

Review

Negotiations between animals and bacteria: the ‘diplomacy’ of the squid-vibrio symbiosis[☆]

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Abstract

A shared characteristic among animals is their propensity to form stable, beneficial relationships with prokaryotes. Usually these associations occur in the form of consortia, i.e. a diverse assemblage of bacteria interacting with a single animal host. These complex communities, while common, have been difficult to characterize. The two-partner symbiosis between the squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* offers the opportunity to study the interaction between animal and bacterial cells, because both partners can be cultured in the laboratory and the symbiosis can be manipulated experimentally. This system is being used to characterize the mechanisms by which animals establish, develop and maintain stable alliances with bacteria. This review summarizes the progress to date on the development of this model. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

The density of prokaryotes in marine environments ranges from 10^5 to 10^6 cells per ml of seawater in nutrient poor regions to over 10^7 cells per ml in high-productivity, nearshore habitats (Austin, 1988). Such concentrations of bacteria not only characterize present-day oceans, but are believed to be similar to the concentrations of bacteria in the oceans over the last 1–2 billion years. During this period, and within this environmental context, not only did the eukaryotic cell

evolve, as a result of alliances created among subsets of the microbial world (Margulis, 1971), but also the metazoans arose and radiated. Whereas biologists have long appreciated that the formation of the eukaryotic cell had a major impact on the evolution of the biosphere, the consequences of evolving in a microbial-rich milieu are poorly understood and have been little explored when considering the evolution of metazoa (Margulis and Fester, 1991). However, every animal examined has been found to maintain stable relationships with a specific array of prokaryotes, most commonly in consortia associated with the integument and alimentary canal (McFall-Ngai, 1998b). Because this specificity persists from generation to generation, it can be hypothesized that these animals have coevolved with their symbionts, and that the speciation of

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an animal host is accompanied by the divergence of its associated microbial species. Conversely, when an animal species becomes extinct, a subset of the microbial world can be also forever lost (Staley, 1997). Thus, animals and their microbial partners can be best viewed as coevolving communities, the nature of which is, at any given time, the result of reciprocal interactions among the members of that community.

The mechanisms by which the most common types of associations, i.e. consortial, are established and maintained, both evolutionarily and through the life history of an individual animal, have been very difficult to define. Technical difficulties arise because microbial consortia present a highly complex subject for analysis. Even when the situation is simplified by the symbiosis being a more approachable, two-partner relationship, it is rare that both partners are culturable outside of the symbiotic state. Recently, several model systems have become available that together promise to provide significant insight into the nature of animal-bacterial associations (Ruby, 1999a,b). One of these models, the subject of this review, is the relationship between the Hawaiian sepiolid squid *Euprymna scolopes* and its marine luminous bacterial partner *Vibrio fischeri* (McFall-Ngai, 1998a; Ruby, 1999a,b). This symbiosis is the most common type of animal-bacterial interaction: the association of specific extracellular bacteria with the apical surfaces of epithelial cells of the animal host in a relationship that begins anew (i.e. horizontally transmitted) between generations. The squid host is abundant and reproduces under laboratory conditions, and the symbiont is culturable. In addition, methods for the genetic manipulation of the microbe have been developed (Ruby, 1999b) as a mechanism by which to analyze gene expression in both partners. Together these features render this association an ideal experimental model for the study of animal-bacterial symbioses (McFall-Ngai, 1998b).

Currently, examination of the squid-vibrio system is being focused on questions that reflect the four principal stages in the establishment and maintenance of horizontally transmitted symbioses. (1) How do a newly hatched host and the presumptively symbiotic, free-living bacteria increase their chances of encounter for the timely, effective onset of the association? (2) By what mechanisms do the partners recognize one another to ensure specificity and exclusivity of their rela-

tionship? (3) What kind of developmental changes in the host and symbiont accompany accommodation to the association? (4) How is a balance created in the fully established association, such that the host does not eliminate the symbiont nor does the symbiont overgrow the host? The answers to these questions promise to provide insight not only into the ontogenic progression of beneficial associations, but also into the salient differences between the biology of cooperative animal-bacterial interactions and pathogenic ones.

2. The general biology of the symbiosis

The host animal, *E. scolopes*, belongs to the coleoid cephalopods in the family Sepiolidae (Nesis, 1987). This nocturnal predatory squid has a complex, bilobed, light-emitting organ in the center of its mantle cavity (McFall-Ngai and Montgomery, 1990). The light generated by the bacterial symbionts in the organ allows the host squid to produce a controlled, diffuse ventral glow, which is thought to be used in antipredatory behavior (McFall-Ngai, 1990). Descriptions of the light organ structure have revealed an integrated set of tissues that serves both to support a culture of a specific luminous bacterium nutritionally, and to control and direct the luminescence produced by this culture.

V. fischeri is the only bacterium persistent in the light organ of *E. scolopes*; in the absence of a sufficient abundance of *V. fischeri* cells, the light organ crypts remain essentially sterile (McFall-Ngai and Ruby, 1991). This gram-negative, marine heterotroph can be easily isolated either from the light organ symbiosis (McFall-Ngai and Ruby, 1991) or from planktonic populations in the environment (Nealson and Hastings, 1991). *V. fischeri* can be readily grown on both minimal and complex media. An extensive literature exists concerning the biology of *V. fischeri* (Nealson and Hastings, 1991), particularly the biochemistry and molecular biology of its bioluminescence capability (Meighen, 1991).

Beginning about halfway through the embryogenesis of the host, the tissues that will constitute the symbiotic light organ begin to develop in the center of the mantle cavity (Montgomery and McFall-Ngai, 1993). At hatching, the light organ is ready to interact with environmental *V. fischeri* cells. Two epithelial tissues characterize the nascent light or-

gan (Fig. 1; McFall-Ngai and Ruby, 1991): (i) each of the two lateral surfaces of the organ is covered by a ciliated, microvillous epithelium that surrounds three, 10- μ m pores; and (ii) each pore leads via a long duct to an epithelium-lined crypt, the eventual site of *V. fischeri* colonization. Once the bacterial symbionts enter the crypt spaces, they encounter at least two types of host cells. In addition to the polarized epithelial lining, *V. fischeri* interacts with a population of free-ranging hemocytes that sample the crypt spaces (Nyholm and McFall-Ngai, 1998). The polarized epithelium circumscribes two regions in each crypt (Fig. 1D), both of which are colonized by symbionts: a larger, or more lateral lacuna, and a series of narrower, more medial, blind-ended diverticula (Visick and McFall-Ngai, 2000). Within ~ 12 h of entry, the initial inoculum of bacteria has proliferated to $\sim 10^6$ cells, which fill the crypt spaces (Ruby and Asato, 1993). The resulting symbiosis follows a dynamic diel rhythm that exhibits two conspicuous

and quantifiable events: a predictable, 1000-fold fluctuation in the level of luminescence emitted from the crypts (Boettcher et al., 1996), and an expulsion of $\sim 95\%$ of the crypt contents at dawn each day (Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998). The daily loss of bacteria from the organ only accounts for about one half of the 1000-fold decrease in luminescence; the other half is due to a fluctuation in the luminescence per *V. fischeri* cell in the host organ, most probably the result of the host's manipulation of oxygen delivery to the crypt environment (Boettcher et al., 1996).

3. The onset of the symbiosis

In habitats containing populations of *E. scolopes*, competent *V. fischeri* cells are typically present at a concentrations averaging 500 cells per ml of seawater, or less than 0.1% of the total bacterial population (Lee and Ruby, 1994, 1995). When presented with these densities of specific

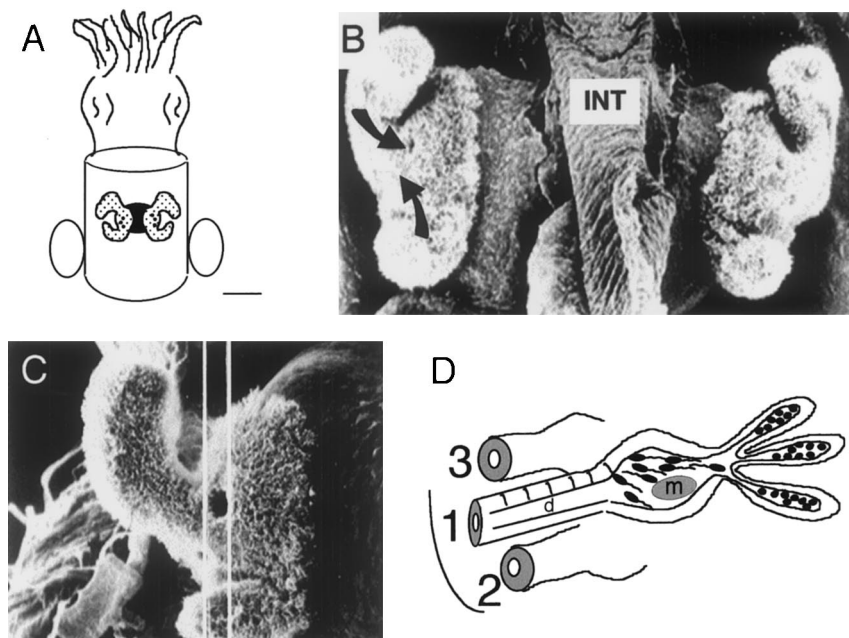


Fig. 1. Tissues and cells of the squid-vibrio symbiosis. (A) A diagram of the host showing the location of the light organ. When viewed through the ventral surface of the animal, the light organ appears as a conspicuous structure in the center of the mantle cavity. Ciliated epithelial fields (stippled) occur on each lateral surface of the organ (bar, 0.5 mm). (B) An SEM of the ventral surface of a hatchling light organ. The activity of the ciliated field entrains symbiont-laden water into the vicinity of three pores at the base of each set of appendages (arrows indicate the region in which the pores are located). (C) One half of the hatchling light organ showing an open pore (vertical lines spanning a distance of 10–15 μ m). (D) A diagram of the internal portion of the hatchling light organ. The crypts of the light organ occur ventral to the ink sac. Each of the three pores (1, 2 and 3) leads by a duct (d) to an independent crypt. The crypt space comprises two principal areas. The lateral, more expansive area houses bacterial symbionts that are larger and motile. Host macrophages (m) are often seen in this region. The more medial, blind-ended area of the crypt consists of three narrow diverticula. In these regions, the bacteria are smaller and apparently non-motile.

and non-specific bacteria under laboratory conditions, the newly hatched host's light organ is colonized by *V. fischeri* within hours. However, a theoretical consideration of the nature of both the 'landscape' associated with colonization and the scale over which the process takes place suggests that encounters between the bacteria and susceptible host tissue must be facilitated. Specifically, during each ventilatory cycle of ambient seawater through the mantle cavity, which occurs three to four times each second, the host squid brings in 1–1.5 μl of fluid (M.J. McFall-Ngai, personal observation). Given the concentration of the symbiont in the squid's habitat, on average, a single *V. fischeri* cell would enter and exit the mantle cavity every 0.3 s! During that period, to colonize the organ, the bacterium must find one of the six pores on the surface of the organ.

Early studies of the development of the association revealed that, following the colonization of the light organ with the symbiont, the superficial ciliated field regresses (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1994). This finding suggested that the activity of the field is somehow involved in the inoculation process. Recent studies of light organ colonization, using confocal microscopy to visualize the interaction of GFP-labeled *V. fischeri* cells with host tissues, have revealed that the cilia of this surface interact with secreted host mucus to harvest the symbiont from the environment (Nyholm, 1999). The precise mechanisms by which these ciliary mucus currents operate to facilitate light organ colonization remain to be determined. However, this activity provides a means to increase the probability of successful colonization by the relatively rare *V. fischeri* cells.

The bacterial symbiont is not a passive player in the colonization process. Studies with *V. fischeri* mutants defective in motility, either due to the absence of the flagellum or a defective 'motor', are not capable of initiating a relationship with the host squid (Graf et al., 1994). Motility may be required to bring the symbionts into the vicinity of the pores. Also, it is very likely to be essential for transmission through the ducts and into the crypts; the ducts are lined by ciliated cells that beat outwardly (McFall-Ngai and Ruby, 1998), i.e. against the path that bacteria must follow to enter the crypts.

4. Recognition and specificity

Because *V. fischeri* is the only bacterium that can establish a relationship with *E. scolopes*, there must be mechanisms by which recognition and specificity are controlled. These mechanisms can be divided into two categories: (i) host and symbiont receptor-ligand interactions between the surfaces of the host and symbiont cells; and (ii) the creation of an environment in which only *V. fischeri* is viable. Our data suggest that both of these mechanisms are operative in this symbiosis and that both partners participate in their function (Visick and McFall-Ngai, 2000). Events occurring in three locations must be considered in an examination of these processes: in the secreted mucus material outside of the pores, in the ducts, and in the crypts. Although discrimination between *V. fischeri* cells and non-specific bacteria may begin as early as during the interaction with the host-secreted mucus that functions to harvest the potential symbionts, any possible enrichment process at this stage remains to be characterized. However, because *V. fischeri* represent such a small proportion of the bacterioplankton (Lee and Ruby, 1994, 1995), it is likely that they either attach to and grow in the mucus, or persist in its matrix while non-specific bacteria are discouraged. Also, the conditions in the ducts that connect the external environment with the crypt spaces have not been fully described, but bacteria are rarely observed in these relatively lengthy ($\sim 20 \mu\text{m}$ long) passages in either juveniles or adult hosts (McFall-Ngai, personal observation). Thus, the duct may represent some kind of hostile gauntlet that is difficult for other bacterial species to negotiate.

While it has not yet been confirmed that an active discrimination process occurs in either the secreted mucus or the light organ ducts, a substantial body of evidence suggests that the crypts themselves represent a significant site of selection for an exclusive relationship between *E. scolopes* and *V. fischeri*. Once in the crypts, the symbionts grow very quickly, with a doubling time of once every 20 min, until they fill the crypt spaces, at which time the growth rate slows (Lee and Ruby, 1994). Even at these early stages of the symbiosis, the bacteria interact both with the microvilli of the crypt epithelium and with host macrophages present within the crypts. Analyses of the surfaces of the symbiotic partners, as well as experiments

with specific lectins, have provided evidence that receptor-ligand interactions are involved with recognition in the squid-vibrio system (McFall-Ngai et al., 1998). Mannose-recognizing adhesins are abundant on the cell surface of *V. fischeri*, and mannose is the most common glycan on the crypt brush border. Coincubation of mannose analogues with the bacteria during experimental infections inhibits colonization, i.e. these reagents appear to compete with *V. fischeri* for receptor sites.

The population of macrophages within the juvenile crypts varies in number from none to several at any given period (Nyholm and McFall-Ngai, 1998). Ultrastructural studies of these macrophages have indicated that they phagocytose bacteria in that environment. Thus, one line of defense against colonization of the crypts that must be avoided by *V. fischeri* cells appears to be the activity of these phagocytic cells in the crypts. The symbiont must either evade attachment to and engulfment by the macrophages, or the cells must grow at a rate that exceeds the rate at which they are cleared.

In addition to the activities of these two classes of crypt cells, the chemical environment of the crypt matrix may also be an important specificity determinant. Interesting, as with the endosymbiosis of the bacteria that gave rise to mitochondria (Margulis, 1971), oxygen and the byproducts of oxidative reactions appear to be key players in the interaction between the partners. Several lines of evidence suggest that the crypts are oxidatively stressful (Ruby and McFall-Ngai, 1999). Elevated levels of the reactive oxygen species superoxide anion and H_2O_2 have been detected in light organ tissue (Small and McFall-Ngai, 1993). Further, a halide peroxidase (HPO), similar to the antimicrobial mammalian enzyme myeloperoxidase, is present in abundance in the crypt space (Weis et al., 1996). This type of enzyme catalyzes the conversion of H_2O_2 and halide ions into microbicidal hypohalous acids (for review see Klebanoff, 1991). High levels of HPO also occur elsewhere in the squid, where pathogenic bacterial infections have arisen (Small and McFall-Ngai, 1999), further implicating this enzyme in the control of animal-bacterial interactions, both cooperative and pathogenic. In the light organ as well as in other cooperative animal-bacterial associations (Ruby and McFall-Ngai, 1999), the HPO may serve to control symbiont number and/or continu-

ally prevent the infection of non-specific bacteria that are susceptible to the activity of the enzyme.

The only known defense that bacteria have against halide peroxidases is either to prevent the production of the H_2O_2 substrate, or remove it once it is produced. Light production by *V. fischeri* cells, an enzymatic process that is the result of the activity of bacterial luciferase, a mixed function oxidase (Meighen, 1991), may keep the levels of H_2O_2 low by effectively removing oxygen from the crypt spaces thereby inhibiting the reactions that produce this toxic oxygen species. Bacteria with mutations in the genes that control luminescence expression show a colonization defect in the organ (Visick and Ruby, 1996; Visick et al., 2000). These data suggest that either the light produced, or more likely, the oxygen utilization by luciferase, is required for a normal phenotype. In addition, *V. fischeri* cells produce a catalase that they transport into their periplasmic space, i.e. between the outer cell membrane and the cell wall. Mutants that lack this catalase activity, which removes H_2O_2 , thereby inhibiting HPO activity, are unable to colonize normally (Visick and Ruby, 1998).

In addition to being oxidatively stressful, the environment may also exhibit nutrient limitation (Ruby, 1999a,b). Studies with amino acid auxotrophs, i.e. bacteria defective in the synthesis of specific amino acids, have indicated that host-derived amino acids constitute a substantial source of nutrients for the symbionts (Graf and Ruby, 1998). It is not yet known whether a specific signal from *V. fischeri* is needed to elicit the host's production and export of the nutrients. However, the swelling of the epithelial cells lining the crypts and an abundance of secretory granules occurring along the apical surfaces of these cells are suggestive that a high level of transport is an critical process in the symbiosis (McFall-Ngai and Montgomery, 1990; Montgomery and McFall-Ngai, 1994).

5. Development of the association

Both host and symbiont exhibit morphological changes in response to the interaction. In the host, at ~12 h following the initiation of colonization, the bacteria signal the regression of the superficial ciliated field on the surface of the organ, a morphogenesis that requires ~4 days to complete

(Montgomery and McFall-Ngai, 1994; Foster and McFall-Ngai, 1998). Experimental manipulation of this process indicates that the bacteria signal from inside the crypts to the remote surface (Doino and McFall-Ngai, 1995). Thus, interactions with either the crypt epithelia or macrophages transduce the signal to the site where it is effective. In addition, the induction is irreversible, i.e. although the program of regression requires 4 days, the symbionts can be eliminated from the organ with antibiotics after 12 h of exposure and the entire program will continue normally (Doino and McFall-Ngai, 1995).

The nature of the bacterial signal(s) and the mechanisms by which these signals exert their developmental effects remain largely unknown. However, analyses of the regression process have suggested that it is at least partially due to the induction of programmed cell death of the superficial epithelium by the bacterial surface molecule lipopolysaccharide (LPS; Visick et al., 2000). The involvement of LPS in the symbiosis is not surprising; this molecule has been implicated in the induction of host cell responses in a number of animal-bacterial interactions (Raetz, 1993; Ulevitch and Tobias, 1995; Fenton and Golenbock, 1998). Most often LPS binds to host-cell receptors belonging to the Toll/IL-1 family. This event leads to changes in host gene expression through the activity of the transcription factor NF- κ B (Belvin and Anderson, 1996; May and Ghosh, 1998). The gene products that result mediate the response of host cells to the presence of bacteria in their environment. Experiments designed to determine whether these pathways are critical in the squid-vibrio association are currently under investigation.

The crypt epithelia also respond to interaction with the bacteria by swelling (Montgomery and McFall-Ngai, 1994; Doino, 1999), which results in a fourfold increase in the volume of the cells, and by an increase in the microvillar density along their brush borders (Lamarcq and McFall-Ngai, 1998). These events are also noticeable during the first day of the symbiosis, at which time the swelling has reached a maximum (Doino, 1999). In contrast, the microvillar density along the brush border continues to increase over the next several days; eventually, almost the entire surface of each bacterial cell is in direct contact with host microvilli. Unlike the developmental changes of the superficial epithelium, the maintenance of

crypt cell edema and a high density of microvilli requires persistent interaction with *V. fischeri* (Lamarcq and McFall-Ngai, 1998; Doino, 1999). When the symbiont is eliminated with antibiotics, the cells gradually return to the state characteristic of the light organs either of newly hatched squids or of animals that have not been exposed to *V. fischeri* (i.e. aposymbiotic hosts).

Mutants of *V. fischeri* deficient in the induction of an increase in microvillar density of the crypt epithelium have not yet been isolated. However, preliminary studies have shown that mutations in the *luxA* gene produce bacteria that do not cause swelling of host cells (Doino, 1999; Foster et al., 1999). The *luxA* gene encodes one of the subunits of bacterial luciferase. Exactly how the activity of this enzyme functions to induce host cell swelling remains to be determined, but if the luminescence reaction does pull down the oxygen tension in the crypts, the epithelium may experience hypoxia, a condition that is known to cause swelling of animal cells under other circumstances (Hierholzer et al., 1997; Mairbaurl et al., 1997).

The symbiont also undergoes marked developmental changes with the onset and progression of the symbiosis. Within 12 h following colonization of the light organ, the bacteria in the narrower, medial portions of the crypts have lost their flagella and become significantly smaller, and the cells in the more capacious portion of the crypt, closer to the duct, retain their flagella and do not decrease in size (Visick and McFall-Ngai, 2000). While these conspicuous changes in the organ occur within hours of colonization, molecular genetic manipulation of the symbiont suggests that continued development and accommodation to the symbiosis occurs over at least the first several days of the association. Specifically, three classes of bacterial mutants defective in the colonization of the light organ have been identified: those that do not infect at all, such as motility mutants; those that do not colonize fully; and those that do not persist past the 2nd or 3rd day of symbiosis (Ruby, 1999a). The existence of these persistence mutants supports the possibility that a series of events is required for continuing bacterial development.

One readily apparent change in the bacteria is the induction of luminescence. Within several hours following the first exposure of the symbiont to the host, the level of bacterial luminescence per cell increases over 1000-fold (McFall-Ngai and

Ruby, 1991). This induction of symbiont light production is due to the accumulation in the crypts of at least one type of *V. fischeri* quorum-sensing, acyl-homoserine lactone (AHL) molecule (Eberhard et al., 1981; Boettcher and Ruby, 1995). *V. fischeri* is one of a group of bacteria that uses this type of molecule as a pheromone, i.e. the bacteria respond to the accumulation of AHL when they are at high density by the induction of genes that are used in their high-density niche (Fuqua et al., 1996).

6. Maintaining a balanced, healthy relationship

The squid nourishes the symbiont and maintains its population within the light organ, which eventually reaches as many as 10^9 cells. This non-pathogenic relationship is characterized by two remarkable traits: (i) the symbiont population remains monospecific, resisting any apparent contamination from other bacteria in the external environment, despite the fact that the crypts stay in communication with the mantle cavity throughout the life of the host (Montgomery and McFall-Ngai, 1994); and (ii) *V. fischeri* cells do not invade other host tissues cells under normal circumstances (McFall-Ngai and Montgomery, 1990; Ruby, 1999b).

Every day at dawn, the squid expels most of the crypt contents, including 90–95% of the bacterial population, through the pores and into the external environment (Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998); the remaining 5–10% of the bacteria continue to be associated with the microvillous epithelium. During the course of the day, these residual *V. fischeri* cells proliferate, repopulating the crypts and restoring a full bioluminescence potential for the host's nocturnal behavior (Boettcher et al., 1996). This continuous cycle of venting and regrowth selects bacterial strains that not only are capable of continued growth within the crypts, but also are competitively dominant under the particular conditions created there.

7. Similarities between tissue colonization by benign and pathogenic *Vibrio* species

Is the above-described array of symbiosis characteristics unique to the squid-vibrio association?

Although little information is presently available on other cooperative, host-microbe relationships, the work of several laboratories has revealed an increasing list of similarities between the colonization of host epithelium by *V. fischeri* and that of a number of pathogenic *Vibrio* species, including *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Vibrio cholerae* (Richardson, 1991; Salyers and Whitt, 1994; Whittaker et al., 1996; Ruby, 1999a,b). These similarities include known virulence determinants that have protein homologs or analogs in *V. fischeri* (Palmer and Colwell, 1991; Reich and Schoolnik, 1994; Reich et al., 1997; Jouravleva et al., 1998; Visick and Ruby, 1998; Graf and Ruby, 2000). Because of these similarities it is reasonable to conclude that a number of properties of their association with host tissue might have arisen from a common evolutionary origin. In fact, some species, like *V. vulnificus*, *V. cholerae*, and *Vibrio harveyi*, are a benign symbiont in one host and a virulent pathogen in another (Tamplin and Capers, 1992; DePaola et al., 1994; Harris et al., 1996). This hypothesis is further supported by additional evidence of the homologous character of tissue colonization by *V. fischeri* and pathogenic species of *Vibrio*. The commensal colonization of invertebrate tissues by *Vibrio* species arose millions of years before members of this genus developed as human pathogens; thus, it is possible that host-colonization mechanisms that were present in these cooperative associations may have been recruited into the development of pathogenic associations.

8. Future directions

Research over the past 12 years on the squid-vibrio association has provided a description of the temporal and spatial framework within which this animal-bacterial association operates. On the horizon of this field is the determination of the precise signals by which the host and symbiont mediate the complex interchange between each other during the development and maintenance of their relationship. To this end, stage-specific cDNA libraries are being created for both symbiotic and aposymbiotic animals. These libraries promise to reveal how and when *V. fischeri* influences gene expression of the tissues of the *E. scolopes* light organ. Similarly, mutant libraries of the bacterial symbiont are being 'mined' to define

how the symbiont responds to interactions with its host. In addition, efforts are being directed toward realizing the sequencing of the *V. fischeri* genome. The resources made available by these efforts should provide insight into the basic nature of the diplomacy between animal hosts and their beneficial bacteria.

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