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Vibrio fischeri Lipopolysaccharide Induces Developmental Apoptosis, but Not Complete Morphogenesis, of the *Euprymna scolopes* Symbiotic Light Organ

J. S. Foster,* M. A. Apicella,† and M. J. McFall-Ngai*,[1]

**Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96813; and*
†Department of Microbiology, School of Medicine, University of Iowa, Iowa City, Iowa 52242

During initiation of the association between the squid host *Euprymna scolopes* and its bacterial partner *Vibrio fischeri,* the bacteria induce dramatic morphogenesis of the host symbiotic organ, a portion of which involves the signaling of widespread apoptosis of the cells in a superficial ciliated epithelium on the colonized organ. In this study, we investigated the role in this process of lipopolysaccharide (LPS), a bacterial cell-surface molecule implicated in the induction of animal cell apoptosis in other systems. Purified *V. fischeri* LPS, as well as the LPS of *V. cholerae, Haemophilus influenzae, Escherichia coli,* and *Shigella flexneri,* added in the concentration range of pg/ml to ng/ml, induced apoptosis in epithelial cells 10- to 100-fold above background levels. The absence of species specificity suggested that the conserved lipid A portion of the LPS was the responsible component of the LPS molecule. Lipid A from *V. fischeri, E. coli,* or *S. flexneri* induced apoptosis. In addition, strains of *H. influenzae* carrying a mutation in the *htrB* gene, which is involved in the synthesis of virulent lipid A, showed a diminished ability to induce apoptosis of host cells. Confocal microscopy using fluorescently labeled LPS indicated that the LPS behaves similar to intact bacterial symbionts, interacting with host cells in the internal crypt spaces and not directly with the superficial epithelium. Although LPS was able to induce apoptosis, it did not induce the full morphogenesis of the ciliated surface, suggesting that multiple signals are necessary to mediate the development of this animal–bacterial mutualism.

INTRODUCTION

The successful outcome of animal embryogenesis relies heavily on finely tuned interactions between and among the animal cells that share a common genome. However, postembryonic developmental programs, during which time the animal's phenotype is refined by interactions with the biotic and abiotic environment (Gilbert, 1997), include the initiation of specific associations with a community of bacterial cells (McFall-Ngai, 1998a). To establish and maintain this community with fidelity between successive host generations, cellular mechanisms must be in place that orchestrate a reciprocal "dialogue" between the cells of these two distinct genomes.

Both embryonic and postembryonic developmental mechanisms are defined by two broad categories of cell-to-cell communication—the export of diffusible factors that affect the activity of surrounding cells and the direct interaction of adjacent cells through specific adhesion molecules or junctional complexes (Edelman, 1986; Ingham, 1994; Clark and Brugge, 1995). Not surprisingly, animal–bacterial cell interactions have been found to rely principally on these same processes (Middlebrook and Dorland, 1984; Ofek and Doyle, 1994; Telford *et al.*, 1998), although the study of such interactions has been largely restricted to pathogenic associations. Of the bacterial cell surface molecules implicated in eukaryotic–prokaryotic interactions, perhaps the most conspicuous and best studied is lipopolysaccharide (LPS) (Raetz *et al.*, 1991; Raetz, 1993; Kielian and Blecha, 1995; Ulevitch and Tobias, 1995; Fenton and Golenbock, 1998; Hamann *et al.*, 1998). This complex molecule, which comprises up to 75% of the outer membrane of gram-negative bacteria (Reitschel *et al.*, 1994), contains both lipid and carbohydrate and consists of three linearly arranged parts: (1) the evolutionarily conserved lipid A

[1] To whom correspondence should be addressed. Fax: (808) 599-4817. E-mail: mcfallng@hawaii.edu.

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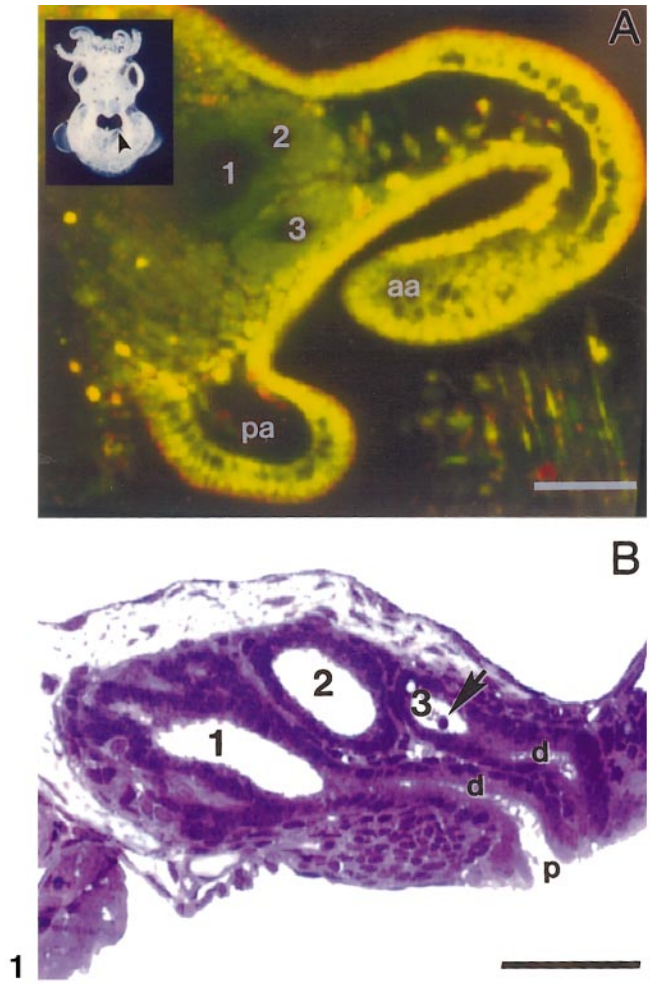
portion, which is embedded in the bacterial cell membrane; (2) the core polysaccharide that is linked to the lipid A; and (3) the O-antigen, a polysaccharide chain of species-specific length and composition that extends outward from the core polysaccharide. This composition renders the LPS an amphipathic molecule that, when purified and dispersed in aqueous environments, forms micelles of varying sizes that retain significant endotoxic activity (Aurell and Wistrom, 1998).

In animal–bacterial interactions, LPS has principally been studied for its role in pathogenesis, most notably as an inducer of host cell apoptosis (Norimatsu *et al.,* 1995; Guichon and Zychlinsky, 1996; Zychlinsky *et al.,* 1996) as well as a mediator of immune-system-regulated septic shock (Xu *et al.,* 1996). The precise biochemical mechanisms by which LPS exerts its effects on host cells to bring about these responses have recently been under intensive investigation. These studies have revealed that LPS first binds to soluble factors in the blood, such as LPS-binding protein (Pugin *et al.,* 1993; Fenton and Golenbock, 1998) or soluble CD14 and its homologs (Lee *et al.,* 1996; Blanco *et al.,* 1997). This complex interacts with well-conserved receptors, belonging to the Toll receptor family (Gerard, 1998; Rock *et al.,* 1998; Yang *et al.,* 1998), that stimulate the similarly well-conserved NF-κB/Rel type transcription factors (Medzhitov *et al.,* 1997; May and Ghosh, 1998). Interestingly, these specific receptor/ligand interactions and the pathways they effect have been shown to play critical roles in both developmental and immune processes in vertebrates and invertebrates (Belvin and Anderson, 1996; Bushdid *et al.,* 1998; Gerard, 1998; Kanegae *et al.,* 1998).

The role of LPS in the induction of a normal developmental program has been extensively characterized only in the symbioses between leguminous plants and nitrogen-fixing soil bacteria (rhizobia) (van Rhijn and Vanderleyden, 1995). LPS is one of a myriad of molecules that mediate the exchange between plant hosts and their rhizobia during formation of root nodules (Fisher and Long, 1992; Hirsch, 1992), and its involvement has been implicated in almost every stage of the developmental program, from formation of the infection thread to the production of functional, nitrogen-fixing nodules (Dazzo *et al.,* 1991; Perotto *et al.,* 1994; Lopez-Lara *et al.,* 1995). Bacterial cell-surface molecules, such as LPS, are likely to mediate interactions between animals and their beneficial bacteria as well. However, because such associations usually involve dynamic relationships among the members of a large, speciose community of microbes, it has been difficult to determine the identity of the specific molecules responsible for the bacteria-induced modifications in host tissues that occur during development (McFall-Ngai, 1998a). The light organ symbiosis between the Hawaiian sepiolid squid *Euprymna scolopes* and its luminous bacterial partner *Vibrio fischeri* offers an opportunity to resolve, in an animal model, the same issues that have been well addressed in the development of the legume–rhizobia symbioses (McFall-Ngai, 1998b; McFall-Ngai and Ruby, 1998). As a two-partner association, the squid–vibrio symbiosis can be experimentally manipulated, providing a system by which to define the role of cooperative bacteria in the postembryonic development of their animal hosts.

During the embryogenesis of *E. scolopes,* an organ forms in the center of the mantle cavity (Montgomery and McFall-Ngai, 1993) that will interact with *V. fischeri,* which are acquired from the environment within hours of the host's hatching (McFall-Ngai and Ruby, 1991). The organ of newly hatched *E. scolopes* has extensive, ciliated epithelial fields on each lateral surface, which appear to facilitate the inoculation process (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993, 1994). Each field comprises an epithelial pad of tissue covering the surface of the nascent light organ; the pad grades into two conspicuous appendages, one anterior and one posterior (Fig. 1A). These appendages consist of a single epithelial layer overlying a blood sinus. The ciliated microvillus fields circulate ambient seawater into the vicinity of three pores at the base of each pair of appendages. *V. fischeri* cells that are present in the seawater migrate into the light organ through these pores and travel along ciliated ducts into crypt spaces, the permanent site of residence of the bacterial symbiont population (Fig. 1B). Once inside the light organ, the bacteria interact with two host cell types, the epithelial cells lining the crypt and dynamic populations of hemocytes (Nyholm and McFall-Ngai, 1998), which migrate into and out of the crypts, appearing to provide an interface between the crypt environment and the circulatory system of the host. The ability to colonize the light organ is restricted to *V. fischeri;* i.e., in its absence, the light organ crypts remain sterile, even though the host may be exposed to countless other bacterial species present in the seawater (McFall-Ngai and Ruby, 1991).

The light organ undergoes a dramatic morphogenesis upon interaction with *V. fischeri* (McFall-Ngai and Ruby, 1998). The most conspicuous change is the bacteria-induced regression of the ciliated epithelial field of the organ over the first 4 days following colonization of the organ (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1994). Experiments in which the light organ was cleared of its symbionts showed that the bacteria send an irreversible, inductive signal about 12 h after first exposure to the symbionts; i.e., if bacteria are removed from the organ at any time before 12 h, regression over the following 4 days will not take place. If the organ is cured at any time after 12 h following inoculation, the full 4-day morphogenetic program is completed (Doino and McFall-Ngai, 1995). Studies with motility mutants of *V. fischeri* (Graf *et al.,* 1994), which cannot enter the light organ crypts (S. V. Nyholm, pers. comm.), showed that direct interaction of the bacteria with the ciliated epithelial field does not induce the morphogenesis (Doino and McFall-Ngai, 1995). *V. fischeri* cells must enter the light organ and interact with either the crypt epithelium or the hemocytes to induce regression. Thus, the induction of morphogenesis occurs



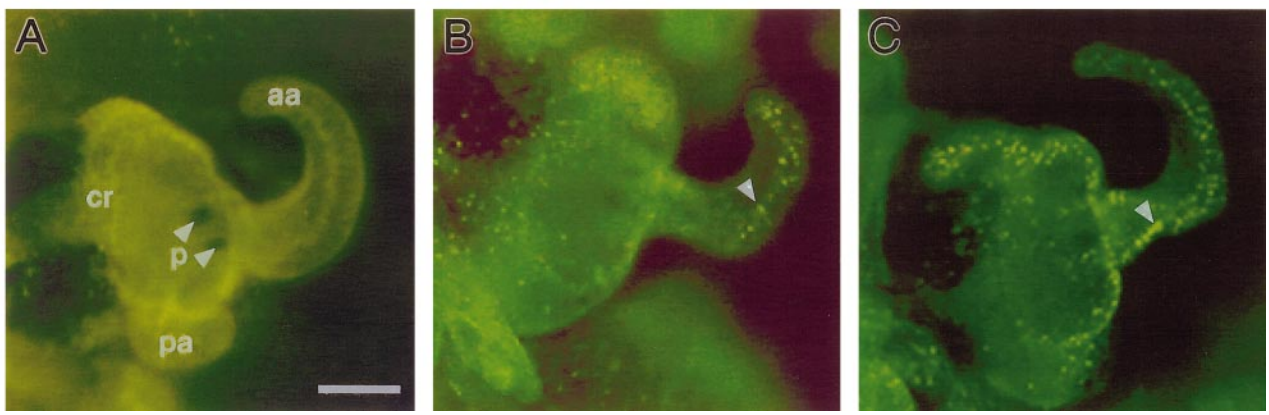


FIG. 1. The morphology and anatomy of the juvenile light organ of *Euprymna scolopes.* (A) The fluorescent, confocal image of the right half (ventral surface) of the light organ of a newly hatched squid stained with acridine orange, which labels the nucleic acids of the cells. A transparent, superficial ciliated field occurs on each lateral face of the organ. The anterior (aa) and posterior (pa) appendage of each field is composed of a single-cell layer over a basement membrane-lined blood sinus. At the base of each set of appendages are three pores (1, 2, 3) through which the bacteria enter the light organ to initiate the symbiosis. Bar, 50 μm. Inset, a ventral dissection of a newly hatched squid (2 mm in total length). The light organ (arrowhead) is invested in the black ink sac in the center of the mantle cavity. (B) A histological section through the left half of the light organ showing the three epithelia-lined crypt spaces (1, 2, 3). To colonize each crypt, the bacteria enter a pore (p) on the surface of the light organ and travel down a ciliated duct (d) to the crypt space. Once inside, the bacteria interact with the epithelial cells lining the crypt and with host hemocytes (arrow) free-floating within the crypt. Bar, 70 μm.

through the presentation of a signal in the crypts that acts on the remote, superficial tissues of the light organ.

Examination of the light organ during morphogenesis revealed an extensive program of bacteria-induced apoptosis that displays a stereotypical pattern (Montgomery and McFall-Ngai, 1994; Foster and McFall-Ngai, 1998). The first visible signs of apoptosis occur along the medial ridge of the ciliated field, which comprises a population of cells with elongated cilia, at 6 h following initial exposure to *V. fischeri*. By 9 h, large numbers of cells along the ridge and throughout the appendages are undergoing apoptosis, and by 12 to 14 h postinoculation the greatest numbers of apoptotic cells are evident. The coincidence of extensive cell death and the triggering of an irreversible signal in the first few hours following colonization suggests that apoptosis is responsible for some, if not all, of the induction of the morphogenetic program that results in regression of the ciliated field.

The studies described above have provided the foundation for elucidation of the signal, or signals, from the bacteria to host animal tissue that results in host light organ morphogenesis. The present study examines the role of *V. fischeri* LPS in this process. The results of our experiments suggest that LPS, and more specifically the lipid A component of the LPS, induces apoptosis in the cells of the ciliated epithelial field. To our knowledge, this report is the first to show involvement of LPS-induced apoptosis in the normal developmental program of an animal. Further, LPS is the first bacterial signal molecule to be identified as critical to the developmental program of the host in the squid–vibrio symbiosis. However, LPS-induced cell death could not account for the full morphogenetic program; i.e., although bacterial LPS caused the extensive and persistent cell death characteristic of the intact symbiosis, it did not induce complete regression of the ciliated field. These data provide evidence that, similar to the legume–rhizobia symbioses that require multiple, reciprocal interactions between the host and the symbiont during development, at least two independent bacterial signals must be involved in the initiation of light organ morphogenesis in the squid–vibrio system.

MATERIALS AND METHODS

General Procedures

A breeding colony of adult *E. scolopes*, collected from the sand flats of O'ahu, Hawaii, was maintained in running seawater tables at the Kewalo Marine Laboratory, Pacific Biomedical Research Center, University of Hawaii, as previously described (Doino and McFall-Ngai, 1995). Upon hatching, juvenile *E. scolopes* were rinsed and maintained in filter-sterilized seawater (FSW) from underground wells of the Waikiki Aquarium, which does not contain symbiosis-competent stains of *V. fischeri*. Each animal was placed in either a 5-ml polystyrene petri dish or the well of a 20-well sterile polystyrene microtiter plate. To initiate the symbiosis, juvenile animals were exposed to 5×10^3 cells/ml of *V. fischeri* ES114, a strain previously isolated from the adult *E. scolopes* light organ (Boettcher and Ruby, 1990). The colonization of the light organ was monitored by measuring bacterial luminescence with a photometer (Turner Designs TD-20/20 luminometer; Sunnydale, CA). In controls, a subset of the animals was maintained aposymbiotically, i.e., in the absence of symbiotically competent *V. fischeri*.

Preparation of LPS and LPS Components

The LPS, lipid A, and O-antigen of *V. fischeri*, as well as the LPS of wild-type and the *htrB* mutant strain B-29 of *Haemophilus influenzae* (Lee *et al.*, 1995), were purified from whole cells as previously described (Apicella *et al.*, 1994). LPS, lipid A, and fluorescently labeled LPS of *V. cholerae*, *Escherichia coli*, and *Shigella flexneri* were purchased from Sigma Chemical Co. (St. Louis, MO). Endotoxin-activity levels of *V. fischeri* LPS and its derivatives were assessed by the limulus amebocyte lysate (LAL) gel-clot test (Associates of Cape Cod, Falmouth, MA) (Tomasulo *et al.*, 1977).

In experiments in which juvenile *E. scolopes* were exposed to LPS and LPS derivatives, stock solutions of 100 μg/ml were prepared in filtered distilled water in polystyrene Falcon tubes. These solutions were sonicated in a water bath for 2 min, at which point they were homogeneous, clear suspensions of micelles of LPS or LPS derivatives. Dilutions of the stock were prepared in FSW and transferred to either polystyrene petri dishes or microtiter plates prior to introduction of the animals.

Detection of Apoptosis/Assessment of Regression

To determine the number of dying cells in the ciliated epithelial surface, animals were anesthetized in a 1:1 solution of 0.37 M $MgCl_2$:seawater, then incubated for 1 min in a 5 ng/ml solution of acridine orange (AO), a fluorochrome that intercalates into the condensed chromatin of dying cells (Delic *et al.*, 1991). After staining, the mantles and funnels of the animals were removed to expose the light organ, and the animals were examined either with a Leica MZFLIII fluorescence stereomicroscope or with a Nikon HFX-11 epifluorescence microscope. Levels of cell death were determined by counting the numbers of stained pycnotic nuclei throughout the epithelial field.

In experiments comparing the potency of the various LPS types, cohorts of animals were exposed to a particular concentration of a

FIG. 2. LPS-induced apoptosis in the host light organ. Fluorescence micrographs of the light organs of 14-h animals stained with acridine orange (only one half of the light organ shown), which intercalates into the condensed chromatin of dead and dying cells (arrowheads). (A) The light organ of an aposymbiotic animal showing no pycnotic nuclei characteristic of apoptotic cells over the entire superficial, ciliated epithelial field. aa, anterior appendage; cr, ciliated ridge; pa, posterior appendage; p, pores. (B) The light organ of an aposymbiotic animal exposed to LPS for 12 h, revealing the stereotypical pattern of apoptosis along the ciliated ridge and throughout the anterior and posterior appendage. (C) The light organ of a symbiotic animal depicting the normal pattern of apoptosis. Bar, 50 μm.

given LPS and the numbers of pycnotic nuclei were determined after the incubation period. These numbers were analyzed as pairwise comparisons in which the response of experimental animals was compared to the response of a cohort of animals that had not been exposed to exogenously added LPS; both the Student *t* test for two samples (parametric) and a Wilcoxon two-sample test (nonparametric) were conducted with the data to assess significance.

We used *V. fischeri* and *V. cholerae* LPS in an extensive set of experiments to assess the within-clutch and between-clutch variability in the response of individual animals, as well as the variability in response to different LPS preparations. For the 18 experiments that were conducted with the LPS of each vibrio species, at each of seven concentrations at least 2 or, for some concentrations, 3 separate experiments were conducted. In each experiment at least 5 (range 5–10) animals were compared to the same number of control, unexposed animals.

To confirm that cells undergoing apoptosis under the influence of LPS exhibited the same type of cell death that is characteristic of *V. fischeri*-induced cell death, we viewed samples by transmission electron microscopy to examine cell ultrastructure. In addition, we analyzed samples for DNA fragmentation using the terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling (TUNEL) method as previously described (Foster and McFall-Ngai, 1998).

Animals were monitored for regression of the ciliated epithelium at 4 days after initial colonization. At this time, they were anesthetized and placed for 12 h in a 5% formalin solution in seawater and prepared for and analyzed by SEM as previously described (Foster and McFall-Ngai, 1998).

Localization of the LPS in the Tissues of the Light Organ

To determine where LPS was interacting with light organ tissues, juvenile animals were exposed to tetramethylrhodamine isothiocyanate (TRITC)-labeled LPS from *E. coli*. The animals were exposed to seawater containing 1 μg of TRITC–LPS per milliliter for 14 and 24 h while being maintained in the dark to prevent the daily expulsion of light organ crypt contents (Graf and Ruby, 1998; Nyholm and McFall-Ngai, 1998). The animals were rinsed for 2 h in FSW to remove excess fluorescent LPS, then counterstained for 30 min with 1 μg of CellTracker green (Molecular Probes, Eugene, OR) per milliliter of seawater. After staining, the animals were anesthetized, dissected, and examined with a Zeiss LSM 510 confocal microscope.

Manipulation of the Timing of the Bacterial Signal

To assess whether the *V. fischeri* signal for programmed cell death was directly linked to epithelial field regression, we used the antibiotic chloramphenicol (Cm) to cure the light organ of symbionts (Doino and McFall-Ngai, 1995) at various times following initial exposure to *V. fischeri*. Briefly, animals were exposed to 10^3 viable *V. fischeri* cells per milliliter of seawater for 3, 6, 9, and 12 h and then treated with 10 μg of Cm per milliliter of seawater, which attenuates viable symbionts from the light organ within a 2- to 3-h period (Doino and McFall-Ngai, 1995). At 14 h after the initial inoculation, a subset of the cured animals was examined for cell death. The remaining animals were maintained in Cm-treated seawater for 4 days and then examined for epithelial field regression. To ensure that the Cm was effectively inhibiting symbiont growth in the light organ over the 4-day period, animal luminescence was monitored twice daily.

To determine whether exposure to purified LPS before exposure to intact symbiont cells (i.e., priming with LPS) would affect the timing of the morphogenetic signal, animals were exposed to 10 ng of *V. fischeri* LPS per milliliter of FSW for 6 h prior to the introduction of symbiotically competent *V. fischeri*. Following 8 h of subsequent exposure of the "LPS-primed" animals to *V. fischeri*, a time interval about 4 h shorter than the reported 12 h required for *V. fischeri* to signal complete morphogenesis, the light organs were cured with 10 μg of Cm per milliliter of seawater. Subsets of the animals were then examined at 14 h for levels of cell death and at 4 days to determine the extent of epithelial cell regression.

RESULTS

LPS and Lipid A Induction of Apoptosis

Examination of the ultrastructure of the cells of the superficial ciliated field, as well as characterization of the mode of DNA fragmentation (TUNEL), demonstrated that *V. fischeri* LPS caused apoptotic cell death that was indistinguishable from the previously characterized apoptosis induced by *V. fischeri* cells (data not shown; Foster and McFall-Ngai, 1998). The spatiotemporal pattern was also similar in both cases (Fig. 2), i.e., apoptosis was first noted at 6 h following exposure, beginning along the ciliated ridges. By 9 h in both treatments, widespread apoptosis was evident along the ridges and throughout the anterior and posterior appendages (Figs. 2B and 2C). However, whereas the time to reach an asymptote of cell death events in animals exposed to *V. fischeri* occurred between 12 and 14 h following exposure as previously reported (Montgomery and McFall-Ngai, 1994), LPS-exposed animals showed a delay to 18 h in reaching this asymptote for all concentrations of LPS tested (see below).

Experimental addition of LPS from other gram-negative bacteria revealed that the induction of apoptosis is not specific to *V. fischeri* LPS. To quantify the response, cohorts of animals were exposed to LPS from other bacterial species for 18 h. The total number of pycnotic nuclei present in the superficial ciliated field was then determined at that time for each animal. The LPS of *V. cholerae*, *E. coli*, *H. influenzae*, and *S. flexneri* all caused apoptosis on average at least 10-fold above background over a range of concentrations from 1 pg to 1 μg of LPS per milliliter.

Although the majority of the animals in all experiments showed apoptosis in response to bacterial LPS, we observed marked variation in the level of the response, ranging from no cell death events detected in a few animals to numbers of pycnotic nuclei in the range characteristic of *V. fischeri*-infected symbiotic animals. This variation was examined in a more extensive set of experiments (see Materials and Methods) that compared responses to the LPS of either *V. fischeri* or *V. cholerae* (Fig. 3). Specifically, when the 18 experiments with *V. fischeri* were analyzed by the Student *t* test, 16 comparisons showed a statistically significant difference (13 at $P < 0.05$; 3 at $P > 0.05$ and < 0.1)

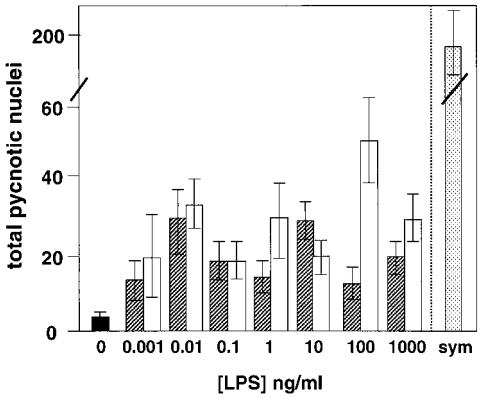


FIG. 3. Induction of apoptosis in the light organs of animals exposed to LPS. The graph is a composite of all experiments performed in a comparison of the response to *V. fischeri* and *V. cholerae* LPS. Bar height represents the average number of cell death events (±SEM) in animals tested at a given concentration of *V. fischeri* (hatched bars) or *V. cholerae* (open bars) LPS. Controls were run with animals exposed to no LPS (black bar), as well as animals exposed to 10^3 *V. fischeri*/ml of seawater (stippled bar; sym). (See text for details.)

between the LPS-treated cohorts and untreated controls; when analyzed by the Wilcoxon two-sample test, 13 comparisons showed significant differences between treated and untreated samples (10 at $P < 0.05$; 3 at $P > 0.05$ and < 0.1). When the 18 experiments with *V. cholerae* were analyzed by the Student *t* test, 10 of 18 comparisons were significant (6 at $P < 0.05$; 4 at $P > 0.05$ and < 0.1), and when analyzed by the Wilcoxon test, 12 of 18 comparisons were significant (8 at $P < 0.05$; 4 at $P > 0.05$ and < 0.1). Significance levels did not reflect the source of the animals (i.e., within a given clutch or between clutches) or variation in the preparation of the LPS between experiments.

On average, *V. cholerae* LPS caused a greater number of apoptotic events than *V. fischeri* LPS at most of the concentrations tested, and the maximum recorded numbers of apoptotic cells were generally higher for *V. cholerae* LPS. However, in the LAL assays, our preparation of *V. fischeri* LPS showed levels of endotoxin activity similar to those of other gram-negative bacteria. Thus, the LAL assays revealed that the endotoxin activity of *V. fischeri* LPS was not relatively low by all measures.

The lack of species specificity in the response to LPS suggested that lipid A was the responsible constituent of the LPS molecule. We tested the response to the purified lipid A or the O-antigen from *V. fischeri* or to the commercially available lipid A from *E. coli* or *S. flexneri.* In addition, we compared the response to the LPS of wild-type *H. influenzae* with that of an *H. influenzae htrB* mutant which is defective in the normal synthesis of the lipid A portion of LPS. Results of these experiments suggested that lipid A was responsible for triggering most, if not all, of the bacteria-induced cell death in the *E. scolopes* light organ, although some variation in the response to the different lipid A types was noted. *V. fischeri* lipid A at concentrations ranging from 0.1 to 1000 ng/ml showed levels of cell death that were significantly higher than background levels ($P < 0.05$ for all concentrations) (Fig. 4A). Purified O-antigen did not induce cell death above background levels. Lipid A from either *E. coli* or *S. flexneri* was also able to induce levels of pycnotic nuclei that were significantly higher than those of untreated animals ($P < 0.0003$) (Fig. 4B). In experiments in which wild-type *H. influenzae* LPS was compared with LPS derived from *htrB* mutants of this species, the mutant LPS did not induce significant apoptosis above background levels at 18 h ($P < 0.4$) (Fig. 4C, top). However, by 48 h, the mutant LPS had induced cell death at levels in the same range as that induced by the wild-type LPS. All of the levels of apoptosis with wild-type LPS at both time points and mutant LPS at 48 h were significantly different from background levels ($P < 0.05$ for all concentrations, except the 48-h *htrB* mutant at 10 ng/ml, for which $P < 0.07$) (Fig. 4C, bottom).

E. coli LPS did not bind and interact with the ciliated apical surfaces of the superficial ciliated epithelium, providing evidence that the micelles of LPS created by sonication were entering the light organ and interacting with receptors on cells in the crypts, similar to the behavior of the intact symbiont, which must enter crypts to effect the cell death signal. The LPS molecules that entered the pores on the surface of the organ (Fig. 5A) were diffused through the ciliated ducts to the

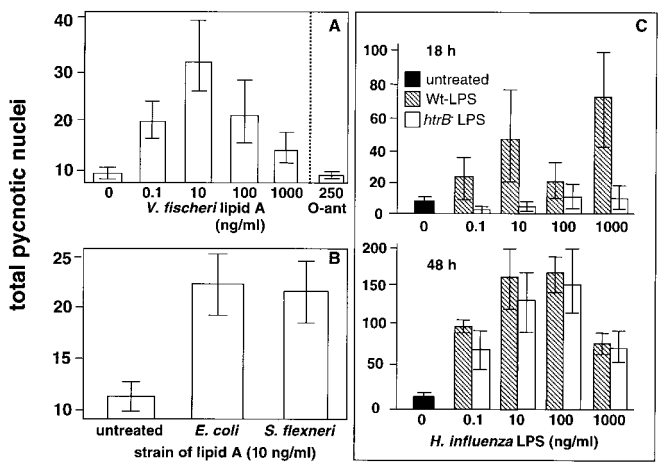


FIG. 4. Induction of apoptosis in the superficial epithelium by LPS and LPS derivatives. (A) The response of the epithelial cells to a range of concentrations of *V. fischeri* lipid A or purified O-antigen (O-ant) from *V. fischeri* LPS. (B) The response to the lipid A of other gram-negative bacteria, *E. coli* and *S. flexneri.* (C) The response to LPS from wild-type *H. influenzae* and to the LPS of an *htrB* mutant of *H. influenzae* which is defective in normal lipid A synthesis, at 18 h (top) and 48 h (bottom). (The bar height represents the mean number of pycnotic nuclei (±SEM) as detected by acridine orange; $n = 6$ animals per treatment group.)

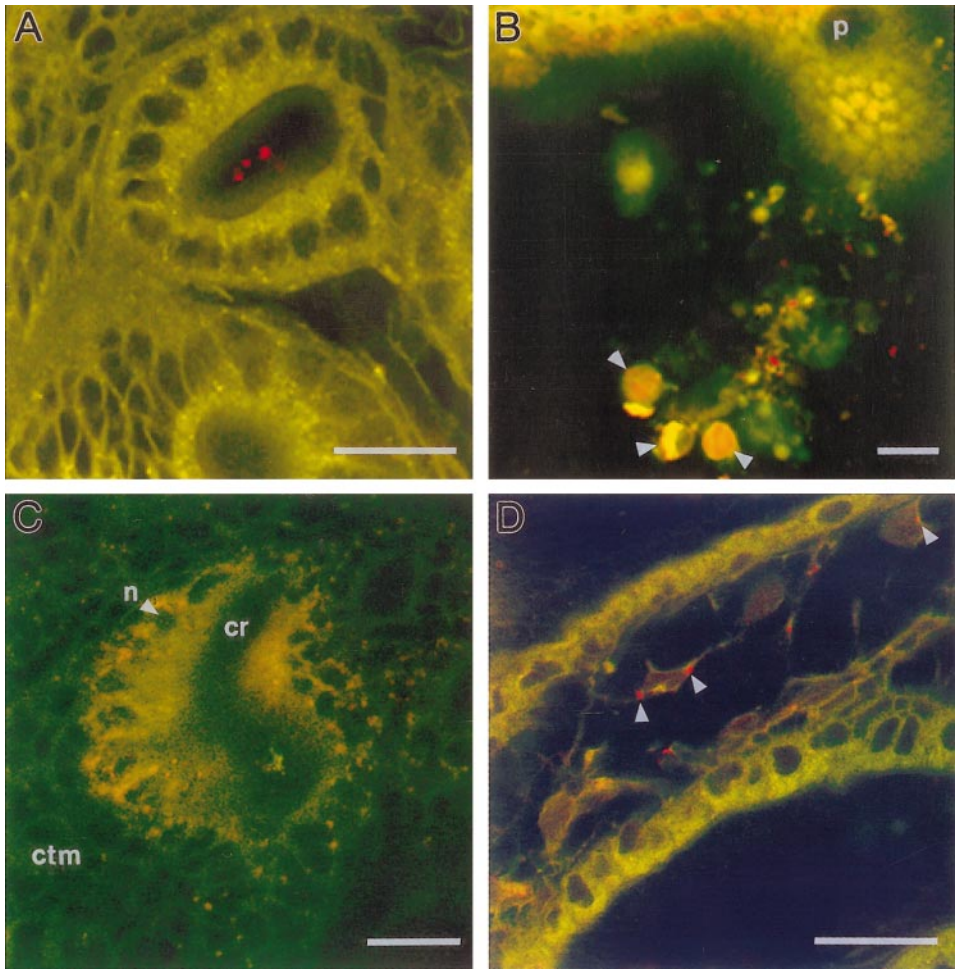


FIG. 5. Confocal micrographs of animals incubated with *E. coli* TRITC-labeled LPS and counterstained with CellTracker green. (A) At the level of the pores on the surface of the light organ at 14 h, labeled LPS was observed in the lumina, but did not interact with the surface of the organ or cells surrounding the pore. Bar, 30 μm. (B) After 24 h of TRITC–LPS exposure, crypt contents, which were expelled from the pore upon light stimulation (Nyholm and McFall-Ngai, 1998) contained hemocytes (arrowheads) stained with both fluorescent markers. Bar, 25 μm. (C) Deeper section at the level of the crypt at 24 h revealed extensive accumulation of LPS within the matrix of the crypt spaces, as well as in the cells comprising the epithelial lining. Bar, 15 μm. (D) Animal cells within the blood sinus stained with the TRITC label suggesting the presence of translocated LPS (arrowheads) at 24 h. Bar, 25 μm.

crypt spaces. Once inside, the fluorescently labeled LPS was found in association with both crypt cell types, the free-floating hemocytes (Fig. 5B) and the epithelial cells lining the crypt spaces (Fig. 5C). Fluorescently labeled LPS molecules were also detected adhering to endothelial cells that line the blood sinus of the anterior and posterior appendages of the light organ (Fig. 5D), indicating that these molecules have access to this internal tissue space.

Effect of either Transient or Continuous Exposure to LPS on Apoptosis and Light Organ Morphogenesis

A transient exposure to *V. fischeri* of 12 h had previously been shown to be sufficient to induce morphogenesis (Doino and McFall-Ngai, 1995). Thus, various exposure times to *V. fischeri* LPS were tested to determine whether the induction of cell death is irreversible and whether LPS alone could induce full morphogenesis. Groups of animals were incubated in 10 ng of *V. fischeri* LPS per milliliter of seawater for 6, 9, or 12 h or continuously for 4 days. A subset of the animals was examined to determine when apoptosis had been triggered and its pattern. The remaining animals were examined at 4 days by SEM to determine the degree of regression of the ciliated field. In animals exposed to LPS for periods as short as 9 h, before being moved to vials containing FSW only, cell death numbers reached an asymptote by 18 h and continued at high levels throughout the remaining 4 days. Thus, similar to the irreversible

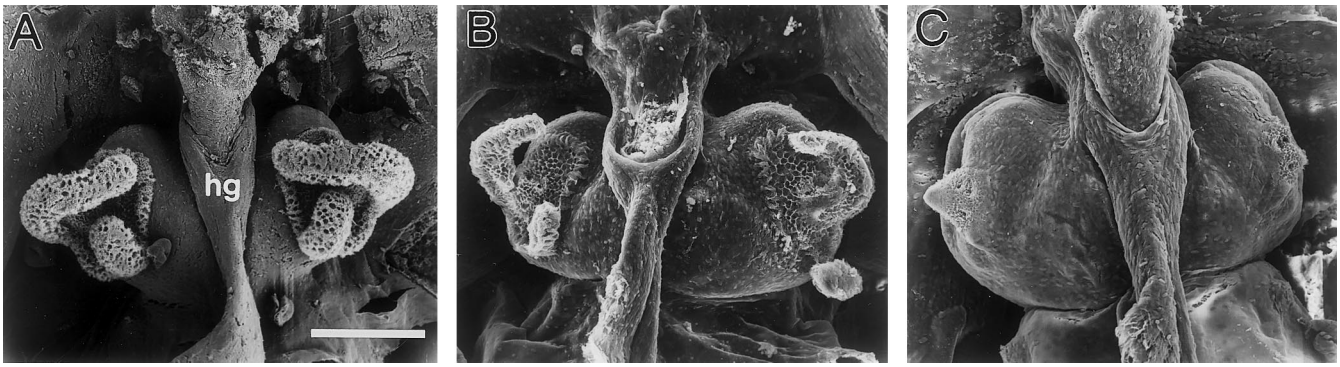


FIG. 6. The effects of LPS on light organ morphogenesis. Scanning electron micrographs of the ventral surface of the light organ of representative 4-day animals revealing the level of ciliated epithelial field regression. (A) Aposymbiotic animals showing no signs of epithelial field regression. The ciliated ridge is intact and both the anterior and the posterior appendages are present and fully functional. hg, hindgut. (B) Aposymbiotic animals exposed to LPS for 12 h exhibiting no marked regression of the ciliated field. (C) Symbiotic controls showing the full extent of regression. Bar, 100 μm.

trigger for morphogenesis induced by the symbiont, the induction of cell death was irreversible, although it occurred a few hours earlier. However, animals treated with LPS alone, either transiently or continuously over the 4 days, showed negligible signs of regression in all samples (Fig. 6). These animals had ciliated fields similar in appearance to those of aposymbiotic animals that had been maintained in FSW throughout the experiment.

Additional Evidence That Multiple Signals Are Required for Complete Light Organ Morphogenesis

The inability of *V. fischeri* LPS to induce the complete morphogenesis of the light organ superficial structures, as well as the difference in the timing of irreversible apoptosis and the induction of full morphogenesis, suggested the presence of multiple bacterial signals in host light organ development. To examine this possibility further, we conducted experiments in which we manipulated the timing of exposure to *V. fischeri* to determine whether the responses of the animals to LPS were similar to those elicited by intact symbionts, i.e., whether the timing of cell death and that of full morphogenesis were uncoupled. Cohorts of animals were exposed to intact *V. fischeri* for 3, 6, 9, or 12 h and then cured with the antibiotic chloramphenicol. A subset of these animals was stained with AO at 14 h and examined for the presence of apoptosis. The remaining animals were maintained until 4 days and examined by SEM (Table 1; Fig. 7). Animals that were maintained in FSW or exposed to bacteria for only 3 h showed no signs of apoptosis at 14 h after the initial exposure. Those animals incubated with competent strains of bacteria for 6 h showed a few pycnotic nuclei along the ciliated ridge at 14 h, but a full cell death pattern was not present. Only those animals exposed for either 9 or 12 h showed evidence of a full cell death pattern at 14 h. These results suggested that intact *V. fischeri* cells produce a signal mediating the full apoptotic program between 6 and 9 h after the symbiosis has been initiated. However, only those animals that maintained a complement of bacteria for at least 12 h exhibited complete regression of the superficial epithelium at 4 days. These data further support the possibility that at least two distinct signals from the bacteria are required to complete morphogenesis.

In experiments in which animals were primed with LPS, prior exposure to LPS did not influence the time required for *V. fischeri*-induced light organ morphogenesis. Only those animals exposed for a full 14 h to intact *V. fischeri* showed complete regression of the superficial epithelium. Animals treated for 6 h to LPS, followed by an 8-h exposure to *V. fischeri*, as well as control animals that were treated with *V. fischeri* for only 8 h or LPS alone for 14 h, showed no signs of epithelial field regression at 4 days, although in

TABLE 1
Development of the Superficial Epithelium with Transient Exposure to *V. fischeri*

Duration of bacterial exposure (h)	PCD[a] at time of Cm treatment[b]	PCD at 14 h	Regression[c] at 4 day
3	−	−	No
6	+/−	−	No
9	++	++++	No
12	++++	++++	Yes

[a] PCD, programmed cell death.
[b] Cm, 10 μg of chloramphenicol per milliliter of seawater.
[c] Complete loss of the ciliated field.

all of these treatments animals showed the characteristic full cell death pattern.

DISCUSSION

The results of this study provide evidence that: (1) bacterial LPS induces apoptosis in the cells of the superficial ciliated field of the *E. scolopes* light organ, (2) the likely candidate component of the LPS molecule is lipid A, and (3) morphogenesis of the light organ requires at least two bacterial signals, only one of which is LPS.

The ultrastructural and molecular characteristics of the LPS-induced apoptosis of the field of ciliated cells were indistinguishable from those observed in *V. fischeri*-induced apoptosis. In addition, the spatiotemporal patterns of cell death triggered by bacteria and purified LPS were similar, although some differences were noted. Specifically, the numbers of cells in the field undergoing apoptosis in response to LPS did not reach an asymptote until 18 h, a delay of between 4 and 6 h, and the response to LPS was somewhat attenuated. These discrepancies may reflect a difference in the manner by which purified LPS and bacteria-associated LPS are presented to the light organ crypt cells, including both the rate of delivery and the mode of presentation to receptors. Certainly not all the LPS of intact cells actually interacts with host cells, but the timing and concentration of LPS delivery during the onset of the natural symbiosis cannot be matched by exposing the animals to micelles of LPS in suspension.

Under our experimental conditions, the response of the animals to purified LPS varied markedly. This variation may be due to differences in the behavior of individual squid, to possible heterogeneity in the LPS suspensions to which the animals were exposed, and/or to variation in the numbers of LPS receptors available for interaction in the crypt spaces. Our studies of *E. scolopes* have revealed significant variation in the animals in aspects related to the onset of the natural symbiosis. For example, colonization levels can vary 100-fold after the first 12 h following exposure to environmental *V. fischeri* (pers. observation), suggesting that delivery of materials into the crypt spaces varies significantly among squid. Further, although every attempt was made to expose animals to homogeneous suspensions of LPS-containing micelles, the sonication procedure forms micelles of differing size; thus, some degree of heterogeneity within the suspension could not be avoided. In addition, we have not yet defined precisely how the signal from the LPS is transferred to the site of apoptosis induction. Confocal microscopy analyses suggest that the LPS can interact with both the epithelial cells and the hemocytes of the crypt and that the LPS may be transported to the sites of apoptosis. If the squid system is similar to that of other animals (Weersink *et al.*, 1990; Fenton and Golenbock, 1998), LPS receptors would be most abundant on the hemocytes, but may also occur on the epithelial and endothelial cells (Pugin *et al.*, 1993). The numbers of hemocytes vary in the crypt spaces (Nyholm and McFall-Ngai, 1998) and blood sinuses of the appendages, and if these cells are key participants for transducing the signal, variation in the response of host tissues to LPS may be related to the number of hemocytes that are sampling the crypt space during the period of incubation with exogenous LPS. Resolution of these issues will require further description of the mechanisms by which presentation of the LPS signal in the crypts elicits a response in the remote tissues of the surface epithelium.

Three lines of evidence suggested that lipid A, the evolutionarily conserved component of the LPS molecule, is responsible for inducing epithelial cell death in the squid light organ: (1) the cell death was not specific to *V. fischeri*, (2) purified lipid A from both *V. fischeri* and other bacterial species caused apoptosis, and (3) LPS purified from *H. influenzae* mutants that are defective in producing lipid A with a normal structure induced an attenuated response in the host in comparison with LPS derived from the parent, wild-type strain of *H. influenzae*. However, although the response to LPS and lipid A is nonspecific, the induction of apoptosis in the natural situation is specific to *V. fischeri*, because other gram-negative bacteria, while abundant in the ambient seawater, do not colonize the light organ crypts. Thus, only the growing culture of *V. fischeri* would present sufficient quantities of LPS to the squid crypt cells to induce widespread cell death.

Under our experimental conditions, purified *V. fischeri* lipid A, as well as its complete LPS, often showed lower levels of cell death than the LPS of other species. Because of the great variation in the animal responses, and the potential for effects on the LPS structure that result from the purification process itself, we believe that caution should be used in the interpretation of these results. However, evidence that the response of an animal to the LPS of its cooperative symbiont is attenuated in comparison with its response to LPS of known pathogens, or nonspecific gram-negative bacteria, would be interesting. Studies are under way to describe the structure of the *V. fischeri* LPS and its components for comparison with these molecules from other gram-negative bacteria. The results of these studies should shed light on whether there is a basis for an attenuated response to *V. fischeri* LPS and its derivatives.

Because lipid A has a structure that is conserved among gram-negative bacteria, it was possible to use mutants in lipid A production from other species (i.e., *H. influenzae* strain B-29) within the context of this study. However, creation of *V. fischeri* mutants defective in normal LPS production is under way and should provide a valuable tool for the study of *V. fischeri* LPS at all stages of light organ development. Recent examination of the *V. fischeri* genome has indicated that there are two *htrB* genes in *V. fischeri* (E. Stabb, pers. comm.). While this feature will make the generation of null mutants more difficult, its raises the intriguing possibility that *V. fischeri* populations use alternative forms of lipid A under the different environmental conditions that they encounter, such as in the light organ

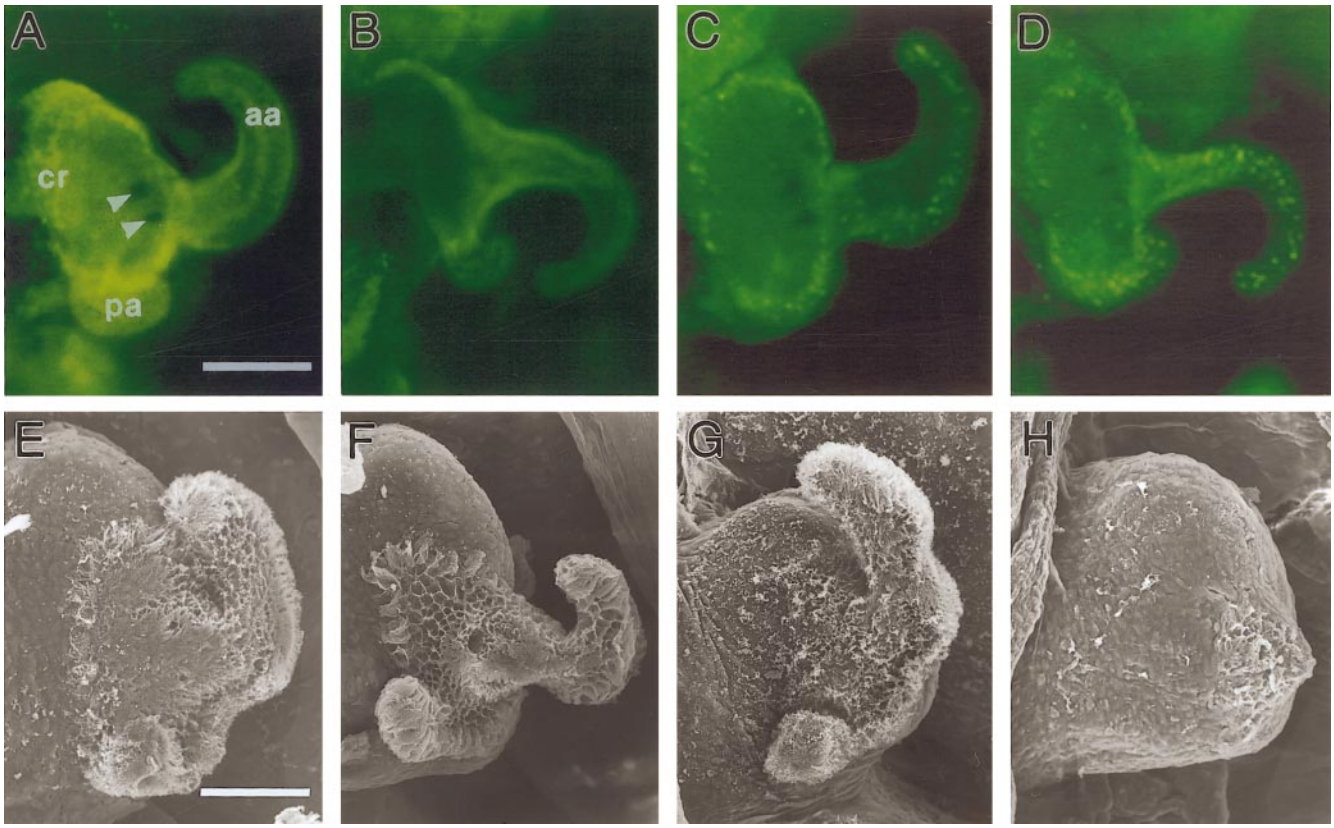


FIG. 7. The uncoupling of the timing of apoptosis and regression of the ciliated field on the light organ. (A–D) Ventral aspect of acridine orange-stained light organs (left half) examined 14 h after initial inoculation with *V. fischeri* for 0 (A), 3 (B), 9 (C), and 12 h (D) and then cured in 10 μg/ml chloramphenicol in seawater. Extensive cell death patterns are visible only in animals exposed to *V. fischeri* for 9 (C) and 12 h (D). aa, anterior appendage; pa, posterior appendage; cr, ciliated ridge; arrowheads indicate pores through which the bacteria enter the light organ. Bar, 100 μm. (E–H) Scanning electron micrographs representative of the ventral aspect of the light organ (left half shown) revealing the extent of ciliated epithelial field regression. Animals were exposed to *V. fischeri* for 0 (E), 3 (F), 9 (G), and 12 h (H) and then cured and subsequently maintained in 10 μg/ml chloramphenicol-treated seawater for 4 days. Only animals incubated for 12 h with symbiosis-competent bacteria exhibited complete morphogenesis of the light organ. Bar, 75 μm.

versus out in the water column, as free-living members of the bacterioplankton.

The finding that the LPS of the *htrB* mutants of *H. influenzae,* while not capable of triggering cell death after the usual interval, did eventually induce apoptosis is in keeping with experiments that compared the responses of animal models and cultured mammalian cells to wild-type *H. influenzae* and mutants of this species defective in the *htrB* gene (DeMaria *et al.,* 1997; Nichols *et al.,* 1997). These *H. influenzae* mutants are significantly less virulent by a variety of measures, including the ability to potentiate persistent infection (DeMaria *et al.,* 1997) as well as induce cytokine release (Nichols *et al.,* 1997). The *htrB* mutation results from the substitution of the wild-type hexaacyl lipid A with a mixture of two lipid A forms, 90% tetraacyl and 10% pentaacyl, through the induction of secondary acyltransferases (Lynn *et al.,* 1991). While tetraacyl lipid A is completely inactive (Golenbock *et al.,* 1991), the pentaacyl form is active (Sunshine *et al.,* 1997) and is likely to be the component that is responsible for the delayed induction of apoptosis in the light organ.

Our results with purified LPS and intact bacteria suggested that the LPS induces an irreversible program of cell death at about 9 h following initial exposure to LPS. One caveat that should be considered regarding the irreversibility of the LPS signal is that we cannot be sure that LPS is not stable in the light organ, i.e., remaining to exert its effects on the cells of the organ after the bacteria are antibiotically attenuated or exposure to LPS is discontinued. This caveat notwithstanding, the bacteria may be exerting more than one irreversible effect, the induction of apoptosis and the complete regression of the ciliated field, which is also irreversibly triggered (Doino and McFall-Ngai, 1995), although a few hours later at around 12 h. This difference in timing, as well as the inability of LPS priming to induce morphogenesis, strongly suggests the presence of

at least one additional signal. The second signal(s) may be one that acts to inhibit further cell proliferation in the superficial epithelium after successful colonization of the light organ by *V. fischeri.* Alternatively, the second signal may be an additional cell death signal. For example, other bacterial surface molecules, specifically lipoproteins and peptidoglycans, have been implicated in the induction of apoptosis in cultured animal cells (Aliprantis *et al.,* 1999; Brightbill *et al.,* 1999). Also, some products of fermentative metabolism in bacteria, such as butyric and propionic acid, have been shown to cause cell death in animal cells (Hague *et al.,* 1995; Kurita-Ochiai *et al.,* 1997), and there is some evidence that *V. fischeri,* a facultative anaerobe, produces organic acids (Ruby and Nealson, 1977).

In summary, the results of this paper suggest that the bacteria-induced developmental program of the squid symbiotic organ is not the response to a single, "silver-bullet" signal, but rather only one of perhaps many signals that control the dramatic morphogenesis of the tissues. Similar to the legume–rhizobia system, this program requires LPS. In the plant–bacterial symbioses, LPS is an important inducer of proper development at a number of stages in the program (Perotto *et al.,* 1994). Thus, in the squid–vibrio system, we may find that LPS not only operates to induce apoptosis of the superficial epithelium of the organ, but also is critical in earlier and later stages of development. In the larger context, the involvement of LPS in the induction of apoptosis in *E. scolopes* light organ morphogenesis suggests the intriguing possibility that these observed responses are mediated through Toll-like receptors that activate NF-κB and modify subsequent gene expression. Further studies of the squid–vibrio association should reveal the extent to which these systems share the mode of LPS presentation (i.e., the involvement of binding proteins) and subsequent signal transduction processes. If these well conserved pathways are involved in host morphogenesis, the *E. scolopes–V. fischeri* symbiosis offers the unusual opportunity to study the concomitant activity of these pathways both in developmental induction and in the response to interactions with bacteria.

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