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The Squid-Vibrio Symbioses: From Demes to Genes[1]

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SYNOPSIS. **The monospecific light organ association between the Hawaiian sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* has been used as a model for the study of the most common type of coevolved animal-bacterial interaction; *i.e.,* the association of Gram-negative bacteria with the extracellular apical surfaces of polarized epithelia. Analysis of the squid-vibrio symbiosis has ranged from characterizations of the harvesting mechanisms by which the host ensures colonization by the appropriate symbiont to identification of bacteria-induced changes in host gene expression that accompany the establishment and maintenance of the relationship. Studies of this model have been enhanced by extensive collaboration with microbiologists, who are able to manipulate the genetics of the bacterial symbiont. The results of our studies have indicated that initiation and persistence of the association requires a complex, reciprocal molecular dialogue between these two phylogenetically distant partners.**

The study of symbiosis is, by its very nature, an integrative discipline both conceptually and technically. Researchers in this field very often analyze the interactions of organisms from different kingdoms or domains, asking the principal question: how does the symbiosis establish and persist over the life span of an individual host, between generations and over evolutionary time? This contribution outlines the results of studies on one type of animal-bacterial association, the cooperative symbioses between the sepiolid squids and their prokaryotic vibrio partners.

THE ECOLOGY AND EVOLUTIONARY BIOLOGY OF SEPIOLID SQUID-VIBRIO SYMBIOSES

Sepiolid squids with bacterial light organs have been documented in as many as 26 species from 5 genera of sepiolids (*Euprymna*, *Sepiola*, *Inioteuthis*, *Rondeletiola*, and *Semirossia*) (McFall-Ngai, 1999). Bacterial strains have been isolated from the light organs of *Euprymna* spp. and *Sepiola* spp. and are from two vibrio species, *Vibrio fischeri* and *V. logei*. Although all *Euprymna* spp., which are confined to the Indo-Pacific region, only have strains of *V. fischeri*, *V. logei* and *V. fischeri* can be found in hosts of the genus *Sepiola*, which occur in the Mediterranean and Eastern Atlantic (Fidopiastis *et al.*, 1998; Nishiguchi, 2000).

Although the host animal has never been observed in nature to occur in the absence of the symbiont, the bacterial species *V. fischeri* and *V. logei* occur in niches other than light organs. They are members of the bacterioplankton and enteric microbiota of vertebrates and invertebrates, as well as saprophytes in marine environments in wounds and on the surfaces of dead animals. In addition, *V. fischeri* also occurs as a light organ symbiont in monocentrid fishes.

Although these vibrio species appear to have broad niches, molecular phylogenetic studies have provided evidence that not all *V. fischeri* strains are alike; *i.e.*, the seeming diversity of niches for a given species may break down with closer analysis, revealing strain specificity for certain habitats. To study this issue, as well as to determine whether evidence exists for co-evolution of the squid-vibrio symbioses, molecular phylogenies were determined for several species of host sepiolid squids and their bacterial symbionts. Sequences from nuclear internal transcribed spacer regions and mitochondrial cytochrome oxidase subunit I were used to derive host phylogenetic trees, and glyceraldehyde-3-phosphate dehydrogenase was used to derive the phylogenies of the bacterial symbionts (Nishiguchi *et al.*, 1998). These data revealed congruence between the resultant phylogenetic trees of the hosts and their symbionts, providing evidence for co-evolution of the partners.

Because the hosts and bacterial partners of the squid-vibrio symbioses can be cultured independently in the laboratory, it has been possible to investigate whether and how well nonnative strains and hosts recognize one another (Nishiguchi *et al.*, 1998). In such experiments, the Hawaiian host *Euprymna scolopes* formed a symbiosis with bacterial strains that had been isolated from any of the sepiolid hosts. However, in competition experiments in which the native strain was presented in an equal ratio with a nonnative strain to the Hawaiian host, the native strain became dominant during the onset and persistence of host colonization. When two nonnative strains were presented to the Hawaiian host, the strain that became dominant was always most closely related to the native strain. A hierarchy of competitive dominance was derived that correlates with the host and symbiont phylogenies. Taken together, these data provide both phylogenetic and experimental evidence that co-evolution of the host and symbiont has occurred in the squid-vibrio associations, and the selection of the appropriate strain of bacterial symbiont occurs immediately following hatching of the host. In addition, the results of these experiments revealed that the squid-vibrio system offers the opportunity to uncover the cellular and mo-

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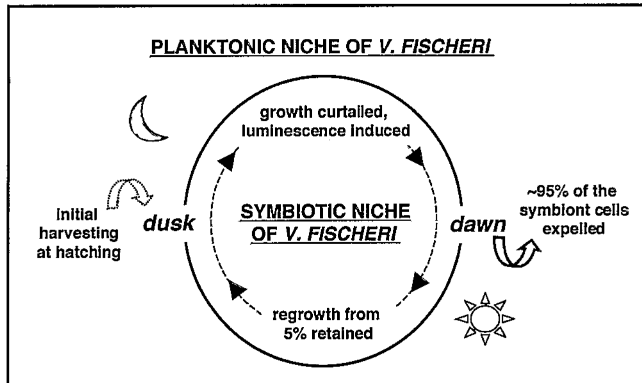

FIG. 1. The dynamic interface between the host *E. scolopes* and the niches of its symbiont *V. fischeri*. The symbiosis begins each generation when the aposymbiotic juvenile host hatches in the hours following dusk and immediately acquires its symbiont cells from the population of *V. fischeri* in the surrounding seawater. Once inside the epithelial crypt spaces of the juvenile, the symbionts grow to colonize the host organ fully within hours. Upon reaching a critical density, growth of the symbionts is curtailed and luminescence is induced. Beginning with the first dawn and dawn each day thereafter, the host expels ~95% of its symbionts into the surrounding seawater as it enters its diurnal quiescent phase, burying in the sandy substrate of the back reef. The behavior of expelling the symbionts maintains *V. fischeri* at high concentrations in the host's environment. The estimated 5% of the *V. fischeri* population remaining in the organ begins the diurnal cycle of re-growth of the symbiont. By dusk, when the host will emerge to become an active forager on the reef, the organ has attained a full complement of luminous symbionts.

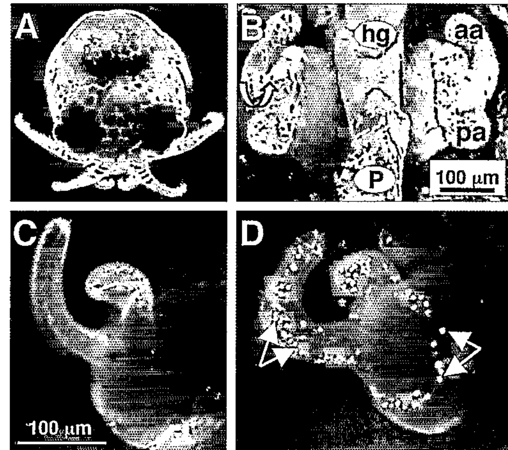

FIG. 2. The juvenile light organ of *Euprymna scolopes*. A. A hatchling *E. scolopes*. Mantle length = 1.8 mm. B. Scanning electron micrograph of the ventral surface of the organ revealing the prominent ciliated fields with associated appendages on each lateral surface. At the base of each set are three pores, the entrance to the sites of symbiont colonization (the arrow points to one of three pores on one ciliated field). aa = anterior appendage; pa = posterior appendage; hg = hindgut; P = posterior. C. One half of an acridine orange-stained (AO), 12-hr aposymbiotic light organ showing no signs of cell death. D. One half of an AO-stained 12-hr symbiotic light organ showing the pycnotic nuclei (arrows) of apoptotic cells, the condensed chromatin of which stain strongly with AO.

lecular changes that underlie the incremental co-evolution of the symbiotic partners.

The above-described experiments were focused principally upon the hosts of *Euprymna* spp., with the Mediterranean *Sepiola* spp. being used for comparison. Recent studies with an emphasis on the *Sepiola* spp. revealed that the members of this genus will preferentially harbor *V. fischeri* at warmer environmental temperatures and *V. logei* at colder temperatures; *i.e.,* a given host species will change its symbiont seasonally and with depth in the water column (Fidopiastis *et al.*, 1998; Nishiguchi, 2000).

Additional studies have focused specifically on the *Euprymna scolopes-Vibrio fischeri* symbiosis, which has become the principal squid-vibrio model for the study of animal-bacterial interactions (McFall-Ngai and Ruby, 1998). *E. scolopes* occurs in the nearshore habitats of the Hawaiian archipelago, where it has been reported from Midway Island to Maui. The *E. scolopes* light organ is exclusively colonized in nature by a strain of *V. fischeri* that can be distinguished from the strains of *V. fischeri* in other *Euprymna* spp. (Nishiguchi *et al.*, 1997). However, studies of populations of the host around Oahu have revealed that the situation may not be as simple as previously thought. Phylogenetic analyses of populations of *E. scolopes* on either side of the island of Oahu (*i.e.,* from Kaneohe Bay on the north side and Maunalua Bay on the south side), using both morphological and molecular data, have revealed that two reproductively isolated populations of this species occur around Oahu (Kimbell *et al.*, 2002). To date, corresponding analyses of the symbiont strains have not been performed, nor has the extent of divergence of the Hawaiian *Euprymna* sp. throughout the islands been characterized. However, studies of the populations of the Hawaiian sepiolid may provide significant insight into the fine-scale coevolution of this species with its bacterial partners. Specifically, they may be useful in answering the questions: at what level of divergence of the host have corresponding population-specific strains of *V. fischeri* evolved, and what are the underlying molecular features of this divergence?

A CLOSER LOOK: THE *EUPRYMNA SCOLOPES-VIBRIO FISCHERI* SYMBIOSIS

For over a dozen years, the relationship between *Euprymna scolopes* and *Vibrio fischeri* has been studied as a model of animal-bacterial symbiosis (Figs. 1 and 2). In this association, the squid host provides a nutrient-rich environment for the symbiont (Graf and Ruby, 1998) and the morphology of the light organ system suggests that light produced by the bacteria is used by the host in anti-predatory behavior (McFall-Ngai and Montgomery, 1990). This association provides an ideal experimental system for the study of animal-bacterial symbiosis because: 1) the association is highly specific; 2) both partners can be cultured independently of one another in the laboratory; 3) the genetics of the bacterial symbiont can be altered (Ruby, 1996); and, 4) the initiation of the association can be manipulated (McFall-Ngai and Ruby, 1991; Visick and McFall-Ngai, 2000). As such, the association is one that can be studied experimentally at a va-

riety of levels, from ecological to molecular. As with most integrative biology, this broadness demands, at the same time, application of a wide variety of the available technical approaches and vigilance that each question is well focused and rigorously addressed.

Each generation of hatchling squid acquires *V. fischeri* from the water column. The extracellular luminous symbionts colonize the light organ within hours of the host's hatching and reside in the organ throughout the life of the host (McFall-Ngai and Ruby, 1991). In the absence of *V. fischeri*, no other environmental bacteria colonize the *E. scolopes* light organ. At the first dawn following colonization, the lifelong diel rhythm of the symbiosis commences in which the host animal vents 95% of the symbionts into the surrounding seawater (Fig. 1; Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998). The establishment of the symbiosis is facilitated by features developed during embryogenesis. During this period, prior to direct exposure to the symbiont, a set of superficial ciliated fields develop on either side of the organ (Fig. 2B; Montgomery and McFall-Ngai, 1993). Each field consists of an epithelial base of tissue with two protruding appendages. In the juvenile, these ciliated fields will function to direct bacteria-rich seawater toward the vicinity of three pores at the base of these appendages. It is through these pores that the *V. fischeri* cells enter and then colonize the light organ, which consists of three epithelium-lined crypt spaces on either side.

How do the host and symbiont form a specific association in a background of a myriad of other bacteria in the surrounding seawater? The light organ is entirely surrounded by the funnel and environmental seawater is passed across the juvenile organ during ventilation of the mantle cavity. However, at about 200 cells/ml of environmental seawater, *V. fischeri* constitutes less than 0.1% of the total bacterial load in the ambient water (Lee and Ruby, 1994). Further, because the hatchling mantle cavity volume is only about 1 μl, on average, no more than a single *V. fischeri* cell, occupying about one-millionth the volume of the juvenile squid mantle cavity will be present in the cavity during each ventilation. These considerations suggested that a mechanism must exist for the enrichment and harvesting of *V. fischeri* from the surrounding environment. Studies of the colonization process revealed that, within a few hours after hatchlings were placed in seawater containing *V. fischeri* cells, currents created by the ciliated appendages aggregated the bacteria in mucus suspended over the light organ pores (Nyholm *et al.*, 2000). Although all Gram-negative bacteria tested could aggregate, only aggregates of *V. fischeri* are able to enter the light organ pores and colonize the host successfully. Further characterization of this process (Nyholm *et al.*, 2002) revealed that the mucus originates from stores in the cells of the ciliated epithelium, demonstrating that this tissue has two functions: the source of the mucus and the mechanism by which symbionts are focused in the aggregates.

Upon colonization, *V. fischeri* induces a series of developmental changes in the light organ. Within 12 hr following first exposure to the host, the symbiont cells cause detectable alterations in the cells of the interfacing epithelial crypts (*i.e.,* cytoskeletal rearrangement and cell swelling); these changes are reversible by removing *V. fischeri* from the light organ crypts with antibiotics (Lamarcq and McFall-Ngai, 1998; Visick *et al.*, 2000). In addition, the symbionts induce a morphogenetic loss of the remote ciliated fields (Montgomery and McFall-Ngai, 1994), which is the most dramatic and well characterized of these bacteria-induced changes. Without the signal from the symbiont, the ciliated fields do not undergo the dramatic cell death or regression program characteristic of normal development of the light organ. This morphogenesis is irreversible, *i.e.,* after a 12-hr exposure to *V. fischeri*, the light organ can be "cured" of the symbiont with antibiotics and the loss of the superficial epithelium continues over the next several days to its conclusion (Doino and McFall-Ngai, 1995). The loss of the ciliated fields is all or partly due to symbiont-induced apoptosis (Fig. 2C and 2D; Foster and McFall-Ngai, 1998), which results from host exposure to the *V. fischeri* lipopolysaccharide, the principal surface molecule of the symbiont (Foster *et al.*, 2000). The onset of the symbiosis not only induces these changes in the morphology of the surface of the light organ but also in the function of this tissue (Nyholm *et al.*, 2002). Specifically, 24 hr following the onset of the symbiosis, the ciliated epithelium ceases shedding mucus and, thus, the aggregation of symbionts no longer occurs. However, unlike the irreversible regression of this field of cells that is induced by the symbionts at 12 hr, if the light organ is cured of its symbionts after mucus shedding ceases, *i.e.,* at 24 hr, curing of the light organ with antibiotics results in a resumption of mucus shedding behavior by the cells of the ciliated epithelium as well as aggregation of bacteria in the mucus.

SYMBIONT-INDUCED CHANGES IN HOST PROTEIN PRODUCTION AND GENE EXPRESSION

These morphological data suggest that specific molecular cross-talk occurs between *E. scolopes* and *V. fischeri* during the early hours of the symbiosis. This host-symbiont dialogue transforms the organ from a morphology associated with the colonization process to one that promotes the mature function of the light organ and persistence of the symbiosis. These underlying interactions are being characterized at the levels of both protein production and gene expression.

What changes do V. fischeri *cells induce in the proteome of the host light organ?*

To identify critical time points in the first several days of symbiont-induced light organ development, we have used 2-D PAGE to characterize differentially expressed host proteins in the *E. scolopes-V. fischeri* symbiosis over this period (Lemus and McFall-Ngai, 2000). Specifically, unlabeled soluble proteins from

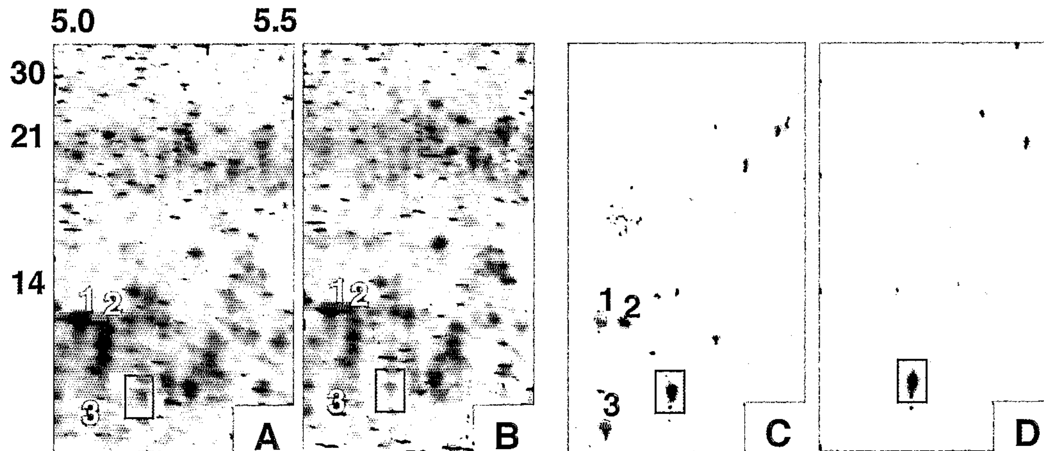

FIG. 3. A sector of a 2-D SDS-PAGE gel and an autoradiogram of total soluble proteins extracted from the light organs of 12-hr aposymbiotic (A, C, respectively) and symbiotic animals (B, D, respectively). Twenty micrograms of protein were loaded on each gel. In the lower left corner of the autoradiograms is a series of spots, three of which show no labeling in symbiotic animals; the boxed protein is similar in both aposymbiotic and symbiotic animals, and serves as an internal control. The corresponding areas of the silver stained gels (A and B-1, 2, 3) show no significant differences in staining between the aposymbiotic and symbiotic protein pools. Molecular mass markers (kD) appear vertically along the left, and pI markers along the top.

12, 24, 48 and 96-hr aposymbiotic and symbiotic light organs were resolved by 2-D PAGE to reveal points when major alterations in the protein pool could be observed by silver staining, a sensitive method for protein detection. These data demonstrated that interactions with the bacteria do not cause detectable changes in the host proteome until around 48 hr, although morphological data suggested that significant molecular changes were induced at or before 12 hr. However, when ^{35}S-labeled proteins from 12-hr aposymbiotic and symbiotic animals were resolved by 2-D PAGE and newly synthesized proteins were visualized by autoradiography, several apparent differences were detectable in this comparison. Specifically, the synthesis of ten proteins was detected only in 12-hr symbiotic light organs and thirteen proteins were more abundant; in 12-hr aposymbiotic light organs three proteins were more abundant (Fig. 3). The next step is to sequence and identify these differentially expressed proteins.

Does V. fischeri *induce changes in the light organ transcriptome?*

Many significant biological processes including stress responses, cell differentiation, tissue development and remodeling, and immune responses are mediated through changes in gene transcription. Until recently, little was known about a cell's transcript expression profile or "transcriptome," but rapid advances in biotechnology (*i.e.,* microarray analysis, differential display, subtractive hybridization, etc.) are rendering such complex characterizations possible. Microbes are well documented inducers of change in host cell gene expression in both beneficial (Nehls *et al.*, 1998; Hooper *et al.*, 2001) and pathogenic symbioses (Diamond *et al.*, 1996; Heller *et al.*, 1997; Yolanda *et al.*, 2000). In microarray analyses of such systems, bacteria have been shown to modulate the expression of hundreds of host genes (Eckmann *et al.*, 2000; Hooper *et al.*, 2001).

To begin characterizing symbiont-induced changes in host gene expression in the *E. scolopes-V. fischeri* symbiosis, 12-hr aposymbiotic and 12-hr symbiotic light organ cDNA libraries were constructed. To assess the quality of these libraries, several random clones (5 from the aposymbiotic library and 5 from the symbiotic library) were sequenced and analyzed (Table 1). Then, subtractive hybridization between these cDNA libraries was performed. This method (Schweinfest, 1995), in which common sequences and aposymbiotic specific cDNAs were "subtracted away," has identified 50 host transcripts as candidate messages that may be more abundant in response to the interaction of the host with the symbiont at this 12-hr time point. Although several of these clones encoded proteins with no match in the gene and protein databases, many had significant sequence similarity to known protein species (Table 1). We have also performed suppressive-subtractive hybridization on 24-hr symbiotic and aposymbiotic animals, a subtractive method that not only reveals clones specific to the symbiotic or aposymbiotic condition, but also enriches for rare transcripts. Finally, the production of a squid light organ microarray is underway. The microarray will be used to determine the changes in gene expression not only in normal development of the symbiosis, but also in response to interactions with *V. fischeri* mutants defective in inducing normal morphogenetic changes in the host light organ.

Real time reverse-transcriptase (RT) PCR is being used to verify and quantify the results obtained thus far by production of the cDNA libraries and manipulations of these libraries. This method is ideal for the study of changes in gene expression when tissue quantity is limiting, such as in the juvenile light organ. In

TABLE 1. *Examples of transcripts of the* Euprymna scolopes *light organ.*

Source	Clone (n)	Derived aa length	% identities/ positives[b]	E values[c]	Function
Random sequencing (12-hr symbiotic cDNA library)	arginine kinase (2)[a]	349	75/89 (Lj)	3×10^{-42}	phosphagen kinase, ATP restoration
	ornithine decarboxylase antizyme (1)	216	56/69 (Xl)	3×10^{-5}	inhibitor of polyamine synthesis
	guanine nucleotide binding protein α subunit (1)	354	88/90 (Ht)	1×10^{-104}	transmembrane signaling
	protein translation SUI1 homolog (1)	113	72/82 (Hs)	3×10^{-26}	protein biosynthesis
12-hr symbiotic subtractive hybridization	proteasome component C8 (13)[a]	255	74/85 (Hs)	3×10^{-5}	protein degradation
	kinesin-related protein (2)	1060	37/48 (Xl)	5×10^{-8}	motor protein
	cytochrome oxidase I (4)	377	49/60 (Tm)	4×10^{-18}	electron transport
	unique clones (30)	NA	NA	NA	NA
24-hr aposymbiotic suppressive subtractive hybridization (SSH)	60S ribosomal protein L9 (1)	192	42/49 (Hs)	2×10^{-23}	protein biosynthesis
	ribosomal protein L18 (1)	188	71/81 (On)	1×10^{-20}	protein biosynthesis
	NADH dehydrogenase subunit I (1)	313	75/85 (Lb)	2×10^{-3}	electron transport
	unique clone (1)	NA	NA	NA	NA
24-hr symbiotic SSH	N33 (1)[a]	348	66/84 (Hs)	7×10^{-17}	putative tumor suppressor
	glyceraldehyde phosphate dehydrogenase (1)	291	65/77 (Be)	2×10^{-15}	glycolysis
	unique clones (5)	NA	NA	NA	NA

[a] Transcripts confirmed to be more abundant in response to *V. fischeri.*

[b] Lj = *Liolophora japonica.* Xl = *Xenopus laevis.* Ht = *Helisoma trivolvis.* Hs = *Homo sapiens.* On = *Oreochromis hiloticus.* Lb = *Loligo bleekeri.* Be = *Boletus edulis.* Tm = *Tinamus major.* NA = not applicable.

[c] Expectation (E) values are given for the highest match using the BLASTX program. The lower the E value the less likely the match is random.

real time RT-PCR, the PCR reaction is monitored throughout the amplification cycles. The amount of product accumulating is detected by a fluorescent reporter, the signal strength of which is directly proportional to the amount of PCR product in the reaction (Fig. 4). Thus, data are collected in the exponential phase of the reaction, where the PCR reagents are not limiting and the polymerase is still working at a high efficiency. Such analyses differ from end point RT-PCR where measurements are taken in the plateau phase of the reactions. Real time RT-PCR has been applied to several of the host transcripts identified by random library sequencing and subtractive hybridization, revealing symbiont-induced modulation of transcript levels (Table 1). Studies using *in situ* hybridization are underway and will enable us to determine the spatial expression patterning of the transcripts of interest.

These early studies of the symbiont-induced changes in the transcriptome suggest that such approaches will yield a rich database. With random sequencing of the libraries and only a single subtraction at two dif-

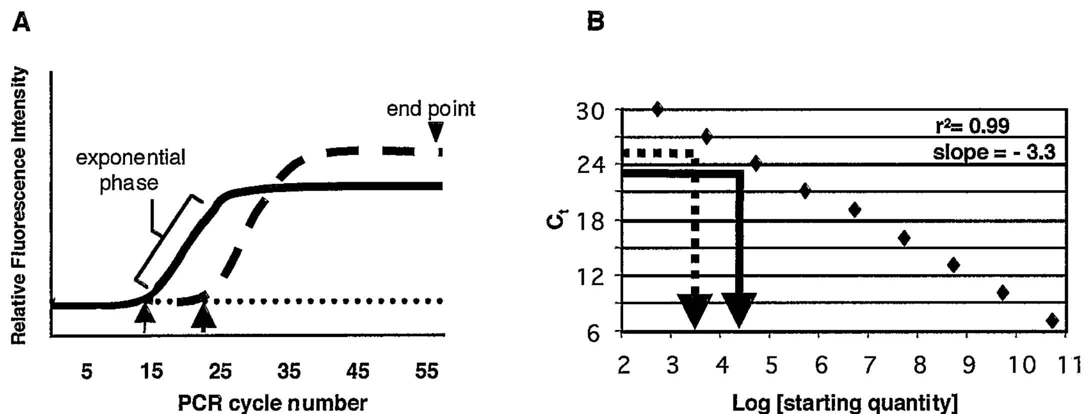

FIG. 4. Representative real time RT-PCR experiment using primers specific for a clone from the 12-hr subtractive hybridization library (iCycler, Bio-Rad). A. PCR-product accumulation over time. Product from symbiotic RNA pool = solid line; product from aposymbiotic RNA pool = dashed line. SYBR green, an intercalating dye, was used to measure fluorescence intensity. The point at which the fluorescence rises above background is termed the threshold cycle (C_t). Background fluorescence is indicated by the straight dotted line. Small arrow = 12-hr symbiotic light organ C_t. Large arrow = 12-hr aposymbiotic light organ C_t. The exponential phase and end point (arrow head) of the PCR reaction are as indicated. B. A graph plotting C_t against the log of the starting quantity of template. Standard curves were constructed using ten-fold dilutions of known concentrations of the clone (diamonds). From the resultant standard curve, the starting quantity of the transcript of interest in each experimental template can be determined. mRNA isolated from 12-hr light organs was used for the experimental template. 12-hr symbiotic template = solid line. 12-hr aposymbiotic template = dashed line. A PCR reaction with 100% efficiency will normally have a slope of −3.32 (technical note #2593, Bio-Rad Laboratories, Hercules, CA).

ferent time points during the onset of the symbiosis, clues to the molecular basis of the host-symbiont interaction have been revealed. For example, transcripts encoding the host arginine kinase, a creatine kinase analog in mollusks, are more abundant in symbiotic light organ tissues. In other invertebrates that have been studied, this enzyme has increased activity in tissues that are experiencing fluctuating metabolic demand or anoxia (Newholme *et al.*, 1978; Shofer *et al.*, 1997). Such conditions characterize the tissue of the symbiotic light organ (Boettcher *et al.*, 1996). In addition, the bacterial symbionts change the expression of the host gene encoding particular subunits of the proteasome, a complex involved in protein turnover in eukaryotic cells (Voges *et al.*, 1999). The changes in expression of this gene may reflect the extensive host tissue remodeling induced by *V. fischeri*. Further rounds of subtraction at the different stages of the symbiosis promise to reveal many additional candidate genes, and the future analyses of changes in the transcriptome using microarray analysis will provide a powerful complement to the results obtained by these subtractive hybridization methods.

CONCLUSION

Advances in technology in recent years have opened for study the vast landscape of animal-bacterial interactions. In research efforts with the *E. scolopes-V. fischeri* association, biochemical and molecular approaches have enabled a better understanding of the association at all levels, from the interface of the symbiosis with its environment to the exclusive molecular interplay between partners. This association is still in the early stages of its development as a system. Discoveries in the coming years should reveal what characteristics of this symbiosis are shared by other extracellular symbiotic associations between animals and bacteria and what characteristics are unique to this specific interaction. In addition, an understanding of cooperative associations between animals and bacteria should provide substantial insight into the nature of pathogenic relationships between animal and bacterial cells.

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