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Detection of the Light Organ Symbiont, *Vibrio fischeri*, in Hawaiian Seawater by Using *lux* Gene Probes†

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Symbiotic bacteria that inhabit the light-emitting organ of the Hawaiian squid *Euprymna scolopes* are distinctive from typical *Vibrio fischeri* organisms in that they are not visibly luminous when grown in laboratory culture. Therefore, the abundance of these bacteria in seawater samples cannot be estimated simply by identifying them among luminous colonies that arise on nutrient agar plates. Instead, we have used *luxR* and polymerase chain reaction generated *luxA* gene probes to identify both luminous and non-visibly luminous *V. fischeri* colonies by DNA-DNA hybridization. The probes were specific, hybridizing at least 50 to 100 times more strongly to immobilized DNAs from *V. fischeri* strains than to those of pure cultures of other related species. Thus, even non-visibly luminous *V. fischeri* colonies could be identified among colonies obtained from natural seawater samples by their probe-positive reaction. Bacteria in seawater samples, obtained either within or distant from squid habitats, were collected on membrane filters and incubated until colonies appeared. The filters were then observed for visibly luminous *V. fischeri* colonies and hybridized with the *lux* gene probes to determine the number of total *V. fischeri* colonies (both luminous and non-visibly luminous). We detected no significant differences in the abundance of luminous *V. fischeri* CFU in any of the water samples observed (≤1 to 3 CFU/100 ml). However, probe-positive colonies of *V. fischeri* (up to 900 CFU/100 ml) were found only in seawater collected from within the natural habitats of the squids. A number of criteria were used to confirm that these probe-positive strains were indistinguishable from symbiotic *V. fischeri*. Therefore, the *luxA* and *luxR* gene probes were species specific and gave a reliable estimate of the number of culturable *V. fischeri* colonies in natural water samples.

The marine bioluminescent bacteria have been the subject of numerous ecological investigations that have led to an appreciation of both the diversity of niches these bacteria occupy and the remarkably predictable patterns of distribution they exhibit (10). Three genera of luminous bacteria have been described from the marine environment: *Vibrio*, *Photobacterium*, and *Shewanella* (3, 13, 18). While these genera include at least nine luminous species, four (*Vibrio harveyi*, *V. fischeri*, *Photobacterium leiognathi*, and *P. phosphoreum*) constitute the majority of all isolates identified. In addition to being frequently encountered members of both the seawater bacterioplankton and the enteric tracts of marine animals, *V. fischeri*, *P. leiognathi*, and *P. phosphoreum* exist as specific symbionts in light-emitting organs of many species of marine fishes and squids (22).

V. fischeri is the specific light organ symbiont of the sepiolid squid, *Euprymna scolopes* (6), which is found throughout its life cycle within certain shallow water reef flats of the Hawaiian Islands (4, 20, 38). In laboratory experiments, adult *E. scolopes* continuously release large numbers of their symbiotic bacteria into the surrounding water (17); thus, environments inhabitated by populations of adult squids might be expected to be relatively enriched in these excreted symbiotic *V. fischeri* organisms. However, no evidence for a causal relationship between the distributions of these (or any other) luminous animals and their symbionts has yet been demonstrated.

When the bacteria are cultured outside the animal, the light emission of symbiotic bacterial strains of *E. scolopes* is depressed more than 1,000-fold (6), and thus their colonies are not visibly luminous and cannot be visually identified on agar medium. Therefore, it was necessary to use another experimental approach to estimate the abundance of *V. fischeri*, including the non-visibly luminous, symbiont-type strains, in and around the natural habitats of the squid. We have applied the technique of DNA-DNA hybridization (12) to enumerate both luminous and non-visibly luminous colonies of *V. fischeri* by using probe sequences found only within this species of luminous bacteria. The *lux* operon of *V. fischeri* is composed of at least seven genes that are responsible for light production (40); of these we chose two, a structural gene for one of the luciferase subunits and a gene in the regulatory region of the *lux* operon. Using these DNA probes, we asked (i) whether it was possible to specifically and reliably detect and enumerate *V. fischeri* organisms from the natural environment and (ii) whether the abundance of symbiont-type *V. fischeri* appeared greater within the host's habitat than elsewhere.

(A preliminary account of this work was presented at the general meeting of the American Society for Microbiology, Dallas, Tex., in 1991 [16].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in these experiments are listed in Table 1. Luminous bacteria were grown on a seawater-tryptone (SWT)-based medium that consisted of (wt/vol) 0.5% tryptone, 0.3% yeast extract, and 0.3% glycerol in 70% natural seawater (21). Luminous bacterial isolates from several environments were taxonomically identified by nutritional characteristics, pigmentation, and luciferase kinetics (6, 21). The identity of *V. fischeri* strains was further confirmed by the presence of sheathed polar flagella (28), the induction of bioluminescence after the

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TABLE 1. Bacterial strains used in this study

Strain	Source[a]
V. fischeri	
ES114	*E. scolopes* light organ (6)
MJ1	*Monocentris japonica* light organ (33)
H905	Hawaiian coastal seawater[b]
PP3	Hawaiian coastal seawater[c]
PP12	Hawaiian coastal seawater[c]
PP28	Hawaiian coastal seawater[c]
PP42	Hawaiian coastal seawater[c]
PP49	Hawaiian coastal seawater[c]
PP55	Hawaiian coastal seawater[c]
PP64	Hawaiian coastal seawater[c]
V. logei	
ATCC 29985	Mussel enteric tract (2)
V. harveyi	
B392	Unknown (28)
H902	Hawaiian coastal seawater[b]
P. leiognathi	
LN1a	*Leiognathus nuchalis* light organ (8)
H9035	Hawaiian coastal seawater[b]
P. phosphoreum	
NZ11D	*Nezumia aequalis* light organ (32)
E. coli	
ML35	29
DH5α(pHK724)	14
Unidentified[d]	
NL11	Hawaiian coastal seawater
NL16	Hawaiian coastal seawater

[a] Isolated in this study (unless otherwise noted by reference numbers in parentheses).
[b] Previously undescribed visibly luminous strain.
[c] Non-visibly luminous, *luxA* probe-positive strain.
[d] Nonluminous, *luxA* probe-negative strain.

addition of *V. fischeri* autoinducer (21), and the ability to colonize the uninfected light organ of a juvenile *E. scolopes* animal (19).

Sampling locations. Water samples were collected at a depth of about 30 cm from two geographical locations: (i) 18 samplings from site 1 and 7 from site 2 or site 3 of Kaneohe Bay, Oahu Island, Hawaii (Fig. 1), during 1990 and 1991, and (ii) 2 samplings from near shore (off Marina del Rey) and 1 from offshore (in Catalina Channel) in southern California in 1990.

Slot blotting. Cell suspensions of 10 different pure cultures of bacteria were layered onto a membrane filter (either nitrocellulose or nylon [Schleicher & Schuell, Keene, N.Y.]) by using a slot-blotting apparatus (Milliblot-S; Millipore Corp., Bedford, Mass.). Suspensions were added to the slots in sets of four concentrations. After the filter had been air dried, the cells were lysed by exposure to 10% sodium dodecyl sulfate (SDS) for 3 min, and the released DNAs were denatured for 5 min with a solution consisting of 0.5 M NaOH and 1.5 M NaCl and neutralized for 5 min with 0.5 M Tris-HCl buffer (pH 8) containing 1.5 M NaCl. Finally, the single-stranded DNAs were treated with 2× SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 7.4]) and baked at 80°C under vacuum to bind them to the filter (35).

Colony blotting. Seawater samples were passed through sterile 0.2- or 0.45-μm-pore-size nitrocellulose filters (type HA; diameter, 47 mm; Millipore Corp.). The filters were placed on SWT agar medium and incubated at 25°C until colonies had formed, usually after about 24 h. No difference was detected in the number of *V. fischeri* colonies obtained by filtration through the two pore sizes of filters used. The DNA from the cells in the bacterial colonies was then released, denatured, and bound to the filter as described for the slot-blotting technique.

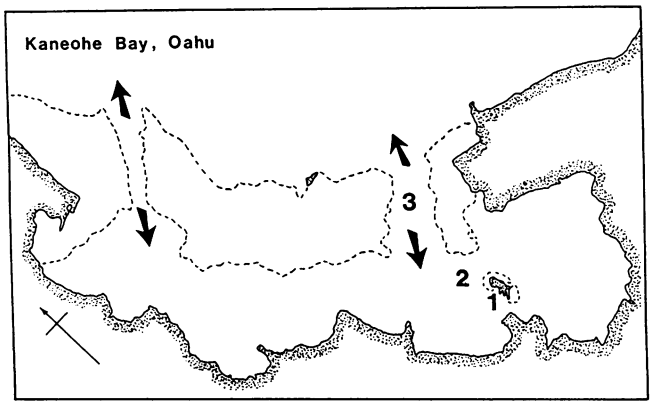

FIG. 1. Seawater sampling locations in Kaneohe Bay, Hawaii. Dashed lines give approximate locations of reef areas within 15 ft (4.5 m) of the surface that create two channels connecting the bay and the sea. Heavy arrows represent the major tidal flows into and out of the bay. Surface seawater for microbiological isolations was obtained from two locations within the bay (sites 1 and 2) and one location within the tidal channel (site 3). The length of the compass arrow represents 2 km.

Preparation of *luxR* and *luxA* DNA probes. Plasmid DNA was isolated (5) from *Escherichia coli* DH5α carrying pHK724, a construct that includes the *luxR* sequence derived from *V. fischeri* MJ1 (14). An approximately 700-bp *luxR*-containing fragment was produced by restriction with *Hin*dIII (Promega, Madison, Wis.) and separated by electrophoresis on a 0.7% agarose gel. This DNA fragment was purified by using a Geneclean II Kit (Bio 101, La Jolla, Calif.), and about 25 ng was labeled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham, Arlington Heights, Ill.) by using the Random Primer DNA Labeling Kit (Bethesda Research Laboratories, Gaithersburg, Md.). Isotopically labeled probes were purified by using a Select-D G-50 column (5′→3′ Inc., Boulder, Col.), and their specific activities were quantified by liquid scintillation counting.

Polymerase chain reaction-amplified *luxA* probe was produced by using total DNA from *V. fischeri* ES114 as reported previously (26, 42). The polymerase chain reaction product was radioactively labeled and purified as described above before hybridization processing.

DNA hybridization. Immobilized DNA samples on membrane filters, either from colony blotting or from slot blotting, were placed at 42°C in a prehybridization solution composed of 5× SSC (0.75 M NaCl, 75 mM sodium citrate [pH 7.0]), 50% formamide, 10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), and 250 μg of herring sperm DNA per ml. After prehybridizing for 4 h, DNA-DNA hybridization was carried out at 42°C for 16 to 20 h in the prehybridization solution to which was added ^{32}P-labeled probe (approximately 10^6 to 10^7 cpm) and dextran sulfate (final concentration, 10%). Nonspecifically bound and unhybridized DNA probe was removed by washing under stringent conditions in 0.1× SSC–0.1% SDS at 65°C for 1 h with shaking to allow DNA-DNA hybridization to be species specific (42). Filters were then exposed to X-Omat AR film (Kodak, Rochester, N.Y.) at −70°C for the required period. The degree of hybridization in the slot blots was quantified by densitometric scanning

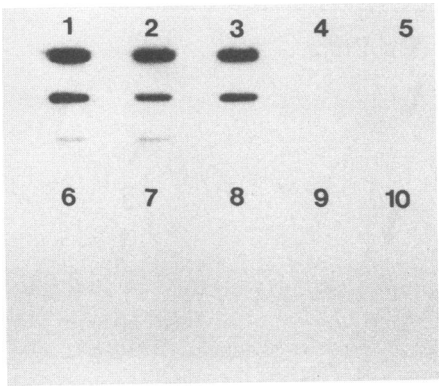

FIG. 2. Autoradiograph illustrating the degree of hybridization of the *luxA* probe to DNA of several bacterial species. Four amounts of cells (8×10^6, 8×10^5, 8×10^4, and 8×10^2 cells) from each of 10 strains were placed in vertical rows of slots with the following designations: 1, *V. fischeri* MJ1; 2, *V. fischeri* ES114; 3, *V. fischeri* H905; 4, unidentified luminous strain; 5, *E. coli* ML35; 6, *V. harveyi* B392; 7, *V. harveyi* H902; 8, *V. logei* ATCC 29985; 9, *P. leiognathi* LN1a; 10, *P. phosphoreum* NZ11D. In no case was hybridization detected with the lowest cell number used (8×10^2).

(model GS 300; Hoefer Scientific Instruments, San Francisco, Calif.).

Isolation of probe-positive bacterial strains. Strains of bacteria from natural water samples that produced hybridizing (probe-positive) colonies were isolated from colony replicas that had been transferred to SWT agar plates from the original isolation filters by using an Accutran Replica Plater (Schleicher & Schuell). During filter hybridization, plates with the replicated bacterial colonies were stored at 4°C. By comparing the hybridization spots on the autoradiogram with the corresponding colony positions on the replica plates, probe-positive colonies were picked and isolated by purity streaking, and their identity as *V. fischeri* was determined as described above.

RESULTS

We tested radioactively labeled DNA probes made from *V. fischeri luxR* and *luxA* genes for their specificity for, and extent of hybridization to, total DNA isolated from 10 strains of luminous and nonluminous bacteria. Detectable hybridization occurred only to DNA from *V. fischeri* strains (Fig. 2). Densitometric scans of autoradiographs indicated that the probes are quite specific, hybridizing at least 50 to 100 times more strongly to DNA from *V. fischeri* strains than to DNA from other related species of luminous bacteria, such as *V. harveyi*, *V. logei*, and *Photobacterium* spp. (Table 2). As would be predicted, the highest hybridization signal was observed between each probe DNA and the particular strain from which it was derived (*luxR* from *V. fischeri* MJ1 or *luxA* from *V. fischeri* ES114). In our experiments, detection by these probes required the presence of the DNA equivalent of 10^4 cells per slot. All of these data support and extend the results of the recently published report by Wimpee et al. (42), which documented the species specificity of *luxA* gene probes.

Using both of these DNA probes, we tested whether we could specifically detect, by colony hybridization, *V. fischeri* colonies in a mixture that also contained *V. harveyi*. Bacterial suspensions containing a known ratio (see Table 3) of *V. fischeri* ES114 and *V. harveyi* H902 cells were passed through nitrocellulose membrane filters, and the filters were incubated on SWT agar medium. Once bacterial colonies formed, each species could be visually differentiated and enumerated (*V. harveyi* colonies are visibly luminous, and *V. fischeri* ES114 colonies are not). The cells in the bacterial colonies were then lysed, and their DNA was fixed onto the filter and hybridized with the radioactively labeled *luxA* or *luxR* probe. After autoradiography the number of hybridization spots was determined and compared with the expected frequency and position of *V. fischeri* colonies (Table 3). Between 93 and 100% of *V. fischeri* colonies were detected, and no false-positive colonies (*V. harveyi*) were observed.

We also examined the specificity and reliability with which the colony hybridization technique detects *V. fischeri* within a bacterial colony assemblage from natural seawater. Twenty-one luminous colonies arose on the surface of a membrane filter through which a 5-ml sample of near-shore southern California seawater had been passed (Table 4). On the basis of the taxonomic identification of luminous colonies isolated from other filters, it was predicted that between 10 and 20 of these colonies were visibly luminous *V. fischeri* colonies; in fact, 16 of these colonies gave probe-positive hybridization spots when probed with the *luxA* DNA probe. A mixture of another 5 ml of the same seawater sample, to which was added an ES114 cell suspension containing an average of 62 CFU, gave rise to a total of 76 colonies that produced probe hybridization spots. This is 97% of the predicted number of 78 *V. fischeri* probe-positive spots (the

TABLE 2. Relative probe hybridization efficiency

Strain	Relative efficiency[a] of:	
	luxR (MJ1)	*luxA* (ES114)
V. fischeri		
ES114	77	100
MJ1	100	56
H905	88	89
V. logei		
ATCC 29985	<1	<1
V. harveyi		
B395	<1	<1
H902	<1	<1
P. leiognathi		
LN1a	<1	<1
P. phosphoreum		
NZ11D	<1	<1
E. coli		
ML35	<1	<1

[a] Autoradiograph exposure densities of the slot blots in Fig. 2, relative to a maximum value of 100.

TABLE 3. Specific detection of *E. fischeri* ES114 colonies in mixed culture

Probe used	No. of CFU detected:			
	V. harveyi (visibly luminous)	*V. fischeri*		Total[b]
		Non-visibly luminous	Probe positive[a]	
luxR	90	28	28 (100%)	118
luxA	9	14	13 (93%)	23

[a] Colonies that produced a positive hybridization signal with the indicated probe. The percentage of non-visibly luminous colonies that were probe positive is indicated in parentheses.

[b] Total number of visibly luminous (*V. harveyi*) and non-visibly luminous (*V. fischeri*) colonies appearing on the filter.

TABLE 4. Specific detection of *V. fischeri* ES114 colonies in natural seawater

Sample[a]	No. of CFU detected:		
	Luminous[b]	*V. fischeri*	
		Predicted	Probe positive[c]
Seawater	21		16
ES114 suspension	0	62	54 (87%)
Seawater + ES114 suspension	22	78[d]	76 (97%)

[a] Colonies of bacteria were cultured on filters through which had been passed 5-ml portions of Marina del Rey seawater, a suspension of cultured ES114 cells, or a combination of the two (see Materials and Methods).
[b] Visibly luminous colonies that were produced by the naturally occurring *V. fischeri* and *V. harveyi* colonies in the seawater sample.
[c] Number of colonies that hybridized with the *luxA* DNA probe. The percentage of predicted *V. fischeri* colonies that hybridized is indicated in parentheses.
[d] Because we added 62 ES114 cells into seawater already containing an estimated 16 *V. fischeri* cells, the number of total *V. fischeri* CFU is expected to be 78.

estimated 16 *V. fischeri* colonies already in the seawater sample, plus the 62 added *V. fischeri* ES114 colonies). Thus, colony hybridization is effective in detecting approximately 87 to 97% of the *V. fischeri* CFU, both visibly luminous and non-visibly luminous, that are present in a seawater sample. It is interesting that there was no evidence from these and other filters tested that either near-shore or offshore (Catalina Channel) southern California seawater naturally contains a detectable number of non-visibly luminous *V. fischeri* CFU.

We next determined the number of visibly luminous bacteria (by observation, isolation, and taxonomic identification), as well as non-visibly luminous (probe-positive) *V. fischeri* colonies (by colony hybridization), from Hawaiian seawater samples taken (i) within a shallow area of Kaneohe Bay that contains an abundance of *E. scolopes* squids, (ii) from bay waters approximately 0.5 km seaward of the squid habitat, and (iii) from channel waters about 3 km further offshore (Fig. 1). The most common visibly luminous *Vibrio* species in Hawaiian waters, *V. harveyi*, occurred at concentrations between 11 and 16 CFU/100 ml of seawater in all of the samples from these locations collected over a 2-year period (Table 5). The concentration of visibly luminous *V. fischeri* colonies was considerably lower, appearing at or below the level of detection (≤1 to 3 CFU/100 ml). In contrast, colony hybridization assays with either *luxR* or *luxA* revealed that probe-positive non-visibly luminous colonies were at least 30 to 200 times more abundant than

TABLE 5. Abundance of luminous bacteria in Kaneohe Bay, Hawaii

Location[a]	Mean abundance (CFU/100 ml)			
	Total[b]	Strain groups[c]		
		V. harveyi	VL *V. fischeri*	NVL *V. fischeri*
Site 1	31 (8–90)	16	<1	211 (28–900)
Site 2	43 (7–160)	11	<1	29 (0–75)
Site 3	40 (14–85)	11	≤3	2 (0–13)

[a] Surface seawater sampling sites as indicated in Fig. 1.
[b] Average total LCFU/100 milliliters; range of values in parentheses.
[c] Abbreviations: VL, visibly luminous; NVL, probe-positive, non-visibly luminous. Average values with range (if given) in parentheses.

TABLE 6. Characteristics of *luxA* probe-positive strains

Strain	*luxA* hybridization[a]	Light emission[b]		Infection of squids[d]
		Without AI[c]	With AI[c]	
ES114	+	83	5,702	+
H902	−	4,428	3,779	−
H9035	−	5,270	4,760	−
NL11	−	1.2	1.2	ND[e]
NL16	−	1.6	1.4	ND
PP3	+	301	7,147	+
PP12	+	3	2,171	+
PP28	+	7	119	+
PP42	+	9	2,535	+
PP49	+	5	172	+
PP55	+	5	1,108	+
PP64	+	5	1,724	+

[a] Strains selected from the primary isolation plates were purified by streaking on agar medium and retested for hybridization with the *luxA* probe.
[b] Luminescence units (quanta per second, 10^3). The photometer background (dark current) was approximately 1.5 units.
[c] Autoinducer (AI) derived from *V. fischeri* MJ1 (21).
[d] Ability to colonize the light organs of juvenile squids in an infection assay, as described previously (19).
[e] ND, not determined.

colonies of visibly luminous *V. fischeri* (Table 5). In addition, there was an indication of a generally elevated concentration of probe-positive CFU in the ambient water of squid habitats relative to offshore waters. This sampling, although limited, suggests the possibility of a gradient in the abundance of symbiotic *V. fischeri*: average values of 211 CFU/100 ml of water in the squid's habitat (site 1) and 29 CFU/100 ml of water at a distance of 0.5 km from their habitat (site 2) were estimated, whereas only a few colonies (never more than 13 CFU/100 ml) were found in more offshore water (site 3).

Even though the *lux* DNA probes were specific for *V. fischeri* among the species we detected (Fig. 2), it was necessary to demonstrate that probe-detected colonies from the natural environments were really *V. fischeri* colonies of the symbiotic type. Representative probe-positive colonies were isolated and found to be symbiont-type *V. fischeri* as indicated by the following three criteria (Table 6). First, they showed extremely low but detectable levels of luminescence, with values ranging between 2 and 200 times the background sensitivity of the photometer. These values are comparable to a value of about 50 times the background for the representative squid symbiont strain, *V. fischeri* ES114. Second, their light emission responded dramatically (20- to 640-fold) to the addition of the *V. fischeri*-specific transcriptional regulator, autoinducer. Third, the probe-positive strains were able to infect juvenile squids in a standard, species-specific symbiosis assay (19), whereas other luminous bacterial isolates from Hawaiian seawater were not.

DISCUSSION

Traditionally, enumeration of the several species of luminescent bacteria occurring in seawater has depended upon the visual observation (in the dark) of light-emitting colonies on nutrient agar media (21); therefore, numerous studies have reported the abundance of these bacteria as luminous CFU (LCFU) per milliliter. As a result of these studies, remarkably predictable geographical and seasonal patterns of distribution and abundance of species of luminous bacteria have been discerned (10). For example, oceanic surface

waters have been reported to contain between 1 and 60 LCFU/100 ml both in the Sargasso Sea (25) and over the Puerto Rico Trench (31), with a consistently observed peak of *P. phosphoreum* abundance at a depth of about 600 m (31). Surface waters from coastal regions typically contain higher concentrations, between 20 and 700 LCFU/100 ml. During the winter months in temperate regions almost all of these colonies are *V. fischeri* (17, 23, 34).

Arguments have been presented previously that these patterns of abundance and distribution of luminous bacteria in seawater are controlled in large part by water temperature, nutrient concentration, salinity, and photooxidation (31, 37, 43). However, in addition to these physicochemical environmental factors, specific associations with marine animals might be important in the ecology of planktonic luminous bacteria. For example, it has been hypothesized that bacteria released from the symbiotic light organs of marine animals may contribute significantly to ambient planktonic luminous bacterial populations (11, 31). However, no test has previously been made of this prediction of such a hypothesis: that the concentration of a symbiotic bacterial species is greater in locations containing an abundance of its animal host.

Enumeration of planktonic luminous bacteria in the nearshore Hawaiian waters inhabited by the squid *E. scolopes*, by using the conventional technique of counting light-producing colonies, suggested that the abundance of these bacteria is comparable to that in other coastal waters examined: we found that between about 10 and 150 LCFU/100 ml was detected in numerous water samples collected over 2 years at three sites in Kaneohe Bay (Table 5). Taxonomic identification revealed that almost all of these colonies were produced by strains of either *V. harveyi* or *P. leiognathi*, consistent with previous reports (28). *V. fischeri* accounted for only a minor percentage of these LCFU, occurring at between ≤ 1 and 3 LCFU/100 ml. These low abundances could have suggested that the presence of *E. scolopes* populations did not have a significant effect on the species composition of the planktonic luminous bacteria in the waters they inhabited. They also could have implied that obtaining an inoculum of *V. fischeri* from the surrounding waters, an essential part of the normal development of juvenile squids (41), must be seriously problematic (19).

As early as 1973, Reichelt and Baumann (28) had pointed out the danger of using the presence of a single colony trait (visible bioluminescence) as the initial and absolute screening criterion for the estimation of the abundance of luminous bacteria in natural samples. Not only had it been reported that certain species would produce dark (non-visibly luminous) mutants under some culture storage conditions (9, 15), but also some non-visibly luminous marine isolates seemed phenotypically similar to the luminous species *V. harveyi* (28). More recently it was reported that symbiotic *V. fischeri* organisms found in the light organ of *E. scolopes* are non-visibly luminous when cultured in typical marine isolation media (6) and that these bacteria are continuously released into the surrounding water by adult animals (17). Therefore, it was apparent that the abundance of visibly luminous *V. fischeri* colonies would not reflect the actual concentration of symbiotic *V. fischeri* colonies in Hawaiian waters where *E. scolopes* reside.

In an effort to identify all *V. fischeri* colonies arising from natural water samples, we have used two *lux* DNA probes for colony hybridization. These probes bind specifically and reliably to DNA from colonies of both visibly luminous and symbiont-type, non-visibly luminous strains of *V. fischeri*. By subtracting the number of visibly luminous *V. fischeri* CFU from the total number of probe-positive CFU, the number of symbiont-type CFU could be calculated. For certain Hawaiian coastal water samples, the resulting estimation of the number of non-visibly luminous *V. fischeri* colonies exceeded the visibly luminous *V. fischeri* colonies by a factor of at least 30 to 200; however, there was no evidence for the presence of non-visibly luminous *V. fischeri* colonies in any southern Californian water sample tested. This latter result explains in part why *E. scolopes* animals reared in southern California seawater do not become infected (19).

This present study joins a number of others in which the advantages of the colony hybridization technique, either for enumeration of microorganisms in natural environments (12, 36) or for the identification of bacteria carrying underexpressed genes (7, 27), have become recognized. However, it should be remembered that, as useful as it is, this approach still requires the formation of colonies on isolation media; therefore, an underestimation of the actual abundance of any bacterial species (or its potential activity) can result from the presence in natural marine environments of cells that are in a viable yet not culturable state (24, 30).

The highest concentrations of symbiont-type (non-visibly luminous) *V. fischeri* CFU were detected in water collected from *E. scolopes* habitats, whereas only rarely were such *V. fischeri* colonies obtained at a site located 3 km seaward from these habitats (Table 5). Because this approximately 100-fold difference in abundance occurs between water samples that show little or no significant variation in the abundance of nonsymbiotic species of luminous bacteria such as *V. harveyi*, the difference appears not to be simply due to a differential ability of the water samples to generally support or maintain *Vibrio* species. Thus, these results are consistent with the notion that host *E. scolopes* not only may be an important biological niche for *V. fischeri* isolated from Hawaiian water but also may be responsible for exerting a major effect on the abundance and distribution of planktonic *V. fischeri* colonies in coastal Hawaiian seawater. In addition, the indication of a gradient in the concentration of symbiont-type *V. fischeri* colonies (Table 5) across Kaneohe Bay, from their potential sources in the inner bay seaward out through the channel waters (Fig. 1), suggests that the daily transport of inner bay water and sediment out of Kaneohe Bay during each tidal cycle (1, 39) may be important in allowing the distribution of symbiotic bacteria among different host populations in the Hawaiian Islands. Conversely, the geographical distribution of the squids may also be dependent on a sufficient abundance of the specific symbiont *V. fischeri* to allow for the proper development and survival of the host (19, 20, 38). Considerable work lies ahead before these suggestive ecological patterns can be confirmed.

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