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Occurrence of Plasmid DNA in the Sepiolid Squid Symbiont *Vibrio fischeri*

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Abstract. Because of the importance of plasmids in many bacterial associations with plants and animals, we determined the occurrence and distribution of plasmid DNA in symbiotic *Vibrio fischeri* from the light organ of the sepiolid squid *Euprymna scolopes*. Analyses of 225 isolates of symbiotic *V. fischeri* from 25 individual squids revealed an overall plasmid-carriage rate of 56%. A large plasmid ($\geq$39 kb) was detected in 96% of those isolates carrying plasmids, and multiple small plasmids were found to co-occur with one of the large plasmids in 81% of plasmid-carrying strains. In addition, these plasmids appear to be restricted to *V. fischeri* strains isolated from *E. scolopes* and from seawater at sites of squid populations. We were unable to assign a role or function to these plasmids, but they do not carry genes required for the establishment of the light organ symbiosis. We conclude that the essential bacterial symbiotic determinants must be encoded on the chromosome and that the plasmids may carry genes that are important for the survival of these *V. fischeri* strains outside of the symbiotic association.

Vibrio fischeri is a luminous bacterium common within the bacterioplankton of many regions of the ocean [29]. In addition to this planktonic, or free-living, niche, members of this species are the specific symbionts in the light-emitting organs of both monocentrid fishes [17, 39] and sepiolid squids [8, 36]. While we still know little about how such complex, cooperative associations with animal hosts are initiated or how their bacterial specificity is maintained in the host tissue, investigations with newly hatched, symbiont-free (aposymbiotic) juveniles of the luminous Hawaiian squid, *Euprymna scolopes*, have demonstrated that this symbiosis is an amenable model system for the study of these processes (reviewed in [37]). In this association the juvenile squid obtains its initial inoculum of *V. fischeri* from the ambient seawater [51] and, once colonized, becomes a source of excess *V. fischeri* that are expelled into the surrounding environment [23, 25, 36]. Thus, these bacteria have an ecological cycle that consists of an alternation between a free-living and a symbiotic niche [25, 36]. Recent work has revealed that not all isolates of *V. fischeri* obtained from seawater are capable of establishing themselves in the *E. scolopes* light organ [30]. To date, no mechanism or basis for the observed differences in symbiotic competency of *V. fischeri* strains has been discovered.

The phenomenon of strain specificity in other bacterial associations is well documented, and in many cases the essential genes required for the interaction of a bacterium with its plant or animal host have been localized to regions of bacterial plasmid DNA. For example, the fast-growing, N_2-fixing species of the genus *Rhizobium* carry large "symbiosis plasmids" necessary for successful nodulation of leguminous plants [21, 30, 35]. Similarly, plant pathogens such as *Agrobacterium* [48, 50] and some species of *Pseudomonas* [6, 10, 11] harbor plasmids with virulence-mediating genes. In addition, a number of species of animal pathogens in the genera *Escherichia* (reviewed in 49]), *Salmonella* [22, 47], *Shigella* [41, 42], *Yersinia* [18, 53], and *Vibrio* [12, 13] are characterized by the presence of virulence plasmids. In view of this pattern, it is surprising that there are few reports of plasmids in bacteria that persist in nonpathogenic associations with animals, and no evidence that plasmids (when present) play an analogous role in mediating these interactions.

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Table 1. Representative *Vibrio fischeri* isolates described in this study

Strain[a]	Native plasmids[b] large	Native plasmids[b] small	Year of isolation
ES12	+	+	1988
ES66	−	−	1988
ES79	+	+	1988
ES114	+	−	1988
ES191	−	−	1989
ES209	+	+	1989
ES213	+	+	1989
ES235	−	+	1989
ES240	−	+	1989
ES324	+	+	1990
ES334	+	+	1990
ES560	−	−	1992
ES566	−	+	1992
ES595	+	+	1992
ES602	+	+	1992
ES620	+	+	1992
ES657	+	−	1992

[a] Strain ES114 has been previously described [8].
[b] Presence (+) or absence (−) of a large plasmid ($\geq$39 kb) and/or one or more small plasmids ($\leq$12 kb) is indicated.

In this paper we describe both the prevalence of plasmid DNA in symbiotic *V. fischeri* and the degree of compositional similarity of these plasmids. Furthermore, we present evidence that such plasmid DNA, while commonly occurring, is not necessary for the early stages of colonization and persistence of bacteria in the *E. scolopes* symbiosis. (A preliminary report of this work has appeared previously [K.J. Boettcher, K.-H. Lee, and E.G. Ruby, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, I-80, p. 211]).

Materials and Methods

Bacterial strains and growth conditions. Symbiotic *V. fischeri* used in this study were isolated from *E. scolopes* light organs and are described in Table 1. Bacterial reference strains were: *Vibrio fischeri* strains MJ1 [39] and B61 [2], *V. anguillarum* 775 [43], *V. harveyi* B392 [32], *V. logei* ATCC 29985 [5], *V. vulnificus* 21 (R. Rosson), *V. orientalis* ATCC 33934 [52], *Photobacterium leiognathi* LN-1a [14], *P. phosphoreum* NZ11D [38], and *Rhizobium meliloti* 1021 [27]. Marine bacteria were routinely grown in a seawater-based medium (SWT) containing 5 g of tryptone, 3 g of yeast extract, and 3 ml of glycerol per liter of 70% (vol/vol) natural seawater, and solidified with 1.3% agar when desired.

DNA isolation and manipulation. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly [7], modified by Rodriguez and Tait [33]. Plasmid DNA from large-scale cultures (250 ml) was further purified by CsCl-ethidium bromide density gradient centrifugation [40]. Total genomic DNA was obtained by the procedure described by Ausubel et al. [3]. Restriction endonuclease analysis and routine agarose gel electrophoresis were performed according to standard methods [40]. For the detection of megaplasmid DNA, the vertical gel electrophoresis method of Eckhardt [16] and the horizontal gel electrophoresis method of Simon [44] were used. After transfer to nylon filters [46], DNA was hybridized as described below.

DNA-DNA hybridization. Dot blots were prepared by transferring equal amounts of cells to Nylon 66 Plus membranes (Hoefer Scientific Instruments, San Francisco, California) before cell lysis and subsequent binding of the released DNA [40]. For production of colony blots, planktonic bacteria were collected by passing between 1 and 20 ml of natural seawater through a 0.2-μm pore-sized membrane filter (Millipore Corporation, Bedford, Massachusetts) that was then placed on SWT agar medium. After colonies appeared, the cells were lysed and the DNA was bound to the filter. DNA probes were generated as previously described [23] and hybridized to prepared filters as described by Sambrook et al. [40]. After washing under high stringency conditions [40], the position and extent of probe hybridization was visualized by autoradiography.

Curing experiments. Attempts to cure *V. fischeri* ES114 of its plasmid DNA were performed by exposure to growth-inhibiting concentrations of acridine orange (10 μg/ml), ethidium bromide (75 μg/ml), and novobiocin (15 μg/ml) in SWT. After 48 h of growth, cells were plated onto SWT agar. For each treatment, between 10 and 20 individual colonies were subsequently screened for the presence of plasmid DNA as described above.

Iron-limited growth experiments. A plasmid-containing *V. fischeri* isolate (ES114) and a plasmidless isolate (ES191) were both tested for their ability to grow in iron-limited media. The iron-chelator, EDDA [ethylenediamine-di(*o*-hydroxyphenyl) acetic acid], deferrated by the method of Rogers [34], was added at concentrations between 5 and 25 μM to an artificial-seawater-based medium containing (per liter) 5 g of peptone, 3 g of yeast extract, and 3 ml of glycerol. Cells grown to mid-log phase in SWT were diluted into 50 ml of each of these iron-limited media to a final A_{600} of 0.01. The cultures were shaken at 25°C for 6 h, during which the optical density and luminescence of the cultures were monitored [8].

Antibiotic resistance screening. To investigate the possibility of plasmid-encoded antibiotic resistance, six different strains of *V. fischeri* (some with plasmids, some without) were screened for sensitivity to 19 antibiotics. After growth to mid-exponential phase in SWT broth, equal amounts of cells were spread onto the surface of an SWT agar plate. Antibiotic-impregnated disks (Difco) were dispensed onto the plates, and zones of inhibition were measured after a 24-h incubation at 27°C. Antibiotics tested were: penicillin, ampicillin, erythromycin, tetracycline, novobiocin, chloramphenicol, cephalothin, colistin, nystatin, gentamicin, cefamandole, bacitracin, nalidixic acid, carbenicillin, methicillin, oxacillin, polymyxin B, tobramycin, and nitrofuratoin.

Experimental infections. Experiments designed to determine the infectivity and competitiveness of different strains of *V. fischeri* were performed with a modification of a symbiotic-infection assay described previously [26]. Clutches of eggs laid by female *E. scolopes* were incubated in an aquarium containing California coastal seawater (CCS). Immediately upon hatching, the juvenile squid were transferred to vials containing 5 ml of CCS. Hatchlings maintained in this water did not become infected because of the low abundance of symbiosis-competent *V. fischeri* in CCS [26]. In the infection assay, SWT-grown cultures of various bacterial strains were diluted in CCS to a final concentration of approximately 10^3 cells/ml. The animals were then transferred into vials containing

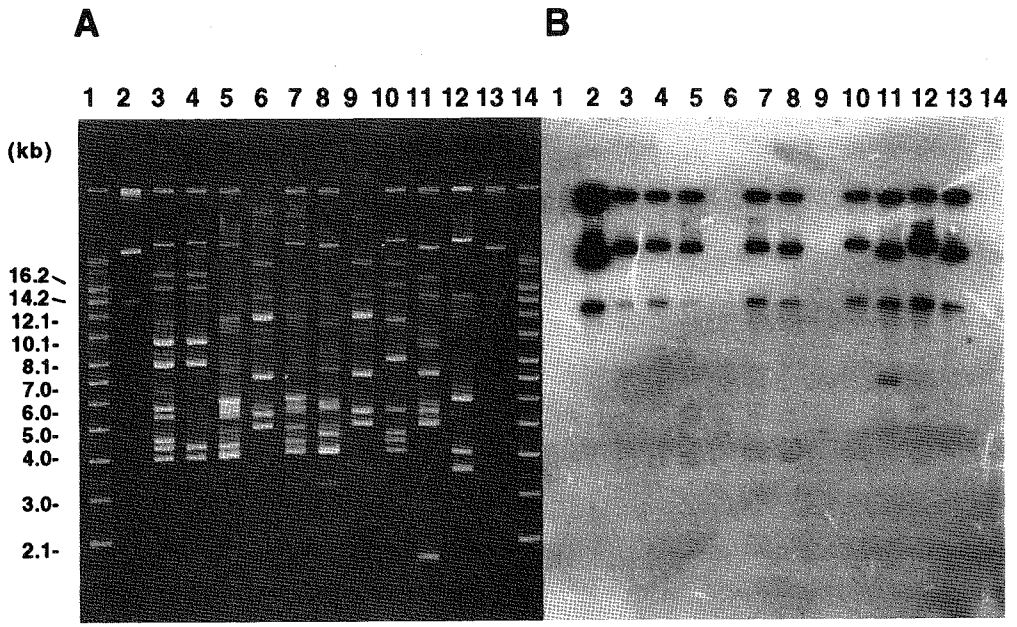

Fig 1. Agarose gel electrophoresis and Southern blot analysis of undigested CsCl-purified, plasmid DNA from 12 strains of symbiotic *V. fischeri*. (A) Ethidium bromide-stained gel. Between one and eight closed circular plasmids are detectable in each of these isolates. When present, the larger plasmids ($\geq$39 kb) also occur in the open circular conformation, which does not migrate out of the loading wells. Occasionally, small amounts of the linear forms of the large plasmids are visible as faint bands below their closed circular forms. Most of the smaller plasmids ($\leq$12 kb) appear both in the closed circular form and, in smaller amounts, as the more slowly migrating, open circular form. Lanes: 1 and 14, supercoiled DNA size-standard ladder; 2, ES114; 3, ES12; 4, ES79; 5, ES213; 6, ES235; 7, ES324; 8, ES334; 9, ES566; 10, ES595; 11, ES602; 12, ES620; 13, ES657. (B) Corresponding autoradiograph of DNA transferred from "A" and probed with radioactively labeled pES100. The top three hybridizing bands in lanes 2 through 5, 7, 8, and 10 through 13, represent the three conformations of the large plasmid present in each of these strains.

the inoculated CCS, and the infection process was monitored with a sensitive photometer. Successful colonization was indicated within a few hours by the initiation of, and rapid exponential increase in, luminescence by the squid [26]. At selected times after infection animals were sacrificed, and the number and identity of colony-forming units (CFU) arising on SWT plates spread with dilutions of homogenates of the colonized light organs were determined [36].

Results and Discussion

Plasmid carriage in symbionts. In total, 225 isolates of *V. fischeri* obtained from the light organs of 25 adult and juvenile *E. scolopes* were screened for the presence of plasmid DNA. Extrapolating from analyses of between 5 and 10 isolates from each light organ, only eight animals were found to harbor plasmid-carrying isolates exclusively, while four of the animals examined appeared to harbor symbiont populations without any plasmids. In sum, 56% of the bacterial isolates carried one or more plasmids. The occurrence of plasmids in planktonic isolates of *V. fischeri* has not been well documented; however, the rate of carriage is estimated to be approximately 30% [20, 45]. The comparatively high incidence of plasmids in the symbiotic *V. fischeri* suggested to us that plasmid DNA might be important at some stage in the development or maintenance of the symbiosis. Therefore, we analyzed the composition and potential role of plasmids in symbiotic *V. fischeri*.

A representative comparison of plasmid profiles obtained from 12 different strains isolated during the years 1988–1992 is shown in Fig. 1A. There was considerable variation both in the number and sizes of plasmids carried by isolates from different individual host squids. In contrast, plasmid banding patterns were generally similar among isolates from a given animal; however, there were instances in which as many as three types of plasmid profiles were detected among isolates from an individual host. A single large plasmid (which ranged in size from 39 to 50 kb) was present in 96% of those isolates carrying plasmids, and multiple small plasmids (12 kb or smaller) co-occurred with a large plasmid in 81% of plasmid-bearing strains. Two in-well lysis gel electrophoresis procedures were also employed and failed to reveal any very large plasmids (greater than 100 kb) in symbiotic *V. fischeri*, although megaplasmids from *R.*

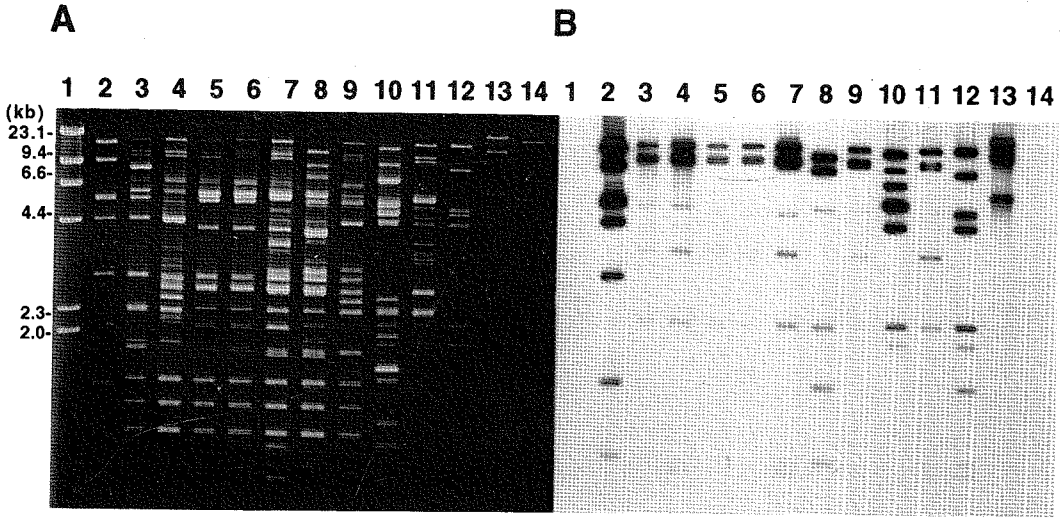

Fig 2. Comparison of the extent of sequence-relatedness of pES100 to the large ($\geq$39 kb) plasmids from 10 other isolates of symbiotic *V. fischeri*. (A) Ethidium bromide-stained gel of plasmid DNA. Lane 1, *Hin*dIII-restricted lambda DNA size markers. Lanes with *Hin*dIII-digested plasmid DNA: 2, ES114; 3, ES12; 4, ES79; 5, ES209; 6, ES213; 7, ES324; 8, ES334; 9, ES595; 10, ES602; 11, ES620; 12, ES657. Lane 13, *Bam*HI-restricted pES100; Lane 14, *Eco*RI-restricted lambda DNA size markers. (B) Corresponding autoradiograph of DNA transferred from "A", and probed with radioactively labeled pES100.

meliloti 1021 (each ~ 1500 kb) were detected (data not shown).

Plasmid characterization. Typically, plasmids involved in symbiotic or pathogenic associations tend to be relatively large in size. For this reason, we became interested in the sequence relatedness and possible role of the large plasmids carried by symbiotic *V. fischeri*. The *E. scolopes* symbiont *V. fischeri* ES114, a strain that harbors a single 39.3-kb plasmid designated pES100 [9], was chosen for the hybridization experiments described below.

Total plasmid DNA from 12 representative strains was separated by agarose gel electrophoresis and transferred to a membrane that was subsequently probed with purified pES100 DNA (Fig. 1B). Between one and eight plasmids are visible in the undigested preparations and do not (on the basis of restriction enzyme analysis) include concatamers. Although all of the large plasmids showed strong hybridization to the probe, only one plasmid of the smaller size class (the 7-kb plasmid from strain ES602) showed any sequence similarity to pES100 as indicated by hybridization. To further determine the extent of sequence-relatedness of these plasmids, the plasmid DNAs from a similar series of strains were digested with *Hin*dIII (Fig. 2A) and probed with labeled pES100 DNA. Despite the strong degree of hybridization evident between pES100 and the other large plasmids (Fig. 1B), the patterns of restriction products suggested that sequence divergence from an ancestral plasmid had occurred (Fig. 2B). Nevertheless, in each case the sum of the lengths of all the products approximated the predicted size of the unrestricted large plasmid from which they were derived. Thus, while some sequence divergence or rearrangements within these plasmids may have taken place, there was no evidence of major deletion or insertion events.

One possible explanation for the absence of plasmid DNA (such as the pES100-like plasmids) in some of the symbiotic *V. fischeri* strains was that such sequences had become integrated into the bacterial chromosome. To investigate this possibility we screened total DNA from cells of both plasmid-bearing and plasmidless strains by dot blot analysis. All but one of the six strains that had previously been shown to contain plasmid DNA also hybridized with the pES100 probe (data not shown). The nonhybridizing strain (ES240) was one of those that carries small plasmids but that lacks a representative of the typical, large plasmids (Table 1). Similarly, none of the 11 plasmidless strains tested exhibited detectable hybridization with pES100 DNA. Thus, it is unlikely that chromosomal integration of some or all of the DNA from a large pES100-like plasmid has occurred in any of the symbiotic *V. fischeri* tested. There was also no evidence of hybridization between pES100 and total DNA isolated from representatives of several other species of *Vibrio* (*V. anguillarum*, *V. logei*, *V. harveyi*, *V. orientalis*, and *V. vulnificus*) or luminous species of

the related genus *Photobacterium* (*P. phosphoreum* and *P. leiognathi*), indicating that this plasmid does not carry sequences that contribute to the general identity of either the species *V. fischeri* or the marine Vibrionaceae (data not shown).

Geographical range of the pES100-like plasmid. The pES100-like sequences are not widely distributed among the naturally occurring, culturable, marine bacteria. Seawater samples collected from various sites around *E. scolopes* habitats (in the Hawaiian Islands), and at sites that do not harbor populations of these animals (along the coasts of southern California and Massachusetts), were used to prepare colony blots that were subsequently hybridized with pES100 DNA. Although filters prepared from Hawaiian, California, and Massachusetts seawater contained many different kinds of colonies, including those arising from indigenous planktonic *V. fischeri* cells, hybridizing colonies were detected almost exclusively on filters prepared from water collected at sites inhabited by the host squid populations (Table 2). Only two colonies that showed a weak degree of hybridization to the probe were detected from water collected at a site that is not an *E. scolopes* habitat. In addition, dot blots were prepared with cells of 17 purified isolates of *V. fischeri* obtained from Hawaiian and Californian waters. In this instance, the only observable hybridization to the pES100 probe was to DNA from isolates obtained from Hawaiian water samples (data not shown). Thus, pES100-like plasmids appear to be harbored predominantly, if not exclusively, by *V. fischeri* (and perhaps other bacteria) in areas where there is a resident *E. scolopes* population.

Experimental infections. The common occurrence of pES100-like plasmids in the natural symbiotic association and their apparent absence in other related strains and species of bacteria suggested that the presence of some or all of the genes carried on this plasmid might confer competency to become a light organ symbiont of *E. scolopes*. To test for the correlation of plasmid presence with colonization effectiveness, eight strains of *V. fischeri* (four of which harbored plasmid DNA) were tested for their ability to initiate a symbiosis with juvenile squids. When presented alone, these strains were similarly effective at initiating a light organ association with juvenile *E. scolopes* (between 9 and 11 animals were included in each test group; the infection success was 92 ± 10% for strains with plasmids and 98 ± 5% for plasmidless strains).

Table 2. Distribution of pES100-like DNA sequences in heterotrophic, colony-forming bacteria from pure culture and coastal seawater sources

	Total colonies screened[a]	Estimated no. of *V. fischeri*[b]	Number with pES100[c]	% *V. fischeri* with pES100
ES114 suspension	150	150	150	100
Hawaiian seawater	1400	30	7	23
Californian seawater	1000	50	0	<2
Massachusetts seawater	4250	170	2[d]	1.2

[a] See Materials and Methods for description of colony blot preparation.
[b] Based on hybridization with *V. fischeri*-specific *luxA* probe (as previously described [31]).
[c] Based on hybridization with pES100 probe (see Materials and Methods).
[d] Colonies showed weak hybridization to the probe.

These results demonstrated that plasmid DNA is not necessary for the initial infection event when only one strain of *V. fischeri* is presented to each potential host. However, in their natural environment, juvenile squid are likely to encounter a number of different strains of planktonic *V. fischeri* before becoming fully colonized [24]. Thus, the possibility remained that, while not an absolute requirement, the carriage of plasmids by certain strains might place them at a competitive advantage over other strains in becoming established symbionts. To test this hypothesis, 20 juvenile squid were exposed to seawater that contained a mixture of equal numbers of bacteria with and without the pES100-like plasmid. There was no significant difference in the ability of plasmid-carrying strains to outcompete plasmid-free strains during the first 48 h of infection and colonization: 5 animals were colonized by the plasmid-carrying strain exclusively, 4 harbored only the plasmidless symbionts, and the remaining 11 animals carried a mixture of plasmid-carrying and plasmidless strains. Thus, as examined in the standard infection assay, pES100-like plasmids do not appear to mediate a significant event in the initial stages of the symbiosis between *V. fischeri* and *E. scolopes*. Futhermore, we consider it unlikely that the plasmids are involved in some later stage (i.e., after the initial 48 h of infection) in the symbiosis because of the lack of any correlation between the size (age) of field-caught host animals and the degree of plasmid carriage by their symbionts (data not shown). It is likely that the essential functions required by *V. fischeri* for its alternate niche as a light organ symbiont are chromosomally en-

coded, and the construction of chromosomal mutants has allowed the identification of these determinants to begin [19].

In spite of the apparent lack of a direct role for plasmid DNA in this symbiosis, several important insights can be gained from the results of this study. First, the dynamics of colonization and maintenance of symbiont populations by *E. scolopes* are more complex than previously assumed. Our results suggest that juvenile squids are capable of being colonized by more than one strain of bacteria. The presence of mixed populations of plasmid-carrying and plasmid-less strains, including populations with up to three distinct plasmid profiles, among symbionts in the light organ of an individual field-caught animal, indicates that multiple infection events do occur in nature. Because we did not observe any evidence for the loss or horizontal transfer of plasmid DNA among symbiotic *V. fischeri*, either in culture or during the initial 48 h after colonization of the light organ, it is unlikely that these differences within natural symbiont populations reflect post-infection events. The hypothesis of multiple inoculations has a structural basis in the fact that there are six pores on the surface of uncolonized juvenile light organs, each leading to a separate crypt that is available for colonization by symbiotic strains of *V. fischeri* [26, 28]. In short, the evidence suggests that the natural light organ association is probably not a "pure culture" in the strictest sense. We propose instead that the term "monospecific" be used to describe the light organ population of *E. scolopes* to distinguish it from bacterial light organ associations of fishes, for which a single inoculation event has been inferred. [29, 39].

Possible functions for plasmids. At present the role or function of plasmids in symbiotic *V. fischeri* remains unknown, although we have been able to eliminate some common plasmid-mediated phenotypes. For example, no difference in the ability to grow on rich media, single carbon source media, or media that were carbon or iron limited was observed between isolates that did or did not harbor a pES100-like plasmid (data not shown). In addition, no correlation was observed between plasmid carriage and resistance to a number of antibiotics. Of the 19 antibiotics tested (see Materials and Methods), both classes of strains were resistant to the same group of six (penicillin, ampicillin, nystatin, bacitracin, methicillin, and oxacillin), suggesting that these are intrinsic chromosomally encoded characteristics of the species *V. fischeri*. Finally, this plasmid does not appear to carry genes required for the normal regulation or expression of the bioluminescent phenotype because there was no detectable hybridization between pES100 and pPD201, a plasmid that apparently contains all of the essential genes of the *V. fischeri lux* regulon [15] (data not shown).

Cryptic plasmids are common in symbiotic *Pseudomonas*, *Agrobacterium*, and *Rhizobium* strains that have an important free-living phase outside of the plant host, and it is believed that these cryptic plasmids contribute in some unknown way to these species' persistence in the soil niche [1, 4, 31]. Similarly, the planktonic niche of Hawaiian *V. fischeri* plays a major role in the ecological cycle of these bacteria [25]. The almost exclusive confinement of the pES100-class of plasmids to *V. fischeri* from light organs and Hawaiian seawater (Table 2), the temporal stability over at least 4 years of these plasmids within the population of bacteria that occur as symbionts of Hawaiian squids, and the recalcitrance of these plasmids to curing by treatment in culture with acridine orange, ethidium bromide, or novobiocin (data not shown) all suggest that these plasmids must contribute in some way to the fitness of these strains when the bacteria find themselves outside of the light organ, perhaps while in the seawater environment between host associations [25].

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