

LESSONS FROM A COOPERATIVE, BACTERIAL-ANIMAL ASSOCIATION: The *Vibrio fischeri*–*Euprymna scolopes* Light Organ Symbiosis

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KEY WORDS: luminescence, mutagenesis, specificity, development, colonization

ABSTRACT

Although the study of microbe-host interactions has been traditionally dominated by an interest in pathogenic associations, there is an increasing awareness of the importance of cooperative symbiotic interactions in the biology of many bacteria and their animal and plant hosts. This review examines a model system for the study of such symbioses, the light organ association between the bobtail squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri*. Specifically, the initiation, establishment, and persistence of the benign bacterial infection of the juvenile host light organ are described, as are efforts to understand the mechanisms underlying this specific colonization program. Using molecular genetic techniques, mutant strains of *V. fischeri* have been constructed that are defective at specific stages of the development of the association. Some of the lessons that these mutants have begun to teach us about the complex and long-term nature of this cooperative venture are summarized.

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INTRODUCTION

Scope and Purpose of this Review

All animals have, and perhaps require, long-term, cooperative associations with a specific complement of bacteria (38, 46, 95). Studies of gnotobiotic animals have revealed not only that the hosts often fail to thrive without the essential nutrients normally supplied by their symbionts, but also that these bacteria participate in the normal maturation of the host tissues with which they closely associate (39, 116, 124). In most cases, such as in the establishment of an enteric flora, the initial interaction begins during the early postembryonic period, when the juvenile animal is first exposed to the bacteria in its natural environment. During this crucial period, a coordinated program of interactions between the proper bacterial species and the developing host must occur (81). The result is a balance between the requirement to establish a healthy, stable set of bacterial associations and the need to maintain the integrity of the animal's non-self-recognition systems. Such an interplay between host and symbiont can be expected to involve a complex signaling program between the two partners. As yet, we understand relatively little about the mechanisms underlying this reciprocal signaling process because of the complexity of the interactions existing among the typically multispecies microbial consortia of most animals.

In this review I examine what has been discovered about the *Vibrio fischeri*–*Euprymna scolopes* light organ symbiosis as a model of nonpathogenic bacterial-animal associations. Interest in this symbiosis has become increasingly active over the past seven years and has entered a phase of rapid development and expansion, the ecological and evolutionary aspects of which have been reviewed elsewhere (112). This article focuses on the factors underlying the initiation, development, and maintenance of this symbiosis, primarily from the point of view of the bacterial partner. However, it is the nature of symbiosis studies that an understanding of the association cannot be adequately achieved by an approach that includes only one side of the relationship. For this reason, the function and activities of the bobtail squid *E. scolopes* will also be considered by drawing on the pioneering work of McFall-Ngai and her coworkers, the principal group studying this animal's symbiotic development.

Table 1 Animal hosts with *V. fischeri* as their light organ symbionts

Host family	Host species	Principal distribution	Reference
Sepiolidae (bobtail squids)			
	<i>Euprymna morsei</i>	Indo-Western Pacific	68, 113
	<i>Euprymna scolopes</i>	Hawaii	10
	<i>Euprymna tasmanica</i>	Australia	^a
Monocentridae (monocentrid fishes)			
	<i>Cleidopus gloriamaris</i>	Australia	34
	<i>Monocentris japonica</i>	Japan	98

^aM Nishiguchi, EG Ruby, MJ McFall-Ngai, unpublished data.

The Symbiotic Partners

THE LUMINOUS BACTERIUM *V. FISCHERI* At least a dozen species of light-emitting bacteria have been cultured and described, most of them marine members of the genus *Vibrio* (98). Whereas much of the earlier work isolating and identifying these species relied upon their formation of visibly luminous colonies on seawater-nutrient agar plates (97), more recently it has become clear that there are strains of several luminous species that, in culture, produce either no luminescence or luminescence that is not visible to the unaided eye (10, 64, 103). Isolates of *V. fischeri* from the light organ of *E. scolopes* are among this group of luminous bacteria that are often termed “not visibly luminous” because in culture they produce colonies that emit only very low levels of luminescence (10). The detection of such strains in natural samples has required the development of specific molecular probe approaches (64, 138).

SPECIES OF SYMBIOTIC LIGHT ORGAN HOSTS Several recent reviews have been published describing the range of animal species that maintains bacteria as the source of bioluminescence in symbiotic associations (26, 51, 84, 98, 114). Light organ associations in which the bacterial partner has been identified as *V. fischeri* include at least five animal species (Table 1) and have evolved at least twice, arising within both the sepiolid cephalopods, commonly called the bobtail squids (74, 99), and the monocentrid fishes (51). Although the monocentrids are confined to coastal regions of the Western Pacific Ocean, the sepiolids are much broader in distribution. Studies of monocentrid symbioses have been recently reviewed (26, 51), and have been centered primarily on electron microscopy of the light organ structure and laboratory studies of the isolated symbionts in culture. The most extensively studied of the sepiolid associations is that involving the endemic Hawaiian bobtail squid *E. scolopes* (8, 114). The host is believed to use the bacterial luminescence in a camouflaging

behavior called counterillumination (79), although no definitive studies have yet been reported.

The E. scolopes Light Organ

Adult *E. scolopes* have a complex light-emitting organ in the center of their mantle cavity (Figure 1). The organ harbors a monospecific culture of *V. fischeri* (10), whose bioluminescence is used by the host during its nocturnal activities (79, 121). The adult light organ consists of two lobes, each with three distinct epithelia-lined crypts (Figure 2) that house the extracellular bacterial symbionts (89) (Figure 3). The three crypts located within each lobe join in a common duct that leads to a single pore on the lateral face of the lobe (Figure 2). A series of accessory tissues serve to control the intensity of light emission from the organ (83). The accessory tissues include a thick reflector, which directs luminescence ventrally (Figure 1) by surrounding the dorsal and lateral faces of the crypts. The ink sac covers the dorsal surface of the organ to absorb stray

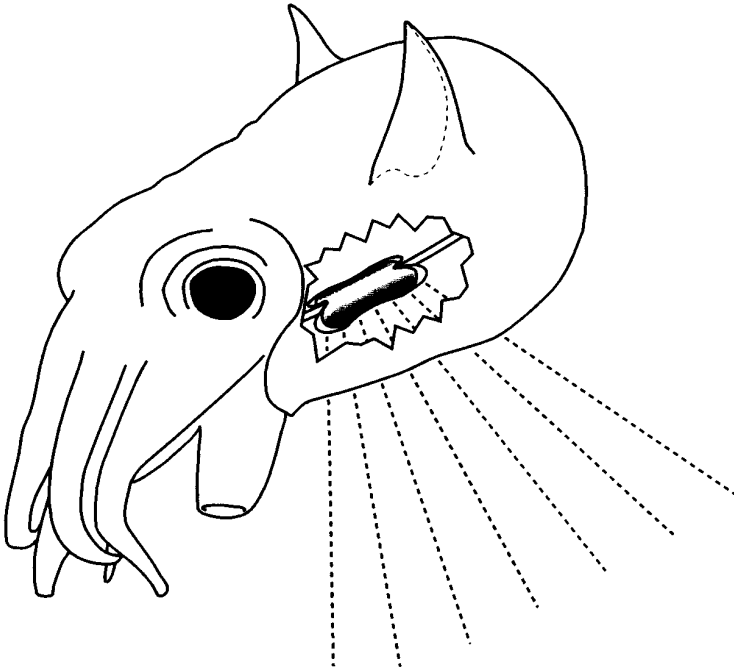


Figure 1 The bioluminescent bobtail squid, *E. scolopes*. A schematic illustrating an adult animal, total length about 4 cm, in its natural swimming behavior. Also illustrated is the position of the saddle-shaped light organ, straddling the hindgut within the mantle cavity. The pattern of ventral light emission from the organ is also indicated.

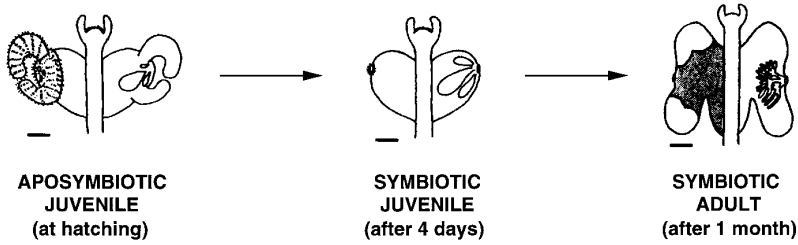


Figure 2 Diagrammatic representations of developmental stages of the *E. scolopes* light organ (left to right): the nascent light organ of a newly hatched, aposymbiotic (bacteria-free) juvenile, the light organ of a juvenile four days after it has been exposed to symbiosis-competent *V. fischeri* cells, and the mature, bilobed light organ of a one-month-old symbiotic animal. The organs are each viewed from the ventral direction and are bisected by the hindgut. The left half of each cartoon indicates an exterior view of the organ, including the ciliated surfaces, pores, and ink sac diverticula. The right half provides an internal view, revealing the increasing degree of complexity of the crypt spaces. The size bars are approximately 100 μm in length for the two juvenile animals and 400 μm in length for the young adult.

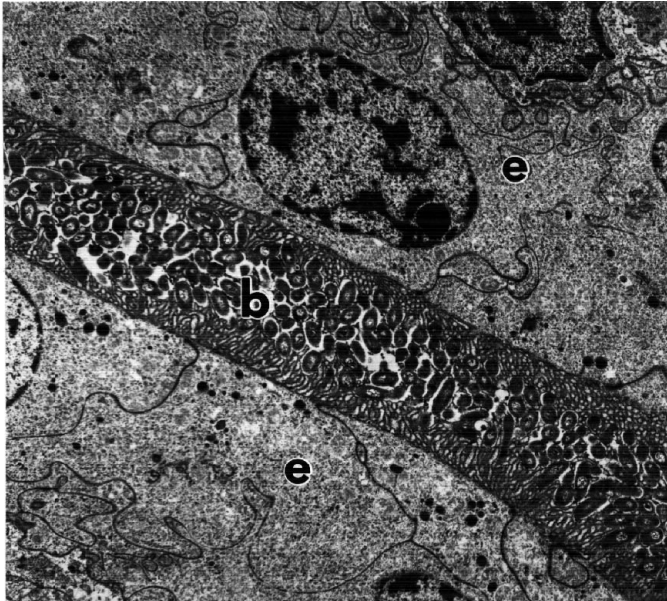
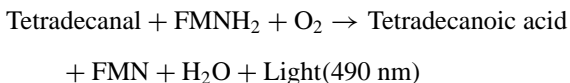


Figure 3 The relationship between symbiotic *V. fischeri* cells and the host epithelial cells lining the light organ crypts of an adult *E. scolopes*. In this transmission electron micrograph of a thin section through the light organ, the bacteria (b) fill the crypt space, and are closely invested within the field of microvilli that covers the surface of the epithelial cell (e) tissue layer. The bacterial cells are approximately 0.6 μm in diameter.

light, and diverticula of the sac dynamically rotate over the crypts, allowing the host to modulate the intensity of emitted light. Finally, the entire ventral surface of the light organ is covered with a thick, muscle-derived lens that refracts the bacterial light into the environment (90, 135). Essentially similar structures are present in the adults of all species of symbiotic bobtail squids examined (99).

Bioluminescence in V. fischeri

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF BACTERIAL LUMINESCENCE The enzyme bacterial luciferase catalyzes light production in the presence of an aliphatic aldehyde substrate, reduced flavin mononucleotide, and molecular oxygen (49, 98), as described by the following mixed-function oxidation reaction:



The biochemistry of this reaction has been extensively studied (85), and exhibits certain analogies to cellular respiration, with luciferase serving as the terminal oxidase (87). The amount of luciferase present in the bacterium is a function of a number of biochemical and physiological factors (22). Although there has been considerable speculation over a possible biochemical function for luciferase (17, 50), as yet the only demonstrated biological function is as a source of luminescence in animal symbioses.

The genes encoding luminescence functions (*lux*) in *V. fischeri* constitute a pair of divergent operons [reviewed in (86)]. To date, *lux* operons of four strains of *V. fischeri* (MJ1, ATCC7744, Y1, and ES114) have been cloned and appear to have an essentially identical genetic structure (44, 86). The *luxAB* genes encode the enzyme luciferase; *luxCDE* encode proteins required for biosynthesis of the aldehyde substrate, and another open reading frame (*luxG*) can be found downstream (86). Finally, two regulatory *lux* genes exist adjacent to each other in divergent operons. The product of one, LuxI, directs the synthesis of a chemical compound, *N*-(3-oxohexanoyl) homoserine lactone (30), called *V. fischeri* autoinducer, or VAI-1. Binding of VAI-1 to LuxR, the product of the second regulatory gene, creates a transcriptional activator of the *lux* operon, and hence induces light production many thousand-fold (36). A second, less dramatic, but potentially important, autoinducer of luminescence, VAI-2, has also been recently discovered elsewhere on the chromosome (61), and the gene encoding it has been cloned (37).

VAI-1 (and presumably VAI-2) are produced at a low constitutive level and freely diffuse through the cell envelope, accumulating in the surrounding

environment (96). If the environment is enclosed and contains a high density of *V. fischeri* cells, the concentration of autoinducer eventually reaches an equilibrium level (outside and inside the cell) that is sufficient to produce a LuxR/VAI-1 complex that results in the induction of the *lux* operon (47, 55). This mechanism, through which bacteria can sense the density of conspecific cells, has been termed quorum sensing (36) and, although first discovered in *V. fischeri* (96), has subsequently been found to function in an increasingly extensive list of gram-negative bacteria (36). The similarities among all these systems, and the fact that they exist in bacteria that predominately occur in either pathogenic or cooperative associations with animal or plant hosts, suggests that quorum sensing is an important and evolutionarily successful paradigm for bacterial symbioses (117).

The cooperative association between the bobtail squid *E. scolopes* and the marine luminous bacterium *V. fischeri* is an unusually tractable system for investigating the mechanisms underlying the establishment of a cooperative symbiosis because (a) the symbiosis is specific, and involves only one bacterial species (10); (b) both partners are culturable in the laboratory (84); (c) the initiation of the association can be experimentally controlled (113, 134); (d) molecular genetics can be applied to the bacterial partner (41, 42, 133); and (e) the association represents the most common type of interaction between animals and bacteria, i.e. host epithelial tissues colonized by extracellular, gram-negative bacteria.

COLONIZATION OF THE NASCENT *E. SCOLOPES* LIGHT ORGAN

To date, the major focus of studies of the *V. fischeri*–*E. scolopes* symbiosis has been on the initiation and development of colonization of the nascent light organ and, in particular, on the accompanying changes in gene expression and morphology in both partners. At the basis of these studies has been the development of an experimental protocol both for initiating the colonization process and for examining its progress by assaying indicators of specific temporal stages in its program.

The Experimental Colonization Assay

The procedure for experimentally initiating a symbiotic association in juvenile *E. scolopes* can be summarized as follows. Newly hatched, symbiont-free (aposymbiotic) bobtail squid are rinsed in either filtered or natural seawater that does not contain symbiosis-competent bacteria (84). The animals are then placed in this seawater, to which has been added 10^3 symbiosis-competent *V. fischeri* cells per ml. Within 3 h an infection is initiated, and the animals can be rinsed to remove the excess *V. fischeri* cells present in the surrounding seawater.

In this way, the subsequent development of colonization is synchronized among the animals to within a 3-h window, thereby allowing a more precise and reproducible study of the temporal unfolding of the colonization program (19, 113). Because newly hatched animals kept in symbiont-free seawater remain uninfected indefinitely, the initiation of the symbiotic infection can be manipulated so that it begins at any time after the juvenile emerges from its egg (84).

After infection, the small (approximately 2 mm total length) *E. scolopes* juveniles are periodically placed in front of a photomultiplier tube, and their light emission quantified as a noninvasive measure of the degree of colonization. Beginning 12 h after inoculation, and continuing for several days thereafter, the amount of light produced is roughly proportional to the number of bacteria in the symbiotic infection (113). At any time after initiation of the symbiosis, the number of bacteria present in the crypts can be easily determined by homogenizing the light organ and spreading an aliquot of the homogenate onto a seawater-based nutrient agar medium. Within 24 h, *V. fischeri* colony-forming-units (CFUs) arise with essentially a 100% plating efficiency (113).

Because the crypt spaces remain in contact with the external environment through the pair of pores that persists, one on either side of the organ (Figure 2), reagents added to the seawater in which the animals are maintained can diffuse into the crypts. In this way, infected light organs can be reversibly cured with several different antibiotics (19), bacterial attachment in the crypt can be inhibited using lectin-binding sugar analogs (81), and antibodies can be introduced to directly interact with cell-surface targets.

This model system has made possible an experimental examination of the physiological and biochemical events accompanying light organ colonization; however, there is still much to be discovered about the role played by signal molecules that send information between the two partners, as well as the underlying genetic mechanisms by which they are controlled.

Species Specificity of the Association

For the past 20 years, it has been recognized that some process dictates which species of luminous bacterium occurs as the light organ symbiont of a given species of host animal. This conclusion was formed as a result of the isolation, and subsequent taxonomic identification, of symbionts from numerous adult host fish and squid species (98). Only with the emergence of the *V. fischeri*-*E. scolopes* model has it become possible to perform controlled infection experiments to determine when and how this specificity is determined and, eventually, to describe its underlying regulation.

The first question that was answered by an examination of the initiation of this association was a fundamental one: Were the bacterial symbionts passed

from generation to generation through the egg (i.e. vertically), or did each new generation of hosts acquire their symbionts from a freeliving population in the environment (i.e. horizontally)? Wei & Young (134) demonstrated that transmission in *E. scolopes* is, in fact, horizontal. Interestingly, this species, and several other sepiolid relatives (M Nishiguchi & M McFall-Ngai, unpublished data), remain to date the only light organ hosts for which the mode of transmission has been demonstrated conclusively, although negative results of studies using molecular probes have provided evidence that vertical transmission does not occur in certain luminous fish species (51).

Horizontal transmission in marine organisms requires the aposymbiotic host to select the correct species of symbiont from among the myriad of bacterial species present in seawater. Does the result of this selection depend only upon which species of luminous bacteria is present (or numerically dominant) in that environment? The first experiments that were aimed at determining the basis for specificity in the *E. scolopes* association definitively demonstrated that *V. fischeri* was the only luminous bacterium that was symbiotically competent (84). Other closely related species of luminous bacteria did not initiate a symbiotic colonization, even when they were presented in the surrounding seawater at concentrations of 10^5 – 10^6 cells per ml, thousands of times the concentration necessary for a *V. fischeri* infection. Most strains of *V. fischeri* tested were capable of colonization, including isolates obtained from the light organs of bobtail squids (*E. scolopes*, *Euprymna morsei*, *Euprymna tasmanica*) (M Nishiguchi, E Ruby & M McFall-Ngai, unpublished data) and recently isolated symbionts from the light organs of monocentrid fishes (*Monocentris japonica* and *Cleidopus gloriamaris*) (Table 1). In addition, *V. fischeri* strains isolated directly from seawater collected from the habitats in which *E. scolopes* are found were also capable of initiating a symbiosis, as were strains from certain other coastal locations (e.g. Woods Hole, Massachusetts). Thus, this work provided the first evidence that specificity is a positively controlled trait and that it is expressed early in the development of the association.

While the presence of a species-specific selection mechanism had been predicted, it was surprising to find that not all strains of *V. fischeri* were capable of colonizing the *E. scolopes* light organ. For instance, *V. fischeri* isolates from Southern California coastal seawater were unable to successfully infect the light organ, as was the well-described laboratory strain, *V. fischeri* MJ1 (84). Strain MJ1, although isolated from a monocentrid fish light organ like the symbiosis-competent strains described in Table 1, has been maintained in culture for over 20 years, much of this time by serial transfer, during which it may have lost an important symbiosis determinant. Thus, symbiosis-competency is restricted to a subset of strains designated as *V. fischeri*. Perhaps this fact suggests either a

closer examination of the criteria for the species designation, or the assignment of subspecies or symbiotic biovars.

An impressive and intriguing characteristic of *V. fischeri*-*E. scolopes* specificity is that, in either the presence or absence of cells of a symbiosis-competent strain of *V. fischeri*, the crypts of the *E. scolopes* light organ remain uncolonized by any of the many other species of marine bacteria present in the surrounding seawater (65, 84). This resistance to nonspecific colonization throughout the lifetime of the host suggests that there is not only a positive selective mechanism (or mechanisms) that recognizes the appropriate bacteria, but also a mechanism of negative selection to exclude unwanted ones. The ability of bacteria to avoid or interfere with this mechanism may rely either on passive features of the cells' surface or metabolism, or on specifically induced cellular modifications (or both).

The primary interaction in the *V. fischeri*-*E. scolopes* symbiosis presumably occurs between the bacterial outer envelope and the host epithelial cells lining the crypts. The investigation of these surface components is in its early stages but is highly promising. Experiments using a mild trypsin treatment to remove superficial outer envelope peptides have revealed that treated *V. fischeri* cells, while remaining fully viable and motile, are no longer capable of infecting the juvenile light organ (53). The results of these experiments further suggested that a surface peptide(s) that was specific to symbiosis-competent strains of *V. fischeri* was released by this trypsinization and, when added to seawater containing hatchling animals, would interfere with the normal colonization process. The identities of the active peptide(s) and the interactions that appear to be blocked are currently being determined. The presence of fimbriae on the surface of *V. fischeri* cells (S Clegg & M McFall-Ngai, unpublished data) and mannose-binding lectins on the surface of light organ cells (V Weis & M McFall-Ngai, unpublished data) provide possible candidates for surface recognition factors.

The expression of an oxygen-radical detoxifying activity may be an important factor in symbiotic competency in the *V. fischeri*-*E. scolopes* symbiosis; light organ tissue has a level of nitroblue-tetrazolium-reducing activity that suggests the presence of high levels of superoxide anion (123). An abundant mRNA that encodes a protein belonging to a family of mammalian peroxidases (POs) has also been reported in the tissues of the adult *E. scolopes* light organ (129, 136). In addition, a high degree of PO activity is present specifically in symbiont-associated tissues of *E. scolopes* and a number of other marine and terrestrial host species (82). This activity may function to regulate symbiont population levels or may serve as a specificity determinant by catalyzing the production of hypochlorous acid (HOCl), a potent antimicrobial oxidant (35).

These results suggest that the host has the potential to produce an oxidatively stressful environment in the light organ and that symbiotically competent *V. fischeri* must possess the means to successfully respond to it (9, 14).

Bacterial Responses

As with a number of bacterial pathogens (88), there is evidence of specialization, or perhaps even cellular differentiation, of *V. fischeri* cells during the colonization of their host. Upon establishing the association, symbiotic *V. fischeri* cells undergo several easily observed changes in their physiology and morphology; whether these changes are only a subset of a number of properties that are acquired by cells in the symbiosis, and whether they result from a coordinated program of modulated gene expression, is yet to be determined. In any case, the host and symbionts undertake a long-term arrangement in which the tissues of the light organ encourage a bacterial population that not only remains monospecific but also is controlled in its number and location within the host.

GROWTH RATE The colonization of a juvenile *E. scolopes* begins as a very rapid proliferation of a few (one to ten) infecting *V. fischeri* cells (113). The first cells to enter the crypts have a growth rate that is remarkably high ($t_{\text{gen}} = 30$ min), approaching the maximum achieved by *V. fischeri* bacteria in culture (10, 113). Two conclusions can be drawn from these results: (a) the cells do not have to pass through a significant lag period in order to adapt to some exotic feature of the crypts, and (b) the conditions presented by the host are sufficiently high in nutrients to support essentially a maximum growth rate.

About 12 h after the colonization has begun, the symbiotic population stabilizes at a level of between 10^5 and 10^6 cells, and the proliferation is curtailed. Thereafter, the cell number gradually increases over the next few months in a manner that parallels the growth of the host animal. It is not yet known what factors limit the maximum population level in the organ, but one possibility is simply a limitation in the rate of delivery of nutrients. Because cell volume is typically proportional to growth rate in many bacteria, the reduced cell size of symbionts obtained from light organs that have been infected for over 24 h is consistent with that of cells experiencing growth-limiting conditions (113).

LUMINESCENCE An even more dramatic response is the change in luminescence output per cell that occurs as the proliferating *V. fischeri* symbionts adjust to the light organ environment. The specific activity of luminescence of the natural symbionts of *E. scolopes* is very low when they are outside of the symbiosis (10, 45). However, within 10 h of initiating a colonization of the juvenile light organ, the progeny of the infecting bacteria induce the synthesis of luciferase, increasing their luminescence per cell over 100-fold (113). This increase in

light emission by bacteria that are enclosed in a symbiotic light organ had been predicted to result from the accumulation of *V. fischeri* autoinducer VAI-1 in the crypt spaces (96). Such a quorum-sensing phenomenon is now also the explanation for the production of species-specific autoinducer molecules by a number of species of host-associated bacteria (36). However, to date, evidence for this hypothesis has been obtained only for the symbioses of *Euprymna* species, for which existence of high concentrations of VAI-1 have been demonstrated in extracts of light organs of adult hosts (12). Measurements of the number of bacterial cells present ($\approx 5,000$) in the nascent light organ crypts (≈ 500 pl total volume) of juvenile animals at the earliest time of induction allow the calculation of an effective density of approximately 10^{10} per ml, well above the experimentally predicted concentration for luminescence induction (12, 113). Therefore, luminescence autoinduction is a natural process in the *V. fischeri* light organ. In addition, it has been demonstrated that VAI-1 diffuses freely into the animal cells surrounding the bacterial symbionts (12), leaving open the possibility that this compound serves to signal host cell functions as well.

In spite of this dramatic increase in light output, the emission per cell of symbiotic bacteria in newly infected juvenile animals (113) is still only 25% that of bacteria in adult animals (10). Perhaps in the adult light organ, physiological factors exist in addition to VAI-1 accumulation, such as iron or oxygen availability (26, 48) or a starvation-induced activation of *groE* (20) that further enhance the symbiont's luminescence activity. Symbiosis-related modulation of *groE* expression is a common characteristic of certain pathogenic and cooperative symbionts when they are associated with their hosts (100, 101). The pattern of expression of *groE* in symbiotic *V. fischeri* during light organ colonization remains to be determined.

FLAGELLATION Besides the simple decrease in their size (113), *V. fischeri* cells undergo another morphological transformation during their development as light organ symbionts. This species is normally characterized by the presence of a tuft of sheathed, polar flagella (3). The motility imparted by these flagella is essential during the colonization process (41) and, during the initial stages of proliferation within the light organ crypts, symbiont cells continue to elaborate them. However, between 12 and 24 h after the colonization has initiated, at approximately the same time rapid growth has ceased, flagella are no longer apparent on the symbionts (113). Repression of bacterial flagella formation as a result of the symbiont's association with host tissues is the typical (21, 126), although not universal (57), condition reported among light organ symbionts, and may result from a coordinately regulated program of gene expression, as has been reported for some microbial pathogens (1). Because of the extremely

Table 2 Effect of growth rate on flagellation of *Vibrio fischeri* cells

Nutrient limitation ^a	Generation time (h)	Cell density (CFU/ml)	Percent motile cells ^b
Phosphate (2)	0.7	5.5×10^8	>95%
Phosphate (2)	5	5.0×10^8	< 5%
Phosphate (1)	20	2.0×10^8	< 5%
Carbon (2)	5	2.0×10^9	< 5%
Carbon (2)	20	1.5×10^9	< 5%

^aCells were grown in a minimal-salts, artificial seawater medium containing ammonium chloride as the nitrogen source. Phosphate limitation was achieved at a concentration of 0.1 mM DL- α -glycerophosphate; carbon limitation was achieved at a concentration of 3.4 mM glycerol. Values in parentheses indicate the number of experiments.

^bAs determined by both light and electron microscopy.

crowded condition of the bacterial population in the enclosed space of the light organs and the apparent absence of a need for movement within this space, repression of flagella elaboration is not surprising. In an attempt to understand what factor(s) might be responsible for this morphological modification in *V. fischeri* and whether the phenomenon is dependent upon a specific factor in the post-24-h light organ, a number of growth conditions were examined for their effects on laboratory cultured *V. fischeri* cells; however, no condition was found under which the cells were nonflagellated (113). More recently, using chemostat-grown cultures of either carbon- or phosphate-limited *V. fischeri* cells, it was shown that maintaining the cells at very low growth rates ($t_{\text{gen}} = 5$ or 20 h) resulted in the absence of flagellar formation (Table 2), suggesting that the change in growth rate characteristic of the pattern of proliferation in the juvenile light organ (113) might itself be sufficient to account for this phenomenon.

The repression of flagella formation in *V. fischeri* is relieved almost immediately when cells are expelled from the light organ either naturally or by homogenization of the organ. Such cells elaborate flagella and become motile within 45 to 60 min of release when placed either in nutrient media or in natural seawater containing no added nutrients (113). This latter observation indicates that even nongrowing cells can become flagellated, presumably by turnover of already existing cellular proteins. A mechanism that induces flagella elaboration should not be surprising considering that (a) such expelled cells are typically present in Hawaiian seawater and appear to be the primary source of inoculum for newly hatched animals (66), and (b) motility is required for successful colonization (41). However, taken together, these results suggest that while both rapidly growing and nongrowing cells form flagella, slow-growing ones do not, a conclusion that predicts an interesting, and perhaps novel, mechanism of control of flagella synthesis in symbiotic *V. fischeri*.

OTHER FACTORS IN SYMBIOTIC DIFFERENTIATION The pattern of communication that occurs between *Rhizobium* spp. and their plant hosts during a developing root nodule symbiosis is an elegant example of how the production of specific signal molecules by one partner can have an effect on gene expression in the other (72, 131, 132). Similarly, virulence regulons, involving numerous genes spread around the chromosome, have been identified in several pathogenic bacteria (88). Could such a coordinated response be active during the initiation of the *V. fischeri*-*E. scolopes* symbiosis?

There is evidence that *V. fischeri* and other luminous bacterial species have the capacity to exhibit pleiotropic responses to changes in their environment. For instance, the formation of certain spontaneous mutant strains, first noted by Beijerinck in 1889 (5) and later called K-variants (58, 98), has been described in the earlier literature. More recently, such variants have been reported to occur at relatively high rates and to differ from their parent strains in such characteristics as colony morphology, luminescence, motility, phage sensitivity, cell adhesion, and outer membrane proteins (27, 117). One of these studies has suggested a connection between this inherent pleiotrophy and symbiosis, but as yet no convincing evidence of a specific relationship has been presented. Almost nothing is known about how, in luminous bacteria, the acquisition of a suite of such diverse physiological and morphological traits could be seemingly regulated by a single heritable change, although variations in autoinducer levels do not seem to be responsible (117). Recently, the phase variation between the brightly and dimly luminescent primary and secondary forms of the terrestrial luminous bacterium *Photorhabdus luminescens* has been shown to be induced in culture by hypoosmotic conditions, suggesting that such an environmental variable might be important in signaling developmental phase variation in their symbiosis with nematode worms (59).

Finally, *V. fischeri* cells, when released from the symbiotic light organ into the ambient seawater, eventually enter a metabolic state in which they no longer are capable of being cultured on typical laboratory media; however, these cells remain capable of colonizing juvenile animals placed in that seawater (67). Differentiation into this state, sometimes referred to as the viable-but-nonculturable condition, may be a common feature of a number of other aquatic bacterial species (102, 107). Because this differentiation appears to be a natural part of the ecology of *V. fischeri*, the *V. fischeri*-*E. scolopes* association is a particularly relevant and attractive system in which to study a bacterium's entry and exit from this state. Taken together, the evidence described in this section suggests that the luminous bacteria have inherent mechanisms for generating multiple, coordinated responses to specific environmental cues.

Host Responses

While the evidence for a pattern of coordinated differentiation in symbiotic *V. fischeri* is as yet rudimentary, the existence of a dramatic program of bacteria-induced symbiotic development in the host light organ is well documented.

THE HOST DEVELOPMENT PROGRAM During embryogenesis, the animal develops a nascent light organ that is free of bacteria (84) but has three pores (see ciliated surface of “aposymbiotic juvenile,” Figure 2) leading to separate, empty, epithelia-lined crypts on each side of the incipient organ (Figure 4a). Upon hatching, the organ appears to be poised to capture *V. fischeri* cells from the surrounding seawater using complex, ciliated, microvillous epithelial structures (CMS) on the organ’s surface (Figure 2) (84, 91). Observation of the CMS by high-speed cinematography has revealed that these surfaces move ambient seawater past the pores, presumably to potentiate inoculation of the organ (81). As such, the CMS may be the only known example, in either animal or plant symbioses, of a tissue whose sole apparent function is to facilitate the initiation of the association by bacteria in the environment (84).

Within hours after hatching, the juvenile animal has been colonized by *V. fischeri* cells, a process that triggers subsequent morphogenesis of the light organ (84, 92). In response to interactions with the bacteria, the cells lining the crypts undergo terminal differentiation, become more cuboidal, and swell to four times their original volume, and their microvilli become lobate and branching, more completely surrounding and supporting the symbiont cells (81, 92). In addition, the crypt spaces themselves continue to grow in size as a result of the proliferation of a few epithelial cells at the blind end of each crypt. Finally, the CMS of the organ regress over a four-day period (Figure 2) as a result of a process of bacteria-induced cell death that first manifests itself at the distal extremes of the surfaces (92). The modification of host tissue through the control of an apoptosis-like event is an exciting example of interspecies signaling that has been described for pathogenic bacteria as well (16, 56).

Studies in which the symbiotic light organ infection by *V. fischeri* was cured with antibiotic treatment have revealed that the host needs to be exposed to the bacteria for only between 8 and 12 h to irreversibly induce the entire four-day morphogenetic program (19). Curing of the organ before 8 h postinfection results in no subsequent morphogenesis. Entry of the bacteria into the crypts is required for the effect (19); thus, contact with the target tissue itself (i.e. the CMS) is apparently not sufficient but must be accompanied by interactions between the bacteria and the internal crypt cells. Similarly, the induction of cell death does not require a complete colonization of the crypt spaces; infection by a strain that produces a population size that is only 10 the typical number of symbiont

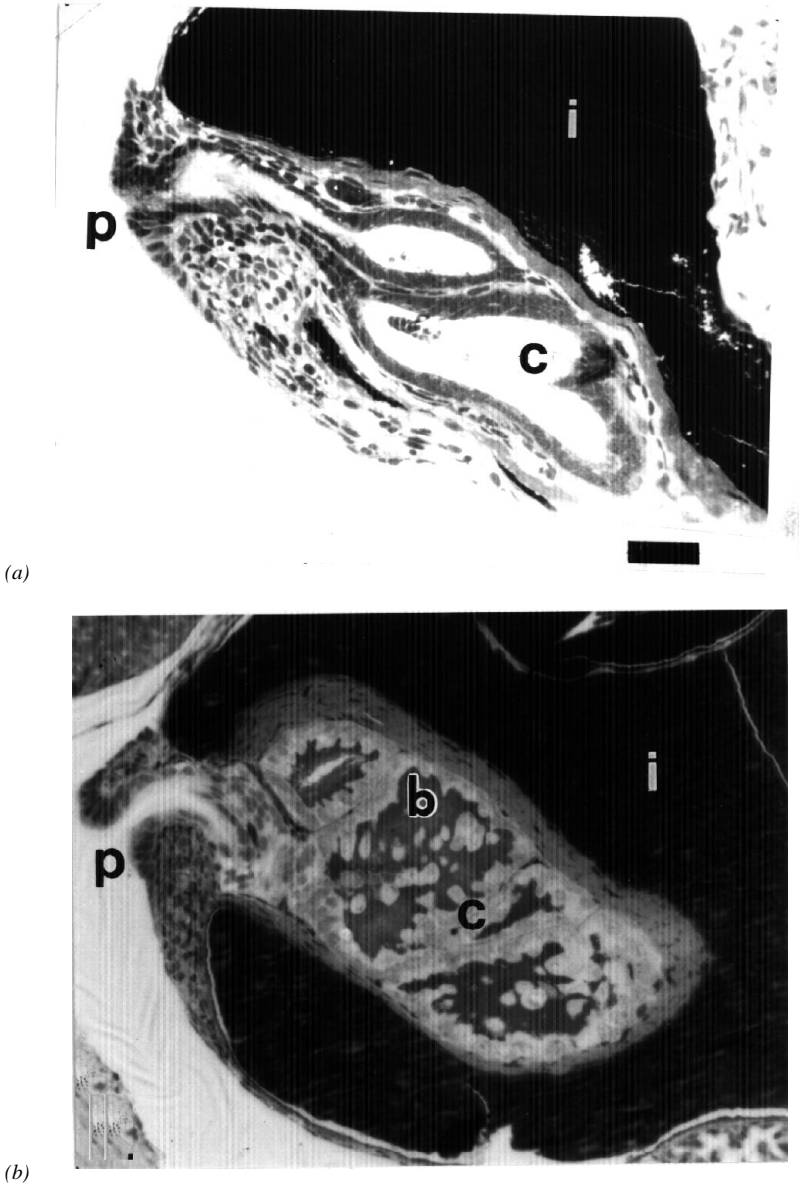


Figure 4 Colonization of the juvenile *E. scolopes* light organ. Histological sections through one side of the nascent light organ of a newly hatched aposymbiotic animal (a) and the equivalent location in a symbiotic animal ten days after colonization by *V. fischeri* cells (b). The sections reveal one of the series of pores (p) leading into the crypt spaces (c). Patches of the bacterial cell population (b) are clearly visible in the colonized animal. By ten days, the ink sac (i) has already begun to encircle the central region of the light organ in the symbiotic animal. The size bar is approximately 20 μm in length.

cells nevertheless leads to CMS regression (19). For this reason, any physical pressure that may result from a complete filling of the crypt spaces by symbiotic cells is unlikely to be responsible for initiating the morphogenic signal.

In addition to these early events, during the subsequent weeks of the symbiosis, the light organ begins to modify and create tissues that will be essential in the mature, functional association. The ink sac becomes capable of surrounding the bacteria-containing tissue (Figure 4*b*) and begins to control the amount of light leaving the organ; the reflector layer thickens; and the light organ lens (90) begins to form over the ventral surface of the organ. The extent to which bacteria are involved in the induction of these late-development events remains to be determined.

MODULATION OF LIGHT ORGAN LUMINESCENCE Each morning the host expels between 90 and 95% of its symbiont population by venting most of its crypt contents into the external seawater environment through pores on the surface of the light organ (66). The bacteria that remain within the crypts are closely attached to the microvilli of the epithelia (92, 93), and their subsequent proliferation repopulates the organ over the next 12 h, thereby restoring the full bioluminescence potential required for the host's nocturnal behavior (13). These investigations using adults have evoked a number of questions about symbiont expulsion that can best be answered with juvenile animals, which are available in much larger numbers. Questions that have been addressed include: (*a*) How soon after colonization does the daily expulsion program begin? (*b*) To what extent does the expulsion decrease the potential for luminescence by the host? (*c*) Does the capacity for luminescence of the remaining cells change?

To address these questions, newly hatched juvenile animals were colonized and their pattern of luminescence emission followed continuously for several days, during which it became clear that the expulsion process begins within the first 24 h and has a direct effect on the intensity of animal bioluminescence (13). In addition, these studies revealed a second phenomenon. The specific activity of light emission by the symbiotic bacteria (i.e. luminescence per cell) is modulated in a photoperiod-dependent behavior, varying by a factor of >10-fold during the course of the day (13). Boettcher et al speculate that the level of symbiont light emission is controlled by regulating the rate of oxygen delivery to the bacterial crypts through the host's vascular control of circulation (13). The possibility that the level of symbiont luminescence could be modulated by the flux of oxygen to the light organ symbionts has been previously suggested for anamniote fishes (57) and experimentally demonstrated in leiognathid fishes (80). Measurements of luminescence and respiration of bacteria immediately after release from a fish light organ have also supported the conclusion that, under some circumstances, oxygen can be the factor limiting symbiont luminescence (21).

The temporal studies of sepiolid luminescence constitute the first confirmed example of a photoperiodic control by the host of both the number and the biological activity of its bacterial symbionts. As yet, it is not known whether this behavior has been recruited from a preexisting diurnally controlled rhythm by which oxygen delivery to tissues is modulated, or if instead it constitutes a specific adaptation that has emerged as a part of the evolution of the symbiotic state. Interestingly, this rhythmic suppression of symbiont luminescence is apparently coordinated with the host's natural biological behavior, i.e. the daily burrowing into the surface sand layer at the beginning of daylight (121), a time at which the value of luminescence is very likely negligible.

Maintenance of the Association

Perhaps the most impressive characteristic of any stable symbiosis is its ability to persist over long periods of time. In the case of bacterial-animal associations, there is a potential that the microbial component may proliferate uncontrollably, even though the host typically has generalized defenses that remove invading bacteria from its tissues. Successful symbioses must function to maintain a balance between these two responses, and a number of examples of such stable relationships have been described (115), although the mechanisms by which the balance is achieved in any association are still poorly understood.

From the perspective of symbiotic *V. fischeri* cells, several factors have the potential to eliminate them from the light organ. Two of these have been discussed above: the daily expulsion of symbiont cells and the production of toxic oxygen species by the host. However, there has also been evidence for displacement of established symbiotic cells by other *V. fischeri* strains through direct intra-strain competition within the light organ crypts.

COMPETITION BETWEEN *V. FISCHERI* STRAINS The diel cycle of expulsion and subsequent regrowth of symbionts, together with the continuous accessibility to the crypts afforded to any bacteria in the ambient seawater by the persistence of the two lateral pores (Figure 2) (65, 92), result in conditions encouraging supernumerary infection and the selection of bacterial strains that are competitively dominant in the symbiotic association. Evidence that more than one strain of *V. fischeri* routinely exists within the light organ crypts has come from comparisons between separate isolates obtained from a single light organ (11, 62, 63), which indicates that the light organ population of a naturally infected host is more accurately considered to be a monospecific culture rather than a pure culture of *V. fischeri* (11).

Experimentally produced mixed-colonizations of a juvenile *E. scolopes* light organ with two *V. fischeri* strains can result in the rapid selection of a single dominant strain that subsequently persists in the light organ. For example,

Lee & Ruby (66) showed that two physiologically distinguishable types of *V. fischeri* strains coexist in the Hawaiian coastal seawater habitat of *E. scolopes*, but that isolates from the organs of mature animals in this location contain only one of them. Exposing aposymbiotic juveniles to seawater containing suspensions of either one or the other type gave rise to pure-culture symbiotic infections that persisted for at least four days. However, exposure of animals to mixtures of the two types initially produced mixed colonizations that, within three days, had always converted to an apparently pure culture of the one type that is typically found in field-caught animals (65). This result suggested that the naturally occurring symbiont of *V. fischeri* possesses a competitively dominant trait that is expressed at some time after the establishment of the colonization. Therefore, the crypts of the *E. scolopes* light organ are a dynamic area of symbiont colonization and competition throughout the lifetime of the host, a phenomenon similar to that reported in the algal-saccoglossan symbiosis (106) and the *Rhizobium*-root nodule associations (130). The ability to perform such competition experiments in juvenile *E. scolopes* provides an excellent method by which to screen mutant phenotypes for the loss of competitiveness relative to the parent strain.

GENETIC DETERMINANTS OF SYMBIOTIC COMPETENCY IN *V. FISCHERI*

A powerful approach to the study of factors controlling the initiation of a bacterial-animal association involves the identification and characterization of genes and genetic elements that mediate these phenomena. Such an approach is usually more easily accomplished with the bacterial partner, in which genes that are nonessential in culture but involved in the colonization of the host can be identified by the following: (a) creation of a library of mutant clones, (b) phenotypic screening of these clones for mutants expressing symbiotic defects, and (c) complementation of the defect with the isolated gene or gene product (31). To apply this paradigm to the identification of *V. fischeri* genes that are active in the *E. scolopes* symbiosis, it was first necessary to adapt and develop new molecular genetic approaches and tools for use in *V. fischeri*.

Molecular Genetic Approaches and Tools for Studies of V. fischeri

Although there have been almost 20 years of analyses of *V. fischeri lux* genes transferred to and expressed in *Escherichia coli*, no molecular genetics studies were performed in *V. fischeri* itself until 1992 (28). Since that time, a number of new methods have become available that allow the transfer of genes into and the mutagenesis of genes in *V. fischeri*.

GENE TRANSFER (CONJUGATION, ELECTROPORATION, TRANSDUCTION) In 1984, Simon, Pühler and their colleagues reported a method of conjugation that utilized a series of RP4-based, broad host-range plasmids that could be delivered to a number of species of gram-negative bacteria by *E. coli* strains carrying a chromosomal copy of the RP4 *tra* gene (119, 120). This technique was used to perform conjugative transfer of plasmid DNA into *V. fischeri* strain MJ1 and its derivatives (28), delivering *lux::lacZ* reporter fusions to monitor the control of luminescence gene expression in the natural host background (23). Subsequently, this method of conjugation has been used by other workers to deliver genetic constructions into several different strains of *V. fischeri* (37, 41, 109).

To expand the number of available gene transfer techniques, as well as to reduce the number of cloning steps involved in preparing a plasmid for delivery, Visick & Ruby (133) established electroporation as a functional approach in *V. fischeri*. The protocol developed was similar to that used with other marine bacteria and required the use of sucrose as a nonionic osmoprotectant during the washes and electroporation steps. Although DNA was easily transferred between *V. fischeri* strains, there was evidence of a restriction barrier between *E. coli* and *V. fischeri*, and it was necessary to pass DNA through a *dam*⁻ *E. coli* strain for efficient transfer to *V. fischeri*. The restriction barrier was overcome by the selection of an electroporation-variant strain of *V. fischeri* that had a >500-fold enhanced rate of transformation with DNA from *E. coli*. This variant remained able to initiate and fully colonize juvenile *E. scolopes*. Interestingly, conditions that were successful in promoting electroporation of two *E. scolopes* light organ isolates were not useful when applied to strain MJ1.

Molecular genetic studies of *V. harveyi* have made use of the well-characterized enteric transducing bacteriophage P1 (118) and have led to important discoveries not only in the mapping and control of luminescence genes, but also in the genetics of flagellation and motility (6) and phosphate and iron regulation (77, 78). Although *V. fischeri* has not proven susceptible to P1 phage, another generalized transducing phage, rp-1, was isolated and shown to infect strain MJ1 (70). Conditions have been recently established under which rp-1 was able to transduce *E. scolopes* light organ isolates as well, and this approach was used to transfer and isolate antibiotic resistance markers in a wild-type background (133). Hidaka & Kobayashi report the isolation of several other *V. fischeri* bacteriophages (54), presenting the availability of distinct transduction vectors for this bacterium that may be valuable in strain-specific selection or for screening of cell-surface mutants. The availability of transduction in *V. fischeri* presents a valuable tool for a number of genetic manipulations that will promote success in the continued study of not only symbiotic determinants but also other genetic aspects of this luminous bacterium.

MUTAGENESIS To date, three classes of *V. fischeri* mutants have been described: spontaneous, transposon insertion, and gene replacement mutants. Spontaneous mutants can be easily isolated from exponential phase cultures of *V. fischeri* by selection on medium containing antibiotics such as rifampicin (40), or novobiocin (28). Such strains, though carrying undefined mutations, have been useful recipients for conjugative transfer of transposons and other plasmid-borne DNA. There is some evidence that these mutants may express subtle alterations in growth characteristics (J Graf, unpublished data) and thus encourage the transduction of transposon insertions into the wild-type genetic background (133).

The Mu derivative *MudII68I*, which contains a transposase under the control of a temperature-sensitive repressor, was easily mobilized into *V. fischeri* strains, and 75% of these transconjugants contained single, apparently random, genomic transposon insertions if the selection was performed at 15°C. One drawback of this transposon was its tendency to produce multiple insertions when the growth temperature was raised to 23°C to cure the donor plasmid (41). A more convenient transposon system for work in *V. fischeri* is now available in the form of a miniTn5Cm that does not carry the gene for its own transposase (43). The transposase is encoded on the suicidal donor plasmid pUT carrying the miniTn5Cm, which can be transferred from *E. coli* to *V. fischeri* by conjugation. Insertion mutants can then be selected by chloramphenicol-(Cm)-resistance. Only a single, apparently random, transposon insertion occurs in each chromosome, although occasionally the plasmid vector can be maintained in *V. fischeri* recipient strains.

Gene replacement has proven to be an effective and controlled method for introducing desired mutations into *lux* genes (28, 133) and the *toxRS* operon (108) of *V. fischeri*. Vectors based on the pACYC184 origin appear relatively stable in symbiotic isolates, and in contrast, a pBR322-based plasmid can be used as a useful suicide vector for gene replacement in at least some *V. fischeri* strains when introduced by electroporation (133). Work with strain ES114, the best studied of the symbiotic isolates from *E. scolopes*, has revealed that relatively long flanking regions (> 1.5 kbp) are needed for efficient homologous recombination (K Reich, unpublished data). The availability of these techniques has opened a new and rapidly developing field of the molecular genetics of symbiotic luminous bacteria.

LOCATION OF SYMBIOTIC DETERMINANTS Carrying genes involved in host association (or virulence) on plasmids or other mobile genetic elements is not uncommon in facultatively symbiotic bacteria (11). Thus, the discovery that different strains of *E. scolopes* symbionts typically carry one to several plasmids, often including a large (> 39 kbp) one with extensive sequence similarity

between strains, suggested that symbiosis determinants might be located on these elements (11). All efforts to cure the large plasmid in culture were unsuccessful, even though it apparently contained no sequences required for *V. fischeri* growth: i.e. they are not found in the chromosome of other, closely related but plasmid-free, symbiotic strains (11). Perhaps this plasmid bears a DNA restriction/modification system that causes death in cells that attempt to rid themselves of the plasmid (60, 94). Carriage of such a system might also explain the electroporation barrier described above (133).

Comparisons of the colonization capacity of strains carrying one of these large plasmids with that of related strains have revealed no significant differences between the strains in either the rate or the extent of colonization of juvenile animals (11). Even competition experiments challenging plasmid-free strains with plasmid-carrying ones gave no suggestion of an increased symbiotic fitness of one over the other. Thus, although these plasmids remain cryptic in function, they most likely will not be the location of significant symbiosis determinants that, instead, should be expected to reside primarily on the chromosome.

Using V. fischeri Mutants to Characterize the Light Organ Crypt Environment

Only two years have passed since the first symbiosis determinants for the *E. scolopes* association were reported. As far as I am aware, this model system continues to be the only bacterial-animal cooperative association in which symbiosis determinants and their functions have been identified by genetic means. The list of phenotypes and/or genes that are required for a normal symbiotic competency in *V. fischeri* continues to grow (Table 3), and three classes of colonization defects have been defined (Figure 5). A few of the members of these various mutant classes are discussed in detail below.

MOTILITY Host-associated microorganisms often must move to specific target tissues that are the sites of colonization. Thus it is not surprising that motility is a behavioral trait that has been associated with colonization success and virulence in a number of pathogenic bacteria (1). To address the question of whether motility is required for successful colonization of the juvenile light organ crypts, mutants of a symbiotically competent strain of *V. fischeri* were produced by transposon insertion in the chromosome, and 18 nonmotile strains were isolated (41). These strains were grouped into two classes based on either the presence or absence of a flagella filament. When tested in the colonization model, none of the strains from either class were capable of initiating a successful colonization, even when presented at 100 times the concentration or for four times the duration typically used for the wild-type parent (41). Thus, not only does

Table 3 Classes of *V. fischeri* symbiosis mutants

Cellular phenotypic defect	Expected genetic defect	Colonization defect ^a	Reference
Motility	<i>mot</i> (?)	Initiation	41
Flagellum	<i>fla</i> (?)	Initiation	41
Luminescence	<i>luxA</i>	Accommodation/persistence	113
Autoinducer synthesis	<i>luxI</i>	Accommodation/persistence	133
Autoinduction	<i>luxR</i>	Accommodation/persistence	133
Siderophore	<i>glnD</i>	Persistence	43
Amino acid synthesis	(various)	Accommodation	42
cAMP utilization	(?)	Initiation	^b
ToxR synthesis	<i>toxR</i>	None	108 ^c

^aBased on their response in the juvenile squid colonization assay (see Fig. 5).

^bEG Ruby, unpublished data.

^cK Reich, EG Ruby, G Schoolnik, unpublished data.

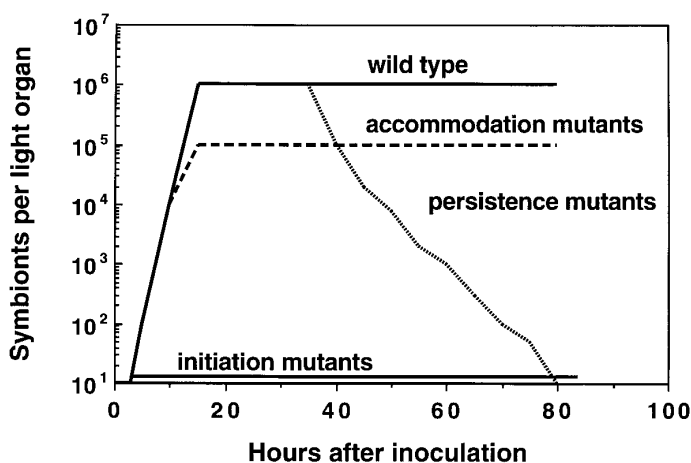


Figure 5 Three stages in the colonization of the *E. scolopes* light organ by *V. fischeri* cells. The process of symbiotic colonization is artificially divided into stages: “initiation” (0 to 3 h), “accommodation” (3 to 12 h), and “persistence” (beyond 12 h). Wild-type *V. fischeri* cells (heavy line) initiate a symbiotic infection within a few hours. This initiation is followed by a rapid period of growth, during which the cells must accommodate themselves to the specific conditions of the light organ crypts, finally achieving a typical population of about one million cells. Subsequently, the symbionts must persist within the crypts for the remainder of the life of the host. Mutant strains of *V. fischeri* (Table 3) have been isolated that demonstrate a defect in one of these three stages of symbiotic colonization as indicated by the additional lines.

flagellation appear to be a required trait for colonization, but also motility itself, and not simply the presence of a flagellar structure, is most likely the crucial factor. As yet there are no results that can address the question of why motility would be required; however, one might imagine that active movement is necessary for *V. fischeri* cells to find and pass through the juvenile light organ pore, down the mucous-filled ciliated passage (Figure 4a), and into the crypt spaces. This process might even require a response to a general or specific chemotactic cue (73) emanating from the crypts themselves. Although there have been no reports of chemotaxis by *V. fischeri*, chemotaxis to both amino acids and cyclic nucleotides has been demonstrated in the related luminous bacterium *V. harveyi* (2).

LUMINESCENCE Mutations in any of at least five genes can lead to the loss of a *V. fischeri* cell's ability to luminesce. Evolutionary theory predicts that without the imposition of a selective pressure, the energetic savings to such a cell would put it at a growth advantage relative to the other light-emitting members of the population. Why then have dark mutants never been isolated from bobtail squid light organs? Perhaps there is a selective advantage in producing light that maintains a fitness advantage for cells that produce luminescence (51). Certainly imposition of such a selection would be useful to the host, assuring that the population of symbionts continues to be a source of luminescence. It has been postulated in the past that such a selection might be imposed if the activity of luciferase had a protective effect, for instance, by reducing the ambient oxygen concentration (50).

To directly test whether luminescence itself has a selective effect in a symbiotic system, a dark, *luxA*-deletion mutant of a symbiosis-competent *V. fischeri* strain was created by gene replacement (133). Colonization of the juvenile light organ by this dark strain was normal for the first 24 h; however, by 48 h postinfection, the number of cells present in the light organ had diminished by a factor of 5–10 (K Visick, unpublished data). Thus, a dark strain of *V. fischeri* appears to have a decreased symbiotic competence in the persistence phase of the symbiosis.

By what mechanism might the functioning of the luciferase enzyme contribute to the ability of luminous bacteria to survive within the light organ? A clue to this question may exist in the evidence for high levels of a host PO (129, 136), as discussed earlier. This enzyme is used by many vertebrates as part of their defense against bacterial pathogens: It catalyzes the terminal step in a cascade that begins with the bacteria-induced oxidative burst using oxygen and NADPH oxidase to produce H_2O_2 and ends with the synthesis of HOCl from H_2O_2 and chloride ion (35). If a population of bacteria could lower the ambient concentration of oxygen to a level below the affinity constant for NADPH

oxidase, then the synthesis of hydrogen peroxide (and thus HOCl) would be inhibited (137). Because bacterial luciferase can have an affinity for oxygen higher than that of both cytochromes and NADPH oxidases (7, 71), one might expect that respiring, light-emitting bacteria would create a lower ambient concentration of oxygen than respiring, dark ones (87). Thus the host could assure that dark mutants would not accumulate in the light organ population.

IRON SEQUESTRATION An essential inorganic nutrient for the growth of most bacteria is iron (usually as Fe^{+3}). For this reason, many animals generally maintain exceptionally low concentrations of free iron in their tissues and fluids by sequestering it in a form bound to a siderophore such as transferrin (111). Bacteria that produce siderophores with a higher binding affinity for iron can proliferate by gaining access to the host's store of this nutrient. *Vibrio* spp. have been shown to produce several different types of siderophores (52, 128), including an α -hydroxamate siderophore synthesized by *V. fischeri* (125). Light organ symbionts from *E. scolopes* also produce a siderophore, as indicated by the colorimetric CAS agar halo reaction (65). This presents the possibility of determining whether the production of a siderophore is essential for successful colonization of this host.

In experiments by Graf & Ruby, a miniTn5Cm mutant (designated SP301) produced practically no halo on CAS agar. Relative to the parent strain, SP301 had a severely depressed growth rate and yield in an iron-limited minimal medium that were reversible by the addition of FeCl_3 (43). Addition of supernatant from a culture of the parent strain, which contained siderophores, did not enhance growth of the mutant. Thus, the mutation affected not only siderophore production but the synthesis of the siderophore receptor as well. Using a more sensitive siderophore assay (18) revealed that SP301 actually could synthesize a small amount of the siderophore produced by the parent strain, suggesting that the transposon interrupts a regulatory gene (40).

Strain SP301 colonized the host as rapidly, and to the same extent, as wild-type *V. fischeri*, suggesting that a normal level of siderophore production is not required for initiation of the association. However, if SP301 cells were pregrown in a medium that was low in iron prior to using them as an inoculum, the number of symbionts present in the crypts 72 h after initiation decreased significantly, suggesting that iron availability became more limiting in the crypts with time, and thus, cells that were iron depleted going into the association were less able to maintain and proliferate than were iron replete cells (40). An alternative reason for the apparent symbiotic requirement for siderophore production might be for the removal of free Fe^{+3} to discourage the Fenton reaction, which produces toxic hydroxyl ions (33). Taken together, these results suggest that the insertion affects the expression of several genes including, but perhaps not restricted to, those for siderophore metabolism.

AMINO ACID AUXOTROPHY Bacteria growing either intracellularly or in contact with host tissue often have access to monomeric building blocks such as amino acids, nucleoside bases, and vitamins (76). One way to determine whether a host supplies such compounds to their symbionts is to construct auxotrophic mutants and determine whether they are capable of colonization in spite of their metabolic defect (42, 69, 75, 110). Nine transposon mutants of *V. fischeri* were isolated that were each auxotrophic for one of the following amino acids: Ala, Arg, Cys, Gly, Leu, Met, Pro, Ser, or Thr. These strains were essentially normal in growth and luminescence induction when grown in a complete nutrient medium (40). All were found to retain the ability to colonize the crypts of juvenile bobtail squid, albeit to reduced levels (between 2% and 93% that of wild-type) (40). Therefore, the crypt environment must provide sufficient levels of these nutrients to allow the proliferation of between 10^4 and 10^6 *V. fischeri* cells. The ability of two of these mutants to persist in the symbiosis was examined, and in both cases the bacterial population maintained its characteristic level for at least 72 h. Because the host expels the majority of its symbionts every 24 h (66), it must be providing a constant ration of each of these amino acids, at least on a daily basis, to allow a repopulation to the characteristic level for that auxotroph (40).

CYCLIC AMP UTILIZATION An unusual, periplasmically located (25, 29), high activity (15), 3':5'-cyclic nucleotide phosphodiesterase found in *V. fischeri* and a handful of other marine bacteria is responsible for the first step in the metabolism of cAMP as a sole source of carbon, nitrogen, phosphorous, and energy. The gene encoding this activity, *cpdP*, has been cloned and used to construct a *cpd* mutation in *V. fischeri* strain MJ1 (25). This enzyme seems to allow *V. fischeri* to exist, perhaps preferentially, in an environment in which cAMP is a regular and important nutrient. Recent reports have demonstrated that cAMP is secreted into pig intestine lumen as a result of the addition of cholera toxin, a potent ADP-ribosyltransferase secreted by *V. cholerae* (105). The discovery that *V. fischeri* produces a secreted ADP-ribosyltransferase in culture (109) has led to the suggestion that this activity may evoke cAMP production by the host epithelium and its secretion into the light organ crypts (24). If such an activity occurs in and is of importance to the developing symbiosis, then the ability to utilize external cAMP might serve as a specificity determinant or provide a competitive advantage to *V. fischeri* strains (65). This hypothesis has yet to be tested because at present the only available *cpdP* mutant is in the symbiotically incompetent strain *V. fischeri* MJ1.

AUTOINDUCERS Ever since the discovery that luminescence gene expression was modulated by the accumulation of an autoinducer (AI) molecule (96),

it has been postulated that genes encoding symbiotic functions in addition to luminescence might be controlled by AI(s). During the past five years, numerous other examples of cell-density-dependent transcription-activating mechanisms have been described among a diversity of bacteria that associate with animal and plant hosts (36). Two important facts have become increasingly evident from studies of these many systems: (a) more than one AI system may exist within a given bacterium (4, 37, 104, 127), and (b) a single AI system may control the expression of multiple, and apparently functionally unrelated, genes (36). Both of these points lend support to the possibility that *V. fischeri* AI-regulated gene expression may include genes of importance to symbiotic competence. To date, however, the only AI-induced genes reported in *V. fischeri* are the VAI-1 and VAI-2-regulated genes in the *lux* regulon (61, 122).

Nevertheless, a number of intriguing observations have been made concerning the possible role of AIs in symbiosis. Light emission by *luxI* and *luxR* mutants of a symbiotic strain of *V. fischeri* is significantly diminished in culture, although luminescence remains detectable (K Visick, unpublished data). The mutant strains are able to colonize juvenile animals normally during the first 24 h after infection; however, by 48 h, their colonization level is only about 10% that of the parent (K Visick, unpublished data). Because the same symbiotic deficiency is observed with the *luxA* mutant (see above), the most parsimonious explanation at the moment is that the diminished light production common to all these mutants is responsible for the phenotype and that it does not require the absence of VAI-1 production.

V. fischeri contains an additional, genetically distinct locus (*ain*) that is responsible for the synthesis of a second AI (VAI-2) (61). Sequence analysis of this locus has revealed the presence of two genes (*ainS* and *ainR*) that encode proteins that appear to be responsible for the synthesis and activity of VAI-2 (37). Continued study of VAI-2 and its possible role in light organ colonization must await the production of *ain*-defective mutants in symbiosis competent strains.

CONCLUSIONS AND FUTURE DIRECTIONS

Recent interest in the *V. fischeri*–*E. scolopes* light organ association has opened a window into the biology of cooperative bacterial-animal symbioses and their underlying biochemical and molecular mechanisms. Clearly, we are in the early days of describing the process of symbiotic colonization in this association, yet already this model has produced some general lessons in the study of benign colonizations of animal tissue.

1. The mechanisms controlling symbiotic specificity can be in force very early in the development of the association, and can distinguish not only between bacterial species, but between strains of a species, as well.
2. Initiation of the association can trigger a complex program of development in each of the partners, and have an especially dramatic effect on the host.
3. Defects in the process of symbiotic colonization exist in loci that encode either regulatory or structural genes.
4. Mutants carrying such defects can be used as bacterial “probes” to help characterize the microecology of the host-symbiont interaction.

Finally, the study of the *V. fischeri*–*E. scolopes* association has reaffirmed an important lesson for both pathogenic and nonpathogenic microbiologists: When examining the biology of a bacterial-host association, one must remember that it very often proceeds through a complex conversation between two specific and well-acquainted partners. As succinctly voiced by Falkow, in the study of such associations, “one cannot neglect the complex role of the host (although microbiologists and molecular biologists often do)” (32). The continued investigation of this symbiosis from the viewpoint of both *V. fischeri* and its host will strengthen the insights this experimental model brings to understanding other cooperative, as well as pathogenic, bacterial-animal associations.

ACKNOWLEDGMENTS

Much of the work covered in this review would not have been possible without the close collaboration of M McFall-Ngai and her laboratory. I also thank F Aeckersberg, L Gilson, J Graf, M Nishiguchi, and K Visick for their advice and critical reading of the manuscript. The data presented in Table 2 were obtained by L Asato, and Figures 3 and 4 were provided by M McFall-Ngai. The following people graciously communicated results prior to publication: S Clegg, L Gilson, J Graf, M McFall-Ngai, M Nishiguchi, K Reich, K Visick, and V Weis.

Much of the research presented here was supported by grants from the National Science Foundation to M McFall-Ngai and EG Ruby (IBN 92-20482), and from the Office of Naval Research to EG Ruby (N00014-93-I-0846) and to M McFall-Ngai (N00014-91-J-1357).

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