APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1995, p. 278–283 Vol. 61, No. 1
0099-2240/95/$04.00+0

Symbiotic Role of the Viable but Nonculturable State of *Vibrio fischeri* in Hawaiian Coastal Seawater†

KYU-HO LEE‡ AND EDWARD G. RUBY*

Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-0371

Received 25 July 1994/Accepted 25 October 1994

To achieve functional bioluminescence, the developing light organ of newly hatched juveniles of the Hawaiian squid *Euprymna scolopes* must become colonized by luminous, symbiosis-competent *Vibrio fischeri* present in the ambient seawater. This benign infection occurs rapidly in animals placed in seawater from the host's natural habitat. Therefore, it was surprising that colony hybridization studies with a *V. fischeri*-specific *luxA* gene probe indicated the presence of only about 2 CFU of *V. fischeri* per ml of this infective seawater. To examine this paradox, we estimated the total concentration of *V. fischeri* cells present in seawater from the host's habitat in two additional ways. In the first approach, the total bacterial assemblage in samples of seawater was collected on polycarbonate membrane filters and used as a source of both a crude cell lysate and purified DNA. These preparations were then assayed by quantitative DNA-DNA hybridization with the *luxA* gene probe. The results suggested the presence of between 200 and 400 cells of *V. fischeri* per ml of natural seawater, a concentration more than 100 times that revealed by colony hybridization. In the second approach, we amplified *V. fischeri*-specific *luxA* sequences from microliter volumes of natural seawater by PCR. Most-probable-number analyses of the frequency of positive PCR results from cell lysates in these small volumes gave an estimate of the concentration of *V. fischeri luxA* gene targets of between 130 and 1,680 copies per ml. From these measurements, we conclude that in their natural seawater environment, the majority of *V. fischeri* cells become nonculturable while remaining viable and symbiotically infective. Experimental studies indicated that *V. fischeri* cells suspended in natural Hawaiian seawater enter such a state within a few days.

The sepiolid squid, *Euprymna scolopes*, has a light-emitting organ containing tens of millions of cells of the bioluminescent bacterium *Vibrio fischeri* (4, 34). This group of symbiotic *V. fischeri* organisms, which are non-visibly luminous (NVL) in laboratory culture (4), is present in the ambient seawater of *E. scolopes* in part because of the daily expulsion of excess symbionts from colonized light organs (3, 24). These planktonic bacteria play an essential role in the life cycle of the squid by initiating the colonization of the aposymbiotic (bacterium-free) nascent light organ of each generation of newly hatched juvenile squid (27, 39).

The symbiotic association is produced when juvenile squid are exposed to seawater from their native Hawaiian habitat (27) in which the abundance of *V. fischeri* (determined by a colony hybridization technique involving specific *luxA* and *luxR* gene probes) averages about 2 CFU/ml (22, 24). Surprisingly, then, to colonize aposymbiotic *E. scolopes* juveniles with 100% success in the laboratory, a minimum of about 240 CFU of *V. fischeri* per ml was required (34). This 2-orders-of-magnitude difference gives rise to the following hypotheses: (i) that naturally occurring cells of *V. fischeri* are vastly more effective at colonizing the squid, or (ii) that the number of culturable *V. fischeri* (i.e., CFU) in Hawaiian seawater is a significant underestimation of the actual abundance of viable cells of this bacterium.

The latter of these two possibilities is suggested by the fact that several pathogenic *Vibrio* species (e.g., *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae*) have been reported to enter a dormant, or "viable but nonculturable," state when either in the natural environment or under stressed conditions (7, 8, 29), although the mechanism or impetus underlying this physiological adaptation is not yet clear. We postulated that Hawaiian seawater may contain viable, and symbiotically infective, *V. fischeri* cells that have lost the ability to form colonies on the typical isolation media. Therefore, we have estimated the actual number of symbionts in natural seawater samples by two independent molecular techniques that did not require the cells to grow and thus would detect and differentiate culturable, nonculturable but viable, and nonculturable and nonviable cells. Our results indicate that in seawater containing populations of *E. scolopes*, *V. fischeri* cells in the nonculturable state greatly outnumber culturable cells and that although nonculturable, the majority of these cells are both viable and capable of initiating a symbiosis with the squid host.

MATERIALS AND METHODS

Host animals and bacterial strains. Male and female squid collected from Kaneohe Bay, Hawaii, were placed together in aquaria to mate and lay eggs. Some of these eggs were transported to the University of Southern California for certain experiments. The nascent light organs of newly hatched juvenile squid become colonized if the animals are placed either in Kaneohe Bay seawater or in aquarium water in which adult animals have been maintained (24). In contrast, the light organs remain uncolonized if the animals are maintained in seawater that contains undetectable numbers of symbiotically competent *V. fischeri* (e.g., seawater collected either offshore from Kaneohe Bay, or near Catalina Island, Calif.) (22). Addition of laboratory-cultured, symbiotically competent *V. fischeri* cells to such seawater will lead to the colonization of the squid light organs (27).

Most of the studies reported here involved *V. fischeri* ES114, a symbiotic strain obtained from the light organ of an *E. scolopes* squid (4). *V. fischeri* cells were routinely grown on a nutrient-rich medium (SWT medium) consisting of 0.5% tryptone, 0.3% yeast extract, and 0.3% glycerol in 70% seawater, and solidified with 1.2% agar when desired (28). Strain ESR1, a spontaneous rifampin-resistant derivative of ES114, is essentially indistinguishable from its parent in both

* Corresponding author. Mailing address: Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-0371. Phone: (213) 740-5803. Fax: (213) 740-8123. Electronic mail address: eruby@molbio.usc.edu.

† Journal paper no. 961 of the Hawaii Institute of Marine Biology.

‡ Present address: Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305-5402.

TABLE 1. Abundance of CFU of VL bacteria and NVL *V. fischeri* in coastal seawater samples

Location (date)	Abundance of:				Infectivity[e]
	Total VL bacteria[a]	VL *V. fischeri*[b]	Probe-positive (total *V. fischeri*)[c]	NVL *V. fischeri*[d]	
Southern California (6/17/89)[f]	2.2	1.65	1.45	0	No
Massachusetts (9/14/92)[f]	25.6	21.5	19.3	0	ND[g]
Site 1 (1990 through 1991)	0.33	0.01	2.1	2.09	Yes
Site 7 (1990 through 1992)	0.10	<0.03	0.032	≤0.032	No

[a] The concentration of VL bacterial colonies was determined by observation in the dark.
[b] The VL *V. fischeri* CFU was determined by colony morphology and nutrient requirements (22).
[c] The total *V. fischeri* CFU was determined by colony hybridization with a *V. fischeri*-specific *luxA* DNA probe.
[d] Calculated as the value in the third column of numbers minus the value in the second column of numbers. This difference was an estimate of the number of the total NVL *V. fischeri* CFU.
[e] Determined as whether exposure of juvenile *E. scolopes* to this seawater led to a colonization of their nascent light organs (see Materials and Methods).
[f] Dates are given as month/day/year.
[g] ND, not determined.

growth rate and light organ infectivity (12). However, ESR1 cells are useful because, even when introduced at very low cell concentrations, their abundance in natural seawater could be easily monitored against the background of indigenous bacteria by plating on selective, rifampin-containing (50 μg/ml) SWT medium.

Decrease in the number of *V. fischeri* CFU during incubation in seawater. Freshly grown ESR1 cells were collected by centrifugation and suspended in seawater that had been sterilized by passage through a 0.2-μm-pore-size polycarbonate membrane filter (Nuclepore Corp., Pleasanton, Calif.). A small volume of this cell suspension was used to inoculate 500 ml of freshly collected (unfiltered) Kaneohe Bay seawater from an offshore location previously designated as site 7 (24) to produce a final concentration of about 3×10^3 ESR1 CFU/ml. At intervals during the subsequent 3-day incubation, diluted samples of the suspension were plated on SWT agar medium, and the number of ES114 CFU that arose was determined by the colony hybridization technique with a *V. fischeri*-specific *luxA* DNA probe as described previously (22). Subsequent changes in the CFU per milliliter value of both ESR1 and the indigenous luminous bacterial population in the sample were monitored by passing between 1 and 100 ml of this seawater through 0.22-μm-pore-size cellulose nitrate filters (Millipore Corp., Bedford, Mass.) and incubating the filters on the surface of either SWT agar medium or SWT agar medium containing rifampin (22).

Infection experiments. Newly hatched (aposymbiotic) juvenile *E. scolopes* squid were exposed to a suspension of cultured *V. fischeri* ES114 cells at a concentration of between 0 and 5×10^3 CFU/ml of filter-sterilized seawater. After 24 h, successful colonization of individual squid light organs was revealed both by the onset of bioluminescence by the animal and by the presence of about 10^5 *V. fischeri* CFU in a homogenate of the light organ (27, 34). Similarly, aposymbiotic juvenile squid were incubated with natural seawater collected from both within Kaneohe Bay (site 1) and offshore from Kaneohe Bay (site 7 [24]), as well as from Catalina Island. In some experiments this seawater was supplemented with suspensions of *V. fischeri* cells.

Seawater from within Kaneohe Bay, which naturally contained about 2.0 symbiotic *V. fischeri* CFU/ml (22), was serially diluted with filter-sterilized seawater. Dilutions were made to produce seawater that contained 2 (i.e., undiluted), 1, 0.5, 0.2, 0.1, 0.02, and 0 (0.2 μm-filtered seawater alone) *V. fischeri* CFU/ml. Between 4 and 12 juvenile squid were placed in each of these inocula to test its effectiveness at initiating a successful colonization of light organs.

To simultaneously monitor both the rate of decrease in CFU and the maintenance of symbiotic infectivity during incubation in seawater, we inoculated ESR1 cells into 10 liters of seawater collected either from Hawaii (site 7) or from Catalina Island, to result in a concentration of approximately 10^3 CFU/ml, and incubated these suspensions at ambient temperature (23 to 25°C) without shaking. Periodically during a 3- to 5-day incubation, a portion of this seawater was taken and (i) checked for its ability to initiate symbiosis in aposymbiotic squid and (ii) plated on rifampin-containing SWT agar medium to determine the concentration of ESR1 CFU per milliliter.

DNA-DNA hybridization. Water samples were collected from Kaneohe Bay site 1 and site 7 on 18 February 1992. The natural microbial assemblages in these samples were harvested by filter concentration as follows: between 1 and 10 liters of water sample was passed through a 45-mm-diameter, 0.2-μm-pore-size polycarbonate membrane filter (Nuclepore Corp.) under a mild vacuum. The cells retained on the surface of the filter were resuspended in 10 ml of filter-sterilized seawater by vortexing. The resulting cell suspension was centrifuged at $13,000 \times g$ for 5 min, the supernatant was discarded, and the cell pellet was resuspended in enough filter-sterilized seawater to make a 1,000-fold cell concentrate (i.e., 1 to 10 ml final volume).

Serial dilutions of the cell concentrates, each equivalent to the bacterial assemblage in 50 to 600 ml of seawater, were spotted onto a nylon membrane (Schleicher & Schuell, Keene, N.H.) along with dilutions of laboratory cultures containing known numbers of *V. fischeri* cells. These natural samples and standards were then processed for dot-blot hybridization with a *V. fischeri*-specific *luxA* DNA probe under high-stringency washing conditions, as described previously (22). The total number of *V. fischeri* cells in the seawater samples was then estimated by a comparison between the degree of hybridization of sample and standard cell preparations. These estimates assumed that on average, each *V. fischeri* cell in the seawater samples contained one copy of the *luxA* gene.

PCR. A second method for determining the presence of *V. fischeri* cells in a seawater sample involved is the use of PCR to identify the presence of the *V. fischeri luxA* gene. The *luxA*-specific PCR primers (forward, 5′GTTCTTAGTT GGATTATTGG3′ [designed by C. Wimpee]; and reverse, 5′TCAGTTCCAT TAGCTTCAAATCC3′ [modified from reference 40]) were used to identify the sequence between nucleotide positions 568 and 995 in the *luxA* coding region (11a). PCR amplification produced a single, specific DNA product of 428 bp, which was synthesized in the presence of a *luxA* template under the following conditions: initial heating at 94°C for 5 min, 40 cycles of 94°C for 1.5 min, 40°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 7 min. These moderately stringent amplification conditions occasionally resulted in some nonspecific products. The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, and 100 pmol of each primer. To a final volume of 95 μl of this mixture was added 2.5 U of *Taq* polymerase. These conditions produced a positive amplification of the *luxA* target from as few as 1 CFU of *V. fischeri* (see Results).

To estimate the number of *V. fischeri* in natural seawater, we collected samples from Kaneohe Bay sites 1 and 7 on 18 and 19 February 1992. The total assemblage of bacterial cells was concentrated by centrifugation (if necessary) and lysed by five cycles of alternating freezing in a dry ice-ethanol bath and thawing in tap water. Ten individual 1-μl subsamples of cell lysate were then used for PCR templates (see above). The amplification products were separated on a 2.5% agarose gel by electrophoresis and viewed after ethidium bromide staining to determine the percentage of subsamples that apparently contained a *luxA* target.

PCR amplifications (of both pure cultures and natural samples) that showed a DNA product of 428 bp were examined by Southern hybridization (22) to verify that the product was an authentic *luxA* fragment. To further confirm that the *luxA* PCR products were derived from symbiotically competent *V. fischeri* present in the seawater sample, a portion of the products was digested with the restriction enzyme *Mbo*I. This resulted in three bands of approximately 70, 100, and 260 bp, a pattern characteristic of squid symbionts (21).

After confirmation of the identity of PCR products, the number of tubes of each dilution that contained positive subsamples of seawater was submitted to a most-probable-number (MPN) analysis (13, 31, 36) to estimate the number of *V. fischeri luxA* copies per milliliter of natural seawater and thus the apparent concentration of *V. fischeri* cells per milliliter.

RESULTS

Water samples collected from a number of coastal locations contained CFU of *V. fischeri* (Table 1), although their actual concentration varied by a factor of at least 10^3. While the vast majority of *V. fischeri* CFU in Hawaiian seawater inhabited by *E. scolopes* squid do not produce visible amounts of luminescence (i.e., they are NVL) and must be identified by DNA-DNA hybridization, seawater obtained from other locations apparently contained only the visibly luminous (VL) type of *V. fischeri* CFU.

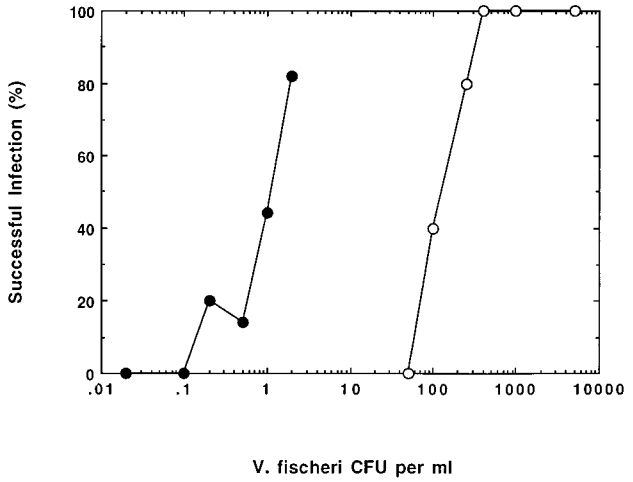

FIG. 1. Comparison of the capability of seawater containing different concentrations of *V. fischeri* CFU to infect and colonize the nascent symbiotic light organ of juvenile *E. scolopes*. Freshly collected nearshore (site 1) Hawaiian seawater, which contains naturally occurring, symbiotically competent (probe-positive [Table 1]) *V. fischeri* (●), and offshore seawater, which does not, but to which a suspension of cultured *V. fischeri* was added (○), were serially diluted with filter-sterilized seawater. The percentage of animals whose light organs became infected after exposure to these dilutions was determined and compared with the concentration of *V. fischeri* (CFU per milliliters) present.

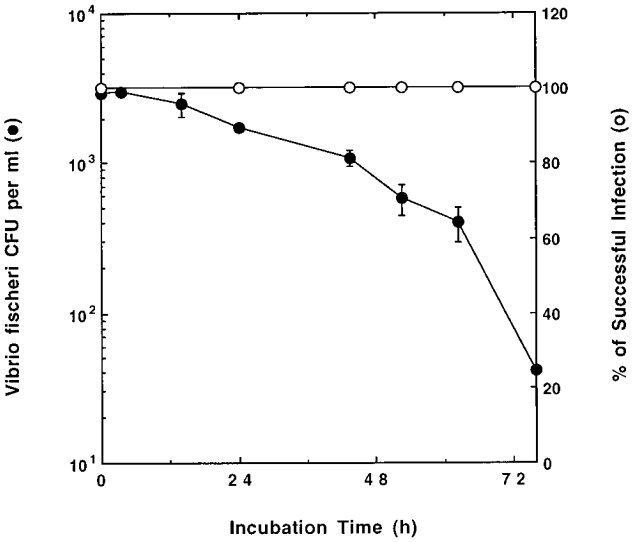

FIG. 2. Change in the number of culturable *V. fischeri* cells remaining in a seawater sample (●) as a function of time. The percentage of juvenile *E. scolopes* whose light organs became symbiotically infected with *V. fischeri* after exposure to this seawater (○) was assayed simultaneously. The error bars indicate one standard deviation.

Coastal Hawaiian seawater collected either from within Kaneohe Bay at a nearshore habitat of *E. scolopes* (site 1) or from a location 3.1 km offshore from the habitat (site 7) was examined for the presence of symbiotic *V. fischeri* by the animal colonization assay. Briefly, newly hatched, uncolonized (aposymbiotic) juvenile squid were exposed to either full-strength natural seawater or serial dilutions made in filter-sterilized seawater. After 24 h, successful colonization of individual squid light organs was determined (see Materials and Methods). Seawater from the offshore location, which is distant from animal habitats, never led to squid colonization, even after a 24-h incubation (Table 1). In contrast, successful colonizations occurred after exposure to as little as 5 ml of a 10-fold dilution of nearshore, site 1 seawater (Fig. 1). Surprisingly, although a concentration of at least 200 CFU of laboratory-cultured *V. fischeri* cells per ml has been shown to be required for successful colonization (Fig. 1) (34), 10-fold-diluted site 1 seawater actually contained only about 0.2 probe-positive (symbiotic) *V. fischeri* CFU/ml (Table 1). This finding suggested that there might be cryptic (i.e., viable and symbiotically infective but no longer culturable) *V. fischeri* symbionts in Hawaiian nearshore seawater.

To see whether a greater number of culturable *V. fischeri* cells could be recovered in laboratory media if the cells were not required to grow on the surface of a membrane filter during primary isolation, we determined the evidence of growth of *V. fischeri* cells in site 1 seawater inoculated directly into SWT overlays containing either 1.5% or 0.5% agar or into SWT broth. None of these methods gave evidence for a significantly greater number of culturable *V. fischeri* cells than could be determined by colony formation on membrane filters. Even when liquid broth was used, the concentration of total culturable *V. fischeri* cells present in site 1 seawater was below the limits of detection (<9.1 cells per ml), which is less than 4.5 times the concentration determined by colony formation (20).

An experimental analysis of the loss of viability of *V. fischeri* cells suspended in seawater revealed that the number of CFU of strain ESR1 present in offshore (i.e., site 7) seawater decreased gradually from 3×10^3 to 40 CFU/ml during 76 h of incubation (Fig. 2). However, all of the juvenile squid exposed to the ESR1 cells in this water sample throughout this incubation became successfully colonized (Fig. 2). In addition, at 43 h, the incubating suspension of ESR1 cells was diluted with symbiont-free water to yield suspensions containing 359, 237, 108, and 36 CFU/ml. All of these dilutions were also able to infect juvenile squid, suggesting that most of the ESR1 cells initially placed in the site 7 seawater remained infective (20). When this dilution procedure was repeated at 54 h, producing suspensions containing 192, 127, 58, and 19 ESR1 CFU/ml, all of the squid exposed to the first two concentrations and 50% of the squid exposed to the last two concentrations became colonized. The lower infectivity of the 54-h-diluted suspension containing 58 CFU/ml (50%) relative to that of the 43-h suspension containing 36 CFU/ml (100%) may suggest that some portion of the *V. fischeri* cells had entered the nonviable or dead state as the incubation proceeded.

Taken together, the data described above indicated the presence of viable but nonculturable cells in natural Hawaiian seawater. Therefore, we used two separate approaches to estimate the abundance of copies of the *V. fischeri* specific *luxA* gene in seawater samples collected from sites 1 and 7. The number of copies of this gene per milliliter of sample could, to a first approximation, be taken as a measure of the concentration of *V. fischeri* cells. In the first approach, the total microbial assemblage present in several liters of each seawater sample was concentrated by filtration to a small volume of liquid and a series of aliquots equivalent to between 10 and 600 ml of the seawater samples were spotted onto a nylon membrane. After lysis of the cells and binding of their DNA to the membrane, labeled *luxA* probe DNA was allowed to hybridize to the sample spots. A positive hybridization was obtained with as little as 150 ml of site 1 seawater, while as much as 600 ml of site 7 seawater had no detectable *luxA* DNA (Table 2). When known amounts of a laboratory culture were used as hybridization standards, the detection limit of this DNA method was estimated at between 3×10^4 and 4×10^4 *V. fischeri* cells. On the

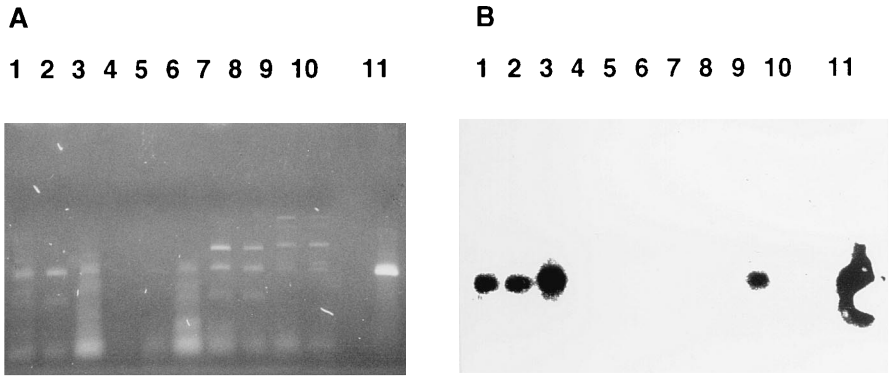

FIG. 3. (A) Agarose gel displaying the DNA products from *V. fischeri luxA* PCR amplifications of 10 individual 1-μl samples of Hawaiian coastal seawater (lanes 1 through 10). Lane 11 contains the expected specific 428-bp product of *luxA* amplification. (B) Identification of authentic *luxA* products by Southern hybridization to a *V. fischeri*-specific *luxA* gene probe.

basis of this approximation, seawater from the animal habitat site contained a total of between 276 and 400 *V. fischeri* cells per ml, while offshore seawater contained fewer than 67 cells per ml (Table 2).

As a second, independent estimate of the number of *V. fischeri* cells present in site 1 and 7 seawater, we used a PCR-based MPN approach. The sensitivity of this method when used with specific primers for *V. fischeri luxA* was tested with aliquots of a suspension of ES114 cells that had been harvested at stationary phase and lysed by five cycles of freezing and thawing. Diluted aliquots, calculated to contain (on average) 100, 50, 10, 5, 2.5, 1, or 0.5 cell equivalents of DNA, were amplified by PCR. The amplification detected the *luxA* sequence from as few as a single *V. fischeri* cell as determined by agarose gel electrophoresis of the PCR products (20). The presence or absence of the *V. fischeri luxA* gene in multiple 1-μl samples of seawater from site 1 was similarly determined; however, unlike the results of PCR of the pure culture of ES114 in distilled water, PCR of natural seawater samples often resulted in nonspecific products in addition to any authentic 428-bp *luxA* amplification product (Fig. 3A). Therefore, the product DNAs from seawater samples were transferred from the agarose gel onto a nylon filter, which was then hybridized with a labelled *luxA* gene probe to confirm the identity of several of the 428-bp products as *V. fischeri luxA* (Fig. 3B).

Of the PCR amplifications performed on 10 separate 1-μl samples of site 1 water (collected at the same time as that used in both the colonization and the DNA hybridization experiments described above), five detected the presence of *luxA* DNA. This frequency translates into a MPN index of 690/ml (95% confidence limits between 210 and 1,680/ml). Of another 10 reactions of 1 μl of site 1 seawater collected the next day, 4 were positive (Fig. 3), suggesting that the number of *luxA* targets in this water sample was a MPN of 510/ml (95% confidence limits between 130 and 1,340/ml). These numbers convert directly into the concentration of *V. fischeri* cells per milliliter (Table 3), assuming that the number of amplifiable *luxA* target DNA sequences corresponds to the number of *V. fischeri* bacterial cells.

TABLE 2. Abundance of *V. fischeri* in seawater samples as estimated by DNA-DNA hybridization

Template source	Maximum for negative hybridization	Minimum for positive hybridization	Estimated range of *V. fischeri* concn (cells/ml)
ES114 culture	3×10^4 cells	4×10^4 cells	
Site 1 seawater	100 ml	150 ml	276–400
Site 7 seawater	600 ml	ND[a]	<67

[a] ND, not determined.

DISCUSSION

The physiological state often described as viable but nonculturable is a well-documented phenomenon among certain obligate intracellular pathogens and cooperative symbionts (1, 6, 14). However, recently this state has gained additional attention as a physiological condition entered into by otherwise easily cultured bacterial species (9). For example, certain pathogenic bacteria have been thought to enter a nonculturable state, perhaps as a strategy that evolved to enhance their survival during periods when they find themselves in environments other than host tissue. Nonculturable forms of some pathogens, such as *Aeromonas salmonicida*, *Campylobacter jejuni*, and *Vibrio vulnificus*, that were subjected to stressful temperature or starvation treatments lost or varied their virulence to host animals (16, 25, 32). This observation may suggest that laboratory and artificial treatments of cells produced nonculturable states different from that of naturally occurring nonculturable cells.

Several marine *Vibrio* species, as well as certain terrestrial enteric bacteria exposed to seawater, have been reported to enter a viable but nonculturable state (18, 33). Although they are no longer recoverable as colonies on isolation media, their presence in the natural environment has been detected by either PCR amplification or DNA hybridization (2, 15, 17, 19, 35, 37) and, in some studies, by their ability to infect a model animal tissue (8). Although the exact mechanism by which such a state is triggered and achieved remains to be described for any species, several factors that frequently occur in the natural

TABLE 3. Abundance of *V. fischeri* in site 1 seawater estimated by positive *luxA* PCR amplification

Expt	No. of positive PCR tubes/ total no.	MPN index (cells/ml)	95% confidence limits
I	4/10	510	130–1,340
II	5/10	690	210–1,680

marine environment (e.g., a temperature change or a decrease in the availability of organic nutrients) have been implicated in the induction of the nonculturable or nongrowing state of heterotrophic bacterial cells (18, 26). Thus, it is possible that many nonculturable bacteria, which make up the vast majority of the bacterioplankton community in seawater, are representatives of known, typically culturable, species that have entered such a state. One approach to exploring this hypothesis has been the phylogenetic identification of the members of this community by 16S rRNA sequencing of cloned DNA (5, 11, 38). A different approach has been to examine the role of the nonculturable state in the natural ecology of a particular species of bacterium.

Throughout its life, the Hawaiian sepiolid squid, *E. scolopes*, maintains a culture of the luminous symbiotic bacterium *V. fischeri* in its light-emitting organ. Studies of the biology of this symbiosis have revealed two major ways in which the ecological cycle of *V. fischeri* is interconnected with and interdependent upon the life cycle of the host squid: (i) the host periodically expels excess light organ symbionts into the surrounding seawater, thereby affecting the abundance and distribution of this particular bacterial species in their habitat (3, 24, 34), and (ii) the colonization of aposymbiotic juvenile squid requires the presence of symbiosis-competent strains of *V. fischeri* in the surrounding seawater (23, 27, 39).

Because of the magnitude of symbiont expulsion from adult squids (24), it is perhaps not surprising that even a short exposure to seawater from the host's habitat is sufficient to result in the successful colonization of juvenile squid (27). Also, when the number of CFU of *V. fischeri* in Hawaiian seawater samples was determined by colony hybridization to the species-specific *luxA* DNA probe, this species was found to be relatively abundant in animal-containing habitats and to decrease in number as an exponential function of the distance from the habitat (24). However, the actual number of symbiotic bacteria that could be cultured from animal-habitat (e.g., site 1) seawater averaged only about 2 *V. fischeri* CFU/ml (range, 0.3 to 9 CFU/ml during a 4-year study). This value is about 100-fold lower than the minimum concentration of *V. fischeri* required for successful colonization of juvenile squid in the laboratory when cultured *V. fischeri* cells are used (Fig. 1). One hypothesis that might explain this paradoxical observation is that Hawaiian seawater contains significant numbers of nonculturable but viable and infective symbiotic *V. fischeri* cells. To determine the presence of such nonculturable *V. fischeri* cells, we used two techniques, DNA-DNA hybridization (Table 2) and PCR amplification (Table 3), to estimate the actual number of symbiotic bacteria in seawater samples collected in different locations.

Extracellular DNA dissolved in seawater or attached to sand or detrital particles might be detected both by PCR amplification of *luxA* sequences from water samples and by *luxA* DNA hybridization to cell concentrates, resulting in an overestimation of the total number of *V. fischeri* cells present. However, while absorption to a solid surface inhibits the hydrolysis of free DNA by DNase activity (30), it also makes the DNA less accessible to PCR amplification. Similarly, rates of dissolved-DNA degradation in aquatic environments have been reported to be very high (10, 17), and therefore only DNA released from lysed cells for less than a few hours can contribute to any possible overestimation of the number of copies of *luxA*. For instance, more than 90% of the DNA released from *V. fischeri* cells by freezing and thawing in natural seawater was degraded within 10 to 30 min (20). For these reasons, we believe that the number of *luxA* copies determined by DNA hybridization (Table 2) and PCR-MPN (Table 3) analyses is a good estimate of the number of intact *V. fischeri* cells.

This estimated concentration of *V. fischeri* in the host animal habitat was in the range of 300 to 700 cells per ml, very similar to that required to successfully initiate a colonization by cultured *V. fischeri* cells (Fig. 1). In addition, about 20% of juveniles exposed to a 10-fold dilution of this water became colonized, again similar to the percentage expected for exposure to 50 to 100 cultured cells per ml. These results support the hypothesis that a large percentage, perhaps greater than 90%, of the viable and symbiotically competent *V. fischeri* cells in seawater from the host habitat are in a nonculturable state. These cells must be converted into a culturable state by some mechanism characteristic of the specific interaction between the two symbiotic partners. Because expelled symbionts are essentially 100% culturable (34), they appear to subsequently enter a nonculturable physiological state in seawater by an as yet unknown mechanism (Fig. 2) while retaining, at least for a while, their ability to colonize the developing squid light organ. These cells appear to be the main inoculum for infection of juvenile squid and appear to be no less effective than culturable cells at colonizing animals. Thus, the light organ symbiosis appears to be an important pathway in the ecological cycle of symbiotic *V. fischeri*, converting viable but nonculturable bacteria into the culturable state. In addition, the *V. fischeri*-squid symbiosis offers a special opportunity for examining the mechanism modulating the entry into the viable but nonculturable state because (i) it allows a direct assay of the recovery of cells that have entered this state and (ii) the recovery system (animal) is an ecologically relevant part of the natural biology of the bacterium.

ACKNOWLEDGMENTS

We thank J. Graf for providing *V. fischeri* ESR1 and C. Wimpee for designing and providing the *luxA* PCR primers. We also thank W. Toller for assistance in rearing animals in Hawaii. This work was supported by grants to E.G.R. from the Office of Naval Research (N0014-93-1-0846) and to M. McFall-Ngai and E.G.R. from the National Science Foundation (IBN 9220482) and a fellowship grant to K.-H.L. from Sea Grant (M-2/94.3).

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