PHENOTYPIC BIOLUMINESCENCE AS AN INDICATOR OF COMPETITIVE DOMINANCE IN THE *EUPRYMNA-VIBRIO* SYMBIOSIS

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Introduction

The study of coevolutionary relationships among symbiotic associations has been an important avenue for understanding the establishment and development of two independent, but closely integrated organisms (1-3). Most studies investigating the evolutionary relatedness amongst host-symbiont pairs have alluded to the parallelisms that occur with the onset of a particular host speciation event that eventually leads to the isolation of the symbiont population (4). Hence, symbionts from an "ancestral" host species may have similar biochemical, physiological, or molecular characteristics that group them within the same "species". Yet, the delineation between strains or biovars within a species is much harder to define; strain characteristics that would normally define a particular bacterial population may be consistent throughout the entire group of symbionts. Although sequencing hypervariable regions of specific loci is a technique that can genetically differentiate between the strains (5), there are few ways that one can phenotypically distinguish similar strains from one other.

The sepiolid squid-bioluminescent bacterial association offers several advantages as a model system to study the coordinated influence of luminous bacteria on the coevolution and speciation between partners (6). Both the bacteria and the host squid can be cultured separately, allowing the specific comparison between different symbiotic strains during colonization of a particular host squid light organ. Initiation, colonization, and persistence of each strain can then be monitored individually, or, in a competition experiment, where two strains compete for dominance in a particular species of host squid (7). Previous research has clearly indicated that symbiotic bacterial strains can be genetically differentiated from each other, either through restriction fragment length polymorphisms from specific DNA fragments (Lee and Ruby, unpubl. data), or through direct sequencing of loci that are variable between strains (8). Although molecular probes designed from these types of experiments can be used to distinguish and identify various strains in each individual light organ, this method is laborious and is limited by the number of colony blots one utilizes per infection assay (most infection/competition experiments utilize approximately 100 juveniles) (9). It has been recently discovered that variation in luminescence intensity of individual strains of symbiotic bacteria can be utilized to phenotypically distinguish strains from each other in an infected juvenile sepiolid light organ (8, Figure 1). This phenotypic

variation allows the direct physiological comparison between different strains of symbiotic bacteria in a particular host squid, and can be compared to other types of data for determining the evolutionary relatedness of different host species and the symbiotic bacteria that have coevolved with them.

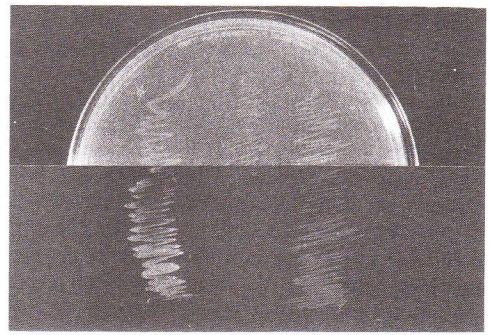

Figure 1. Light organ symbionts (left to right: EM17, ES114, and ET101) from three species of sepiolid squid (*Euprymna morsei*, *E. scolopes*, and *E. tasmanica*, respectively) have similar growth characteristics on agar plates. However, when observed in the dark, it can be seen that the light output per bacterial cell differs significantly between the strains.

Methods and Materials

For colonization assays, luminescent symbiotic bacteria were isolated from frozen or freshly collected light organs of several species of squid: *Euprymna scolopes* (Hawaii), *E. morsei* (Japan), *E. tasmanica* (Australia), *Sepiola affinis* (France), and *Loliolus noctiluca* (Australia) (10, Table 1).

Table 1. Sepiolid and loliginid squids and their respective light organ symbionts

Species	Representative light organ symbiont strain	Concentration in *E. scolopes* light organ	Luminescence on seawater nutrient agar medium
Euprymna scolopes	ES114*	2.6×10^6	non-visible
Euprymna morsei	EM17*	5×10^6	bright
Euprymna tasmanica	ET101*	3×10^6	dim
Sepiola affinis	SA1*	4×10^6	moderately bright
Loliolus noctiluca	LN101†	0	bright

* = *Vibrio sp.*
† = *Photobacterium leiognathi*

All strains isolated from the sepiolid squids were identified as *Vibrio* species; the bacterium isolated from the loliginid squid was identified as *Photobacterium leiognathi*. For experiments, cell suspensions consisting of approximately equal concentrations [about 10^3 colony forming units (CFU's)] were used as the bacterial inocula. Using isolates of symbiotically competent bacteria from *Euprymna*, *Sepiola*, and *Loliolus* species, infection experiments were completed to determine whether non-native strains could infect juvniles from a closely related sepiolid host, *E. scolopes* . The number of symbionts of each strain that

had colonized a light organ 48 hours after infection was determined by simply observing the percentage of brightly, dimly, or non-visibly luminous CFU's arising from platings of light organ homogenates on seawater tryptone (SWT) agar medium (Table 1). The relative luminescence of the colonies was easily distinguished by viewing them in a dark room.

Results and Discussion

When presented individually, all strains of sepiolid symbionts, whether isolated from *Euprymna* or *Sepiola*, were capable of initiating and maintaining a symbiotic population in *E. scolopes* juvenile light organs. The only strain that did not infect *E. scolopes* juveniles was the light organ symbiont of *L. noctiluca*, which belongs to the species *Photobacterium leiognathi*. Although concentrations of bacteria homogenized from light organs were similar between the symbiotic strains, luminescence from these isolates on agar plates varied between visibly luminous and non-visibly luminous phenotypes (Table 1). This indicates that a symbiont associated with a particular *Euprymna* host may vary in the luminescence it produces outside the light organ; once colonization has occurred luminescence in the squid has little variation. Thus, once the symbiosis is established it can be deduced that the light organ has a direct effect on the production of light and luminescence of each bacterial strain, regardless of which host it has evolved. Previous results of luminescence variation have shown direct correlation with the amount of autoinducer present in the light organ (11), irrespective of the evolutionary or selective agents that differentiate luminous symbiotic bacteria.

The evidence of bacterial specificity and phenotypic variability between symbiotically competent strains that are "genetically" the same species (<2% 16s sequence identity) provides new insights into the speciation and evolutionary events that have occurred after the origin of the association (1, 8). Bacterial speciation within symbiotic populations is difficult to monitor; unless the molecular clock of either host or symbiont can be traced, there is little evidence that alludes to the predisposition of the individual partners before the onset of the symbioses (12). Present day models of symbiosis can only reveal the biological mechanisms that control the interactions, and unless there are distinct methods for defining characteristics of similar strains or biovars, evolutionary tracking is obscure. Thus, the use of bioluminescence as a phenotypic character in the sepiolid squid-bioluminescent bacteria symbioses is a unique and informative quality that enables one to distinguish the competency of individual strains, and provides evidence that speciation and diversification have occurred after the evolutionary origin of the symbioses (8).

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