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Title

Environmental Effects on Anti-Microbial Activity of Bacterial Symbionts in the Reproductive System of Squid

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FINAL YEAR NARRATIVE —R/MP-89—David Epel
Environmental Effects on Anti-Microbial Activity of Bacterial Symbionts in the Reproductive
System of Squid

The goals of this grant were:

- 1). To **identify** and characterize the symbiotic bacteria associated with the accessory nidamental gland and egg sheath of squid and cuttlefish.
- 2). To describe the changes in **quantity and distribution** of these bacteria in the egg sheath during development of the squid embryo.
- 3). To **grow** these bacteria in **culture**.
- 4). To determine **culture conditions** that permit production of secondary products that are anti-bacterial and anti-fungal.
- 5). To determine if **synthesis of secondary products** is related to quorum sensing, to microaerophily, to surface effects and/or to interspecies interactions referred to as syntrophy.
- 6). To **develop methods** to determine which bacteria are responsible for secondary **products should it prove impossible to grow the candidate bacteria in culture**.
- 7). Once the conditions are worked out for identification of the bacteria and their growth, then to **identify and characterize these bacterial secondary products** through collaborative research with natural products chemists.

We have made exceptional progress on most of these goals as will be described in this final report. Below I describe accomplishments on each of the above goals.

Goals 1-3 were to identify symbiotic bacteria associated with the accessory nidamental gland (ANG) and egg sheath, to describe changes during development and to attempt to grow these bacteria in culture.

We identified and characterized bacteria associated with the ANG and egg cases of one loligonid and three species of sepioid squid and cuttlefish. For identification, we used molecular approaches using the sequence of the bacterial small subunit ribosomal RNA genes as an indicator of relatedness. This approach is accepted as the best method to ascertain taxonomic identity.

We examined the loligonid squid [*Loligo opalescens* from the Pacific Ocean (California coast)]; and three species of sepioid cephalopods, the Hawaiian bob-tail squid, *Euprymna scolopes* (Pacific, from Hawaii)] and two species of cuttlefish [*Sepia officinalis* (Atlantic Ocean, originally from Plymouth UK but grown in the Monterey Bay Aquarium) and *Sepia pharonis* (Pacific Ocean, coast of Thailand)].

All species harbored a consortium of symbiotic bacteria in the ANG or in the egg case. We confirmed that similar bacteria were present in the ANG and egg case, indicating transfer from the ANG to the egg case. The majority of bacteria were alpha-protobacteria and most of the bacteria represent new genera and new species.

A phylogenetic tree is presented in Figure 1 and shows the relatedness of the symbionts from the different squid. There are some remarkable similarities. For example, *L. opalescens* and *L. peali* share the same bacteria, since *L. peali* D47 and *L. opalescens* cultures 3-5 appear to be identical.

This is remarkable as these two squid reside in different oceans and have been separated for millions of years. There is also relatedness in the cuttlefish bacteria with *S. officinalis* and *S. pharaonis* having highly related bacteria even though they also reside in separate oceans and presumably have been separated for millions of years also.

Comparison of microbial communities of ANG/EC from 4 cephalopod species.

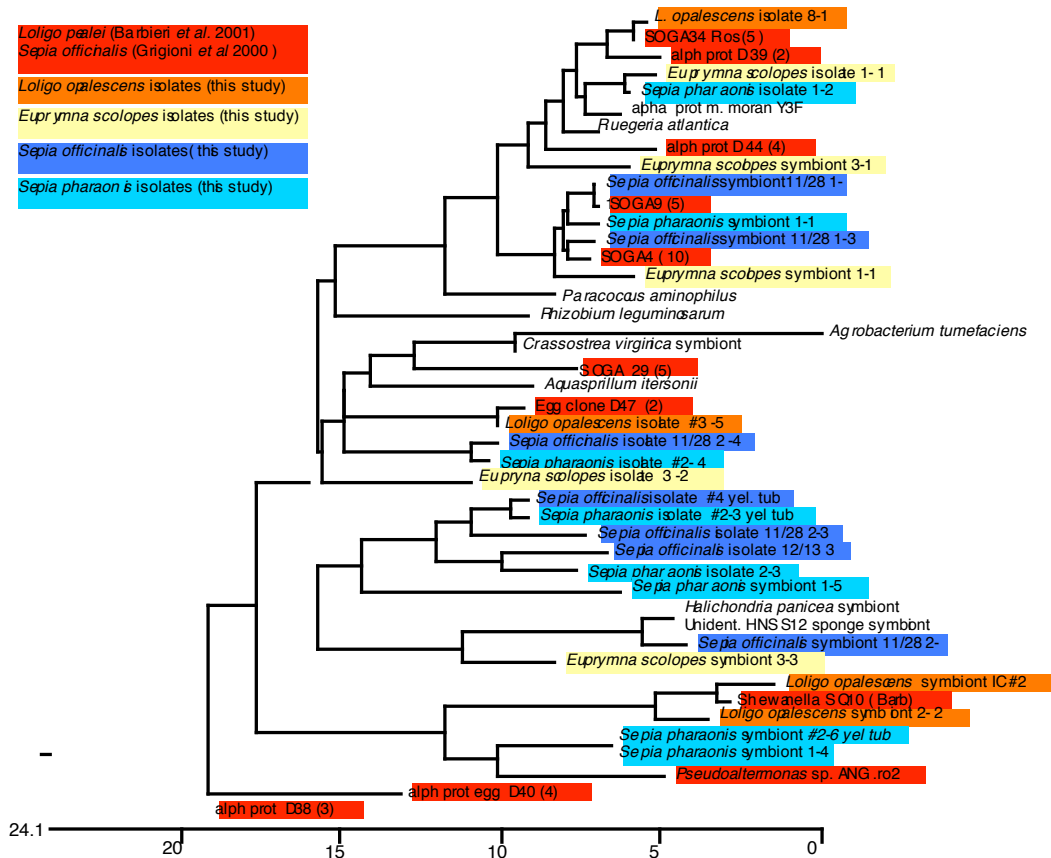


FIGURE 1. A phylogenetic tree showing relationships of the different bacteria found in the consortia living in the ANG or egg cases of various squid.

Many bacteria cannot be grown in culture and can only be identified by their unique rDNA sequence. Our earlier work suggested this would be a problem since we only cultivated two species of bacteria (a *Roseobacter* and a *Shewanella* species). It therefore was a challenge to be able to grow these bacteria but we have succeeded by using minimal culture media and growing the bacteria for long periods of time. To date, we have succeeded in growing 21 species in culture, which is quite remarkable and represents ~31-55% of the species we have identified on the basis of rDNA sequence.

Goal 4 was to determine if the bacteria in culture produce antibiotics. We observed positive results with the eggcase of the cuttlefish *S. officinalis* (see Figure 2). and also found activity in cultured bacteria from this squid **Goal 7 was to characterize the nature of this antibiotic activity** and we were not able to do this during the course of the grant period. However, we are working with Crescent Biologics, a biotech firm here on the Monterey Peninsula, to identify the anti-bacterial agent. This work, if successful, will be licensed by the Office of Technology Transfer at Stanford University.

Antibiotic activity from egg sheath extr acts.



Figure 2. Antibiotic assay using an overlay of *Micrococcus luteus* as the sensitive species of bacteria and extracts of the egg case of the cuttlefish, *S. officinalis*. The clearing around the wells shows the presence of antibiotic activity.

Goal 5 was to determine if quorum-sensing molecules were part of this bacterial consortium and to determine if these quorum-sensing molecules might control synthesis of other secondary metabolites. These molecules are typically acyl homoserine lactone (AHL) compounds and we worked in collaboration with Professor E. P. Greenberg of the University of Iowa who is the world's expert on this important group of signaling molecules. We adapted known methods to separate various AHLs and were able to identify a number of quorum sensing molecules that are produced by consortia members. The methodology and structure of the various compounds is shown in Figure 3.

Elution profiles of acyl-HSLs on a reverse phase-HPLC C18 column (10-100% MeOH gradient for 158mls)













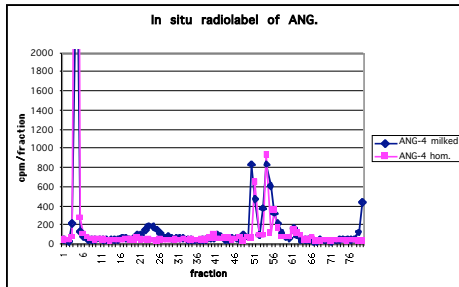
Acyl-HSL	%MeOH	fraction	
C4-HSL	23	9-10	
3oxoC6-HSL	27	12-13	
C6-HSL	46	32-33	
3oxoC8-HSL	50	36-37	
C8-HSL	63	43-44	
3oxoC10-HSL	67	51-52	
C10-HSL	75	57-58	
3oxoC12-HSL	77-79	58-59	
C12-HSL	84	65-66	
3oxoC14-HSL	85-86	67-68	
C14-HSL	89-91	70-71	
C16-HSL	95	75-76	

Figure 3. Elution characteristics of the various candidate quorum sensing molecules

Assays for these in the AN gland of *S. officinalis* and also from a cultured symbiont from *S. pharaonis* (isolate #10, an alpha-proteoebacteria) are shown in Figures 4 and 5. We were able to identify C10 and 2-oxo-C10 HSLs as dominant products of six of the cultured bacteria. We also found a larger than C10 molecule produced by symbiont 11 and detected autoinducer 2 activity, presumably due to the production of bromylated furanosyl diester compound by the gamma-proteoebacteria cultures 3,4 and 15.

Two dominant putative acyl-HSLs are found in the *S. officinalis* ANG



Acyl-HSL from a cultivated symbiont.

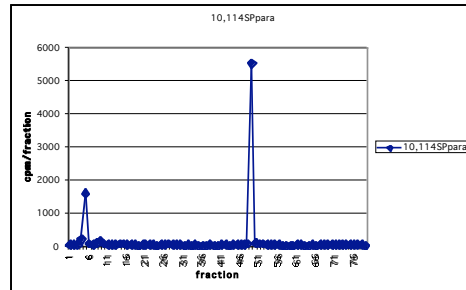


Figure 4 and 5 respectively show the elution characteristics of acyl-HSLs from *S. officinalis* ANG and from a cultivated symbiont # 10 which is an alpha-proteobacteria.

Future work will determine if any of these compounds regulate the synthesis of bacterial secondary metabolites. Based on precedent, we expect this to be the case.

Goal 6 (to develop methods to assay secondary product genes if we could not grow the bacteria) was not pursued since we were able to culture most of the bacterial species that were identified on the basis of rDNA sequences.

Summary: We succeeded in identifying and characterizing the symbiotic bacteria in the ANG and egg cases of five species of squid and cuttlefish on the basis of rDNA sequences and found strong relationships between the bacteria in these different squid. This conservation suggests an important role of these bacteria, something that evolved millions of years ago, and which has not been lost through drift. We were able to culture 21 of these species, which is a remarkable achievement. We also identified anti-microbial activity from *Sepia* egg cases and from species of cultured bacteria and are working with private industry to characterize and identify the nature of this antibiotic activity. The bacterial consortia also produce quorum-sensing molecules and we demonstrated their synthesis as well as identified the major molecules. Future work will determine if they regulate synthesis of other secondary metabolites.