrio light-organ symbiosis is the host's creation of a habitat in which only *V. fischeri* is able successfully to initiate and maintain a stable association. Data also exist that implicate receptor-ligand interactions in the positive selection of *V. fischeri* in the light organ (46).

The Organ as a Stressful Environment Studies of the cell biology, biochemistry, and molecular biology of the *E. scolopes* light organ have revealed that the crypt environment has many features typically associated with innate immune responses in mammals. Recently, mollusk macrophage-like hemocytes have been reported in the crypt spaces (Figure 1), and phagocytosed bacterial cells have been seen in transmission electron micrographs of these crypt hemocytes (58). The mechanisms by which these cells are transported to the crypt spaces and their exact function there remain to be determined. It is not yet known whether these cells engulf nonspecific bacteria, thus maintaining *V. fischeri* as the sole inhabitant of the organ, or whether they instead provide a mechanism to control symbiont number. Further analysis of the activity of these cells in adult hosts, as well as experimental manipulations of aposymbiotic and symbiotic juvenile squids, should contribute to an understanding of their precise role in the dynamics of the symbiosis.

In addition to the presence of macrophage-like cells in the light-organ crypts, a library derived from the mRNA of the light organ had unusually high concentrations of messages that encode a peroxidase with sequence similarity and biochemical affinities to mammalian myeloperoxidase (MPO) (72, 75, 82). MPO occurs abundantly in neutrophils where it catalyzes the production of hypohalous acid from halide ions and hydrogen peroxide (30). The presence of abundant hydrogen peroxide substrate is the result of a respiratory burst that occurs in response to the phagocytosis of a pathogen. The hypohalous acids that are produced by MPO are potent microbicides, and their activity provides a first line of defense in mammalian immune systems (30).

Since its discovery in the light organ, research on the squid peroxidase and the possible responses of *V. fischeri* have provided valuable insight into the nature of the "dialogue" between these symbiotic partners. Biochemical studies of the squid peroxidase revealed that it is also halide-dependent and that the light organ exhibits relatively high activity for the enzyme (72, 82). Antibodies to mammalian MPO recognize the squid peroxidase in immunocytochemical analyses and in protein-antibody hybridizations (Western blots). In addition, antibodies generated to the expressed gene product of the squid peroxidase cDNA recognize human MPO in

Western blots. Immunocytochemical localization of the squid peroxidase revealed that it is abundant along the apical surfaces of the crypt epithelia as well as in association with both host and bacterial cells in the crypt spaces.

The finding of an enzyme that catalyzes the production of a microbicide in a cooperative association presented a seeming contradiction, but its abundance suggested that it may be pivotal in the dynamics of this symbiosis. Because no bacteria have been described thus far that have mechanisms by which to resist the effects of hypohalous acids, it was reasoned that *V. fischeri* must be in some way inhibiting the activity of this enzyme, either directly or indirectly.

Two principal mechanisms have been described by which pathogens resist the activity of halide peroxidases (18, 74): (i) by inhibiting the respiratory burst activity of the cell; or (ii) by competing for the substrate of the enzyme, hydrogen peroxide. There is some evidence that *V. fischeri* may use both of these mechanisms. Reich & Schoolnik (64) determined that *V. fischeri* has two enzymes which they called halovibrin α and β (63, 64) with ADP-ribosyltransferase activity. Halovibrin α is produced by *V. fischeri* during log-phase growth, whereas the gene encoding halovibrin β is expressed primarily in stationary phase (63, 64). The activity of these enzymes is analogous to that of cholera toxin, the agent of *V. cholerae* that perturbs the physiology of mammalian host enterocytes. Among other activities, cholera toxin inhibits host cell respiratory burst activity and thus the production of superoxide anion that would have been catalyzed by superoxide dismutase to hydrogen peroxide, the MPO substrate (69). Comparisons of superoxide anion production in symbiotic and aposymbiotic light organs revealed that symbiotic light organs have significantly lower levels of this reactive oxygen species (71). Further, when aposymbiotic animals are incubated in seawater containing cholera toxin, they produce superoxide anion at the decreased levels that are characteristic of symbiotic animals. Presently, the halovibrin proteins are being isolated from *V. fischeri* to determine whether they experimentally induce similar oxidative signatures in the light organ. In addition, *V. fischeri* mutants defective in the production of their halovibrins are being generated (64; EV Stabb, EG Ruby, personal communication). As in the case of cholera toxin, which has multiple effects on host cell function, the halovibrins may participate in a variety of aspects of the squid-vibrio association, only one component of which may be the inhibition of respiratory burst activity in host cells.

Data also exist to suggest that *V. fischeri* inhibits the host peroxidase by competing for its hydrogen peroxide substrate. Visick and Ruby recently described a high level of catalase activity in the periplasmic space of *V. fischeri* (77). The periplasmic space lies between the cell wall and the cell membrane, and periplasmic catalases appear to occur most frequently in organisms that encounter high levels of environmentally imposed oxidative stress (36). Visick and Ruby found that the *V. fischeri* catalase gene is most fully induced during stationary phase. Because, with the daily venting of the light organ at dawn, the symbiont population goes through both log phase and stationary phase growth each day, the induction

of the catalase late in the growth cycle of the bacterium may track fluctuations in the host's contribution to the organ's oxidative state.

In addition to the possibility that *V. fischeri* may prevent the production of and compete for the substrate of the peroxidase, preliminary data indicate that the symbiont influences the host's expression of the gene encoding the squid peroxidase. Specifically, at 48 h post inoculation, mRNA levels for the squid peroxidase in symbiotic animals are approximately half that of aposymbiotic animals, suggesting that the presence of the symbiotic *V. fischeri* results in a reduction of the expression of this gene (AL Small, AM Hirsch, MJ McFall-Ngai, unpublished results).

Additional studies of the pattern of occurrence of this peroxidase in a variety of tissues in *E. scolopes* suggest that it plays a pivotal role in a variety of interactions, and that the regulation of this protein may be crucial in defining the precise nature of the symbiosis, i.e., whether it is cooperative or pathogenic. For example, the accessory nidamental gland of the female, which contains a consortium of bacteria thought to be involved in successful development of eggs, also contains high levels of this enzyme, whereas the eye and digestive gland, tissues which do not typically interact with microbes, do not (72). In addition, as in certain insects, in which a similar peroxidase has a dual function in antimicrobial activity and melanin production, the enzyme is also present in the ink gland of *E. scolopes*, the site of melanin synthesis (38, 52).

The most compelling evidence that this enzyme is involved in modulation of pathogenic interactions with bacteria comes from studies of its production in the gills. Cephalopod gills are the site where potential pathogens that have invaded the blood stream are cleared from the circulatory system (5). At the gills, these bacteria are sequestered into cysts. In studies of adult *E. scolopes*, gills without cysts show very low activity of the peroxidase and very low levels of peroxidase gene expression (72). However, in gills with abundant cysts, both activity for this type of peroxidase and high levels of peroxidase gene expression are evident (72). These observations are supported by recent studies of the response of *E. scolopes* to elevated concentrations of *V. fischeri* in the ambient seawater. In newly hatched squid, when the light-organ symbiont is presented at levels normally experienced in the host's environment (i.e., 200 to 1000 cells $\cdot$ ml^{-1}), the light organ becomes colonized and no gill cysts form (AL Small, AM Hirsch, MJ McFall-Ngai, unpublished results). Under these conditions, *V. fischeri* appears to turn down peroxidase message expression, as mentioned above, and there are undetectable levels of peroxidase in the gills. However, in experiments in which *V. fischeri* is presented at levels of 10^5 to 10^7 cells $\cdot$ ml^{-1} in the seawater surrounding a juvenile, in addition to a light organ being colonized, gill cysts containing *V. fischeri* form in the animal (AL Small, AM Hirsch, MJ McFall-Ngai, unpublished results). Under these conditions, *V. fischeri* causes a dramatic increase in peroxidase gene expression in the gills, suggesting that the host is responding to *V. fischeri* as a pathogen.

The occurrence in the cooperative squid-vibrio symbiosis of macrophage-like cells and an enzyme heretofore associated only with antimicrobial activity present the possibility that the same genes, gene products, and cells are involved all types of

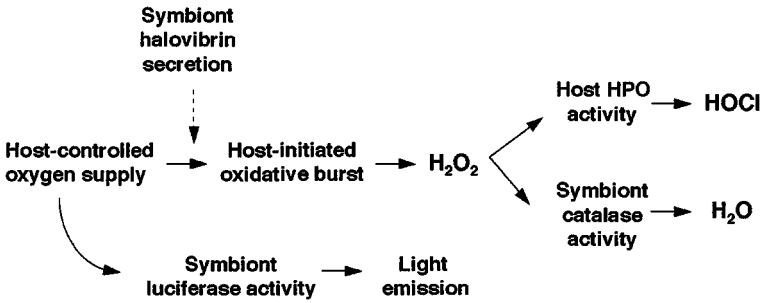

Figure 2 Model of potential oxidative-stress reactions in the squid-vibrio symbiosis. The circulatory system of the host supplies the crypt environment with oxygen, which provides the substrate for host respiratory burst activity in response to microbes. The respiratory burst leads to the eventual production of hydrogen peroxide (H_2O_2), which is the substrate for the squid halide peroxidase (HPO). The activity of the halide peroxidase creates hypochlorous acid (HOCl), which is toxic to bacteria. The symbionts can respond by lowering oxygen availability through the oxygen-requiring luminescence reaction. In addition, halovibrin secretion may inhibit respiratory burst activity. The action of either, or both, of these processes would lower the production of HOCl by inhibiting the creation of substrate for the HPO. As another defense, the bacteria may compete for existing H_2O_2 substrate of HPO with a highly active, periplasmic catalase.

animal-microbial interactions and that modulation of these components defines the outcome. Further, these data suggest that these genes may have evolved for animal-bacterial interactions, no matter what sort, rather than being strictly associated with defense against nonself.

Taken together, the data available on the oxidative environment of the host light organ, and the possible responses of the specific symbionts to this environment, suggest a model of this aspect of the squid-vibrio association (Figure 2). In this model, *V. fischeri* would enter the light organ and initially restrict the activity of the peroxidase by inhibiting a respiratory burst of host cells through the activity of the halovibrins, thus depriving the peroxidase of substrate. A second line of defense would be available to *V. fischeri* by the activity of its periplasmic catalase, which would compete with the squid peroxidase for the hydrogen peroxide substrate. The bacteria would also turn down expression of the host peroxidase gene. Some level of host peroxidase expression would remain throughout the life of the host, perhaps either to control the invasion of nonspecific bacteria into the light organ or to control the numbers of *V. fischeri*, or both. There is evidence that both these functions may be important in the biology of the symbiosis. When adult squid are incubated in water with antibiotically tagged bacteria, these bacteria are later found in the light organ, indicating that it remains open to colonization after the initial infection event. In addition,the studies with supernumerary *V. fischeri* indicate that the host can respond to them as pathogens, and that they have the potential to overgrow.

Receptor-Ligand Interactions In addition to the creation of an environment in which *V. fischeri* is able to persist, while other bacteria are not, there is evidence that specific receptor-ligand interactions may be important in the determination of specificity in the squid-vibrio symbiosis (46). Animal-bacterial cell interactions are often mediated by the recognition of sugars on the host cell membrane by bacterial surface proteins called adhesins (59). Many gram-negative bacteria have mannose-recognizing adhesins, and specificity of an interaction is conferred by variations in the bacterial adhesins that correspond to differences in the microenvironment of the mannose residues on the host receptor (59). Assays with *V. fischeri* have shown that they hemagglutinate guinea-pig red blood cells, a behavior characteristic of bacteria that have mannose-recognizing adhesins associated with their cell surfaces (46). Histochemical analyses of juvenile host light organs showed that mannose residues are abundant along the crypt brush border (46). Thus, both partners had elements of the lock-and-key mechanisms associated with this type of recognition system. Further, mannose analogs, but not other sugars and sugar analogs, introduced into the environment during the infection process, fully block colonization by *V. fischeri* (46), suggesting that these interactions are essential for successful infection of the light organ by the symbiont. Although *V. fischeri* strains mutant in mannose recognition are not yet available, a homolog of a gene encoding the mannose adhesin in *V. cholerae* (*mshA*) has recently been identified, and strains of *V. fischeri* mutant in this gene are currently being generated (EVStabb, EG Ruby, personal communication).

These adhesin-glycan interactions in the squid-vibrio symbiosis may vary through ontogeny. In studies of the development of the mammalian intestine (29), bacteria have been shown to induce increased expression of glycans on the host cell surfaces to which they are adhering. This augmentation of host binding sites results in reinforcement of the association between the bacteria and these host cells. Mutant bacterial cells that are defective in adhesion to host cells do not induce an increase in the production of host cell glycans. This type of interaction may also occur in the squid-vibrio association. Over the first few days of the symbiosis, the bacteria induce an increase in their intimacy with host cells; i.e., the percentage of the bacterial surface in contact with the host cell increases dramatically during this time (31; see below). Studies with *V. fischeri* strains defective in adhesion to host crypt cells should reveal whether the increase in intimacy is achieved in a similar manner as demonstrated in the mammalian intestine.

Other types of receptor-ligand interactions have also been suggested as essential in the initiation of the squid-vibrio association. In some bacteria, outer membrane proteins (Omps) called porins participate in adhesion to host cells. Aeckersberg & Ruby (1) found that antibodies to a homolog (OmpL of *Photobacterium profundus*) of a *V. fischeri* porin inhibit infection, and mutants generated in the *V. fischeri* gene (*ompV*), were found to colonize the host squid less efficiently (2).

Evolution of Specificity Determinants Another mechanism by which to study specificity is to ask how specificity determinants express themselves in related

host species, i.e., ask what kinds of experiments nature has done over evolutionary time that resulted in specific symbiotic associations. The phylogenetic trees derived from gene sequence data of several of the sepiolid hosts and their light-organ symbionts are congruent (55, 56). For some other symbioses, this type of data has provided evidence that the host and symbiont have coevolved (15, 28). Additional support for coevolution in the squid-vibrio symbioses has been obtained by studying the response of the newly hatched Hawaiian squid to interaction with vibrio symbionts from other sepiolid species (55). The Hawaiian squid are colonized by these heterologous symbionts when they are presented to the hatchling in the absence of any other potential symbionts. However, when the native symbiont and a strain from another host sepiolid are presented in the inoculum at equal concentrations, the native symbiont outcompetes those isolated from the light organs of all other sepiolid species. When two nonnative strains are presented to the host in such competition experiments, the strain from the host most closely related to the Hawaiian host always prevails. Thus, not only are the phylogenetic trees of the hosts and symbionts congruent, the ability of the bacterial symbionts to compete in colonization experiments with *E. scolopes* directly reflects their relative position on the phylogenetic tree.

The mechanisms underlying these differences in symbiont competitiveness remain to be determined. However, the data suggest that (i) selection of a particular symbiont occurs by processes that express themselves in the early stages of the symbiosis; and (ii) for the first time in any animal-bacterial symbiotic association, analyses of the differences between the host and their symbionts may reveal the precise nature of the specificity determinants, i.e., those biochemical and molecular features of the host and symbiont upon which selection has acted to bring about evolution of these symbioses.

DEVELOPMENT OF THE LIGHT ORGAN OF *E. SCOLOPES*

The host squid *E. scolopes* hatches with a light-organ morphology and anatomy markedly different from that of the adult (42, 51). The bacteria-containing epithelial core of the organ is surrounded by a thick reflector that serves to direct light ventrally; the ink sac has diverticula that act as an iris to control the intensity of emission; yellow filters are present over the ventral surface that may act to shift the wavelength of luminescence closer to that of downwelling moonlight and starlight; and, a thick, muscle-derived lens, with striking biochemical similarities to the squid eye lens (47, 81), covers the entire ventral surface of the organ and appears to function as a diffuser of the bacteria-produced light.

The periods of light-organ development correlate with functional stages of the symbiotic association (40, 51). In addition to the embryonic organogenesis, dramatic changes occur in the light organ over the first few hours to days posthatch that are associated with the establishment of the relationship (44, 49). This early stage is followed by late development, or maturation, of the organ, in which

the components associated with modulating bacterial light emission–the reflector, lens, filters, and ink sac diverticula–are elaborated (51). Whereas specific interactions with *V. fischeri* are essential for early posthatch morphogenesis of the organ (17, 20, 31, 49, 50), the elaboration of tissues associated with the maturation of the *E. scolopes* light organ do not appear to require interaction with the bacterial symbionts. These results are supported by research on the development of the light organs of the Mediterranean sepiolids (JS Foster, SV Boletzky, and MJ McFall-Ngai, unpublished results). The embryonic period of these species is two to three times that of *E. scolopes*, and upon hatching they also have a complex, superficial, ciliated field, the loss of which is under bacterial induction. However, the shape of the light organ resembles that of *E. scolopes* at 1-month posthatching, and all of the components that modulate bacterial light (lens, reflector, and ink sac diverticula) are well developed.

Three principal changes occur in the normal, early posthatch development of the *E. scolopes* light organ. They include a loss of the superficial, ciliated, microvillous field of epithelial cells at 4-5 days following hatching (49), an increase in the microvillar density along the apical surfaces of the crypt cells (31), and a swelling of the crypt epithelial cells (49). Studies in which development of the organ is compared in symbiotic and aposymbiotic animals have shown that all of these changes are under symbiont induction; i.e., *V. fischeri* is required for the full expression of each facet of this early posthatch developmental program.

Bacteria-Induced Loss of the Superficial Field of Cells

The first experimental manipulations of the squid-vibrio developmental program showed that interaction with *V. fischeri* induces death in the cells of the superficial epithelium (Figure 3 see color insert; 43, 49). Ultrastructural, biochemical, and molecular analyses of the dying cells showed that the process of death is classic programmed cell death, or apoptosis (20). These cells die in a characteristic pattern. About 9 h following first exposure to *V. fischeri*, the cells along the medial margin of the ciliated field that bear long cilia and the cells at the tips of the appendages die. In the following hours, cells die over the entire field. By between 18 h and 24 h, the blood sinus in the center of each appendage collapses. In aposymbiotic animals, occasional cell death events sometimes occur in this field of cells, but no regression occurs and the sinus remains intact.

Experimental manipulations of the system have revealed the timing of the bacterial induction and the location in the organ where the induction takes place. Studies in which light organs were antibiotically cured of *V. fischeri* at various times following inoculation revealed that the bacteria deliver an irreversible signal at about 12 h following the initial exposure to the symbionts (17). This signaling triggers the 4-d developmental program that results in loss of the superficial field of cells. In addition, when large numbers of nonmotile mutants of *V. fischeri*, either lacking flagella or with a disabled "motor," were coincubated with the squid host, no cell death or regression was noted (17). These data suggested that the bacteria

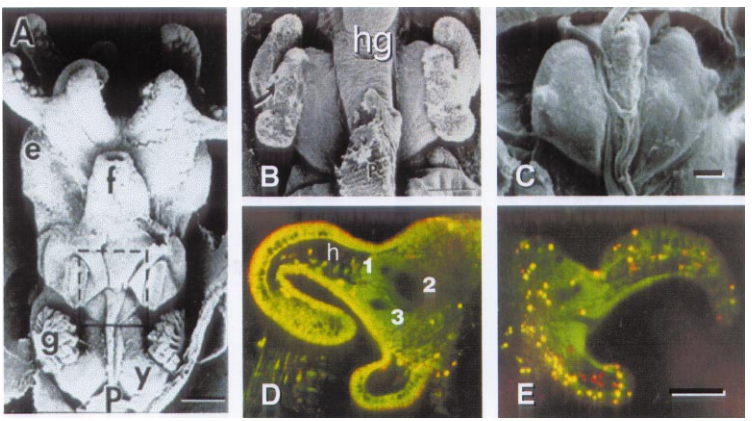

Figure 3 The juvenile *E. scolopes* light organ. (*A*) SEM of ventral dissection, showing the eye (*e*), funnel (*f*), gill (*g*), and yolk sac (*y*). The light organ is under the dotted square. Bar, 200 μm. (*B*) SEM of the ventral surface of a hatchling organ revealing the dense ciliated, microvillous field (CMS). Pores on the surface of the organ (arrow), lead to the internal crypts that will house the bacterial symbionts, hindgut (*hg*), posterior (*p*). (*C*) SEM of the light organ of a 4-d symbiotic animal showing complete regression of the CMS. Bar, μm. (*D*) A confocal image of an acridine-orange (AO) stained light organ of a 14-h aposymbiotic animal revealing that the CMS is a single layer of epithelial cells over a blood sinus. Hemocytes can be seen freely floating in the sinus of the CMS appendages (arrowhead). The three pores (1, 2, 3) lead to the crypt spaces. (*E*) 14-h symbiotic organ under same conditions as (*D*). The condensed chromatin of apoptotic cells can be seen as bright spots of AO staining. Bar, 50 μm.

must get into the crypts and interact with cells there, rather than directly interacting with the superficial field of cells, to trigger the morphogenesis.

These findings present two questions: What is the bacterial signal(s)? How is the signal(s) transmitted to the responsive cells? The most common bacterial inducer of animal cell death is the bacterial lipopolysaccharide (LPS) (57, 62), and specifically, the lipid A portion of LPS, which is the most conserved component of the molecule. Experiments with newly hatched *E. scolopes* revealed that when either purified *V. fischeri* holo-LPS or the lipid A fraction are coincubated with the host, the typical cell death pattern is induced (JS Foster, MA Apicella, MJ McFall-Ngai, unpublished results). In addition, commercially available LPS and lipid A of other bacteria are also effective in inducing the characteristic pattern on the *E. scolopes* light organ. These responses were dose dependent, with levels of LPS in the range of pg $\cdot$ ml^{-1} of seawater causing death in only a few cells in the field, whereas levels in the range of ng to μg $\cdot$ ml^{-1} induced the full pattern characteristic of the infected light organ. The finding that the signal triggering cell death may be something conserved on the surface of gram-negative bacteria helped explain the observation that cell death can occasionally be seen in the superficial field of cells of aposymbiotic animals but not in animals kept axenically (personal observation). Because experiments with GFP-labeled bacteria have shown that other bacterial species get into the organ, but do not colonize, bacterial LPS as a component of the cell surface of these nonspecific bacteria is likely to occur at low levels in the light organs of aposymbiotic juveniles. Thus, the triggering of the extensive cell death pattern characteristic of the colonization of the organ by *V. fischeri* may be the result of the high doses of LPS that would be presented over a short time period by the growing culture of symbionts.

Although all of the available data suggest that symbiont LPS induces cell death, when the light organs of juveniles that have been exposed to LPS are viewed at 4 d, they do not show signs of regression of the ciliated field (JS Foster, MA Apicella, MJ McFall-Ngai, unpublished results). This finding suggested that a second bacterial signal may be required for morphogenesis of the organ. The existence of a second signal was also suggested by the fact that the full pattern of symbiont-induced and LPS-induced cell death occurs at around 9 h post-exposure (20; JS Foster, MA Apicella, MJ McFall-Ngai, unpublished results), whereas the irreversible signal for complete morphogenesis does not occur until about 12 h. One possible explanation is that the bacteria inhibit further cell proliferation, as well as inducing cell death. This hypothesis was supported by experiments with a cell proliferation inhibitor, colchicine. By itself, colchicine does not cause full regression of the superficial field, but when the animals are incubated in seawater containing both LPS and colchicine, full regression of the superficial field occurs. The nature of this second signal remains to be determined.

The mechanism by which the bacterial signals are transmitted to their site of action and the specific cell-death pathways triggered by the bacteria also remain to be determined. The bacterial cells interact with cells of two types in the crypts (Figure 1B), the epithelial cells lining the space and the macrophage-like cells free

in the crypt space (58). It is likely that, following interactions with cells of one or both of these types, the signals are either carried into the circulatory system and up to the sinus of the appendages or there is a cascade of signals through the cells that connect the superficial epithelium with the deeper tissue. One interesting observation is that, if the crypt spaces are loaded with fluorescently labeled dextran, macrophage-like cells carrying dextran can be seen in the sinus of the arms a few hours later (DJ Park, MJ McFall-Ngai, personal observation). A great deal more study of the system must be undertaken before these issues can be resolved.

Bacteria-Induced Changes in Light-Organ Crypt Cells

Whereas the bacteria send an irreversible trigger that induces cell death and regression of a remote set of cells, the epithelial cells with which they directly interact in the crypt do not undergo cell death. However, significant developmental changes do occur in the crypt epithelium. Over the first four days following inoculation of the organ, the microvillar density along the apical surfaces of these cells increases fourfold (31) and the host cells swell (49). Significant differences in the number of microvilli per unit area are detectable by 12 h postinoculation with *V. fischeri*. Transmission electron micrographs of the crypt cells of adult animals reveal highly complex, lobate microvilli (42), indicating that the elaboration of the brush border continues after this initial increase. Light micrographs of the crypts have demonstrated that the crypt cells also exhibit a fourfold increase in volume over the first few days following colonization (49). A quantifiable difference in cell volume is apparent within 24 h (16). Experiments involving the antibiotic curing of the organ showed that, unlike the triggering of the regression of the superficial epithelium, the increase in microvillar density and cell swelling in the host cells in direct contact with the bacteria are reversible (16). In cured animals, the microvillar density and cell volume return to the condition characteristic of hatchling or aposymbiotic animals. The bacterial cells also become much more intimately associated with the host cells; the percent of a given bacterial cell's surface in contact with microvilli increases from less than 30% to more than 80% over the first several days (31). The increase in both microvillar density and cell swelling appears to contribute to this increase in the intimacy of the partners' cells.

Bacterial Development

While far less dramatic than those of the host, developmental changes in *V. fischeri* cells occur soon after the symbionts initiate the association. Within 6 to 12 h of entry into the light-organ crypts, the bacteria have undergone cell differentiation. Two morphological changes have been reported: (i) a loss of flagellation, and (ii) a decrease in cell volume. Neither of these phenomena occurs during the first 12 h when the inoculating cells are undergoing a period of rapid growth; however, after the light-organ population has reached its maximum level of about a million cells, the polar flagella that are typically present on *V. fischeri* cells disappear, and the cells decrease to a volume about one-seventh that of the inoculating bacteria

(67). The cause of these events remains unknown, although similar events have been reported for cells responding to a severe decrease in growth rate (65).

At the biochemical level, a large induction of bacterial luciferase becomes evident within 6 to 8 h postinoculation (67) and results in the normally dimly luminescing *V. fischeri* cells (7) producing a biologically useful amount of light for its host. This induction is due to the accumulation around the bacteria in the light-organ crypts of an acyl-homoserine lactone signal molecule called autoinducer (8), which serves as an activating cofactor when it binds to a transcriptional regulator of the *lux* operon. The synthesis of proteins besides those encoded by the *lux* genes (e.g., luciferase) are positively regulated by autoinducer accumulation (12). Discovery of the functions of these proteins may suggest the processes that change in the developing symbiont.

By applying a technology that identifies *V. fischeri* promoters that are activated specifically in cells as a response to growth in the light organ, the molecular genetics underlying the initiation of the symbiosis is being revealed (78). To date, five *V. fischeri* genes have been identified as symbiosis-induced: three are homologs of *Escherichia coli* genes that have known metabolic functions, including amino acid metabolism (23), while two others are novel and may prove to be our best route to uncovering future insights into specific events in the regulation of this association.

MAINTENANCE OF A STABLE SYMBIOSIS

Less information is presently available about the long-term maintenance of the association between *E. scolopes* and *V. fischeri* than about other aspects of the relationship. One key aspect of the dynamics of this association appears to be a complex diel rhythm (9, 23, 58). Beginning immediately after hatching and persisting throughout the life history of the host, highest levels of light are emitted from the host around dusk and through the early hours of the night, and the lowest luminescence is observed around dawn and the early hours of the morning (9). These variations in luminescence appear to be under host control. Bacteria experimentally removed from the light organ at different times of day show the same luminescence per cell, which demonstrates that expression of components the *lux* operon, including the aldehyde substrate of the luminescence reaction, do not vary in concentration within *V. fischeri* cells over the course of the day (9). Because the light organ is well vascularized and the luminescence reaction requires oxygen, the most parsimonious explanation is that the host modulates oxygen delivery to the crypt space (9).

Superimposed on this diel rhythm of luminescence is a daily release of the crypt contents into the environment at dawn (23, 58). The material exits through the lateral pores of the light organ into the mantle cavity as a thick paste, which can be collected intact for analysis. The constituents of this exudate include, on average, 95% of the bacterial symbionts (34), a population of host macrophage-like cells (58), and a dense proteinaceous matrix (23, 58). All of the bacterial cells

appear to be alive and culturable (67), while not all of the host cells are viable (58). The venting of the bacteria likely serves several functions in the symbiosis, the most obvious being the control of symbiont numbers in the light organ. In addition, it has been hypothesized that this behavior also serves to seed the environment with *V. fischeri*, which must be acquired by newly hatched juveniles (34, 68).

As discussed earlier, the role of the macrophage-like cells in the crypt space is poorly understood, but some data are available on the role of the matrix material. The matrix is rich in a mixture of proteins (SV Nyholm, MJ McFall-Ngai, unpublished results). Studies with amino acid auxotroph mutants of *V. fischeri* have demonstrated that in the light organ, the symbionts obtain amino acids as a carbon and nitrogen source (23). Further analyses of the constituents of this matrix are expected to provide valuable insight into the microenvironment of the crypt space.

CONCLUSIONS

Studies of the squid-vibrio association have revealed a rich frontier for future research. The past ten years of work on this symbiosis have principally focused on describing the association and building the tools, such as microbial genetics of *V. fischeri*, for the development of this model. With this framework in place, researchers in the field are now poised to determine some of the underlying biochemical and molecular mechanisms by which bacteria form long-term, beneficial associations with animals.

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LITERATURE CITED

1. Aeckersberg FT, Welch T, Ruby EG. 1997. Possible participation of an outer membrane protein in the symbiotic infection of the *Euprymna scolopes* light organ. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 97: 387
2. Aeckersberg FT, Welch T, Ruby EG. 1998. Possible role of an outer membrane protein of *Vibrio fischeri* in its symbiotic infection of *Euprymna scolopes*. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 98:374
3. Arnold J, Singley C, Williams-Arnold L. 1972. Embryonic development and post-hatch survival of the sepiolid squid *Eu-*

prymna scolopes under laboratory conditions. *Veliger* 14:361–64
4. Bang SS, Baumann P, Nealson KH. 1978. Phenotypic characterization of *Photobacterium logei* (sp. nov.), a species related to *P. fischeri*. *Curr. Microbiol.* 1:285–88
5. Bayne C. 1974. Molluscan immunobiology. In *The Mollusca*, Vol. 5, ed. ASM Saleuddin, EL Wilbur, pp. 408–69. New York: Academic
6. Berry S. 1912. The Cephalopoda of the Hawaiian islands. *Bull. US Bur. Fish.* 32:255–362
7. Boettcher KJ, Ruby EG. 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid, *Euprymna scolopes*. *J. Bacteriol.* 172:3701–6
8. Boettcher KJ, Ruby EG. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. *J. Bacteriol.* 177:1053–58
9. Boettcher K, Ruby EG, McFall-Ngai MJ. 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J. Comp. Physiol.* 179:65–73
10. Boletzky SV. 1995. The systematic position of the Sepiolidae (Mollusca: Cephalopoda). In *Mediterranean Sepiolidae, Bulletin de l' Institut oceanographique, Monaco, Numero special 16*, ed. SV Boletzky, pp. 99–104. Monaco: Musee Oceanographique
11. Bonnaud L, Boucherrodoni R, Monnerot M. 1997. Phylogeny of Cephalopoda inferred from mitochondrial-DNA sequences. *Mol. Phylogenet. Evol.* 7:44–54
12. Callahan S, Dunlap PV. 1998. The autoinduction regulon of *Vibrio fischeri*: identification and partial characterization of five novel LuxR/autoinducer-regulated proteins. *Abstr. Ann. Meet. Am. Soc. Microbiol.* 98:282
13. Colwell RR, Huq A. 1994. Vibrios in the environment: viable but nonculturable *Vibrio cholerae*. In *Vibrio cholerae and Cholera*, ed. IK Wachsmuth, PA Blake, O Olsvik, pp.117–134. Washington: Am. Soc. Microbiol.
14. Dilly PN, Herring PJ. 1978. The light organ and ink sac of *Heteroteuthis dispar* (Mollusca: Celphalopoda). *J. Zool., Lond.* 172:81–100
15. Distel DL, Felbeck H, Cavanaugh CM. 1994. Evidence for phylogenetic congruence among sulfur-oxidizing chemoautotrophic bacterial endosymbionts and their bivalve hosts. *J. Mol. Evol.* 38:533–42
16. Doino JA. 1998. The role of light organ symbionts in signaling early morphological and biochemical events in the sepiolid squid *Euprymna scolopes*. PhD diss., Univ. Southern Calif. 123 pp
17. Doino JA, McFall-Ngai MJ. 1995. Transient exposure to competent bacteria initiates symbiosis-specific squid light organ morphogenesis. *Biol. Bull.* 189:347–55
18. Dukan S, Touati D. 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. *J. Bacteriol.* 178: 6145–50
19. Fidopiastis PM, Boletzky SV, Ruby EG. 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *J. Bacteriol.* 180:59–64
20. Foster JS, McFall-Ngai MJ. 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev. Genes Evol.* 208:295–303
21. Fukusawa ST, Dunlap PV. 1986. Identification of luminous bacteria from the light organ of the squid, *Doryteuthis kensaki*. *Agric. Bio. Chem.* 50:1645–46
22. Gabel-Deickert A. 1995. Reproductive patterns in *Sepiola affinis* and other Sepiolidae (Mollusca,Cephalopoda). In *Mediterranean Sepiolidae, Bull. de l'Institut oceanographique, Monaco, Numero special 16*, ed. SV Boletzky, pp. 73–83. Monaco: Musee Oceanographique
23. Graf J, Ruby EG. 1998. Characterization of the nutritional environment of a symbiotic light organ using bacterial mutants and bio-

chemical analyses. *Proc. Natl. Acad. Sci. USA* 95:1818–22
24. Graf J, Dunlap PV, Ruby EG. 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. *J. Bacteriol.* 176:6986–91
25. Hanlon RT, Claes MF, Ashcraft SE, Dunlap PV. 1997. Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacterial-animal symbioses. *Biol. Bull.* 192:364–74
26. Haygood MG. 1993. Light organ symbioses in fishes. *Crit. Rev. Microbiol.* 19:191–216
27. Herring PJ. 1988. Luminescent organs. In *The Mollusca*, Vol. 11, ed. P Hochachka, pp. 449–89. London: Academic
28. Hinkle G, Wetterer JK, Schultz TR, Sogin ML. 1994. Phylogeny of attine ant fungi based on analysis of small subunit rRNA gene sequences. *Science* 266:1695–97
29. Hooper LV, Bry L, Falk PG, Gordon JI. 1998. Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. *BioEssays* 20:336–43
30. Klebanoff SJ. 1991. Myeloperoxidase: occurrence and biological function. In *Peroxidases in Chemistry and Biology*, ed. J Everse, KE Everse, MB Grisham, pp. 2–35. Boca Raton, FL: CRC
31. Lamarcq LH, McFall-Ngai MJ. 1998. Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. *Infect. Immun.* 66:777–85
32. Lee K-H, Ruby EG. 1992. Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater using *lux* gene probes. *Appl. Environ. Microbiol.* 58:942–47
33. Lee K-H, Ruby EG. 1994. Competition between *Vibrio fischeri* strains during the initiation and maintenance of a light organ symbiosis. *J. Bacteriol.* 176:1985–91
34. Lee K-H, Ruby EG. 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl. Environ. Microbiol.* 60:1565–71
35. Lee K-H, Ruby EG. 1995. Symbiotic role of the nonculturable, but viable, state of *Vibrio fischeri* in Hawaiian seawater. *Appl. Environ. Microbiol.* 61:278–83
36. Loewen PC. 1997. Bacterial catalases. In *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, ed. JG Scandalios, pp. 273–308. New York: Cold Spring Harbor Lab.
37. Margulis L, Fester R. 1991. *Symbiosis as a Source of Evolutionary Innovation*. Cambridge, MA: Mass. Inst. Technol. Press. 454 pp
38. Marmaras VJ, Charalambidis ND, Zervas CG. 1996. Immune response in insects: the role of phenoloxidase in defense reactions in relation to melanization and sclerotization. *Arch. Insect Biochem. Physiol.* 31:119–33
39. McFall-Ngai MJ. 1994. Evolutionary morphology of a squid symbiosis. *Am. Zool.* 34:554–61
40. McFall-Ngai MJ. 1998. The development of cooperative associations between animals and bacteria: establishing détente among Domains. *Am. Zool.* 38:3–18
41. McFall-Ngai MJ. 1998. The adventure of pioneering a biological model: the squid-vibrio association. *ASM News* 64:639–45
42. McFall-Ngai MJ, Montgomery MK. 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae). *Biol. Bull.* 179:332–39
43. McFall-Ngai MJ, Ruby EG. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254:1491–94
44. McFall-Ngai MJ, Ruby EG. 1998. Bobtail squids and their luminous bacteria: when first they meet. *BioScience* 48:257–65
45. McFall-Ngai MJ, Toller WW. 1991. Frontiers in the study of the biochemistry and molecular biology of vision and luminescence in fishes. In *The Molecular Biology and Biochemistry of Fishes*, Vol. 1, ed.

P Hochachka, T Mommsen, pp. 77–107. New York: Elsevier
46. McFall-Ngai MJ, Brennan C, Weis VM, Lamarcq LH. 1998. Mannose adhesin-glycan interactions in the *Euprymna scolopes–Vibrio fischeri* symbiosis. In *New Developments in Marine Biology*, ed. Y LeGal, HO Halvorson, pp. 273–77. New York: Plenum
47. Montgomery MK, McFall-Ngai MJ. 1992. The muscle-derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. Evidence for recruitment of ALDH as a predominant structural protein. *J. Biol. Chem.* 267:20,999–21,003
48. Montgomery MK, McFall-Ngai MJ. 1993. Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes*. *Biol. Bull.* 184:296–308
49. Montgomery MK, McFall-Ngai MJ. 1994. The effect of bacterial symbionts on early post-embryonic development of a squid light organ. *Development* 120:1719–29
50. Montgomery MK, McFall-Ngai MJ. 1995. The inductive role of bacterial symbionts in the morphogenesis of a squid light organ. *Am. Zool.* 35:372–80
51. Montgomery MK, McFall-Ngai MJ. 1998. Late postembryonic development of the symbiotic light organ of *Euprymna scolopes* (Cephalopoda:Sepiolidae). *Biol. Bull.* 195:326–36
52. Nappi AJ, Vass E. 1993. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment Cell Res.* 6:117–26
53. Nesis KN. 1982. *Cephalopods of the World*. Neptune City, NJ: TFH Publ. 351 pp.
54. Niner BM, Hirsch AM. 1998. How many rhizobium genes in addition to the *nod*, *nif/fix* and *exo* are needed for nodule development and function. *Symbiosis* 24:51–102
55. Nishiguchi MK, Ruby EG, McFall-Ngai MJ. 1997. Phenotypic bioluminescence as an indicator of competitive dominance in the *Euprymna-Vibrio* symbiosis. In *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons*, ed. JW Hastings, LJ Krick, PE Stanley, pp. 123–26. New York: Wiley & Sons
56. Nishiguchi MK, Ruby EG, McFall-Ngai MJ. 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in the sepiolid–*Vibrio fischeri* symbioses. *Appl. Environ. Microbiol.* 64:3209–13
57. Norimatsu M, Ono T, Aoki A, Ohishi K, Takahashi T, et al. 1995. Lipopolysaccharide–induced apoptosis in swine lympocytes in vivo. *Infect. Immun.* 63: 1122–1126
58. Nyholm SV, McFall-Ngai MJ. 1998. Sampling the microenvironment of the *Euprymna scolopes* light organ: description of a population of host cells with the bacterial symbiont *Vibrio fischeri*. *Biol. Bull.* 195:89–97
59. Ofek I, Doyle RJ. 1994. *Bacterial Adhesion to Cells and Tissues*. New York: Chapman & Hall
60. Oliver JD. 1995. The viable but nonculturable state in the human pathogen *Vibrio vulnificus*. *FEMS Microbiol. Lett.* 133:203–8
61. Pierantioni U. 1918. Gli organi simbiotici e la luminescenza batterica dei Cefalopodi. *Publ. Staz. Zool. Napol.* 20:15–21
62. Placido R, Mancino G, Amendola A, Mariani F, Vendetti S, et al. 1997. Apoptosis of human monocytes/macrophages in *Mycobacterium tuberculosis* infections. *J. Pathol.* 181:31–38
63. Reich KA, Schoolnik GK. 1996. Halovibrin, secreted from the light organ symbiont *Vibrio fischeri*, is a member of a new class of ADP–ribosyltransferases. *J. Bacteriol.* 178:209–15
64. Reich KA, Beigel T, Schoolnik GK. 1997. The light organ symbiont *Vibrio fischeri* possesses two distinct secre-

ted ADP-ribosyltransferases. *J. Bacteriol.* 179:1591–97
65. Ruby EG. 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. *Annu. Rev. Microbiol.* 50:591–624
66. Ruby EG. 1998. Ecology of a benign "infection": colonization of the squid luminous organ by *Vibrio fischeri*. In *Microbial Ecology and Infectious Disease*, ed. E Rosenberg, pp. 217–31. Washington: Am. Soc. Microbiol.
67. Ruby EG, Asato LM. 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch. Microbiol.* 159:160–67
68. Ruby EG, Lee K-H. 1998. The *Vibrio fischeri–Euprymna scolopes* light organ association: current ecological paradigms. *Appl. Environ. Microbiol.* 64:805–12
69. Seifert R, Schultz G. 1991. *The Superoxide-Forming NADPH Oxidase in Phagocytes*. New York: Springer. 338 pp
70. Singley CT. 1983. *Euprymna scolopes*. In *Cephalopod Life Cycles*, Vol. 1, ed. PR Boyle, pp. 69–74. London: Academic
71. Small Al, McFall-Ngai MJ. 1993. Changes in the oxygen environment of a symbiotic light organ in response to infection by its luminous bacterial symbionts. *Am. Zool.* 33:61A
72. Small AL, McFall-Ngai MJ. 1998. A halide peroxidase in tissues interacting with bacteria in the squid *Euprymna scolopes*. *J. Cellul. Biochem.* 72:445–57
73. Staley JT. 1997. Biodiversity: Are microbial species threatened? *Curr. Opin. Biotechnol.* 8:340–45
74. Tartaglia LA, Storz G, Ames BN. 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. *J. Mol. Biol.* 210:709–19
75. Tomarev SI, Zinovieva RD, Weis VM, Chepelinsky AB, Piatigorsky J, McFall-Ngai MJ. 1993. Abundant mRNAs in the bacterial light organ of a squid encode a protein with high similarity to mammalian antimicrobial peroxidases: implications for mutualistic symbioses. *Gene* 132:219–26
76. Van Rhijn P, Vanderleyden J. 1995. The *Rhizobium*-plant symbiosis. *Microbiol. Rev.* 59:124–42
77. Visick KL, Ruby EG. 1998. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence, and is induced both by oxidative stress and by approach to stationary phase. *J. Bacteriol.* 180:2087–92
78. Visick KL, Ruby EG. 1998. Tn*luxAB* insertion mutants of *Vibrio fischeri* with symbiosis-regulated phenotypes. *Abstr. Ann. Meet. Am. Soc. Microbiol.* 98:277
79. Volpi C, Borri M, Boletzky SV. 1995. Mediterranean sepiolidae: An introduction. In *Mediterranean Sepiolidae, Bulletin de l'Institut oceanographique, Monaco, Numero special 16*, ed. SV Boletzky, pp.7–14. Monaco: Musee Oceanographique
80. Wei SL, Young RE. 1989. Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*. *Mar. Biol.* 103:541–546
81. Weis VM, Montgomery MK, McFall-Ngai MJ. 1993. Enhanced production of ALDH-like protein in the bacterial light organ of the sepiolid squid *Euprymna Scolopes*. *Biol. Bull.* 184:309–321
82. Weis VM, Small AL, McFall-Ngai MJ. 1996. A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the *Euprymna-Vibrio* mutualism. *Proc. Natl. Acad. Sci. USA* 93:13683–88