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Molecular and Biochemical Characterization of Microbial Symbiont Bioactivities in
Sepioid and Loliginid Squids.

Analysis of the bacterial community associated with the accessory nidamental gland and egg sheath from four species of cephalopods.

Previous research on characterization of the microbial community associated with the accessory nidamental gland (ANG) and egg sheath (ES) of the squid relied mostly on molecular techniques (i.e. analysis of 16S ribosomal DNA sequence) with the majority of the symbionts being uncultured. Although these studies and previous work in our lab provided valuable initial descriptions of the ANG microbial community, it was evident that a more extensive attempt at cultivation and molecular characterization of the ANG and ES bacteria was warranted and also necessary for describing any potent bioactivities against fungi or other bacteria.

We took a comparative approach by analyzing the microbial community from four different cephalopod species. We analyzed the microbial community of *Loligo opalescens* because it is locally available, a significant fishery in California and the results can be compared to a recent molecular analysis of the Atlantic species *Loligo pealei* (Barbieri et al., 2001); *Euprymna scolopes* because it is an emerging model for symbiosis research (McFall-Ngai, 1999); and *Sepia officinalis* and *S. pharaonis* for heartiness, availability and to compare to recent analysis (Grigioni et al., 2000). We expected this approach to complement previous studies on ANG/ES bacteria, provide insights in the biology of the symbiosis and provide a greater chance of success in developing a model for studies of consortial bacterial interactions with animal epithelium.

Diversity of bacteria associated with the ANG and ES from four cephalopod species.

Our progress to date is our successful cultivation of many of the bacterial symbionts, some of which are likely to be new species or new genera, and a more extensive analysis of the microbial community by molecular techniques than was done previously. The most significant new bacteria we were able to characterize and cultivate was a red carotenoid-producing alpha-proteobacteria from *L. opalescens*. This achievement is significant since this bacteria has not been cultivated, is likely to represent a new genus since its 16S rDNA sequence is 10% divergent from the nearest cultivated relative (*Stappia stelulata*), and is related to the *Rhizobiaceae* that include dinitrogen fixing rhizobial symbionts of legumes and other bacteria that are interact intimately with eukaryotic organisms. We also cultivated two *Shewenalla* sp. and one *Roseobacter* sp. symbiont. All these symbionts were found repeatedly in

both the ANG and ES from different individuals and by molecular analysis of the ANG/ES community.

Molecular analysis of the ANG/ES bacteria revealed that 65% of 16S rDNA sequences obtained from the ANGs and ESs were members of the alpha proteobacteria and 9% being gamma proteobacteria. Of the alpha proteobacteria 40% are most closely related to *Roseobacter*, including the cultivated species. The rhizobia-like bacteria were also found by molecular analysis to comprise 7% of the alpha proteobacteria sequences and 3% of the total sequences. There are at least two types of alpha proteobacteria that remain uncultured. The overall diversity of the ANG and ES bacteria is that majority (58%) of the 16S sequences are represented by 5 ribotypes, and 85% represented by 13 ribotypes and remaining 15% of the sequences were seen only one time. We are in the process of analyzing 100 additional 16S rDNA sequences from other individual squid ANGs and ES to determine which bacteria are reproducibly found in association with the ANG.

The results of these studies are indicated in Table 1. The sequence data is not included in this table, but of particular significance is that the microbial community in *Loligo opalescens* is nearly identical at the level of 16S rDNA sequence diversity to the Atlantic species, *Loligo pealei*.

Table 1. Symbionts of *Loligo opalescens*

Symbiont (type-nearest relative)	Cultivated?	Present in ANG/ES?
α -proteobacteria, <i>Roseobacter</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Stappia stellulata</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Rhodobacter</i> sp.	No	Yes/Yes
α -proteobacteria, <i>Roseobacter</i> sp.	No	Yes/Yes
γ -proteobacteria, <i>Shewenella pealeana</i>	Yes	Yes/Yes
γ -proteobacteria, <i>Shewenella</i> sp.	Yes	Yes/Yes
γ -proteobacteria, mollusk gill symbiont.	No	Yes/Yes
cytophaga/flavobacterium, <i>Cytophaga</i> sp.	No	Yes/Yes

In *Sepia officinalis* we cultivated alpha proteobacterial symbionts that are also abundant by molecular analysis (72% of 16S rDNA sequences). One ribotype has 16S rDNA identical to the cultivated alpha proteobacterium *Ruegaria atlantica*, another is 2% divergent to the nearest cultivated *Ruegaria* species and might constitute a new species. Bacteria 10% divergent from the closest cultivated alpha proteobacteria *Aquaspirillum* and 5% divergent from the closest cultivated *Spingomonas* sp. also were isolated. Members of the Cytophaga/Flavobacterium group of bacteria were also cultivated and identified by molecular analysis of the ANG and ES. *Pseudoaltermonas* sp., members of the gamma proteobacteria, were found by molecular analysis but are not yet cultivated (a closely related bacteria was isolated from *S. pharaonis*, see below). Results from this sepioid squid are presented in Table 2 below.

Table 2. Symbionts of *Sepia officinalis*

Symbiont (type-nearest relative)	Cultivated?	Present in ANG/ES?
α -proteobacteria, <i>Ruegaria atlantica</i>	Yes	Yes/Yes
α -proteobacteria, <i>Ruegaria</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Sphingomonas</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Aquaspirillum</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Paracoccus</i> sp.	Yes	Yes/Yes
γ -proteobacteria, <i>Pseudoalteromonas</i> sp.	No	Yes/Yes
γ -proteobacteria, <i>Pseudomonas</i> clade	No	Yes/No
cytophaga/flavobacterium, <i>Cytophaga</i> sp.	Yes	Yes/Yes

In *Sepia pharaonis*, like *Sepia officinalis*, two species of *Ruegaria* were cultivated but here a *Pseudoalteromonas* sp. was also cultivated. Four types of bacteria were cultivated which are more than 7% divergent to cultivated members; a rhizobia-like bacterium, a cytophagalflavobacteria type bacterium, a *Paracoccus* type bacterium, and a *Hyphomonas* type organism. Alpha proteobacteria of the *Ruegaria* sp., *Sphingomonas*, *Paracoccus*, and cytophaga/flavobacteria were also found by molecular analysis of the ANG and ES bacteria. The results of these studies are reported in Table 3 below.

Table 3. Symbionts of *Sepia pharaonis*

Symbiont (type-nearest relative)	Cultivated?	Present in ANG/ES?
α -proteobacteria, <i>Ruegaria atlantica</i>	Yes	Yes/Yes
α -proteobacteria, <i>Ruegaria</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Sphingomonas</i> sp.	No	Yes/Yes
α -proteobacteria, <i>Aquaspirillum</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>Paracoccus</i> sp.	Yes	Yes/Yes
γ -proteobacteria, <i>Pseudoalteromonas</i> sp.	Yes	Yes/Yes
cytophaga/flavobacterium, <i>Cytophaga</i> sp.	Yes	Yes/Yes
Verrucomicrobiales	No	No/Yes

In the Hawaiian bobtail squid, *Euprymna scolopes*, 6 types of alpha proto bacteria, 2 *Reugaria* sp., a *Roseobacter* sp., a rhizobia-like bacterium, an *Erythrobacter* sp. and two cytophago/flavobacterium species were cultivated. Among the cultivated bacteria only the *Erythrobacter* and rhizobia-like bacteria were not found by molecular analysis of the ANG/ES community. We are currently analyzing 100 additional 16S rDNA sequences from the ANG and ES from other individual cephalopods. The findings of these analyses are summarized in Table 4.

Table 4. Symbionts of *Euprymna scolopes*

Symbiont (type-nearest relative)	Cultivated?	Present in ANG/ES?
α -proteobacteria, <i>Ruegaria atlantica</i>	Yes	Yes/Yes
α -proteobacteria, <i>Ruegaria</i> sp.	Yes	Yes/Yes
α -proteobacteria, <i>rhizobia</i> type	Yes	No/Yes
α -proteobacteria, <i>Erythrobacter</i> sp.	Yes	Yes/No
α -proteobacteria, <i>Paracoccus</i> sp.	Yes	Yes/Yes
γ -proteobacteria, <i>Aeromonas</i> sp.	Yes	Yes/No
cytophaga/flavobacterium, <i>Flexibacter</i> sp.	Yes	Yes/Yes
cytophaga/flavobacterium, <i>Muricauda</i> sp.	Yes	Yes/Yes
Verrucomicrobiales	No	No/ES

In summary, continued analysis of the microbial consortium associated with the ANG and ES by classical and molecular methods is revealing important similarities and also important differences in the microbial communities from these four species of cephalopods. All cephalopods have communities dominated by members of the alpha subclass of the proteobacteria, namely the *Roseobacter/Ruegaria* clade. Related squid have related bacterial communities. Bacteria associated with the *Loligo opalescens* are nearly identical to an independent analysis of *Loligo pealei* (Barbieri et al., 2001). *Sepia officinalis* has also has a nearly identical bacterial community to another recent analysis of the same species (Grigioni et al., 2000) which is also similar to the community found in *Sepia pharaonis*. Finally, *Euprymna scolopes* has a community related to but distinct from *Sepia* sp. The number of bacterial species associated with the ANG and ES is approximately 10, but additional analysis of the community is required to distinguish between the symbionts and the tourists.

We have obtained full length sequence of the 16S rDNA and in continuing work under Sea Grant RIMP-89 plan to use signature sequences so that the communities of the ANG/ES can be rapidly profiled from different individuals and at different stages of development of the ANG/ES. We also plan to employ the fluorescent in situ hybridization technique to verify that similar symbionts are found in both the ANG/ES and to determine spatial relationships among the members.

A summary of this work is indicated in Figure 1, which presents a phylogenetic tree of the bacteria from these four species of squid and their imputed phylogenetic relationships.

Function of the bacteria associated with the ANG/ES.

One hypothetical function of the ANG/ES bacteria is to provide protection to the developing squid embryos by the production of secondary metabolites. We have detected antibiotic activity in extracts from *Sepia officinalis* and the *Pseudoaltermonas* symbiont of *Sepia pharaonis*. We are collaborating with William Fenical at the Scripps Institute of Oceanography to purify and identify the antibiotic molecules. Using the same extraction procedures, we did not detect antibiotic activity from the ES obtained from *L. opalescens*. These ESs might have different antibiotics that require different extraction procedures (or they might contain no antibiotics).

Another avenue of research is to identify acyl-homo serine lactones (HSLs) that might be present in the ANG and ES. HSLs are signal molecules involved in inter- and intra species quorum sensing abundant in the proteobacteria. Under our new grant RIMP- 89 we are collaborating with E.P. Greenberg at University of Iowa to use a technique to identify HSLs produced in situ in the ANG/ES. This will reveal the HSLs that the bacteria use to communicate with each other in the ANG and ES and reveal who's speaking to who and in what language. This work could find signaling molecules that control production of secondary metabolites such as antibiotics or fungicides.

References:

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Fig. 1. Bacteria associated with the ANG and ES from five cephalopod species.

