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# Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis

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**Abstract.** A pure culture of the luminous bacterium *Vibrio fischeri* is maintained in the light-emitting organ of the sepiolid squid *Euprymna scolopes*. When the juvenile squid emerges from its egg it is symbiont-free and, because bioluminescence is part of an anti-predatory behavior, therefore must obtain a bacterial inoculum from the surrounding environment. We document here the kinetics of the process by which newly hatched juvenile squids become infected by symbiosis-competent *V. fischeri*. When placed in seawater containing as few as 240 colony-forming-units (CFU) per ml, the juvenile became detectably bioluminescent within a few hours. Colonization of the nascent light organ was initiated with as few as 1 to 10 bacteria, which rapidly began to grow at an exponential rate until they reached a population size of approximately $10^5$ cells by 12 h after the initial infection. Subsequently, the number of bacteria in the established symbiosis was maintained essentially constant by a combination of both a >20-fold reduction in bacterial growth rate, and an expulsion of excess bacteria into the surrounding seawater. While *V. fischeri* cells are normally flagellated and motile, these bacteria did not elaborate these appendages once the symbiosis was established; however, they quickly began to synthesize flagella when they were removed from the light organ environment. Thus, two important biological characteristics, growth rate and flagellation, were modulated during establishment of the association, perhaps as part of a coordinated series of symbiotic responses.

**Key words:** *Vibrio fischeri* – *Euprymna scolopes* – Symbiosis – Growth – Flagellation

A common characteristic of symbiotic associations between microorganisms and their animal or plant hosts is a coordinated regulation of both physiological processes and morphological structures. One of the most important adjustments that must be coordinated in non-pathogenic symbioses is the relative rates of growth of the two members of the association. In many cases this means that proliferation of the microorganism must be limited to a level well-below its actual potential (Smith and Douglas 1987). To maintain the number of symbionts in proportion to the host's size, animals harboring beneficial intracellular microorganisms often rely upon digestion or expulsion of a certain portion of a growing symbiont population (Colley and Trench 1985; Nardon and Grenier 1989; Fiala-Medioni et al. 1990). Conversely, in some extracellular symbioses, restriction of the growth of symbionts within the host's tissues is achieved as a result of the symbiont entering a severely growth-restricted, differentiated state (Smith and Douglas 1987). The basis of this latter process has been best studied in certain nitrogen-fixing *Rhizobium* spp., which differentiate into metabolically distinct cells called bacteroids soon after colonizing the induced root nodule of their plant hosts (Sutton et al. 1981; Vasse et al. 1990).

Bacteroids are also morphologically differentiated, expressing a distinct surface biochemistry, and repressing the synthesis of flagella (Vincent 1980; Long 1989). Similarly, in a number of both pathogenic and non-pathogenic associations between animals and motile bacteria, flagella are no longer elaborated by the symbionts after they have taken up residence within host cells or tissues (Tebo et al. 1979; Dunlap 1984; Felbeck and Distel 1991; S. Minnich personal communication). In at least some cases this repression is the result of modulation of a coordinated genetic regulon (Neidhardt 1987), such as that controlling both flagella synthesis and several virulence determinants in *Bordetella* and, perhaps, other pathogens (Akerley et al. 1992).

There are numerous non-pathogenic associations in which an animal host uses the unusual metabolic capabilities (such as bioluminescence, antibiotic production, cellulose degradation, or nitrogen fixation) of a single bacterial species maintained within a specialized tissue (e.g., Waterbury et al. 1983; Dunlap and McFall-Ngai 1987; Nealson et al. 1990). However, in contrast to the details of development either in animal pathogenesis or in plant root nodules, there is little known about the

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initiation and control of bacterial growth and differentiation in any beneficial animal symbioses. Such studies require a model system that allows the examinition of the initial events in development of the symbiosis.

The luminous bacterium *Vibrio fischeri* is the specific light organ symbiont of the sepiolid squid *Euprymna scolopes* (Boettcher and Ruby 1990). Upon hatching, each juvenile squid must obtain an inoculum of symbiosis-competent *V. fischeri*, which then colonizes the developing light organ and initiates the animal's ability to become bioluminescent (Wei and Young 1989; McFall-Ngai and Ruby 1991; Ruby and McFall-Ngai 1992). We report here the changing pattern of bacterial growth and luminescence, and the disappearance of flagellation, as physiological and morphological adjustments during the initiation and establishment of the association. These adaptations may reflect a coordinated differentiation of *V. fischeri* in response to conditions within the host's developing light organ.

## Materials and methods

### *Bacterial strains and growth media*

Symbiotic luminous bacteria were maintained on a seawater-based nutrient medium (SWT) containing (per liter) 5 g of Bacto-Tryptone (Difco Laboratories, Detroit, Mich., USA), 3 g of yeast extract (Difco), 3 ml of glycerol, 700 ml of filtered seawater, and 300 ml of distilled water. A minimal seawater medium (SWM) contained (per liter of 70% seawater) 3 ml of glycerol, 1 g of $NH_4Cl$, 58 mg of $KH_2PO_4$ and 50 ml of a 1 M Tris-HCl solution (pH 7.5). TLMS medium contained (per liter of tap water) 10 g of Bacto-Tryptone, 5 g of yeast extract, 10 g of NaCl and 200 mg of streptomycin. The pH was adjusted to 7.5 with a 1 M solution of KOH. Media were solidified when necessary by the addition (per liter) of 14 g of Bacto-Agar (Difco).

### *Animal collection and bacterial isolation*

Adult specimens of *Euprymna scolopes* were collected in Kane'ohe Bay, O'ahu, Hawaii, and commercially caught *E. morsei* were obtained in Hiroshima, Japan. Animals were either maintained live in aquaria before use, or stored frozen at −70 °C. Symbiotic bacteria were released by homogenizing a dissected light organ in a small volume of either SWT or autoclaved seawater as previously described (Boettcher and Ruby 1990). The suspension was vigorously vortexed and aliquots taken either for experiments, for dilution plating on SWT agar medium, or for microscopy. Estimations of bacterial abundances in cell suspensions either by direct counts of cells stained with acridine orange (Hobbie et al. 1977), or by enumeration of colony-forming-units (CFU) on SWT agar medium, were comparable (see Fig. 4, and Boettcher and Ruby 1990). Other experiments showed that the extent of viability and patterns of physiological responses of bacteria obtained either from live or from frozen animal tissue were indistinguishable.

Bacteria obtained from the light organs of *E. morsei* were subjected to a series of taxonomic tests as previously described (Boettcher and Ruby 1990). All 50 strains isolated from 15 specimens of this species of squid were unambiguously identified as *V. fischeri*, consistant with early observations (Kishitani 1928). Interestingly, all of the isolates produced a strongly visible luminescence in culture, a characteristic not shared with the symbionts of *E. scolopes*, which are not visibly luminous on laboratory media (Boettcher and Ruby 1990).

### *Initiation of the symbiosis in juvenile E. scolopes*

Symbiotic infections were generally initiated using *Vibrio fischeri* strain ES114, an isolate from the light organ of a specimen of *E. scolopes* (Boettcher and Ruby 1990). The bacteria were grown to mid-log phase in SWM and diluted to approximately $10^3$ CFU per ml in California coastal seawater before addition of juvenile squids (McFall-Ngai and Ruby 1991). Axenic animals were taken within 12 h of hatching and placed in California coastal seawater to which had been added a known concentration of strain ES114. After an exposure of either 2 or 3 h at 25 °C, the animals were rinsed by serial passage through, and finally placed in, uninoculated seawater maintained at 25 °C. California coastal seawater does not naturally contain sufficient symbiosis-competent *V. fischeri* to initiate an infection of axenic squids (McFall-Ngai and Ruby 1991; Lee and Ruby 1992).

To determine the total symbiotic CFU present in the light organ of a juvenile squid the animal was rinsed 4 times in sterilized seawater and the entire body homogenized for dilution plating. When the juvenile light organ was dissected away from the rest of the body, and both the light organ and the remaining body tissue were homogenizied and plated separately, CFU were detected only in the light organ homogenate, and this homogenate contained the same total CFUs as the whole animal homogenate (data not shown).

### *Light measurement*

Light emission of cell suspensions, recorded as quanta per second, was measured by using a model 110 laboratory photometer (Pacific Instruments, Concord, Calif., USA) calibrated with the light standard of Hastings and Weber (1963). Low levels of light emission were detected by using a model 2000 photometer (Lab-Line Instruments, Melrose Park, Ill., USA).

### *Expulsion of symbionts from infected juvenile E. scolopes*

(Experiment I) Three pairs of juvenile squids were exposed to seawater containing *V. fischeri* strain ES114 to initiate a symbiotic infection as described above. After a 3 h exposure, the animals were rinsed well and each pair placed in 20 ml of seawater that had been filter-sterilized by passage through a 0.2-μm-pore-sized membrane filter (Millipore, New Bradford, Mass., USA). The animals were removed from the seawater every 3 to 6 h and transferred to a fresh 20 ml volume of sterile seawater. The spent seawater was vortexed and aliquots immediately filtered through a 0.45-μm-pore-sized, 45 mm diameter, membrane filter, which was then placed on SWT agar medium. Colonies with the pigmentation and morphology of *V. fischeri* were enumerated after 24 h incubation at 30 °C. Confirmation of their identity was obtained by colony hybridization with a *V. fischeri*-specific DNA probe (Lee and Ruby 1992). Additional incubation and examination of the spent seawater suggested that the expelled symbionts did not undergo significant post-release proliferation.

To assure that conducting the experiment in sterile seawater did not introduce a significant artifact, the symbiosis was initiated in a second experiment (Experiment II) by exposing squids instead to a streptomycin-resistant mutant of *V. fischeri* (strain ES114S) for 3 h. These animals were then rinsed and placed in natural seawater, and the experiment carried out as described above with the following exception: the membrane filters were placed on TLMS agar medium to allow only the streptomycin-resistant cells in the seawater (almost exclusively expelled ES114S) to develop into colonies.

### *Development of motility by released symbionts*

Symbiotic *V. fischeri* were released from adult *E. scolopes* light organs by homogenization as described above. These cells were diluted into either SWM or SWT media containing the following additions (per ml): glucose (1 mg), cAMP (3.6 μg), synthetic *V. fischeri* autoinducer (0.1 μg), or ethylenediamine-di(*o*-hydroxy-

phenyl acetic acid), an iron chelator (36 ng). Alternatively the released cells were spread on SWM or SWT agar media to form colonies on a solid surface. The cells in the suspensions and colonies were observed for motility by phase-contrast microscopy after incubation at 25 °C for 3 and 24 h.

### *Visualization of flagella by electron microscopy*

Droplets of bacterial suspensions were placed on Formvar-coated carbon grids (Ted Pella Company, Tustin, Calif., USA), stained for 15 s with a 1% uranyl acetate solution, washed twice with water, and air-dried. The specimens were then examined on a JEOL transmission electron microscope at a magnification of 20000×.

## Results

### *Growth kinetics during symbiotic infection*

After exposing an axenic juvenile squid for 2 h to seawater containing an inoculum of symbiosis-competent *Vibrio fischeri*, the number of bacteria present within the juvenile light organ was found to increase at roughly an exponential rate for several hours (Fig. 1). In spite of the variation between individual animals, it is evident that the infection had begun within 3 h, and was apparently initiated by an inoculum of between 1 and 10 bacteria. Within 12 h the light organ contained approximately $10^5$ cells, indicating an exponential growth rate with a generation time ($t_{gen}$) of approximately 30 min. Similar results have been obtained with two other strains of symbiosis-competent *V. fischeri* (K.-H. Lee, personal communication).

Using the onset of luminescence as another indication of the time course of colonization (McFall-Ngai and Ruby 1991), we found that the kinetics of light organ development were similar over a wide range of bacterial concentrations in the inoculating seawater (Fig. 2). Placing juvenile squids either in California coastal seawater,

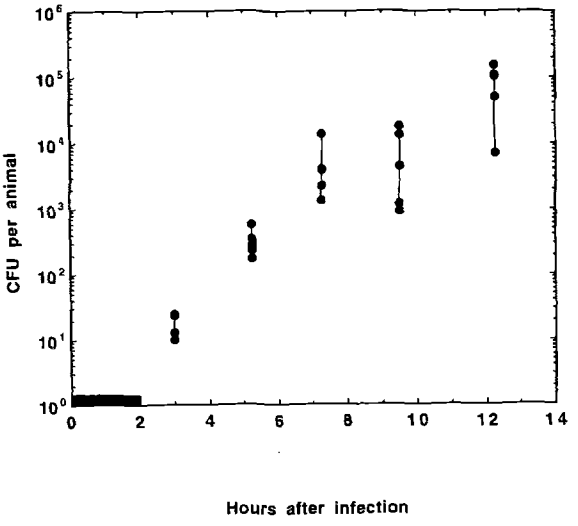


**Fig. 1.** Early progress of the symbiotic infection of the juvenile *E. scolopes* light organ by *V. fischeri* ES114. Infection was initiated by placing animals in seawater inoculated with symbiosis-competent bacteria for a period of 2 h *(horizontal black bar)*. At several subsequent time points the total number of symbiont CFU (●) in light organ homogenates of five individual squids was determined

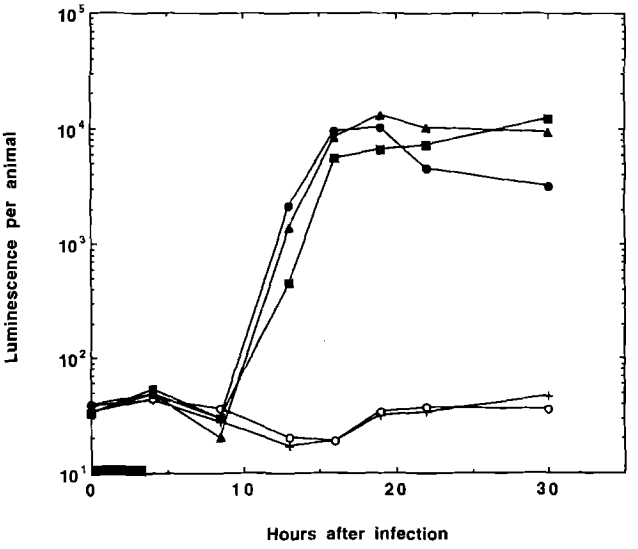


**Fig. 2.** Expression of symbiotic luminescence by juvenile *E. scolopes*. Newly hatched animals were placed in symbiont-free seawater (+), or seawater inoculated with (per ml) 24 (○), 240 (●), 2400 (■), or 24000 (▲) CFU of symbiosis-competent bacteria for a period of 3 h *(black bar)*. Luminescence (in quanta per s) of the squids was monitored periodically as an indication of the development of the symbiosis. Essentially identical results were obtained in two other repetitions of this experiment

or in such seawater to which was added only 24 CFU of strain ES114 per ml, did not lead to a symbiotic infection under the assay conditions described. However, a maximal rate of infection occurred when animals were exposed to California coastal seawater to which had been added between 240 and 24000 CFU of ES114 per ml. Thus, the infection process apparently was not enhanced by the addition of as much as a hundred-fold excess of symbiotic bacteria in the inoculating seawater. These rapid colonization rates were indistinguishable from the rate observed when juveniles were placed in Hawaiian seawater from the squid's habitat (McFall-Ngai and Ruby 1991), which naturally contains symbiosis-competent bacteria (Lee and Ruby 1992).

The initial rapid burst of colonization of the light organ was followed by the maintenance, without a significant net increase, of approximately 1 to $2\times10^5$ symbionts per host animal for at least 80 h (Fig. 3). This period coincided with an apparent plateau in the level of symbiotic luminescence (Fig. 2). Examination of electron micrographs of thin sections of the light organs of both juvenile and adult animals revealed no evidence of digestion or lysis of symbiotic bacteria within the squid tissue (M. Montgomery, personal communication). Instead, the infecting strain of bacterium eventually reappears in seawater containing juvenile squids, suggesting that after the light organ is fully colonized, it becomes a significant source of released symbiont cells (Table 1). Thus, expulsion of excess symbionts at least partially accounted for the absence of an uncontrolled increase in the bacterial population. Based upon the number of cells expelled over a 24-h period, and the population's steady-state level within the juvenile light organ (in this experiment, between 1.6 and $2.2\times10^5$ CFU over the period

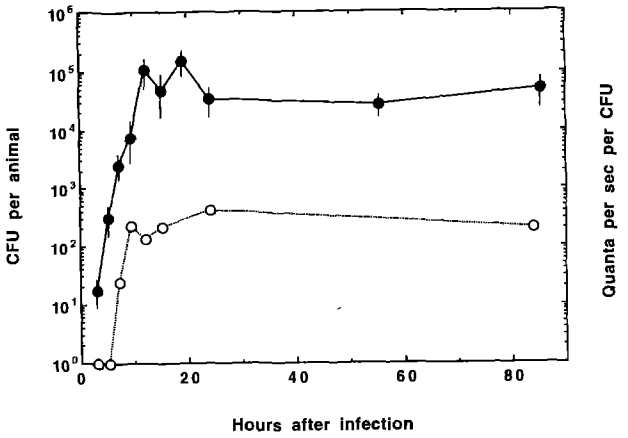


**Fig. 3.** Kinetics of the establishment of *E. scolopes* light organ symbiosis. The total number of symbiont CFU (●) in light organ homogenates of squids was determined as described in Fig. 1. *Error bars* indicate ±1 SD. The specific activity of light emission (○) by the bacteria in the symbiosis was calculated from measurements of squid luminescence

**Table 1.** Estimation of symbiont growth rate from the rate of expulsion of light organ bacteria during initiation and establishment of the association

| Experiment[a] | Time interval after infection (h) | No. of *V. fischeri* appearing in water ($\times 10^5$) | Generation time in hours ($t_{gen}$) |
|---|---|---|---|
| I | 3–12 | 0.002 | 0.5[b] |
| | 24–48 | 7.6 ± 4.6 | 10 ± 2[c] |
| II | 24–48 | 2.7 ± 1.1 | 18 ± 4[c] |
| | 42–66 | 3.6 ± 1.1 | 17 ± 3[d] |

[a] Experiment I used *E. scolopes* infected with *V. fischeri* ES114, and maintained in filter-sterilized seawater; Experiment II used *E. scolopes* infected with *V. fischeri* ES114S, and maintained in natural seawater (see Materials and methods). Values are the means of 3 replicates ±1 SD

[b] Estimated from data in Fig. 3

[c] Calculated based upon a constant population density of $1.6 \times 10^5$ cells per organ in this experiment during the 24 to 48 h interval after infection

[d] Calculated based upon a constant population density of $2.2 \times 10^5$ cells per organ in this experiment during the 42 to 66 h interval after infection

examined), an average $t_{gen}$ of between 10 and 18 h can be estimated for symbionts during the 24 to 66 h period following establishment of the symbiotic population. Thus, the maintenance of a constant level of bacterial colonization in the juvenile squid apparently results from the combination of a significantly restricted proliferation of the symbionts, in conjunction with the continued expulsion of a portion of the symbiont population, However, as a squid grows in size over a period of several months, the symbiont population within its light organ does increase, as a linear function of the animal's length (Fig. 4), to between $10^7$ and $10^8$ cells in a sexually mature adult.

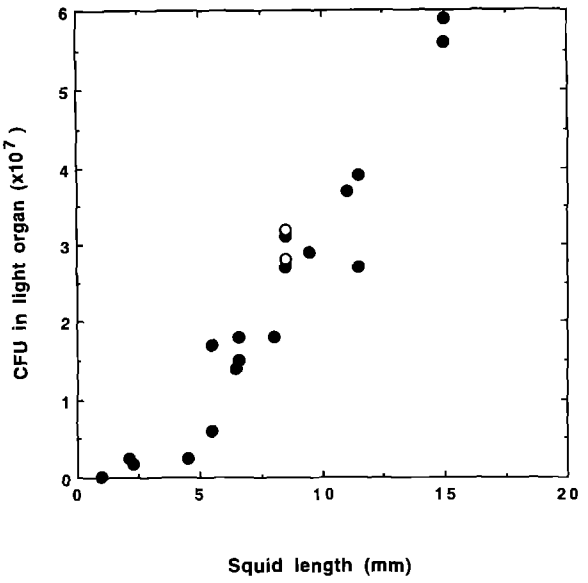


**Fig. 4.** The relationship between an *E. scolopes* specimen's mantle length and the population size of its symbiotic bacteria. The number of bacteria in the light organs of a size range of animals were determined by either the total CFU in (●) or direct microscopic counts of (○) light organ homogenates

*Luminescence induction during symbiotic infection*

During the first few hours of their proliferation in the light organ the luminescence of the infecting bacteria increased approximately 250-fold, reaching a specific activity of about 200 quanta per s per CFU by 9 h (Fig. 3). This level of luminescence is about 25% that reported for bacteria released from adult squids (Boettcher and Ruby 1990), and appears to remain generally constant during the first few days of infection. Because an increase in luminescence specific activity was first detected within 7 h, when less than 5000 bacteria inhabit the nascent light organ, the induction appeared to be triggered during the period of initial rapid colonization, well before the establishment of the symbiotic population.

*Size and motility during symbiotic infection*

Laboratory-cultured cells of *V. fischeri*, such as those used to initiate the symbiosis, are relatively large cells that possess a characteristic tuft of polar, sheathed flagella (Allen and Baumann 1971) (Fig. 5A). Electron microscopic examination of preparations of *V. fischeri* cells released from the adult squid light organ revealed that the symbionts are by comparison small and non-flagellated (Fig. 5B). This latter cell morphology was not characteristic of light organ symbionts during the initial 12 h of rapid colonization but, within 24 h of the establishment of the juvenile light organ association, >95% of the population had become small and non-flagellated. In an attempt to determine culture conditions that result in the inhibition of flagella elaboration, isolated symbionts were grown in the laboratory under environmental

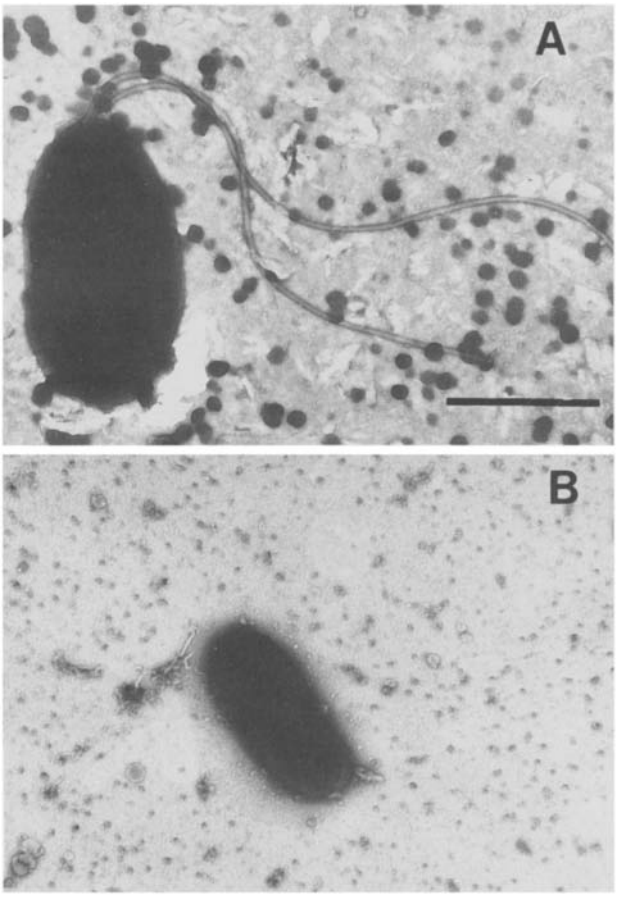


**Fig. 5A, B.** Electron-microscopic images of *V. fischeri* cells either grown in SWT medium for inoculation of juvenile *E. scolopes* light organs **A**, or immediately after release from an adult light organ **B**. Three hours after release and incubation in SWT medium, the bacteria from **(B)** are morphologically indistinguishable from those depicted in **(A)**. *Size bar* is 1 μm in length and applies to both panels

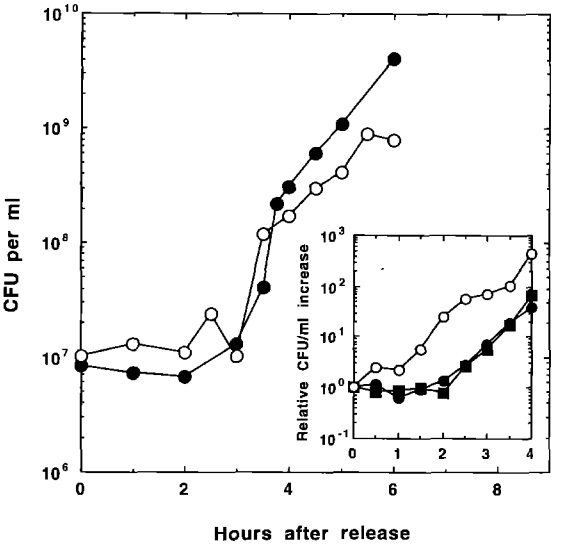


**Fig. 6.** Proliferation of symbiotic bacteria after release from the light organ. Bacteria from homogenates of either adult. *E. scolopes* (●) or *E. morsei* (○) light organs were suspended in SWT medium and monitored for the initiation of cell number increase. The same 3-h lag in the onset of proliferation seen here was observed when bacteria were suspended instead in SWM medium. *Inset*: Appearance of the lag effect in symbiotic bacteria as a function of the establishment of the association in juvenile squids. Bacteria were released from host light organs at 6 h (○), 12 h (●), or 24 h (■) after initation of the symbiosis. The cells were suspended in SWT medium and monitored for the onset of proliferation. Because there are different total numbers of bacteria in the light organ at the three release times (Fig. 3), the increase in cells is graphed relative to the initial cell concentration to emphasize the absence of a lag in cells released at 6 h

conditions that are known to effect gene expression in *Vibrio* spp. (see Materials and methods). The preponderance of motile cells in these cultures (as determined by phase-contrast microscopy) revealed that none of the growth conditions examined resulted in a significant decrease in the number of cells bearing functional flagella (data not shown).

### *Response of released symbionts*

The greatly reduced rate of proliferation in the post-12 h-infected light organ (Table 1) suggests that the symbionts are in a growth-restricted (or nutrient-limited) condition. When symbionts were released from adults of either *E. scolopes*, or the related *E. morsei*, and suspended in a rich nutrient medium, a 3-h lag was observed before the number of cells in the suspensions began to increase logarithmically at a rate typical for *V. fischeri* under these culture conditions (Fig. 6). Symbiotic bacteria released from 12-h or 24-h associations with juvenile squids also exhibited the lag; however, bacteria released at 6 h after initiation of the association began proliferating immediately after release (Fig. 6 inset). Observation of the released bacteria under phase-contrast microscopy demonstrated that the establishment of the motile, flagellated condition is achieved within 45 to 60 min of release and suspension either in SWT or, surprisingly, in natural (unsupplemented) seawater. Thus, initiation of flagella elaboration can occur even in non-growing cells.

## Discussion

The host tissues of an animal that requires a symbiotic colonization as part of its normal life cycle must be able to encourage the initiation of the association and selectively permit the proliferation of a specific bacterial species. During the remainder of its life span the ability of the host to control and restrict the net growth of the symbiont population becomes critical not only because a rapidly growing microbial infection is metabolically costly, but also because there is the potential threat that an opportunistic pathogenesis will arise through the spread of the bacterium to other, inappropriate tissues of the host.

The *V. fischeri* – *E. scolopes* association can be initiated by as few as 1 to 10 symbiosis-competent bacteria. Under laboratory conditions, concentrations of *V. fischeri* in excess of about 200 cells per ml of seawater are sufficient to bring about an infection after only a few hours of exposure. Interestingly, this bacterial concentra-

tion is at least two orders of magnitude greater than that reported for the natural seawater in which the animals are typically found (Lee and Ruby 1992). These facts suggests that: 1) the juvenile usually becomes infected in an environment with a higher *V. fischeri* concentration than has yet been reported; and/or, 2) the infectivity of naturally occurring *V. fischeri* is significantly greater than that of bacteria cultured under laboratory conditions, a phenomenon often observed among pathogenic bacteria (Sokal and Woods 1984; Wei et al. 1992). The validity of these possibilities is currently under investigation.

Initiation of the symbiotic association consists of two distinct phases of bacterial growth: an initial, very rapid proliferation ($t_{gen}$ = 0.5 h) that lasts for about 12 h, and results in the colonization of all or most of the available lumenal space in the light organ rudiment (McFall-Ngai and Ruby 1991; Montgomery and McFall-Ngai 1991); and a second, longer-term period of restricted growth at a significantly limited rate (average $t_{gen}$ = 10 to 18 h). The kinetics of the initial phase indicate both that the infecting bacteria undergo at most a 1 to 2 h lag period before initiating growth, and that the environment of the nascent light organ lacunae is conducive to remarkably rapid (and thus apparently aerobic and nutrient-sufficient) proliferation. The manner by which the animal tissue restricts growth in the second phase of symbiosis establishment is as yet unknown, but may simply be due to the eventual limitation in the rate of supply of nutrients and oxygen, a common circumstance of bacterial growth at high cell density in animal tissues (Matin et al. 1989).

In any case, the result is a controlled infection, whose slowly-growing, established bacterial population can be expected to place less of a nutrient demand upon the host then do the rapidly-growing cells that initiate the infection. The strategy of coupling growth rate restriction of light organ bacteria with the expulsion of symbionts has been previously reported for established symbioses in adults of several luminous fish species (Haygood et al. 1984). Similarly, in *E. scolopes*, coordinating the bacterial population size with the animal's growing organ size, even at their restricted growth rate, relies heavily upon the expulsion of excess bacteria, presumably by way of the lacunal pores (McFall-Ngai and Ruby 1991). Such expulsion will also lead to the introduction of significant numbers of symbiosis-competent *V. fischeri* into the ambient seawater, and may be an important part of the host's life cycle, and the bacterium's ecology (Lee and Ruby 1992).

The induction of light emission by symbiotic bacteria after entry into and growth within a light organ has been postulated (Nealson 1977), but the kinetics of this process has not previously been demonstrated. During their initial infection of *E. scolopes*, the bacteria rapidly increase the specific activity of light production by at least two orders of magnitude (Fig. 3). The factor(s) bringing about luminescence induction probably include the build-up of the transcriptional regulator autoinducer (Kaplan and Greenberg 1985) in the restricted volume of the organ (Boettcher and Ruby 1990). The effect becomes evident at a time when the bacterial concentration is approximately 3000 CFU in 300 pl, a lacunal volume estimated from micrographic, three-dimensional reconstructions of the juvenile organ (Montgomery and McFall-Ngai 1991). Laboratory experiments (Gray and Greenberg 1992) suggest that this effective concentration (about $10^{10}$ CFU per ml) should be sufficient to bring about induction, even in these autoinducer-underproducing symbiotic strains (Boettcher and Ruby 1990).

Symbiotic *V. fischeri* from adults of two species of *Euprymna* are not capable of proliferation for between 2 and 3 h after release from the light organ environment (Fig. 6). Bacteria initiating the symbiosis in juvenile light organs enter this state only after the symbiotic association is 12 to 24 h old, at about the time that growth restriction is becoming apparent in the light organ. This lag in the ability of established symbionts to proliferate is reminiscent of a recovery from a starvation condition (Amy et al. 1983; Baker et al. 1983). An analogous response has been reported for *Vibrio* strain S14 as a result of simple nutrient deprivation (Malmcrona-Friberg et al. 1990). Interestingly, starvation can activate stress-induced regulons that lead to cellular differentiation (Jenkins et al. 1991). In addition, some of the genes induced by starvation (e.g., *gro*EL) are coordinately regulated by, and required for, the association of bacterial pathogens and mutualists with their animal hosts (Buchmeier and Heffron 1990; Ohtaka et al. 1992). Perhaps, in the light organ, conditions of high autoinducer concentration (Boettcher and Ruby 1990) and a starvation-induced activation of *gro*EL result in the full induction of bioluminescence by established symbionts (Dolan and Greenberg 1992).

During the second (post-12-h) phase of infection flagella (and thus motility) are no longer apparent in a significant portion of the bacterial population. This phenomenon is not uncommon among bacteria that are tightly packed in tissues or vacuoles. The lack of flagellation may result from the repression of the synthesis of the appendage in progeny cells during colonization of the target tissue. Such a response has been reported as part of a coordinately regulated program exhibited by some animal pathogens (Akerley et al. 1992). Whatever factors are responsible for flagella repression in symbiotic *V. fischeri*, this effect is lost quickly: within an hour of release from the organ, and dilution in either nutrient medium or seawater, functional flagella become apparent. As yet, however, such a repression of flagella elaboration can not be mimicked in laboratory culture, even in the presence of conditions or chemicals that are known to affect gene expression in these and other symbiotic bacteria (Yokota and Gots 1970; Belas et al. 1986; McCarter and Silverman 1987, 1989; Dunlap 1991).

Unlike the induction of luminescence, the repression of flagella elaboration does not occur early in the association of bacteria with the host tissues. Instead, the absence of motility becomes evident only between 12 and 48 h after initiation of the infection, a period during which growth rate modulation is also occurring. It is too early to say whether or not these two features that accompany the transformation of colonizing bacteria into established symbionts are under a regulated system

of gene expression. However, coordinated control of luminescence and other physiological characteristics has been reported in terrestrial luminous bacteria (Nealson and Hastings 1991), and the presence of animal tissue often induces such regulated responses in pathogenic bacteria (Mekalanos 1992). At this point, the question of a symbiosis-induced differentiation by *V. fischeri* remains open; however, it is a particularly intriguing possibility in light of evidence that it is the onset of bacterial infection that in turn triggers differentiation of certain tissues of the juvenile squid (McFall-Ngai and Montgomery 1990; McFall-Ngai and Ruby 1991; Ruby and McFall-Ngai 1992).

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