APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2000, p. 3550–3555 Vol. 66, No. 8
0099-2240/00/$04.00+0

Temperature Affects Species Distribution in Symbiotic Populations of *Vibrio* spp.

MICHELE K. NISHIGUCHI*

Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003-8001

Received 17 March 2000/Accepted 13 May 2000

The genus *Sepiola* (Cephalopoda: Sepiolidae) contains 10 known species that occur in the Mediterranean Sea today. All *Sepiola* species have a light organ that contains at least one of two species of luminous bacteria, *Vibrio fischeri* and *Vibrio logei*. The two *Vibrio* species coexist in at least four *Sepiola* species (*S. affinis*, *S. intermedia*, *S. ligulata*, and *S. robusta*), and their concentrations in the light organ depend on changes in certain abiotic factors, including temperature. Strains of *V. fischeri* grew faster in vitro and in *Sepiola* juveniles when they were incubated at 26°C. In contrast, strains of *V. logei* grew faster at 18°C in culture and in *Sepiola* juveniles. When aposymbiotic *S. affinis* or *S. ligulata* juveniles were inoculated with one *Vibrio* species, all strains of *V. fischeri* and *V. logei* were capable of infecting both squid species at the optimum growth temperatures, regardless of the squid host from which the bacteria were initially isolated. However, when two different strains of *V. fischeri* and *V. logei* were placed in direct competition with each other at either 18 or 26°C, strains of *V. fischeri* were present in sepiolid light organs in greater concentrations at 26°C, whereas strains of *V. logei* were present in greater concentrations at 18°C. In addition to the competition experiments, the ratios of the two bacterial species in adult *Sepiola* specimens caught throughout the season at various depths differed, and these differences were correlated with the temperature in the surrounding environment. My findings contribute additional data concerning the ecological and environmental factors that affect host-symbiont recognition and may provide insight into the evolution of animal-bacterium specificity.

The existence of symbiotic associations between eukaryotic hosts and microbial partners has long intrigued biologists (1, 8, 9). The diversity and broad range of each host-symbiont pair demonstrate how each partner has coevolved to exploit new ecological niches and to provide a new capability or function for the newly adapted individual or population (10, 25, 34). Although a number of symbiotic partnerships have evolved as parts of adaptations to particular ecological niches, there has always been the question of whether the ecology or the specificity of the partnership drives the evolutionary radiation of the hosts and their symbionts. Do environmental factors contribute to the evolution of independent populations that eventually leads to speciation? If a preference exists between a symbiont and a host, how is specificity maintained in associations in which the partnership is either promiscuous or environmentally transferred?

Studies investigating the nature of symbiotic associations are beginning to answer some of these questions and to provide clues to the underlying mechanisms of host-symbiont cospeciation (11, 15, 16, 26). One example that is shedding light on the evolutionary history of symbiotic associations is the partnership between a group of shallow-water benthic squids (family Sepiolidae) and their luminous bacterial symbionts (genus *Vibrio*) (22, 23). This system has provided molecular and physiological evidence for the coordinated influence of bacteria on the parallel evolution and specificity of closely related host species (20, 31). Until recently, all luminous bacterium-animal mutualisms were thought to be monotypic; i.e., it was thought that only one species of bacterium was associated with a particular host taxon (27, 33). However, Fidopiastis et al. (12) discovered a new species of luminous bacteria that resides in the light organs of several species of Mediterranean sepiolid squids (genus *Sepiola*), which results in a two-species consortium (21) (*Vibrio fischeri* and *Vibrio logei*). This unique and interesting finding is the first observation of two luminous species of bacteria residing in the light organs of sepiolid squids. What is peculiar about the two-symbiont relationship is that the two *Vibrio* species differ not only in their 16S ribosomal DNA (rDNA) genotypes but also in some of their physiological characteristics (12). Most notably, the in vitro growth rates of *V. logei* isolates are higher than the in vitro growth rates of *V. fischeri* isolates at lower temperatures (18°C), whereas at higher temperatures (26°C) *V. fischeri* isolates grow faster. Therefore, *V. logei* symbionts are psychrophilic compared to *V. fischeri* symbionts found in *Sepiola* species. There are several Mediterranean *Sepiola* species that live in the same coastal habitat, and this provides an opportunity to test the present state and nature of the symbiont population. Does the variety of *Sepiola* species have an effect on the concentration and abundance of *Vibrio* symbionts in the sepiolid light organs, or do ecological factors (e.g., temperature) have a greater effect on the presence and dominance of either symbiont in sepiolid light organs?

MATERIALS AND METHODS

Collection of specimens of *Sepiola* species and isolation of *Vibrio* light organ symbionts. Specimens of sepiolid squids (Cephalopoda: Sepiolidae) (Table 1) (28) were collected from depths of 20 to 75 m within 10 km of shore near the Laboratoire Arago, Banyuls-sur-Mer, France. The water temperature and depth were recorded at the time of collection in each case. The adult *Sepiola* specimens were collected during May (*S. affinis*) and during July and September (*S. affinis*, *S. intermedia*, *S. ligulata*, and *S. robusta*) (Table 2). The mantle lengths in adult specimens ranged from 10 to 30 mm. All specimens were identified by using the criteria described by Bello (2). Squids were either flash frozen in liquid nitrogen to preserve the bacteria in the light organs or kept alive until dissection and isolation of the light organ symbionts. Once frozen, the bacterial light organ maintains the integrity of the bacteria and can be used later for culturing bacteria in future analyses (12, 29, 33).

Once animals were collected, individual strains of *V. fischeri* and *V. logei* were isolated from adult specimens of *S. affinis*, *S. intermedia*, *S. ligulata*, and *S. robusta*

* Mailing address: Department of Biology, New Mexico State University, Box 30001, MSC 3AF, Las Cruces, NM 88003-8001. Phone: (505) 646-3721. Fax: (505) 646-5665. E-mail: nish@nmsu.edu.

TABLE 1. European *Sepiola* species[a]

Species	Habitat	Location	Symbiont	Strains isolated
Sepiola affinis Naef, 1912	Shelf	Mediterranean Sea	*V. fischeri*	SA1, SA8
			V. logei	SA6, SA12
Sepiola atlantica d'Orbigny, 1839	Shelf-edge of slope	Northeast Atlantic Ocean	?	NA[b]
Sepiola aurantiaca Jatta, 1896	Outer shelf and upper bathyal	Southern Norway and Mediterranean Sea	?	NA
Sepiola intermedia Naef, 1912	Shelf	Mediterranean Sea	*V. fischeri*	SI2, SI4
			V. logei	SI7, SI5
Sepiola knudseni Adam, 1984	Inner shelf	Northwest and West Africa	?	NA
Sepiola ligulata Naef, 1912	Shelf-edge of slope	Mediterranean Sea and Adriatic Sea	*V. fischeri*	SL2, SL8
			V. logei	SL4, SL12
Sepiola pfefferi Grimpe, 1921	Shelf	West Africa and Mediterranean Sea	?	NA
Sepiola robusta Naef, 1912	Outer shelf	Mediterranean Sea	*V. fischeri*	SR5, SR10
			V. logei	SR1, SR18
Sepiola rondoleti Steenstrup, 1856	Shelf and upper bathyal	Mediterranean Sea	?	NA
Sepiola steenstrupiana Levy, 1912	Upper sublittoral	Mediterranean Sea	?	NA

[a] Adapted from the data of Nesis (28) with permission from the publisher (TFH Publications, Inc.).
[b] NA, not available or collected in this study.

as previously described (5, 12) (Table 1). For live specimens, each squid was anesthetized, and the light organ was removed and subsequently homogenized in sterile seawater. Aliquots of the homogenate were spread onto seawater-tryptone-yeast extract (SWT) agar medium (5), and duplicates were incubated at 18 and 26°C overnight. Frozen light organs were also homogenized in this manner, and diluted homogenates were spread onto SWT media to determine the concentrations of individual *Vibrio* species. Both *V. fischeri* and *V. logei* strains were isolated from the preparations incubated at 18 and 26°C, and all strains used in the competition experiments were isolated at the same temperature so that the relative growth abilities of the strains were not biased. Once organisms were isolated, golden yellow colonies were observed on all plates, and differences in colony size were observed for some of the isolates.

Chelex DNA isolation methods for the *Vibrio* symbionts were used to obtain templates for PCR amplification (31). Briefly, DNA from an individual isolate was extracted by placing a single colony in 200 μl of 20 mM Trisbase–0.05 mM EDTA buffer (pH 7.4) containing 5% (wt/vol) Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.). The cells were homogenized with an autoclaved pestle to prevent contamination, and the homogenate was incubated at 80°C for 25 min and then boiled for 10 min to denature proteins and lyse the cells. The cell debris and Chelex 100 were pelleted by centrifugation, and the supernatant fluid containing the DNA was used as the bacterial DNA template for PCR. The species identities of individual *Vibrio* colonies were confirmed by PCR amplification of a region of the 16S rDNA (designated region V1) that differentiates *V. fischeri* from *V. logei* (12). The PCR products were visualized by ethidium bromide staining by using a UV illuminator, and size differences were determined by using the amplified bands (*V. fischeri*, 121 bp; *V. logei*, 111 bp). Once identified, individual strains were isolated and stored for use in infection and competition studies. The remaining colonies were used for colony lifts for identification of *V. fischeri* and *V. logei* strains.

Growth studies. All of the bacterial strains used in this study were inoculated into 5-ml starter cultures, which were then transferred into flasks containing 50 ml of SWT broth so that the final optical density at 600 nm was approximately 0.01 (5×10^7 cells/ml). The cultures were shaken at 225 rpm and were grown at either 18 or 26°C. Optical density was measured periodically throughout the logarithmic growth phase. Each strain of *V. fischeri* or *V. logei* used in this study was isolated from a *Sepiola* adult obtained from the Mediterranean Sea (Table 1) and was identified by using the methods of Fidopiastis et al. (12) and the methods described above.

Competition and infection experiments. To determine whether temperature has a direct effect on colonization potential and symbiont composition, several symbiotic strains were analyzed to determine their competitive abilities to infect light organs of juvenile *Sepiola* squids (*S. affinis* and *S. ligulata*) by using a standard colonization method (19, 32). These isolates were used to test whether the species of host determined the symbiotic composition or whether temperature was a significant factor in establishing symbiotic competence. Newly hatched *S. affinis* or *S. ligulata* juveniles were placed in vials containing 5 ml of seawater that was inoculated with 10^3 CFU of either one strain or, in competition experiments, two strains of symbiotically competent bacteria. The bacteria and squids were incubated at 18 and 26°C. After 12 h of incubation, the juvenile squids were transferred to vials containing 5 ml of seawater without symbiotic bacteria. Since bacterial cells from light organ homogenates have 100% plating efficiency (32), the actual extent of colonization could be calculated from the number of CFU arising from aliquots of light organ homogenates that were plated onto seawater nutrient agar medium (5, 6). After 48 h of incubation, juvenile squids were homogenized, and dilutions of the homogenates were plated onto seawater-tryptone agar to determine the number of *Vibrio* cells resulting from either single-strain infections or two-strain competition infections (19). The relative abundance of each strain (inter- or intraspecies) in a light organ was determined

TABLE 2. Total light organ concentrations of *V. fischeri* and *V. logei* in field-caught *Sepiola* adults

Squid species[a]	Depth (m)	Temp (°C)	CFU/adult light organ[b]		Ratio of *V. fischeri* to *V. logei*	Month and yr caught
			V. fischeri	*V. logei*		
S. affinis (1)	15	18	6.1×10^9	2.4×10^9	72:28	May 1999
S. affinis (1)	20	22	2.4×10^9	6.3×10^8	79:21	July 1995
S. affinis (7)	10	22	$7.5 \times 10^9 \pm 6.6 \times 10^8$	$9.1 \times 10^8 \pm 2.1 \times 10^7$	89:11	July 1999
S. affinis (2)	20	18	$5.1 \times 10^8 \pm 4.3 \times 10^8$	$3.2 \times 10^8 \pm 1.2 \times 10^7$	61:39	September 1995
S. intermedia (2)	75	16	$4.8 \times 10^8 \pm 5.4 \times 10^7$	$8.3 \times 10^9 \pm 3.4 \times 10^9$	6:94	July 1999
S. intermedia (2)	60	12	$8.4 \times 10^7 \pm 4.6 \times 10^2$	$2.1 \times 10^9 \pm 1.2 \times 10^8$	4:96	September 1995
S. intermedia (1)	55	12	1.0×10^9	3.8×10^9	21:79	September 1998
S. ligulata (1)	40	16	2.1×10^{10}	7.9×10^{10}	21:79	July 1999
S. ligulata (3)	55	10	$7.1 \times 10^{10} \pm 3.2 \times 10^7$	$1.8 \times 10^{11} \pm 2.9 \times 10^6$	28:72	September 1998
S. robusta (1)	60	12	5.1×10^9	4.2×10^{10}	11:89	July 1995
S. robusta (2)	60	10	$5.4 \times 10^9 \pm 1.5 \times 10^9$	$7.1 \times 10^{10} \pm 4.5 \times 10^9$	7:93	September 1998

[a] The numbers in parentheses are the numbers of adult animals used to calculate bacterial concentrations.
[b] When more than one animal was used to calculate concentrations, the values are means ± standard deviations.

TABLE 3. Growth constants for *V. fischeri* and *V. logei* strains isolated from European *Sepiola* species

Bacterial strain	Species	Host squid	Growth rate constant at 18°C (h^{-1})	Growth rate constant at 26°C (h^{-1})
SA1	*V. fischeri*	*S. affinis*	0.6	0.8
SA8	*V. fischeri*	*S. affinis*	0.6	1.0
SA6	*V. logei*	*S. affinis*	0.9	0.7
SA12	*V. logei*	*S. affinis*	0.8	0.7
SL2	*V. fischeri*	*S. ligulata*	0.4	1.3
SL8	*V. fischeri*	*S. ligulata*	0.5	1.3
SL4	*V. logei*	*S. ligulata*	1.2	0.9
SL12	*V. logei*	*S. ligulata*	1.2	0.9
SI2	*V. fischeri*	*S. intermedia*	0.6	1.1
SI4	*V. fischeri*	*S. intermedia*	0.6	1.0
SI5	*V. logei*	*S. intermedia*	0.9	0.7
SI7	*V. logei*	*S. intermedia*	0.9	0.7
SR5	*V. fischeri*	*S. robusta*	0.4	0.9
SR10	*V. fischeri*	*S. robusta*	0.5	0.9
SR1	*V. logei*	*S. robusta*	1.4	1.0
SR18	*V. logei*	*S. robusta*	1.3	0.9

by either visual luminescence of the CFU (30, 31) or by probing for the V1 variable region of the 16S rDNA (12) by using direct colony lifts of the plated light organ homogenates (18). The V1 region probe was labeled by tailing oligonucleotide primers (digoxigenin-dUTP; Boehringer Mannheim) and was hybridized to the colony blots at 58°C overnight. The blots were washed under stringent conditions (15 mM NaCl, 0.1 mM EDTA, 1% sodium dodecyl sulfate; 60°C), and colonies were detected with the fluorescent substrate Vistra ECF (Amersham). All imaging in this study was performed with a Molecular Dynamics model Storm 860 PhosphorImager. The percentages in each competition experiment were calculated for each juvenile squid (30 squids/competition experiment), and the values were averaged and arcsine transformed to test for significance (36).

Determination of symbiont composition in wild *Sepiola* specimens. Adult *Sepiola* specimens were collected at various times throughout the year (Table 2), and the light organ composition of each specimen was examined. All adult specimens were identified by using morphological characteristics, as described by Bello (2). The adult light organs and ink sac were dissected out of the body cavity of each squid and frozen in liquid nitrogen until proper analysis could be completed at the home institution. After freezing, each adult light organ was placed in sterile seawater or SWT medium and homogenized. Serial dilutions were made in order to obtain a reasonable number of CFU to plate onto SWT agar. The light organ homogenates were incubated at both 18 and 26°C to determine whether temperature had a pronounced effect on the relative abundance of each *Vibrio* species. Individual CFU were isolated from the homogenates, and colony lifts were used for analysis with the V1 region 16S rDNA probe in order to distinguish between *V. fischeri* and *V. logei* from individual light organs (as in the competition assays). In addition to the V1 region 16S rDNA probe analysis, phenotypic typing of either *V. fischeri* or *V. logei* strains was accomplished by using the protocol described by Nishiguchi et al. (30).

RESULTS

Comparison of populations of *V. fischeri* and *V. logei* in adult sepiolids. An analysis of the adult light organ contents of four *Sepiola* species revealed that both *Vibrio* species were present in all of the sepiolid species examined (Table 2). The light organ populations of all *S. affinis* specimens were primarily composed of *V. fischeri*, independent of depth or temperature. The concentrations of *V. fischeri* in *S. affinis* squids ranged from 98 to 99% of the total light organ isolates. The water temperatures at the time of collection for all of the *S. affinis* adults ranged from 18 to 22°C, and individuals were collected at depths of 20 m or less (Table 2). For all other species of squids (*S. intermedia*, *S. ligulata*, and *S. robusta*), the majority of the symbiotic bacteria found in the light organs were *V. logei* (Table 2). The *V. logei* strains isolated from these species of squids accounted for between 95 and 99% of the total light organ contents during all collection periods (May to September). These squid species were collected at depths ranging from 40 to 75 m, and the temperatures ranged from 10 to 16°C. During the collecting season, the surface temperatures ranged from 24°C at the height of summer to 10°C in the winter months. Although summer sea surface temperatures are much higher than winter surface temperatures, a distinct thermocline was formed between June and September in all years that samples were obtained. At this time, the water temperatures at depths below 25 m ranged from 12 to 16°C.

Growth of *V. logei* and *V. fischeri* in vitro. Table 1 shows all of the host squids and the bacterial strains isolated and used in this study. Each strain of *V. fischeri* or *V. logei* was tested for growth at two different temperatures (18 and 26°C) in SWT medium. Due to the relative psychrophily of *V. logei* (12), the growth constants were expected to be higher for *V. logei* at colder temperatures (<20°C) and higher for *V. fischeri* at warmer temperatures (>22°C). The growth constants for all strains of *V. fischeri* ranged from 0.4 to 0.6 h^{-1} at 18°C, whereas the growth constants were between 0.8 and 1.3 h^{-1} for the same strains grown at 26°C (Table 3). The *V. logei* strains used in this study had optimal growth constants that ranged from 0.9 to 1.4 h^{-1} at 18°C and from 0.7 to 1.0 h^{-1} at 26°C in culture. All strains were initially isolated from different species of squid hosts on SWT agar at 18 and 26°C in order to avoid any bias which favored specific temperature-acclimated strains.

Host specificity of *Vibrio* strains. In addition to the individual tests of bacterial strains in situ, I performed experiments to determine whether strains isolated from one species of squid were specific to their native host and if host specificity provided a competitive advantage when the native squid species or a nonnative species was infected. No differences in infection competency were observed between *S. affinis* and *S. ligulata* juveniles with *V. fischeri* native strains SA1, SA8, SL2, and SL8 at either 26 or 18°C (Table 4). *V. fischeri* strains isolated from *S. intermedia* or *S. robusta* squids (designated SI and SR strains) did not exhibit any preference for either of the two nonnative juvenile squid hosts at these temperatures (Table 4). Similarly, no significant differences in specificity were observed with *S. affinis* and *S. ligulata* juveniles when they were infected

TABLE 4. Infection of *Sepiola* juveniles with *V. fischeri* strains

Temp (°C)[a]	*V. fischeri* strain	Concn in squid host species (10^5 CFU/squid) after 48 h	
		S. affinis	*S. ligulata*
26	SA1	4.1	4.5
	SA8	4.0	4.0
	SL2	4.1	3.9
	SL8	3.7	3.9
	SI2	4.4	4.0
	SI4	4.2	4.1
	SR5	3.8	4.2
	SR10	3.9	3.9
18	SA1	0.24	0.20
	SA8	0.31	0.35
	SL2	0.30	0.34
	SL8	0.31	0.34
	SI2	0.28	0.25
	SI4	0.31	0.33
	SR5	0.19	0.22
	SR10	0.24	0.23

[a] Preparations were incubated at 18 or 26°C.

TABLE 5. Infection of *Sepiola* juveniles with *V. logei* strains

Temp (°C)[a]	*V. logei* strain	Concn in squid host species (10^5 CFU/squid) after 48 h	
		S. affinis	*S. ligulata*
26	SA6	0.09	0.07
	SA12	0.09	0.09
	SL4	0.08	0.07
	SL12	0.07	0.09
	SI5	0.07	0.07
	SI7	0.06	0.08
	SR1	0.07	0.08
	SR18	0.07	0.06
18	SA6	2.2	2.1
	SA12	2.4	2.3
	SL4	1.8	1.7
	SL12	1.9	1.7
	SI5	1.7	1.5
	SI7	1.6	1.9
	SR1	2.0	1.8
	SR18	2.0	1.9

[a] Preparations were incubated at 18 or 26°C.

with the native strains *V. logei* SA6, SA12, SL4, and SL12 at 26 or 18°C (Table 5). Again, *V. logei* strains from *S. intermedia* and *S. robusta* (SI5, SI7, SR1, and SR18) did not exhibit any specificity for juveniles of either of the two squid species examined (Table 5).

In addition to strain specificity, all *V. fischeri* and *V. logei* strains were tested to determine their infectivity at 18 and 26°C (Tables 4 and 5) at the end of a 48-h incubation period. All of the *V. fischeri* strains inoculated and incubated at 26°C infected both *S. affinis* and *S. ligulata* juveniles at concentrations at least 10-fold higher than the concentrations of the strains inoculated and incubated at 18°C (Table 4). No significant differences were observed with the *V. fischeri* strains examined at the same temperature (Table 4). Conversely, *V. logei* strains inoculated and incubated at 26°C were present at concentrations that were approximately 10-fold lower than the concentrations of the strains tested at 18°C (Table 5). Again, no differences were observed with *V. logei* strains tested at the same temperature (18 or 26°C) (Table 5). *V. fischeri* strains from *S. intermedia* and *S. robusta* were also tested at different temperatures with nonnative host squid species (Table 4), and no difference was observed between these strains and the native strains tested at the same temperature (either 18 or 26°C). Similarly, no differences were observed for *V. logei* strains isolated from *S. intermedia* or *S. robusta* when they were measured at the temperatures used for strains obtained from *S. affinis* or *S. ligulata* (Table 5).

Competition within and between *V. fischeri* and *V. logei* strains in *Sepiola* juveniles. Since no intraspecies differences in growth and infection were observed for the *V. fischeri* or *V. logei* strains tested, only one representative strain was used for the following competition experiments. *V. fischeri* native strain SA1 isolated from *S. affinis* was used in competition experiments with all of the other *V. fischeri* strains (SL8, SI2, SR5) at 26°C, and no discernable preference was observed among the *V. fischeri* strains examined (Table 6). Similarly, *V. fischeri* native strain SL8 from *S. ligulata* was not preferred over other nonnative strains when it was used in competition experiments performed with *S. ligulata* juveniles (Table 6). *V. logei* native strains SA6 and SL12 isolated from *S. affinis* and *S. ligulata*, respectively, were also tested at 18°C and exhibited no preference for either squid species during competition with all other *V. logei* strains used in this study (Table 7). None of the SI and SR strains exhibited specificity for either *S. affinis* or *S. ligulata* juveniles when competition experiments were performed at 26 or 18°C.

TABLE 6. Competition between isolates of *V. fischeri* in *Sepiola* juveniles, as determined at 26°C[a]

V. fischeri strains	Ratio of bacterial strains in squid host species	
	S. affinis	*S. ligulata*
SA1 and SL8	49:51	58:42
SA1 and SI2	51:49	44:56
SA1 and SR5	52:48	50:50
SA1 and SA8	56:44	49:51
SL8 and SR5	47:53	51:59
SL8 and SI2	44:56	55:45
SL8 and SL2	50:50	54:46
SI2 and SR5	49:51	43:57

[a] The results of individual competition experiments are average percentages based on 30 individuals. All percentages were arcsine transformed by methods described by Sokal and Rohlf (36).

In competition experiments performed at 26°C, all juveniles, regardless of species, were infected primarily with *V. fischeri* strains regardless of the species from which they were isolated (Table 8). The majority of *V. fischeri* colonies were much larger than the *V. logei* colonies arising from the homogenized juvenile light organs. The *V. fischeri* in both *S. affinis* and *S. ligulata* juvenile light organs accounted for 78 to 95% of the total light organ population in squids incubated at 26°C (Table 8). Conversely, all light organ competition experiments performed at 18°C resulted in colonization primarily by *V. logei* isolates from all four squid species sampled (Table 8). In these competition experiments, *V. logei* symbionts accounted for 77 to 96% of the total light organ population (Table 8). The colonies were primarily the same size, and only colony blots or visibly luminous colony morphology (30) could indicate the difference between *V. logei* and *V. fischeri* symbionts.

TABLE 7. Competition between isolates of *V. logei* in *Sepiola* juveniles, as determined at 18°C[a]

V. logei strains	Ratio of bacterial strains in squid host species	
	S. affinis	*S. ligulata*
SA6 and SL12	44:56	50:50
SA6 and SI7	54:46	48:52
SA6 and SR1	50:50	52:48
SA6 and SA12	49:51	53:47
SL12 and SR1	66:44	49:51
SL12 and SI7	56:44	50:50
SL12 and SL4	45:55	53:47
SI7 and SR1	45:65	43:57

[a] The results of individual competition experiments are average percentages based on 30 individuals. All percentages were arcsine transformed by methods described by Sokal and Rohlf (36).

TABLE 8. Effect of temperature on the ratio of *V. fischeri* cells to *V. logei* cells present in juvenile *Sepiola* light organs after 48 h

Competition assay temp (°C)	Bacterial strains		Ratio of *V. fischeri* strain to *V. logei* strain in squid host species[a]	
	V. fischeri strain	*V. logei* strain	*S. affinis*	*S. ligulata*
26	SA1	SA6	85:15	91:9
	SL8	SL12	89:11	95:5
	SI2	SI7	88:12	80:20
	SR5	SR1	82:18	78:22
18	SA1	SA6	21:79	14:86
	SL8	SL12	4:96	8:92
	SI2	SI7	10:90	12:88
	SR5	SR1	15:85	23:77

[a] All percentages are averages based on data from 30 juvenile squids for each competition experiment. The percentages were arcsine transformed (36) to test for significance in each competition experiment.

DISCUSSION

Previous studies of luminous bacterium-squid symbioses showed that host light organs were monotypic (22). Until recently, no other species of luminous bacteria were known to exist in members of the Sepiolidae. Recently, Fidopiastis et al. (12) reported that a second *Vibrio* species is present only in Mediterranean species of sepiolids. This finding suggests that luminous bacterium-squid mutualisms are not species specific but may depend on environmental factors. In this study I investigated the abiotic factors that affect this two-symbiont association and whether temperature influences the degree to which different symbiotic vibrios infect sepiolid squid hosts.

Sepiolid light organ pores are continually open to the surrounding seawater, which potentially provides access for any type of bacterium in the squid habitat. The presence of only *V. fischeri* or *V. logei* in the *Sepiola* light organ shows that there are strong species-specific interactions between these bacteria and their hosts. In the related genus *Euprymna*, the numbers of bacterial cells are controlled in part by diel venting of approximately 90% of bacteria through the lateral pores of the light organ into the mantle cavity (5, 6). This venting behavior results in a sufficiently high density of symbiotically competent *Vibrio* cells in the water column to promote colonization of the next generation of juvenile squids (19). Thus, symbiotically competent bacteria that are present in the environment can influence the composition of the squid light organ, particularly if more than one species is present. Strict species recognition does not occur for *V. fischeri* or *V. logei* in Mediterranean *Sepiola* squids, and it appears that one of the more influential factors determining light organ composition is the temperature at which the host is infected and persists. In previous studies to investigate the influence of temperature acclimation in *Escherichia coli* (3, 24), workers have shown that the genetics of a particular strain has no discernable effect on adaptation to a different environment but does influence the fitness of the strain (4). One may conclude that the sepiolid symbiosis is species specific (*V. fischeri* and/or *V. logei*), but the variability in light organ composition and the ability of both symbiont species to infect different host squids at different temperatures indicate how environmental factors can influence the distribution and population dynamics of symbiont colonization.

The degree of relatedness and diversity among numerous symbiotic taxa has traditionally been thought of as a good predictor of cospeciation (17). However, recently, a number of examples have demonstrated that the systematics underlying symbiosis is not as clear as we have traditionally thought (1, 10, 11, 15, 16, 25, 31). Changes in the host association with a particular symbiont may be related to selection pressure on the host's fitness and how the symbiont may or may not affect future generations of host-symbiont pairs (13, 35). Population size and the availability of hosts that can be infected also affect the pairing of host-symbiont associations and possibly the specificity that a mutualism eventually expresses (7, 37). The presence of two species of symbionts that can both infect different host species may be an initial step in establishing a monotypic host-symbiont association. However, how the natural balance between host fitness and symbiont competence is established remains to be determined (14). Because there are several sympatric *Sepiola* host species living in the area sampled, the possibility of host switching between squids and the establishment of host-symbiont specificity can be studied. In future experiments researchers should examine the degree to which ecological and/or genetic factors control patterns of cospeciation and evolution and how we can better predict which patterns arise from these factors. Whether the symbionts determine new avenues for host evolution and radiation into different ecological habitats in this family of squids is just one of the many questions that should be pursued in future studies.

ACKNOWLEDGMENTS

I thank S. von Boletzky for help with collecting *Sepiola* adults and egg masses at Laboratoire Arago, Banyuls-sur-mer, France, and for providing insight and knowledge of the Mediterranean Sepiolidae. I also thank J. L. Botsford, D. J. Howard, and J. Randall for reviewing the manuscript prior to submission and M. J. McFall-Ngai and E. G. Ruby of the University of Hawaii for providing insight and supporting my work on the sepiolid-*Vibrio* symbiosis. I thank C. L. Lickliter of my laboratory at New Mexico State University for providing bacterial growth data.

This work was supported by a summer research grant from the Arts and Sciences research center at New Mexico State University and by the University Research Expeditions Program (UREP), University of California.

REFERENCES

1. **Baumann, P., N. A. Moran, and L. Baumann.** 1997. The evolution and genetics of aphid endosymbionts. BioScience **47:**12–20.
2. **Bello, G.** 1995. A key for identification of the Mediterranean sepiolids (Mollusca: Cephalopoda). Bull. Inst. Oceanogr. (Monaco) **16:**41–55.
3. **Bennett, A. F., and R. E. Lenski.** 1993. Evolutionary adaptation to temperature. II. Thermal niches of experimental lines of *Escherichia coli*. Evolution **47:**1–12.
4. **Bennett, A. F., and R. E. Lenski.** 1998. Evolutionary adaptation to temperature. VI. Phenotypic acclimation and its evolution in *Escherichia coli*. Evolution **51:**36–44.
5. **Boettcher, K. J., and E. G. Ruby.** 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J. Bacteriol. **172:** 3701–3706.
6. **Boettcher, K. J., E. G. Ruby, and M. J. McFall-Ngai.** 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. J. Comp. Physiol. **179:**65–73.
7. **Bull, J. J., I. J. Molineux, and W. R. Rice.** 1991. Selection of benevolence in a host-parasite system. Evolution **45:**875–882.
8. **Cary, S. C.** 1994. Vertical transmission of a chemoautotrophic symbiont in the photobranch bivalve, *Solemya reidi*. Mol. Biol. Biotechnol. **3:**121–130.
9. **Cavanaugh, C. M.** 1994. Microbial symbiosis: patterns of diversity in the marine environment. Am. Zool. **34:**79–89.
10. **Distel, D. L., H. Felbeck, and C. M. Cavanaugh.** 1994. Evidence for phylogenetic congruence among sulfur-oxidizing chemoautotrophic bacterial endosymbionts and their bivalve hosts. J. Mol. Evol. **38:**533–542.
11. **Distel, D. L.** 1998. Evolution of chemoautotrophic endosymbioses in bivalves. BioScience **48:**277–286.
12. **Fidopiastis, P. M., S. von Boletzky, and E. G. Ruby.** 1998. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J. Bacteriol. **180:**59–64.
13. **Frank, S. A.** 1996. Host-symbiont conflict over the mixing of symbiotic lineages. Proc. R. Soc. London Biol. Sci. **263:**339–344.
14. **Frank, S. A.** 1996. Host control of symbiont transmission: the separation of

symbionts into germ and soma. Am. Nat. **148:**1113–1124.
15. **Haygood, M. G., and D. L. Distel.** 1993. Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes from unique lineages related to the genus *Vibrio*. Nature **363:**154–156.
16. **Hinkle, G., J. K. Wetterer, T. R. Schultz, and M. L. Sogin.** 1994. Phylogeny of the attine ant fungi based on analysis of small subunit rRNA gene sequences. Science **266:**1695–1697.
17. **Law, R.** 1985. Evolution in a mutualistic environment, p. 145–170. *In* D. H. Boucher (ed.), The biology of mutualism: ecology and evolution. Oxford University Press, New York, N.Y.
18. **Lee, K.-H., and E. G. Ruby.** 1992. Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater by using *lux* gene probes. Appl. Environ. Microbiol. **58:**942–947.
19. **Lee, K.-H., and E. G. Ruby.** 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. **60:**1565–1571.
20. **Lee, K.-H., and E. G. Ruby.** 1994. Competition between *Vibrio fischeri* strains during the initiation and maintenance of a light organ symbiosis. J. Bacteriol. **176:**1985–1991.
21. **Mangold, K., and S. von Boletzky.** 1988. Mediterranean cephalopod fauna, p. 315–330. *In* M. R. Clarke and E. R. Trueman (ed.), The Mollusca, vol. 12. Academic Press, San Diego, Calif.
22. **McFall-Ngai, M. J., and E. G. Ruby.** 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science **254:**1491–1494.
23. **McFall-Ngai, M. J., and E. G. Ruby.** 1998. Sepiolids and vibrios: when they first meet. BioScience **48:**257–265.
24. **Mongold, J. A., A. F. Bennett, and R. E. Lenski.** 1996. Evolutionary adaptation to temperature. IV. Adaptation of *Escherichia coli* at a niche boundary. Evolution **50:**35–43.
25. **Moran, N. A., and A. Telang.** 1998. Bacteriocyte-associated symbionts of insects. BioScience **48:**295–304.
26. **Munson, M. A., P. Baumann, M. A. Clark, L. Baumann, N. A. Moran, and D. J. Voegtlin.** 1991. Evidence for the establishment of aphid-eubacterial endosymbiosis in an ancestor of four aphid families. J. Bacteriol. **173:**6321–6324.
27. **Nealson, K. H., and J. W. Hastings.** 1991. The luminous bacteria, p. 625–639. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag, New York, N.Y.
28. **Nesis, K. N.** 1987. Cephalopods of the world. T.F.H. Publications, Neptune City, N.J.
29. **Nishiguchi, M. K., L. H. Lamarcq, E. G. Ruby, and M. J. McFall-Ngai.** 1996. Competitive dominance of native strain symbiotic bacteria measured by colonization and *in situ* hybridization. Am. Zool. **36:**41A.
30. **Nishiguchi, M. K., E. G. Ruby, and M. J. McFall-Ngai.** 1997. Phenotypic bioluminescence as an indicator of competitive dominance in the *Euprymna-Vibrio* symbiosis, p. 123–126. *In* J. W. Hastings, J. W. Kricka, and P. E. Stanley (ed.), Bioluminescence and chemiluminescence: molecular reporting with photons. J. Wiley and Sons, New York, N.Y.
31. **Nishiguchi, M. K., E. G. Ruby, and M. J. McFall-Ngai.** 1998. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-*Vibrio* symbioses. Appl. Environ. Microbiol. **64:**3209–3213.
32. **Ruby, E. G., and L. M. Asato.** 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. **159:**160–167.
33. **Ruby, E. G.** 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu. Rev. Microbiol. **50:**591–624.
34. **Saffo, M. B.** 1992. Invertebrates in endosymbiotic associations. Am. Zool. **32:**557–565.
35. **Sniegowski, P. D., P. J. Gerish, and R. E. Lenski.** 1997. Evolution of high mutation rates in experimental populations of *Escherichia coli*. Nature **387:**703–705.
36. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry. W. H. Freeman and Co., New York, N.Y.
37. **Turner, P. E., F. S. Cooper, and R. E. Lenski.** 1998. Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. Evolution **52:**315–329.