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Competition between *Vibrio fischeri* Strains during Initiation and Maintenance of a Light Organ Symbiosis†

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Colonization of the light-emitting organ of the Hawaiian squid *Euprymna scolopes* is initiated when the nascent organ of a newly hatched squid becomes inoculated with *Vibrio fischeri* cells present in the ambient seawater. Although they are induced for luminescence in the light organ, these symbiotic strains are characteristically non-visibly luminous (NVL) when grown in laboratory culture. The more typical visibly luminous (VL) type of *V. fischeri* co-occurs in Hawaiian seawater with these NVL strains; thus, two phenotypically distinct groups of this species potentially have access to the symbiotic niche, yet only the NVL ones are found there. In laboratory inoculation experiments, VL strains, when presented in pure culture, showed the same capability for colonizing the light organ as NVL strains. However, in experiments with mixed cultures composed of both VL and NVL strains, the VL ones were unable to compete with the NVL ones and did not persist within the light organ as the symbiosis became established. In addition, NVL strains entered light organs that had already been colonized by VL strains and displaced them. The mechanism underlying the symbiotic competitiveness exhibited by NVL strains remains unknown; however, it does not appear to be due to a higher potential for siderophore activity. While a difference in luminescence phenotype between VL and NVL strains in culture is not likely to be significant in the symbiosis, it has helped identify two distinct groups of *V. fischeri* that express different colonization capabilities in the squid light organ. This competitive difference provides a useful indication of important traits in light organ colonization.

Vibrio fischeri is the specific bacterial symbiont colonizing the complex, light-emitting organs of a number of marine squid and fish species (6, 22, 23). The physiological and genetic bases for this example of host-symbiont specificity can be examined in the model association between *V. fischeri* and *Euprymna scolopes* (24), a sepiolid squid occurring in sandy-bottom reef habitats of the Hawaiian archipelago (29). It is believed that the animal host provides nutrients to its bacterial symbionts, which in turn produce the bioluminescence used for the nocturnal behavior of the squid (19). Because the bacteria are continuously growing within the light organ, the host is a source of expelled symbionts that appear in the ambient seawater (16, 23) and are thus available to provide the required inoculum for the initiation of the colonization of newly hatched aposymbiotic squids (14, 19, 34).

On the basis of their degree of bioluminescence in culture, two classes of *V. fischeri* isolates from Kaneohe Bay, Hawaii, a typical habitat for *E. scolopes*, have been described. One class includes the visibly luminous (VL) strains that are often present in coastal seawater (22) and that produce the brightly luminous colonies typical of this species. The second class of isolates is composed of non-visibly luminous (NVL) *V. fischeri* strains that can be either obtained directly from squid light organs (6) or isolated from seawater inhabited by the squids (15). The NVL strains do not produce VL colonies on laboratory media, because they are underproducers of autoinducer, the transcriptional activator of the *lux* regulon (6, 11). However, NVL strains are as brightly luminescent as VL strains in the light organ association (19). Other than their intrinsic levels of bioluminescence in culture, the VL- and NVL-type strains have no reported differences in growth characteristics, flagellation, or other physiological or biochemical traits (15, 16).

The absence of examples of VL *V. fischeri* strains among the several thousand symbiotic bacteria isolated from the light organs of numerous squids suggests that only the NVL strains of *V. fischeri* are recruited from the ambient seawater to become light organ symbionts of juvenile *E. scolopes*, even though VL *V. fischeri* strains are also present in the same environment. The basis for this prevalence of NVL strains over VL strains might be that (i) VL strains either are not effective or are much less effective than NVL strains at colonizing the light organs of juvenile squids, or (ii) if a light organ is colonized by representatives of both types of strains, the VL strain fails to compete with the NVL strain during their subsequent growth within the light organ. In this paper, we present results illustrating the dominance of NVL over VL *V. fischeri* strains in the light organ symbiosis and discuss the possibility that competition for limiting substrates provided by the host animal underlies this phenomenon. These studies demonstrate the dynamic and cyclical interactions of NVL *V. fischeri* strains with their symbiotic hosts throughout the life cycle of *E. scolopes*.

MATERIALS AND METHODS

Bacterial strains and media. *V. fischeri* isolates were obtained either from *E. scolopes* light organs or from seawater and were identified as described previously (6, 15). ESR1, a derivative of *V. fischeri* ES114 that is resistant to rifampin, was isolated and provided by J. Graf (University of Southern California). This stable mutant is indistinguishable from ES114 in symbiotic competence (10). Other *V. fischeri* strains used in this study, as well as their sources and relevant characteristics,

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TABLE 1. Bacterial strains used in this study

Strain	Source or feature	Reference or source
NVL *V. fischeri* strains		
ES114	*E. scolopes* light organ	6
ESR1	ES114 Rif^+ mutant	10
PP3	Hawaiian seawater	15
PP64	Hawaiian seawater	15
VL *V. fischeri* strains		
H905	Hawaiian seawater	15
H906	Hawaiian seawater	15
P. leiognathi		
H9035	Hawaiian seawater	This study

are listed in Table 1. Strain H9035 was isolated from Hawaiian seawater and was identified as *Photobacterium leiognathi.*

Bacterial cells were grown and maintained in a nutrient medium (SWT) consisting of tryptone (0.5% [wt/wt]), yeast extract (0.3% [wt/wt]), glycerol (0.3% [vol/vol]), and seawater (70% [vol/vol]) (21). When desired, the medium was solidified by the addition of 1.5% agar. A basal minimal medium (MM) modified from that of Dunlap et al. (8) contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 19 mM NH_4Cl, 0.33 mM KH_2PO_4, and 0.002% (wt/vol) ferric ammonium citrate in an artificial seawater solution consisting of 100 mM $MgSO_4$, 20 mM $CaCl_2$, 400 mM NaCl, and 20 mM KCl (21). Either glucose (final concentration of 3.4 mM) or cyclic AMP (cAMP) (final concentration of 2 mM) was added as the carbon substrate. After all of the components had been combined, the medium was filter sterilized by passage through a 0.2-μm-pore-size membrane filter. Comparisons of the VL and NVL strains of *V. fischeri* used in this study revealed no significant difference in growth rates in either the complex nutrient medium (SWT) or the glucose MM (16).

Infection experiments with pure cultures. Juvenile *E. scolopes* that are hatched and maintained in coastal Southern California seawater (CSW) remain uninfected, because this water does not intrinsically contain symbiosis-competent *V. fischeri* (19). Thus, colonization of the light organ can be experimentally initiated by the addition of an inoculum of symbiosis-competent *V. fischeri* cells to CSW containing juvenile animals (24).

Suspensions of laboratory-cultured *V. fischeri* cells were added to CSW to give a final concentration of approximately 10^3 CFU/ml. To initiate a colonization of the nascent light organ, individual uninfected juvenile squids were each transferred into 5 ml of this seawater in a glass scintillation vial and were maintained for a total of 12 h. The seawater was replaced with a fresh bacterial inoculum at 3-h intervals, during which time there was no significant change in the concentration of the *V. fischeri* inoculum (16). The kinetics of the subsequent colonization process were determined at 1- to 3-h intervals by monitoring both (i) the light production of juvenile animals with a sensitive photometer (Luminescence Photometer model 3000; Biospherical Instruments Inc.) (19) and (ii) the total CFU of symbiotic bacteria within homogenates of light organs after spreading a dilution of the homogenate on SWT agar medium.

Infection experiments with mixed cultures. For competition experiments, cell suspensions consisting of approximately equal concentrations (about 10^3 CFU/ml) of VL and NVL strains of *V. fischeri* were used as the bacterial inocula. Otherwise, the procedure for individually infecting juvenile squids was the same as that described above. The proportion of VL and NVL symbionts subsequently colonizing a light organ was determined by simply observing the percentage of visibly luminescent CFU arising from platings of light organ homogenates on SWT agar medium. As described previously (6, 15, 22), VL and NVL colonies are easily distinguished by viewing them in a darkened room. There has been no instance of phenotypic interconversion among the VL and NVL strains used in this study during 5 years of culture (16). Similar competition experiments were performed with two different pairs of VL and NVL strains: H905 and PP3 in one case and H906 and PP64 in another. In experiments involving strains H906 and PP64, several different ratios of mixed inocula were used for infection experiments; the 10:1 ratio contained 10^4 and 10^3 CFU of each strain, the 5:1 ratio contained 5×10^3 and 10^3 CFU of each strain, and the 1:1 ratio contained 10^3 and 10^3 CFU of each strain per ml.

Reinfection experiments. The ability of VL and NVL strains of *V. fischeri* to displace each other in an established symbiosis was determined as follows. Fourteen juvenile squids were inoculated with pure cultures of either a VL (H905) or an NVL (PP3) strain. At 12 h after this infection was established, pairs of animals, one colonized with a VL strain and the other colonized with an NVL strain, were placed in a single glass vial containing 5 ml of CSW. Because juvenile squids infected with VL and NVL strains release bacteria into the surrounding seawater at essentially the same rate (16), each animal was subsequently exposed to a mixture of these bacteria. At 13, 26, and 50 h after combination of the sets of juvenile pairs, the light organs of the animals in several vials were homogenized separately in sterile seawater, and serial dilutions were plated on SWT agar medium. The identities of the colonizing bacteria present in each light organ were determined by observing the proportion of visibly luminescent CFU that subsequently arose.

To examine whether juvenile squids colonized by one NVL *V. fischeri* strain could be secondarily infected by another NVL strain, 11 newly hatched juvenile squids were exposed to aquarium seawater that contained natural populations of NVL *V. fischeri* strains that had been expelled from the light organs of several adult squids. Subsequently, at 3, 4, and 5 days after they had been initially colonized, the symbiotic juvenile squids were challenged by placing them for 16 h in CSW containing strain ESR1 at a concentration of 2.4×10^4 CFU/ml. After an additional 24 or 48 h of incubation in fresh CSW, the light organs of these squids were homogenized, and serial dilutions of the homogenate were spread on agar medium of both SWT and SWT containing 50 μg of rifampin per ml. The latter medium was used to estimate the proportion of total symbionts that were the secondarily infecting, rifampin-resistant strain ESR1.

Similarly, to determine whether adult squids could be reinfected by ambient *V. fischeri*, four *E. scolopes* (mantle lengths of between 9 and 11 mm) collected from Kaneohe Bay were incubated in 4 liters of CSW, to which was added strain ESR1 at a concentration of between 2.4×10^4 and 4.3×10^6 CFU/ml. After 3 days, the squids were placed in fresh CSW for another day, after which they were rinsed in sterile seawater and aseptically dissected to obtain the light organs. The light organs were rinsed several times and homogenized in sterile seawater, and serial dilutions were plated on both SWT and rifampin-containing SWT agar medium.

Measurement of siderophore production. The production of siderophores by both NVL and VL *V. fischeri* strains was

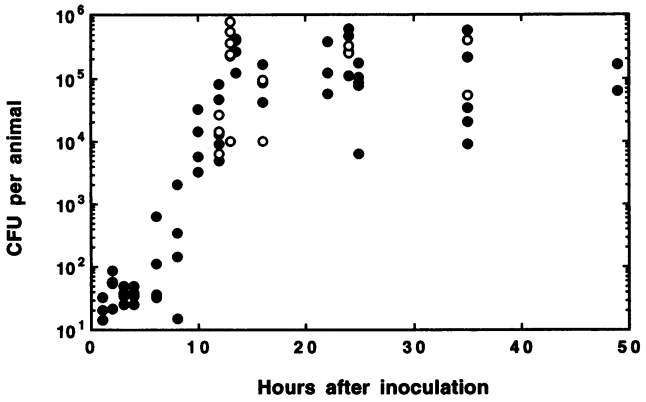

FIG. 1. Kinetics of colonization of juvenile *E. scolopes* light organs by pure cultures of *V. fischeri* PP3 or H905. The numbers of PP3 CFU (●) and H905 CFU (○) in each juvenile light organ were determined at various times after initiation of the colonization.

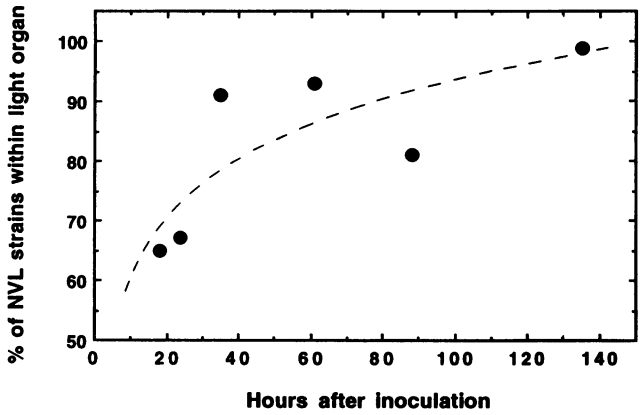

FIG. 2. Percentage of NVL strain of *V. fischeri* within the light organs after exposure to seawater containing equal numbers of NVL and VL strains during the development of the light organ symbiosis of juvenile *E. scolopes* hosts. At each time point, the composition of the symbiont population of each of the light organs of eight juvenile animals was determined as described in Materials and Methods. Immediately after exposure, approximately 50% of the total CFU recovered from the light organs of animals were the NVL strain PP3, while the remaining CFU were the VL strain H905. At various times during the subsequent 5 days, groups of animals were similarly assayed for the percentages of total CFU that were strain PP3.

detected on an agar medium containing chrome azurol S indicator (CAS agar) (26). The relative affinities of iron-chelating siderophores produced by both types of *V. fischeri* were determined by spotting a cell suspension (10 μl containing 1.5×10^7 CFU) onto CAS agar. Similarly, 10-μl portions of *V. fischeri* cells were spotted onto a lawn of cells of *P. leiognathi* H9035 (which does not produce a siderophore) growing on SWT agar. After 20 h of incubation at 28°C, the zone of color change on CAS agar and the zone of inhibition of lawn growth of H9035 on SWT agar were measured for each strain of *V. fischeri*.

Spent medium was prepared for cross-feeding assays from cells of PP3 or H905 grown in cAMP-based MM. At an optical density at 600 nm of 0.2, two cultures (50 ml each) were centrifuged at 8,000 × *g* for 5 min, and the supernatants were passed through a 0.2-μm-pore-size membrane filter to remove any remaining cells from the spent medium. PP3 cells pregrown in cAMP-based MM were inoculated into the spent medium of H905, and H905 cells pregrown in cAMP-based MM were inoculated into the spent medium of PP3. The subsequent growth of these cultures was monitored spectrophotometrically.

RESULTS

We compared the abilities of VL and NVL strains to individually colonize aposymbiotic, newly hatched juvenile squids. The kinetics of light organ colonization were determined by measuring the number of CFU present in the organ at 1- to 2-h intervals after the animals were exposed to seawater containing either the VL strain H905 or the NVL strain PP3. The colonization process observed when animals were inoculated with strain PP3 demonstrated the typical exponential pattern of growth previously observed for symbiosis-competent *V. fischeri* strains that had been isolated from adult squid light organs (19, 23). Colonization began with an initial 12 to 16 h of growth that matched these strains' typical rapid doubling times in culture (about 30 min at 23°C), producing a population of between 10^5 and 10^6 cells and was followed by a subsequent maintenance or slow increase in the maximum level of CFU in the light organ (Fig. 1). Strain H905 colonized newly hatched juvenile animals at least as rapidly (i.e., with an apparent doubling time of about 40 min), and to the same degree, as did strain PP3. Successful initial growth by H905 alone, PP3 alone, or a mixture of both strains was not significantly different, on the basis of a Kruskal-Wallis analysis (30). Thus, there was no evidence that the intrinsic colonization capability of a VL strain is less than that of a NVL strain.

To determine whether there was any competitive dominance in the colonization effectiveness of these two types of *V. fischeri*, 47 juvenile squids were individually exposed to seawater containing equal concentrations of both strains H905 and PP3. After the initial 12-h period of colonization, neither strain demonstrated an enhanced ability to initiate the symbiosis; e.g., there were approximately equal numbers of animals that appeared to be solely (i.e., >99% of the total light organ inhabitants) colonized by each strain, and in the light organs that had mixed populations, neither strain was consistently numerically dominant. However, during the subsequent 5 days of symbiotic development, a clear trend emerged that indicated the developing dominance of strain PP3 (Fig. 2). Continued examination of the symbiont populations during this period revealed that an increasing number of the CFU found in the light organs of juvenile squids was strain PP3. This result suggested that because exposure to the external mixed inoculum was experimentally terminated at 12 h, all of the animals must have had a small (although sometimes initially undetected by dilution plating [i.e., <5,000 cells per light organ]) level of colonization by PP3 at that time.

Similar data were obtained when another pair of VL (strain H906) and NVL (strain PP64) bacteria were compared in the same kind of experiment. Forty-one hours after infection, a time by which strain PP3 had exhibited dominance over strain H905 (Fig. 2), juvenile squids exposed to seawater containing a mixture of equal numbers of cells of PP64 and H906 were apparently colonized only by PP64. Only when animals were incubated with a 10-fold excess of H906 cells to PP64 cells were any juvenile light organs found to contain detectable numbers of H906 cells (Table 2), indicating that NVL strains can outcompete VL ones even when at a numerical disadvantage during the early stages of colonization.

TABLE 2. Infection of juvenile squids with mixed inocula of PP64 and H906 cells

Relative proportion of cells in the mixture (PP64-to-H906 ratio)	No. of juvenile squids colonized by[a]		
	PP64 only	H906 only	Both strains
10:1	3	0	0
5:1	3	0	0
1:1	8	0	0
1:5	3	0	0
1:10	2	0	1[b]

[a] Bacterial strains within the light organs of juvenile squids were checked and enumerated by plating dilutions of homogenates 41 h after infection with inocula consisting of different ratios of NVL PP64 and VL H906 cells (see Materials and Methods).

[b] This one juvenile squid had been infected by both strain PP64 and strain H906; only 29% of the total CFU within its light organ were strain H906.

To determine whether an NVL strain could enter an already colonized light organ and displace the cells of a VL strain, even after the VL strain had established a symbiosis with juvenile squids (i.e., 12 h after the initiation of colonization), we combined pairs of animals, one of which had been infected with PP3 and the other of which had been infected with H905. At 13, 26, and 50 h after the combination (i.e., 25, 38, and 62 h after initial symbiotic infection), the identities of symbionts inside light organs were determined by looking at the degree of luminescence of colonies arising from platings of serial dilutions of light organ homogenates. Thirteen hours after combination, there appeared to be no change in the composition of symbionts in either group of animals (because individual animals were not identifiable, it is formally possible that all of the PP3-infected animals could have become H905 infected at the same time that all of the H905-infected animals became PP3 infected, giving the appearance that no change had occurred by 13 h). Twenty-six hours after the combination, both of the initially H905-colonized squids must have become at least partially colonized by cells of strain PP3 that, presumably, had been expelled into the ambient seawater by the co-occurring PP3-colonized squid. In contrast, the initially PP3-colonized squids retained their original light organ populations, apparently pure, for at least an additional 50 h (Table 3).

Although NVL-type strains were not replaced by VL-type symbionts in these experiments, NVL-colonized squids could be secondarily colonized by other NVL *V. fischeri* strains. Two days after establishment of a symbiosis with NVL symbiont strains, squids were exposed to seawater containing a rifampin-resistant (Rif^+) mutant derivative of strain ES114, designated ESR1. This resistance marker made it possible to distinguish between CFU arising from cells of the initial colonization and those of cells that had secondarily infected the 2-day-old light organ. Light organ homogenates of 7 of the 11 animals secondarily exposed to the Rif^+ strain contained these cells within 48 h of the exposure. The proportions of Rif^+ cells varied considerably between light organs, ranging between 0.002 and 15% of the total CFU in the organ homogenates (Table 4). Thus, there is a continuing capacity for symbiosis-competent cells to enter and proliferate within the developing juvenile light organ.

TABLE 3. Secondary infection by either NVL or VL *V. fischeri* strains of established light organ symbioses[a]

H after combination	Result for initially PP3-colonized squids[b]		Result for initially H905-colonized squids[b]	
	H905 infected/ total	% H905	PP3 infected/ total	% PP3
13	0/2	<1	0/2	<1
26	0/2	<1	2/2	89
50	0/3	<1	3/3	33

[a] Pairs of juvenile squids, previously colonized by either strain PP3 or strain H905, were placed together to determine the ability of each strain to secondarily infect a light organ colonized by the other strain (see Materials and Methods).

[b] The extent of secondary infection is reported both as the number of light organs in which the second strain was detected, and as the percentage of the total symbiont population that was the second strain, at the indicated times after combination of pairs of animals. The sensitivity of detection was well below 1% in all cases.

TABLE 4. Secondary infection of light organs by Rif^+ *V. fischeri* ESR1

Type and size of animal	No. of animals tested	No. secondarily infected (%)[a]	% Rif^+ symbionts in light organ[b]
Juvenile (1–2 mm)	11	7 (63)	0.002, 0.01, 0.03, 0.53, 3.5, 8.0, 14.9
Adult (9–11 mm)	4	2 (50)	0.0001, 0.0004

[a] Numbers of animals (and the percentages of the total numbers tested) in which secondarily infecting Rif^+ symbionts were detected (see Materials and Methods).

[b] Percentage of the total symbiont population of each secondarily infected animal that was Rif^+.

The potential for *V. fischeri* in the ambient seawater to produce such a secondary colonization extends well beyond the juvenile developmental stage. Two of four adult squids exposed to the Rif^+ strain for 3 days showed evidence of secondary colonization (Table 4). However, in contrast to the case with juvenile squids, this colonization had occurred to only a minor extent (i.e., between 0.0001 and 0.0004% of the total light organ symbionts were Rif^+ cells). This much smaller degree of secondary colonization could reflect both the 100- to 1,000-fold-greater number of established symbionts in an adult (relative to a juvenile) light organ and the diminished access provided by the two pores connecting the ambient environment to the adult organ's symbiotic *V. fischeri* compared with the six pores present in the juvenile light organ (18, 19). These Rif^+ cells present in the adult organs were not simply the result of a spontaneous resistance mutation arising among the original light organ symbionts because (i) the estimated frequency of the spontaneous mutation was very small (less than 3×10^{-8}) and (ii) the Rif^+ cells isolated from these light organs at the end of the 3-day exposure carried the 39-kbp plasmid (pES100) that is characteristic of ES114, the parent strain of the Rif^+ mutant (5) used in the experiment (16). This plasmid (which does not bear the rifampin resistance) was not present in the endemic population of symbionts of these two adult squids. These data indicated that the light organ of *E. scolopes* continues to be an active, dynamic area of colonization and competition well into the development and maturity of the symbiotic association, a situation also reported for *Rhizobium* symbionts in the root nodule (27).

The competitive advantage exhibited by NVL strains of *V. fischeri* relative to VL strains could have its basis in a difference between the intrinsic abilities of the two groups of strains to obtain and utilize a nutritional substrate that is provided by the host squid, but whose availability is limited in the light organ environment. To determine whether these two groups of strains have different abilities to sequester free iron, which might be expected to be in short supply in host tissues, VL and

TABLE 5. Siderophore production by *V. fischeri* strains

Strain spotted onto agar	Zone of CAS color change (mm^2)[a]	Zone of H9035 growth inhibition (mm^2)[b]
V. fischeri		
PP3	7.1	2.5
PP64	7.1	3.1
H905	10.2	19.6
H906	8.0	15.2
P. leiognathi		
H9035	<0.01	<0.01

[a] Equivalent numbers of cells of the indicated strains were placed in each 10-μl spot. The area of apparently siderophore-mediated color change (from blue-green to orange) of CAS agar medium was calculated from a measurement of the diameter of the zone of orange around the spot of test cells (see Materials and Methods).

[b] The area of apparently siderophore-mediated inhibition of the growth of a lawn of cells of *P. leiognathi* H9035 by a spot of either *V. fischeri* cells or its own cells was calculated from a measurement of the distance from the center of the spot to the edge of the beginning of lawn growth.

NVL strains were grown on CAS agar. Both types of strains changed the color of the indicator (CAS) from blue-green to orange, which suggested that they both produced a siderophore. In addition, the growth of the Hawaiian isolate *P. leiognathi* H9035, a strain that did not produce the color reaction on CAS agar, was inhibited by the presence of all four strains of Hawaiian *V. fischeri* tested (Table 5), further suggesting that the production of *V. fischeri* siderophores decreased the local concentration of available iron below the growth-limiting level for strain H9035. Both the 29% greater area of CAS agar that changed color and the 537% greater area of *P. leiognathi* growth inhibition (Table 5) indicated that the VL strains produced even more siderophore activity during growth on either SWT or CAS agar media than did the NVL strains.

Additional evidence that VL strains produce even more siderophore activity than NVL strains was found from the cross-feeding experiments. Because siderophores produced by a bacterial strain often can be utilized by the same or related species (2, 13), we investigated the effects of inoculating PP3 or H905 cells into the spent medium of H905 or PP3, respectively. In the first few hours after inoculation, the culture density of strain PP3 placed in spent medium from H905 increased 3.1 times more than did strain PP3 placed in fresh, low-iron medium. In contrast, the culture density of strain H905 placed in spent medium from PP3 increased only 1.2 times more than H905 cells in the fresh medium. Therefore, the spent medium of strain H905 provided better growth conditions than that of strain PP3, probably because of the presence of more siderophore activity, which made free iron more available during the early phase of bacterial growth. Thus, the competitive advantage of NVL strains over VL ones during light organ colonization does not appear to be due to a greater iron-sequestering capability of NVL strains.

DISCUSSION

Two distinct types of *V. fischeri* strains have been described on the basis of the intensity of their luminescence in laboratory culture: the typical VL strains and the more recently discovered NVL ones (6). Although both types of *V. fischeri* occur in the natural environment of the symbiotic squid *E. scolopes* (15), during a 3-year survey of more than 100 field-collected animals (from 1989 to 1991), none of these hosts was found to have light organs colonized by VL strains of *V. fischeri*. At first we hypothesized that these VL-type strains might be symbiosis incompetent, as has been reported for certain other strains of *V. fischeri* (19). However, when individually tested in the standard laboratory infection assay, VL strains were as effective and rapid as NVL strains at colonizing the light organs of newly hatched juvenile squids (Fig. 1). Taken together, these observations suggested that the absence of VL bacteria within host light organs in nature might result from the presence of competing NVL *V. fischeri*.

There have been numerous studies of the competitive dominance of different strains of nitrogen-fixing bacteria of the genera *Rhizobium* and *Bradyrhizobium* in the symbiotic root nodule association. The results have indicated that certain strains are able to dominate the nodulation of legume roots in the presence of other congeneric strains that co-occur in the rhizosphere (1, 17). Among the many phenotypic characteristics of root nodule bacteria that have been reported to be important in nodulation competitiveness are bacterial surface exopolysaccharides (4), motility (20), and trifolitoxin production (32). Transport and utilization of C_4-dicarboxylic acids produced by host plants' photosynthesis have been found to be essential for effective nodulation by nitrogen-fixing symbionts (11), although there is no information about competition among *Rhizobium* strains or mutants with different abilities to use the host-derived nutrient. Some unknown function, associated with the gene *nifA*, has also been documented to affect nodulation competitiveness in *Rhizobium meliloti* (33). Much less has been discovered about the mechanisms underlying competitive dominance in other cooperative bacterial symbioses.

While we found no evidence that the presence of NVL strains of *V. fischeri* could affect the ability of VL strains to initiate the early events in light organ colonization, subsequent development of the symbiosis uniformly led to an eventual dominance of all of the light organ associations by NVL strains. Moreover, even when a VL strain had established itself in a light organ as a pure culture, within a few days it would be subsequently displaced (either partially or entirely) by an NVL strain to which the association was secondarily exposed. Conversely, the reciprocal replacement of an NVL strain by a VL one was never observed. Therefore, we sought to define the underlying mechanism(s) that might explain the competitive dominance expressed by NVL strains.

Free iron is an important and essential nutrient for the growth of *Vibrio* species interacting with animals, especially because its availability to bacteria in animal tissues is typically limited (2, 28, 31). Thus, it is not surprising that symbiotic *V. fischeri* strains produce siderophore activity that allows them to grow in conditions of reduced iron availability (Table 5). While iron depletion has also been hypothesized as a mechanism for regulating the luminescence of *V. fischeri* in the monocentrid fish light organ symbiosis (12), iron limitation has no apparent effect on the induction of light production by symbionts of *E. scolopes* (6). In addition, iron limitation does not appear to be an important factor underlying the competition among different *V. fischeri* bacteria for colonization of the squid light organ because, at least in culture, the competitively dominant NVL symbiotic strains actually produce less siderophore activity than VL strains of *V. fischeri*.

The source(s) of carbon and energy supplied by any animal host to its symbiotic light organ bacteria has yet to be determined. Glucose and cAMP have both been hypothesized to be host-provided nutrients for symbiotic *V. fischeri* within the light organs of monocentrid fishes (8, 25). A comparison of the growth rates of *V. fischeri* cells in a glucose MM revealed no significant difference between the two pairs of VL and NVL

strains (1.1 ± 0.1 and 1.1 ± 0.04 doublings per h, respectively [16]), suggesting that while it might be provided by the squid host, there is no evidence that glucose could function as a competitively limiting substrate in this symbiosis.

cAMP also has been hypothesized to be the host-supplied nutrient for *V. fischeri*-containing light organ symbioses (7, 8). This ability appears to result from a high level of activity of the novel periplasmic enzyme, 3′:5′-cAMP phosphodiesterase in *V. fischeri* strains (3, 8). Preliminary physiological growth data suggest that NVL strains have a competitive growth advantage in a cAMP-containing MM relative to VL strains (16). To test the validity of this hypothesis in the *E. scolopes* symbiosis, a mutant NVL strain with a decreased capacity for cAMP utilization will be essential.

In the natural habitat of *E. scolopes* (e.g., Kaneohe Bay, Oahu, Hawaii), the average abundance of NVL and VL *V. fischeri* strains has been estimated to be approximately 200 and 1 CFU/100 ml of seawater, respectively (16). This predominance of NVL *V. fischeri* strains is believed to be driven by the expulsion of large numbers of symbionts by the intrinsic population of *E. scolopes* (14). The resulting numerical dominance of NVL strains in seawater and sediments in squid habitats could in turn potentiate, and further tend to maintain, the natural propensity of *E. scolopes* to harbor this group of *V. fischeri*. Nevertheless, in the laboratory we occasionally obtain squids that continue to be colonized by both VL and NVL *V. fischeri* strains, even 41 h after the establishment of the symbiosis (Table 2). Thus, although it is rare, colonization of an occasional light organ by a VL *V. fischeri* strain might be expected to occur in the natural environment. However, such a symbiont population probably would be subject to eventual displacement by secondarily colonizing NVL strains.

We propose that there are at least two ecologically distinct populations of *V. fischeri* in habitats of the squid *E. scolopes*. NVL-type strains have an ecological cycle consisting of both a symbiotic phase and a planktonic phase, while the VL-type strains are essentially confined to the planktonic niche. Newly hatched, aposymbiotic squids must be colonized by *V. fischeri*, and the resulting symbiotic animals subsequently expel excess symbionts into the surrounding seawater and sediments. These expelled symbionts serve as the important bacterial inoculum that initiates the symbiosis of new generations of *E. scolopes* and may continuously interact with later stages of the *E. scolopes* life cycle. Thus, the ecology of an NVL *V. fischeri* strain in habitats containing *E. scolopes* may be not only interconnected with but also interdependent on the life cycle of its host animal.

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