A Transient Exposure to Symbiosis-Competent Bacteria Induces Light Organ Morphogenesis in the Host Squid

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Abstract. Recent studies of the symbiotic association between the Hawaiian sepiolid squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* have shown that colonization of juvenile squid with symbiosis-competent bacteria induces morphogenetic changes of the light organ. These changes occur over a 4-day period and include cell death and tissue regression of the external ciliated epithelium. In the absence of bacterial colonization, morphogenesis does not occur. To determine whether the bacteria must be present throughout the morphogenetic process, we used the antibiotic chloramphenicol to clear the light organ of bacteria at various times during the initial colonization. We provide evidence in this study that a transient, 12-hour exposure to symbiosis-competent bacteria is necessary and sufficient to induce tissue regression in the light organ over the next several days. Further, we show that successful entrance into the light organ is necessary to induce morphogenesis, suggesting that induction results from bacterial interaction with internal crypt cells and not with the external ciliated epithelium. Finally, no difference in development was observed when the light organ was colonized by a mutant strain of *V. fischeri* that did not produce autoinducer, a potential light organ morphogen.

Introduction

Prolonged associations with bacterial symbionts are now recognized as important phenomena in the developmental program of many plant and animal hosts (for reviews see Schwemmler, 1989; Hirsch, 1992; Saffo, 1992). In some cases, bacterial symbioses may even be required for normal host development or survival. For example, enteric bacteria provide essential enzymes and vitamins to their mammalian hosts, and associations with bacteria are required for normal development of the mammalian immune system (Gordon and Pesti, 1971). In other cases, though essential only under nutrient-poor conditions, the association is highly beneficial to the host's fitness in its natural environment, such as the symbioses between leguminous plants with nitrogen-fixing bacteria or between the weevil *Sitophilus oryzae* and its associated gram-negative bacteria. In these partnerships, the bacteria provide nutritional metabolites to their host (Nardon and Grenier, 1991) as well as influence its development.

Of the known prokaryote-eukaryote associations, much progress has been made toward the understanding of the development of plant-bacterial symbioses, both because the plant hosts are easily maintained and manipulated in the laboratory and the bacterial symbionts are culturable. An animal-bacterial association offering similar experimental benefits is the highly specific association between the Hawaiian sepiolid squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri*. This symbiosis provides an experimental system to study the effect of bacterial symbionts on host animal development (McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). In the host squid, the bacteria are always contained within epithelia-lined crypts inside the light organ, which is housed within the mantle cavity. However, the morphology of the light organ in juvenile squid is much different from that of the adult (McFall-Ngai and Montgomery, 1990), and the light organ undergoes complex de-
developmental changes following bacterial colonization (Montgomery and McFall-Ngai, 1994).

Upon hatching, juvenile squid are aposymbiotic (without bacterial symbionts) and normally acquire free-living *V. fischeri* from the surrounding seawater within hours (Wei and Young, 1989; McFall-Ngai and Ruby, 1991). A substantial portion of the juvenile light organ epithelium is microvillous and ciliated, bearing two lateral pairs of appendages (ciliated epithelial appendages; CEA) that appear to facilitate inoculation of bacteria into the light organ (Fig. 1a; McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993). Microscopy and high-speed cinematography have revealed that the two appendages on each side of the light organ form a ring, at the base of which are three pores leading into three independent crypts (Fig. 1b). Beating of the cilia entrains passing seawater within the ring, potentially increasing the probability that symbionts within the water will enter the pores (M. McFall-Ngai and R. Emlet, unpub. results). When the light organ has been successfully colonized by *V. fischeri*, cell death is observed in the CEA and regression of these appendages occurs over a period of four days. Four-day-old squid that are not infected with *V. fischeri* do not show cell death nor regression of the CEA (Montgomery and McFall-Ngai, 1994). Therefore, the presence of symbiosis-competent bacteria somehow induces host tissues that are several cell layers away to initiate light organ morphogenesis. Cell death and the resulting regression of the CEA are the first observable events of light organ morphogenesis and therefore the first developmental evidence that induction has occurred.

In this study we have asked whether the presence of bacteria within the light organ is required continuously for 4 days to induce CEA regression. Additionally, we used noninfective strains of *V. fischeri* to determine whether colonization of the light organ is necessary for induction. Finally, we tested whether *V. fischeri* autoinducer, a cell density-dependent factor secreted by the bacteria and involved in the production of light, is required to induce light organ morphogenesis.

**Materials and Methods**

**Animal care and maintenance**

Adult squid were collected at night from Kaneohe Bay, Oahu, HI, with dipnets and were transported back to the University of Southern California, Los Angeles within one week of collection. Animals were maintained in a 265-liter recirculating aquarium at 23°C, and females were mated approximately once a week. Egg clutches, attached to coral rocks or other hard surfaces by the females, were transferred for hatching to smaller temperature-controlled 23°C aquaria. To ensure that juvenile squid did not become prematurely infected with any residual bacteria that might be associated with the egg clutch, squid were transferred immediately upon hatching through three rinses with California coastal seawater (CSW), which does not contain infective strains of *V. fischeri* (McFall-Ngai and Ruby, 1991). Juveniles were used for infection studies within 6 h of hatching.

**Inoculation of squid with *V. fischeri* bacteria**

Bacteria were grown to log phase in a seawater-based minimal medium (Ruby and Asato, 1993) and diluted to between $10^3$ and $10^5$ cells/ml for inoculation of squid. After inoculation, squid were rinsed in CSW and transferred to either CSW or chloramphenicol-treated CSW (as described below). For all experiments, positive (infected) controls were exposed to symbiosis-competent bacteria in CSW for the entire 4 days and negative (uninfected) controls were exposed to CSW alone.

**Monitoring bacterial colonization**

Because *V. fischeri* is luminous in the light organ, successful colonization of the organ can be monitored by measuring the bioluminescence of the squid with a photomultiplier tube (Luminescence Photometer, Model 3600, Biospherical Instruments, Inc.). For these measurements, individual squid were kept in 5 ml of seawater in glass scintillation vials. Seawater in the vials was changed daily throughout the 4-day experiments, just prior to each luminescence measurement.
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Figure 2. Time series of CEA regression in symbiotic (sym) and aposymbiotic (apo) juvenile squid over 4 days (d). Symbiotic animals were inoculated with $\sim 10^4$ cells/ml of *V. fischeri* ES114 within 6 h of hatching. The sym-4d panel represents a fully regressed CEA. Only the right half of each light organ is shown. Scale bar = 100 $\mu$m. Differences in size reflect individual variation.

**Determination of Ciliated Epithelial Appendage (CEA) regression**

Regression of the CEA was determined at the end of 4 days for each experiment. Squid were fixed for 24 h in seawater containing either 5% formalin or 2% paraformaldehyde/2% glutaraldehyde. Samples were subsequently rinsed twice for 30 min in 50 mM sodium phosphate buffer with 0.45 M NaCl (pH 7.2), followed by a dehydration series with 15%-100% ethanol. Samples were critical-point dried with liquid CO$_2$, or desiccated with hexamethyldisilazane (Polysciences, Inc.). Dried squid were mounted onto aluminum stubs and the ventral portions of the mantle and siphon were dissected away to reveal the juvenile light organs. These samples were then sputter coated with gold and the light organs were examined with a Cambridge 360 scanning electron microscope (SEM). The presence or absence of CEA was scored and recorded, and photomicrographs were taken of representative samples.

CEA regression of symbiotic juveniles was compared to that of aposymbiotic juveniles for 4 days following inoculation with bacteria (Fig. 2). For experiments involving variable exposure times, CEA regression at day 4 was divided into five stages (0, 1, 2, 3, and 4), which correspond to the regression seen at 0 (Fig. 1A), 1, 2, 3, and 4 days of uninterrupted symbiosis (Fig. 2). CEA of light organs were considered regressed if they were at or beyond stage 3. Due to the high variability between individual squid, even within control groups, regression is reported as an E/C index, which is defined as the percentage of experimental animals with regressed CEA divided by that of the symbiotic controls in a given experiment (experimental and control animals for a given experiment were always from the same clutch of eggs).

**Manipulation of exposure times and colonization levels**

Transient vs. continuous exposure to *V. fischeri* strain ES114. To determine first whether a continuous exposure
to competent bacteria is necessary for initiating morphogenesis of the light organ. We used the bacteriostatic antibiotic chloramphenicol (Cm) to clear the light organ of viable symbionts. Squid were exposed to one of two symbiosis-competent strains of *Vibrio fischeri*: ES114, a chloramphenicol-sensitive light organ isolate (Boettcher and Ruby, 1990) or ES114-U2, a spontaneous chloramphenicol-resistant mutant of ES114 (donated by J. Graf).

The inoculations were performed for two time periods: (1) continuously for 4 days, or (2) for 12 h. Those exposed continuously were inoculated with *V. fischeri* and then transferred to CSW after 12 h for the remainder of the 4 days (Fig. 3a, top bar). These squid remained infected for the duration of the experiment. Following incubation with *V. fischeri*, squid exposed for only 12 hours were transferred to CSW treated with 10 μg/ml Cm in seawater for the remainder of the experiment (Fig. 3a, second bar). The transient time period of 12 h was chosen because successful colonization of the light organ by bacteria can be confirmed by the appearance of luminescence between 10 and 12 h after exposure. The Cm-resistant strain ES114-U2 was used as a control for any inhibitory pharmacological effects that Cm may have on CEA regression. Squid were monitored for luminescence before exposure to bacteria, every 2 h during initial colonization and every 12 h thereafter. Uninfected controls were exposed to noninfective CSW with or without Cm (Fig. 3a, third and fourth bars) and monitored for luminescence every 12 h.

To insure that Cm treatment was effectively clearing the light organ of viable bacteria, the decrease of both bacteria colony forming units (CFU) and luminescence was monitored in squid treated with 10 μg Cm/ml CSW following exposure to bacteria for 12 h.

**Variable exposure times to *V. fischeri* strain ES114.** To determine the minimum time period required to induce morphogenesis, hatchling squid were exposed to *V. fischeri* for variable lengths of time (Fig. 3b). At time 0, all squid were placed in a single bowl with of CSW containing ~5 × 10^3 ES114 cells/ml. Groups of 10–20 animals were removed from the bowl at 1, 4, 8, and 12 h. Upon removal from the bowl at each time period, half of the squid were rinsed twice and transferred to vials with Cm-treated CSW (Fig. 3b, top), while the other half were transferred to vials with Cm-free CSW (Fig. 3b, bottom). Groups transferred to Cm-free CSW became infected within 12 h. Luminescence was measured immediately before and after exposure to bacteria and once per day thereafter.

**Exposure to other strains of *V. fischeri***

To determine whether colonization of the light organ by the bacteria is necessary to induce CEA regression, squid were exposed to three noninfective strains of *V. fischeri* (M101, MdR12, and MJ1). A fourth strain of *V. fischeri* (MJ11), which is not normally associated with the *E. scolopes* light organ but is capable of colonization, was also tested for its ability to induce morphogenesis. Following the inoculation period, squid were transferred to CSW for the remainder of the 4 days. Possible colonization of squid exposed to noninfective strains was determined by both luminescence measurements and bacterial plate counts. Colonization of positive and negative controls was determined by luminescence only.

**Noninfective strains.** Strain M101 was produced by transposon (Mu dI-168 1) mutagenesis of symbiosis-competent strain ES114, resulting in a nonmotile mutant. Nonmotile mutants of *V. fischeri* have previously been shown to be noninfective in *E. scolopes* (Graf et al., 1994). Squid were exposed to ~10^4 M101 cells/ml for 12 h. Strain MdR12 is a non-symbiotic wild type isolate from Southern California coastal seawater. Strain MJ1 was originally isolated from the light organ of the Japanese pinecone fish *Monocentris japonica*, but has been in culture for 21 years (Ruby and Nealson, 1976) and does not infect *E. scolopes*. Squid were exposed to ~10^5 cells/ml of this strain for 24 h.

**Infective strain.** Strain MJ11 was isolated from the light organ of *M. japonica* in 1992 and is infective to *E. scolopes*. Squid were exposed to ~10^5 MJ11 cells/ml for 12 h. This strain was of interest because, although it is capable of colonization, bacterial numbers inside the light organ reach only 10% of the levels seen with ES114 (K.H. Lee and E.G. Ruby, pers. comm.).

**Exposure to an autoinducer mutant strain of *V. fischeri***

Symbiont bioluminescence in the *E. scolopes* light organ is induced via a well studied mechanism involving the secreted *V. fischeri* molecule autoinducer (VAI), a homoserine lactone. Normally VAI is expressed constitutively at a low level, but when cell densities become high, such as in the light organ (Boettcher and Ruby, 1990), the build up of VAI in the extracellular medium positively regulates VAI gene expression and in turn activates expression of the *lux* operon, which encodes for those genes responsible for bacterial light production (for review, see Dunlap and Greenberg, 1991). To determine whether VAI was a morphogen of the squid light organ, we used a mutant strain (3100) of *V. fischeri* (provided by Kendall Gray) containing an insertion in the autoinducer gene, which renders the cells incapable of making autoinducer.

One-day-old squid were exposed to symbiosis-competent *V. fischeri* strain 3102 or to ES114 at a concentration of ~10^5 cells/ml for approximately 20 h. Because the 3102 strain is nonluminous, successful colonization of the squid could not be monitored with a photometer. Instead, at the end of 4 days, two of the squid that had been
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Figure 3. Experimental design for transient and variable exposure to bacteria. n = 5–20 per experiment for each treatment group in A and B. At the end of 4 days (96 h), squid were fixed for SEM to score regression. A. Hatchling squid were exposed to ~10^4 cells/ml of ES114 or ES114-U2 for 12 h, at which time the bacteria-containing seawater was changed to CSW (top bar) to allow the infection to ensue, or to Cm-treated seawater (second bar) to stop the infection and cure the light organ. Controls were exposed to CSW alone (third bar) or to CSW followed by Cm-treated CSW (fourth bar). B. Hatchling squid were exposed to ~10^4 cells/ml ES114 for 1, 4, 8, or 12 hours (started at time 0), at which time the bacteria-containing seawater was changed to Cm-treated seawater (top bar) or to CSW (lower bar).

Results

Transient vs. continuous exposure to V. fischeri strain ES114

The number of viable bacteria in the light organ declines sharply after only 2 hours in Cm-treated CSW to 487 CFU (approximately 1% of the initial value), concomitant with a decline in luminescence (Fig. 4). After 10 hours of Cm treatment, all of the squid monitored had no viable bacteria detectable in their light organs. Additionally, if the Cm treatment was removed after 4 days and replaced with CSW alone, the light organs of the squid did not become reinfected, confirming that there were no viable V. fischeri in the light organ after treatment with Cm for 4 days. Squid exposed to Cm appeared as healthy as those not exposed to Cm and there was no adverse effect on the ability of the squid to infect after a 4-day exposure to Cm; i.e., if the Cm treatment was lifted after 4 days and a new inoculum of V. fischeri was introduced into the seawater the squid became luminescent within 24 h, indicating that they were still capable of being infected.

Figure 4. Decrease in colony forming units (CFU) of bacteria per squid (open squares, solid line) and relative luminescence (in photometer units) per squid (closed circles, dashed line) over time following Cm treatment. Squid were exposed to V. fischeri bacteria for 12 h prior to Cm treatment (0 h). Data points are the averages from n = 5 squid. Vertical bars represent standard deviations.
Squid exposed to *V. fischeri* for 12 h showed regression of the CEA similar to that of squid exposed for 4 days (Fig. 5b). Negative control animals exposed for 4 days to CSW (Fig. 5a) or Cm-treated CSW (not shown) showed no regression of CEA. Additionally, Cm did not have an inhibitory effect on CEA regression, as evidenced by complete regression of CEA from squid infected with the Cm-resistant strain ES114-U2 and treated with Cm (Fig. 5c).

Variable transient exposure to *V. fischeri* strain ES114

Exposure of squid to bacteria for 1, 4, 8, 12, or 14 hours to determine the minimum time requirement for induction of CEA regression revealed that only those squid exposed to bacteria for at least 12 h showed CEA regression (E/C = 0.96) comparable to that of continuously exposed control squid (Fig. 6; Table I). These squid were visibly luminous at the time of Cm treatment. Squid exposed to bacteria for 1 or 4 h were not luminous and showed no CEA regression. Those individuals exposed for 8 h were only occasionally luminous and the E/C ratio was only 0.24. Although some animals died during the experiments, the death rate was not greater than that normally seen in animals 4 days post-hatching (averaging less than 10%) and the incidence of death appeared random with respect to experimental groups. Animals that died were not used in the calculation of CEA regression percentages. The level of infection, measured by plating light organ homogenates after exposure to bacteria, was significantly higher at 12 h.
than at 8 h (Fig. 7). Although CEA regression was higher at 12 h than at 14 h (Fig. 6), the values at 14 h are within the range of values for 12 h. These results suggest that the minimum exposure time for complete regression of the CEA must lie between 8 and 12 h.

**Exposure to other strains of V. fischeri**

When squid were exposed to the nonmotile strain of V. fischeri, M101, neither colonization nor CEA regression was observed, supporting the above evidence that the bacteria must be within the light organ to induce morphogenesis. Additionally, of the natural isolates tested, only the infective strain, MJ11, induced CEA regression (Table II).

**Exposure to an autoinducer mutant of V. fischeri**

Squid exposed to the autoinducer mutant of V. fischeri, 3100, were infected and showed complete regression of CEA after 4 days (Table II), thus eliminating the possibility that autoinducer is required for light organ morphogenesis.

**Discussion**

The results of this study show that light organ morphogenesis of the squid Euprymna scolopes in response to the presence of symbiotic bacteria (1) requires only a 12-h exposure to symbiosis-competent bacteria; (2) requires colonization of the light organ by bacteria; (3) does not require V. fischeri autoinducer.

The finding that a transient exposure to symbiosis-competent bacteria is sufficient to induce morphogenesis of the squid light organ (i.e., the bacteria are not required throughout the 4-day morphogenetic process) suggests that the signal from the bacteria may trigger an irreversible cascade of events that eventually results in CEA regression. Signal transduction through some host cell surface receptor in the light organ crypts is an attractive model for this type of response. This model is supported by evidence that colonization of the light organ crypts is required to elicit CEA regression (see below), and by the recent finding that cell death and regression events are first seen at the tips of the ciliated appendages, several cell layers away from the crypt epithelium adjacent to the bacteria (Montgomery and McFall-Ngai, 1994). Thus, cells that are in direct contact with the bacteria must somehow pass the signal through several layers of adjacent host cells to effect cell death in the ciliary appendages, presumably through one or more signal transduction pathways.

The results of experiments with various strains of V. fischeri indicate that noninfective strains are not morphogenic and that the bacteria must enter the light organ to induce morphogenesis. Also, because nonmotile V. fischeri are unable to infect the light organ, motility is indirectly required for induction of morphogenesis. Similarly, motility is required for infection and virulence in many pathogenic bacteria, such as V. cholerae (Guentzel and Berry, 1975; Yancey et al., 1978), Pseudomonas spp. (Drake and Montie, 1988), Helicobacter pylori (Eaton et al., 1989; Dunn, 1993), and Salmonella typhi (Liu et al., 1988).

The requirement for infection suggests that transduction of the signal occurs through interactions with the light organ crypt epithelial cells, and not through interactions with the external ciliated microvillous cells. Unlike the Rhizobium legume symbiosis, in which a diffusible morphogen excreted by the bacteria induces cell division

### Table II

| V.f. Strain | Source | Phenotype | Infection | Morphogenesis |
|-------------|--------|-----------|-----------|---------------|
| ES114       | squid light organ | wildtype | +         | +             |
| U2          | ES114 Cm resistant derivative | - | -         | -             |
| M101        | ES114 motility derivative | - | -         | -             |
| 3100        | ES114 lux I" derivative | - | -         | -             |
| MJ1         | fish light organ (1974) | wildtype | -         | -             |
| MJ11        | fish light organ (1992) | wildtype | +         | +             |
| MdR12       | seawater isolate | wildtype | -         | -             |

**Figure 7.** Average colony forming units (CFU) of bacteria per squid after 8 and 12 h of exposure to V. fischeri. Vertical bars represent standard deviations (n = 5).
and morphological changes in the plant without colonization of the host by the symbiont (Long, 1989; Appelbaum, 1990; Hirsch, 1992), morphogenetic induction in the *Vibrio-Euprymna* symbiosis requires that the bacteria be within the confined space of the light organ. Studies using strain MJ11, which produces a colonization consisting of only about 10% of the typical cell number yet induces morphogenesis, indicate that the actual bacterial volume is probably not exerting a physical pressure or stretching of the light organ to induce morphogenesis. It is unclear at this time whether the signal is secreted from the bacteria into the light organ crypt lumen or the signal is presented directly on the surface of the bacteria. In either case, the observed time window of between 8 and 12 hours necessary to induce CEA regression, may reflect the need for an accumulation of bacteria, or their products, to a critical density within the light organ. If the morphogenic signal is secreted, there are a few possible scenarios: (1) secretion of the morphogen is induced and only occurs within the environment of the light organ, (2) the morphogen is produced constitutively and light organ crypts provide a barrier to diffusion of bacterial products such that within the light organ the signal reaches a critical concentration required for transduction, or (3) the presence of bacteria within the light organ (perhaps through direct cell-cell contact) renders the host cells competent to “accept” the secreted signal from the bacteria.

Rather than secreted, the bacterial signal may be a molecule presented on the bacterial cell surface that interacts directly with a receptor on the animal cell membrane. Direct interactions via glycan adhesin binding have been implicated in many symbioses and there is evidence for a mannose lectin in *E. scolopes*: when squid are inoculated with bacteria in the presence of mannose, colonization is significantly inhibited (V. Weis, K. Brennan and M. McFall-Ngai, unpublished data). In the *Rhizobium*-legume symbiosis, plant lectins that recognize specific bacterial surface oligosaccharides have been suggested to play a major role in attachment and invasion mechanisms (Dazzo and Truchet, 1983). Further, in pathogenic associations, bacterial adhesins on pili often are involved in recognizing specific sugar receptors on the animal cell surface (Finlay and Falkow, 1989).

Recently it has been shown that various other autoinducer molecules regulate the production of exoenzyme virulence determinants in *Pseudomonas aeruginosa* and *Erwinia carotovora* (Jones et al., 1993). Also, these autoinducers are structural analogs of actinomycetes A-factor, which has been implicated as an autoregulator of cellular differentiation between different *Streptomyces* species (Beppu, 1992). However, the results of this study indicate that *V. fischeri* autoinducer is not required for light organ morphogenesis. Other secreted bacterial molecules such as *Vibrio* spp. endotoxins are known to interact with animal cells (Lin et al., 1993) and may be potential morphogens. For example, cholera toxin has been shown experimentally to induce metamorphosis in certain marine larvae (Hofmann and Brand, 1987). In addition, Reich and Schoolnik recently found that *V. fischeri* carries a gene homologous to toxR (1994), which regulates cholera toxin production in *V. cholerae*, and also synthesizes a cholera toxin-like ADP-ribosylating protein (1995). However, while commercially available cholera toxin mimics some aspects of the symbiotic state, by itself it does not cause morphogenesis in *E. scolopes* (Small and McFall-Ngai, 1993), suggesting that if an endotoxin-like molecule is the squid morphogen, it is significantly different from cholera toxin, or that additional molecules (perhaps on the surface of the bacteria) are also required. Other bacterial factors that have been demonstrated to affect metamorphosis or morphogenesis in various host organisms include oligopeptides (Hofmann and Brand, 1987), phorbol esters (Muller, 1985), diacylglycerols (Leitz and Muller, 1987), and lipo-oligosaccharides (Lerouge et al., 1990; van Brussel et al., 1992), any of which may prove important in our system.

In conclusion, we have shown that a transient colonization of *E. scolopes* with symbiosis-competent *V. fischeri* induces morphogenesis of the squid light organ. Transduction of the morphogenic signal requires the presence of the bacteria within the light organ for approximately 12 h. Further investigations are necessary to determine the nature of the bacterial signal, the role of colonization in the generation of the signal, and the transduction pathway within the host squid.

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