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The object of our research was to characterize symbiotic chemoautotrophic bacteria using molecular techniques. We are concentrating on the sequencing of their 16S rRNA to establish phylogenetic relationships between symbionts from different invertebrate hosts. In addition, we compare genes for ribulose-1, 5-bisphosphate-carboxylase in a number of symbiotic systems using molecular probes of various origin.

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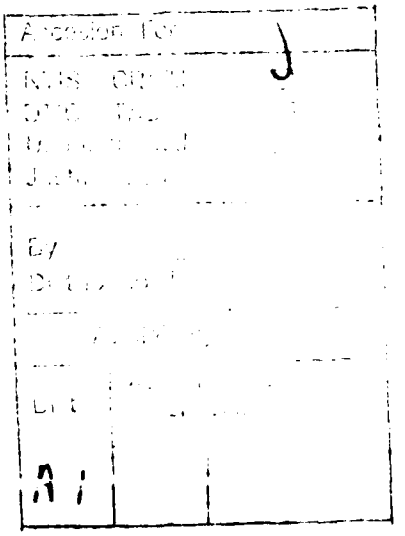


FINAL REPORT ON CONTRACT N00014-88-K-0079; R&T 4412026

PRINCIPAL INVESTIGATOR: Horst Felbeck

CONTRACTOR: University of California

CONTRACT TITLE: Biology of Symbioses between Marine Invertebraies and Intracellular Bacteria

START DATE: 1 January 1988

TIME PERIOD: 1/1/1988 - 12/31/1990

RESEARCH OBJECTIVE: To study the properties of symbiotic bacteria and to characterize them by 16S rRNA sequencing

PROGRESS:

Our research focused mainly on three subjects:

a) The characterization and sequencing of the gene for ribulose bisphosphate carboxylase (RubisCO) from symbiotic bacteria of various origins,
b) To continue methods development for 16S rRNA sequencing from symbionts in frozen and badly preserved specimens, and
c) To use these new techniques to sequence 16s DNA from a variety of symbionts

a) RubisCO

We have cloned the gene coding for RubisCO from the sulfur oxidixing symbiont of the gastropod Alvinochoncha hessleri. Nucleotide sequence analysis of the cloned fragment revealed that the large (*rbcL*) and small (*rbcS*) subunits of the symbiont RubisCO were organized similarly to the RubisCO operons of free-living photo- and chemoautotrophic prokaryotes. A comparison of aligned sequences showed that the symbiont rbcL gene shared the highest degree of sequence identity with the cyanobacterium Anabaena (69%) while the nucleotide sequence of small subunit was 61% identical to that of the green alga Chlamydomonas rheinhardti. A partial sequence analysis, which included a portion of the *rbcL* sequence from a symbiont of the bivalve Solemya reidi, revealed that the two symbiont sequences were highly identical -- 85 % at the nucleotide level and 93% at the amino acid level, suggesting a recent common origin. RubisCO activity was expressed in Escherichia coli transformed with a plasmid carrying the RubisCO fragment of the gastropod symbiont in the proper orientation for transcription off of the plasmid lac promoter. The level of activity suggests proper assembly of this deep-sea RubisCO into the holoenzyme.

A reprint of our paper published in the Proceedings of the National Academy of Sciences is included.

b) Methods for 16S rRNA sequencing

After many delays and problems we finally succeeded to develop a method to amplify the genes for 16s rRNA from mixed (i.e. host and symbiont) DNA extracted from frozen tissues and to sequence these genes directly from the amplification product.

We first extract the samples in Guanidinium isothiocyanate followed by further purification through CsCl gradient centrifugation. After an additional purification step (dissolving and re-precipitating) one obtains a preparation suitable for amplification.

We have had severe difficulties in the past sequencing directly from PCR amplified DNA. Within the last year of this contract, however, we have overcome these problems and are now routinely using this approach for sequencing. We are able to obtain sequence information usable for phylogenetic comparisons within a few days without prior cloning. The basic protocol is to sequence the cleaned PCR product directly using the Sequenase ™ (United States Biochemical) protocol with either the PCR primer or other internal primer as sequencing primer. With these primers we can sequence two thirds of a 16s gene, i.e., approximately a thousand base pairs, which allows a very thorough and detailed comparison of genes for 16s rRNA from different symbionts. Using this technique we are currently able to determine 350 to 400 bases from each primer for double stranded DNA.

c) Sequencing of 16S rRNA from symbiotic bacteria

The host species of the symbionts whose sequences we have obtained are listed in Table 1.

A phylogenetic tree based on most of these sequences (and additional reference species) is also attached.

PUBLICATIONS AND REPORTS

Stein, J.L., Haygood, M., and H. Felbeck: Diversity of ribulose bisphosphate carboxylase genes in sulfur - oxidizing symbioses. In: Endocytobiology IV, P. Nardon, V. Gianinazzi-Pearson, A.M. Grenier, L. Margulis, D.C. Smith (eds.), Institut National de la Recherche Agronomique, Paris, 343 - 348 (1990)

Distel, D.L.: Detection, identification and phylogenetic analysis of endosymbiotic bacteria using ribosomal RNA sequences. In: Endocytobiology IV, P. Nardon, V. Gianinazzi-Pearson, A.M. Grenier, L. Margulis, D.C. Smith (eds.), Institut National de la Recherche Agronomique, Paris, 339 - 342 (1990)

Felbeck, H.: Symbiosis of bacteria with invertebrates in the deep sea. In: Endocytobiology IV, P. Nardon, V. Gianinazzi-Pearson, A.M. Grenier, L. Margulis, D.C. Smith (eds.), Institut National de la Recherche Agronomique, Paris, 327 - 334 (1990)

Felbeck, H., and D. L. Distel: Prokaryotic symbionts in marine invertebrates. In: The Prokaryotes, 2nd edition (eds. A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K. H. Schleifer), Springer Verl., in press

Stein, J.L., Haygood, M., and H. Felbeck: Nucleotide sequence and expression of a deep sea ribulose 1,5 bisphosphate carboxylase gene cloned from a chemoautotrophic bacterial endosymbiont. PNAS 87:8850-8854 (1990)

Distel, D.L., DeLong, E., and J.B. Waterbury: Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) using 16S rRNA sequence analysis and oligonucleotide probe hybridization. Appl. Env. Microbiol., submitted

Distel, D.L., and A.P. Wood: Phylogenetic characterization and comparison of Thiobacillus thyasiris and the bacterial endosymbiont in the gill of Thyasira flexuosa (Thyasiridae: Bivalvia) by 16S rRNA sequence analysis. Arch. Microbiol, in prep.

Distel, D.L., and H. Felbeck: Analysis of the phylogenetic origins of autotrophic bacterial symbioses in marine bivalves by 16S rRNA sequence analysis. In prep.

TRAINING ACTIVITIES:

Research assistantships for Ute Hentschel, Tristan Darland, and Connie Woolfe (50%, part of the year) and salary for postdoctoral researcher Dr. Daniel L. Distel.

Women or Minorities - 1 (U.H.)
Non-citizens - 1 (german, U. H.)

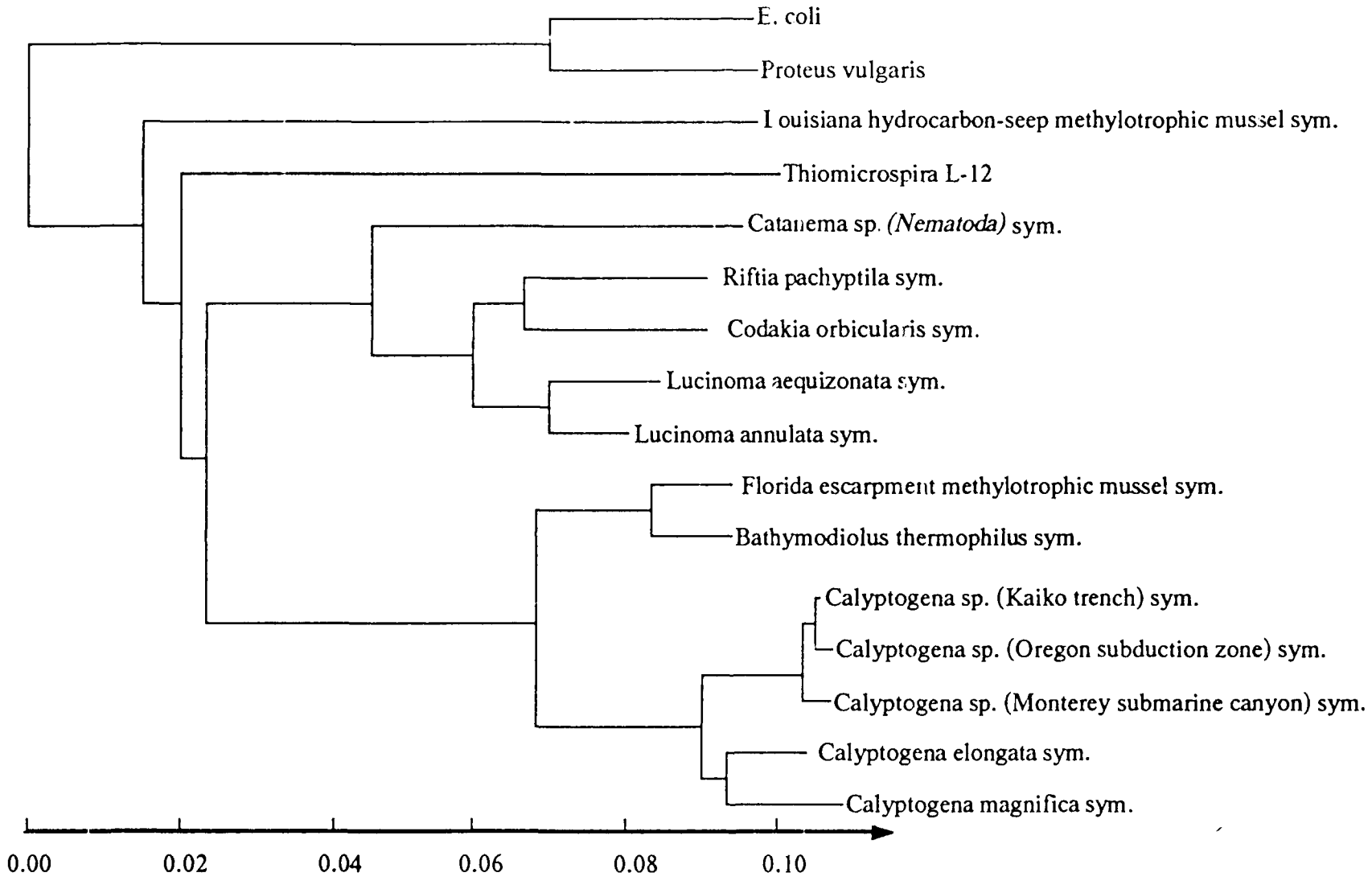
AWARDS

Graduate student Jeffrey Stein has been awarded a NASA graduate student fellowship.

Table 1. Species with symbionts whose 16S rRNA sequences have been determined by us.

Bivalves

Superfamily	Family	Genus/Species	Location
Lucinacea	Lucinidae	Lucinoma aequizonata	Santa Barbara Basin, CA
		Codakia orbicularis	Nassau, Bahamas
		Anodontia philippiana	Mangrove Bay, Bermuda
		Divaricella quadrisulcata	Waderick Wells, Exumas
	Thyasiridae	Thyasira flexuosa	Brest Harbor, France
Glossacea	Vesicomyidae	Calyptogena sp C1KN7	Kaiko trench, Japan
		Calyptogena kaikoi	Kaiko trench, Japan
		Calyptogena laubieri	Kaiko trench, Japan
		Calyptogena magnifica	Rose Garden, East Pacific Rise
		Calyptogena sp	Oregon Subduction Zone
		Calyptogena sp	Monterey Submarine Canyon
		Calyptogena elongata	Santa Barbara Channel, CA
Mytilacea	Mytilidae	Bathymodiolus thermophilus	Rose Garden, East Pacific Rise
		Bathymodiolus sp	Louisiana Hydrocarbon Seeps
		Bathymodiolus sp	Florida Escarpment Seeps
Pholadaceae	Teredinidae	Lyrodus pedicellatus	San Diego, CA
		Bankia gouldi	Fort Pierce, FL
		Dicyathifer manni	Australia
		Teredora malleolus	Massachusetts
Other Symbiont-Containing Taxa			
Riftiida	Riftiidae	Riftia pachyptila	Rose Garden, East Pacific Rise
Nematoda	Stilbonematinae	Catanema sp.	Carrie Bow, Belize
Gastropoda		Alvinochonca hessleri	Marianas Back Arc basin



Phylogenetic relationships among known sulfur and methane oxidizing symbiotic bacteria based on comparison of 16S rRNA sequences. The horizontal axis represents evolutionary distance (nucleotide substitutions per sequence position). Also included as reference species are the enteric bacteria E. coli and P. vulgaris and the obligate sulfur chemoautotroph Thiomicrospira (strain L-12).