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Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis

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Abstract

Nearly complete 16S rDNA sequences were determined for three tropical Lucinid endosymbionts and compared with previously examined symbiotic thioautotrophs. These symbionts include one previously analyzed, *Codakia orbicularis,* and two new gill symbionts of *Lingua pensylvanica* and *Lucina pectinata.* Only one type of bacterial 16S rDNA was detected in the two analyzed specimens of each host species, showing that the symbiont population within a Bivalve species appears to be composed of a single species. The three symbiont sequences analyzed fall within the γ-subdivision of the Proteobacteria, in the cluster containing previously examined symbiotic thioautotrophs from the bivalve superfamily Lucinacea. The two bivalves from shallow-water sea-grass beds, *Codakia orbicularis* and *Lingua pensylvanica,* seem to be colonized by the same symbiotic bacteria. However, the symbiont of *Lucina pectinata,* which that lives in sulfide-rich muddy mangrove areas, seems to be a deeply branching microorganism of the Lucinacea cluster.

Introduction

Symbioses between bivalves and intracellular ctenidial bacteria were first described in deep-sea hydrothermal vent environment (Felbeck et al., 1981), then in a gutless species of Solemyidae inhabiting reducing sediment (Felbeck, 1983) and in shallow-water sea-grass bed Lucinidae (Berg and Alatalo, 1982, 1984). The sulfur-oxidizing symbiosis has been found in all species of eight genera of Lucinidae that have been examined so far, and this symbiosis is considered as the most important factor in the evolution of the Lucinacea (Reid, 1990). Some evidence of phylogenetic congruence has been emphasized by Distel et al. (1994) between sulfur-oxidizing bacterial endosymbionts and their hosts, showing that symbionts of the bivalve superfamily Lucinacea and the family Vesicomyidae each form distinct monophyletic lineages.

We tried to cultivate the endocellular bacteria collected from the large species *Codakia orbicularis,* but without success, probably because of unknown conditions found in host bacteriocytes which have not been reproduced in vitro. Therefore, the chemoautotrophic symbionts remain inaccessible by traditional microbiological techniques. In this investigation, three large tropical Lucinidae, *Codakia orbicularis* (Linné, 1758), *Lucina pectinata* (Gmelin, 1791), and *Lingua pensylvanica* (Linné, 1758) were analyzed. We compared sequences of 16S rRNA genes amplified directly from symbiont-containing gill tissues of these Lucinidae by polymerase chain reaction (PCR). The goal of this investigation was, by using comparative analysis of 16S rDNA sequences, to compare the sequence obtained from *Codakia orbicularis* collected in Guadeloupe with the sequence previously proposed by Distel et al. (1988) on a single *Codakia orbicularis* from the Bahamas; identify and characterize the bacterial endosymbionts of *Lingua pensylvanica* and *Lucina pectinata;* and examine the phylogenetic relations of these new symbionts among the variety of known sulfur-oxidizing chemoautotrophic bacterial endosymbionts.

Results

PCR amplification performed with bacterial 16S rRNA primers on each gill tissue produced DNA

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fragments of the expected size (approx. 1500 bp). No amplification products were detected using DNA from symbiont-free foot tissues of each host species. Direct sequence analysis indicated that PCR products from the gill tissues of each host species contained a single detectable sequence. Each of these symbiont 16S rDNA samples was then sequenced independently. The almost-complete sequence of a continuous stretch of 1502 nucleotide positions of the 16S rDNA from the three symbionts was determined, corresponding to positions 9 to 1510 of the *Escherichia coli* nomenclature (Brosius et al., 1981). In each case, 16S rDNA sequences from the symbionts of two specimens of the same host species were identical at all positions determined.

Sequence analysis of the 16S rDNA of *Codakia orbicularis* and *Lingua pensylvanica* symbionts showed them to be identical at all 1502 nucleotides positions determined (Table 1). Thus, it is highly probable that these two symbionts represent a single bacterial species (Fox et al., 1992). A partial sequence (1271 nucleotides with numerous uncertainties) was available for *Codakia orbicularis* symbiont (Distel et al., 1988). After this sequence was aligned with our database, we found some phylogenetic discrepancies that led us to assume that the published sequence contained a few errors (for example, differences were observed in conserved domains, and secondary idiosyncrasies relative to other 16S rRNA were noted). For confirmation, we always obtained the same sequence for *Codakia orbicularis* symbiont 16S rDNA from different specimens and PCR amplifications.

Phylogenetic analysis placed, as expected, the new symbiont sequence in the γ-subdivision of the Proteobacteria (Figure 1). Analyses performed with either distance (Figure 1) or parsimony methods (data not shown) gave identical topology, indicating that the correct phylogeny has been determined (Kim, 1993).

Codakia orbicularis and *Lingua pensylvanica* symbionts are well included in the Lucinidae branching. On the other hand, the *Lucina pectinata* symbiont seems to be included as a basal member of the Lucinacea cluster that contains the symbionts of Lucinidae and Thyasiridae species. *Lucina pectinata* symbiont represents an individual line of descent, showing no close relations to any of the previously described symbiont sequences.

Discussion

All gill endosymbionts studied here are phylogenetically related to the cluster of Lucinidae symbionts. Our analysis revealed a robust monophyletic unit formed by the Lucinidae symbionts, which agrees with previous small-subunit rRNA analysis (Distel et al., 1994). Our sequence of *Codakia orbicularis* contains approximately 1500 bp against 1271 bp for the sequence of Distel et al. (1988); all differences detected between these two sequences are attributable to sequence ambiguities. All specimens of *Codakia orbicularis* collected in different Caribbean areas (Bahamas and Guadeloupe) and at different times (1988 to 1995) have identical 16S rDNA sequences for the symbionts. No specimen-to-specimen variation in 16S rDNA sequences is found in the many different animals that were independently analyzed. Thus, these observations provide further support for the conclusion that host-symbiont relations appear to be unique and invariant within *Codakia orbicularis*.

Lingua pensylvanica and *Codakia orbicularis* symbionts have indistinguishable 16S rDNA sequences, supporting the conclusion that they host the same intracellular gill symbiont. Both bivalve species occur in the same type of shallow-water sea-grass beds (*Thallassia testudinum* environment) but exclude each other.

Lucina pectinata hosts a symbiont that seems to be a deeply branching microorganism from the cluster of Lucinacea symbionts. This bivalve is an unusual species in comparison with the other Lucinidae: it is the most abundant species living deeply burrowed in black reducing mud of mangrove swamps and the most resistant to flood rains and to stagnant conditions that imply anaerobic conditions and H_2S accumulations (Read, 1962; Jackson, 1973). Symbionts are located mostly in the apical region of the bacteriocytes (personal observations). These features are important enough to suppose metabolic pathways to be quite different in *Lucina pectinata* than in other Lucinacea.

Owing to the fact that the bivalve classification is an old one based on shell characteristics, only a comparison of 16S rRNA gene symbiont sequences and 18S rRNA gene (eukaryotic) sequences from bivalve hosts can resolve questions of phylogenetic relations between symbionts and their hosts.

The finding that *Codakia orbicularis* and *Lingua pensylvanica* host the same symbiont led us to revise the assumption that symbionts are unique to each host species in the superfamily Lucinacea. But more results need to be obtained for other symbiont-bearing bivalves from the same environment (shallow-water sea-grass bed) to clarify the situation.

Vertical transmission of bacterial endosymbi-

Table 1. Percentage identity matrix based on comparison of 16S rRNA gene sequences from currently known bivalve symbionts and free-living bacteria.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1: Codakia orbicularis sym.																								
2: Lingua pensylvanica sym.	100.0																							
3: Lucina floridana sym.	97.2	97.2																						
4: Codakia costata sym.	97.5	97.5	99.6																					
5: Lucinoma aequizonata sym.	96.2	96.2	96.6	97.0																				
6: Thyasira flexuosa sym.	94.2	94.2	94.9	94.9	94.1																			
7: Anodontia philipiana sym.	94.5	94.5	95.1	94.7	93.9	94.7																		
8: Lucina pectinata sym.	94.4	94.4	93.9	93.9	92.8	94.3	94.0																	
9: Solemya velum sym.	92.5	92.5	92.2	91.8	91.2	91.9	92.5	91.9																
10: Solemya reidi sym.	91.3	91.3	91.8	91.4	90.7	90.6	90.8	90.4	91.7															
11: Laxus sp. sym.	92.0	92.0	91.4	91.4	90.4	89.9	89.3	91.8	89.9	90.3														
12: Thiomicrospira L12	88.8	88.8	88.4	88.4	88.2	89.1	88.5	88.9	87.7	87.8	87.5													
13: Thiomicrospira thyasiris	86.9	86.9	87.2	87.2	86.7	87.3	86.4	87.8	86.7	87.1	86.9	92.2												
14: Calyptogena magnifica sym.	87.8	87.8	87.6	88.0	87.4	88.1	86.4	87.3	85.6	86.4	86.7	85.1	85.2											
15: Vesicomya chordata sym.	87.4	87.4	87.6	88.0	87.3	87.7	86.3	87.0	85.4	86.3	86.6	85.2	85.2	97.5										
16: Calyptogena elongata sym.	88.1	88.1	88.1	88.4	87.9	88.5	87.0	87.5	85.6	86.8	86.7	85.4	85.4	96.7	96.8									
17: Bathymodiolus thermophilus sym.	89.1	89.1	90.0	89.6	88.9	89.4	89.3	87.9	87.6	87.9	86.9	87.1	85.9	93.6	94.4	94.0								
18: Escherichia coli	83.8	83.8	84.7	84.3	83.3	83.9	84.1	85.5	84.3	84.7	84.2	83.0	82.0	82.6	83.0	82.8	84.3							
19: Thiobacillus hydrothermalis	87.5	87.5	87.5	87.2	86.1	85.7	87.0	87.1	86.3	88.8	87.4	83.3	84.5	85.2	85.3	85.2	86.2	83.9						
20: Vibrio harveyi	84.5	84.5	85.3	85.3	85.0	85.4	84.7	85.7	85.4	85.4	84.3	84.4	84.3	84.3	84.1	84.6	86.1	89.6	83.5					
21: Chromatium vinosum	90.0	90.0	89.9	89.5	88.1	88.4	89.5	89.4	89.2	89.4	91.0	85.6	85.6	84.4	84.2	84.5	85.8	84.7	88.9	84.0				
22: Neisseria gonorrhoeae	83.0	83.0	83.4	83.1	82.1	82.6	82.7	84.1	83.0	82.7	82.9	80.6	80.3	80.4	80.9	81.0	81.9	81.6	83.5	79.5	83.5			
23: Pseudomonas testosteroni	83.8	83.8	83.5	83.3	83.1	83.2	84.4	83.9	83.7	83.1	82.2	80.8	81.7	80.0	80.3	80.3	81.7	81.1	82.8	81.0	83.3	84.5		
24: Agrobacterium tumefaciens	84.0	84.0	84.0	84.0	83.4	83.5	83.3	84.0	82.7	81.7	83.8	81.3	80.4	81.0	81.3	81.7	81.2	78.8	81.9	78.7	82.9	81.1	77.4	
25: Rickettsia ricketsii	80.2	80.2	80.3	80.5	81.0	81.7	80.1	80.7	80.8	81.0	80.4	79.0	79.4	80.1	79.9	80.3	79.6	77.3	80.3	79.2	79.7	77.4	78.4	83.5

Analysis was restricted to 1099 nucleotide homologous positions of sequence from each microorganism.

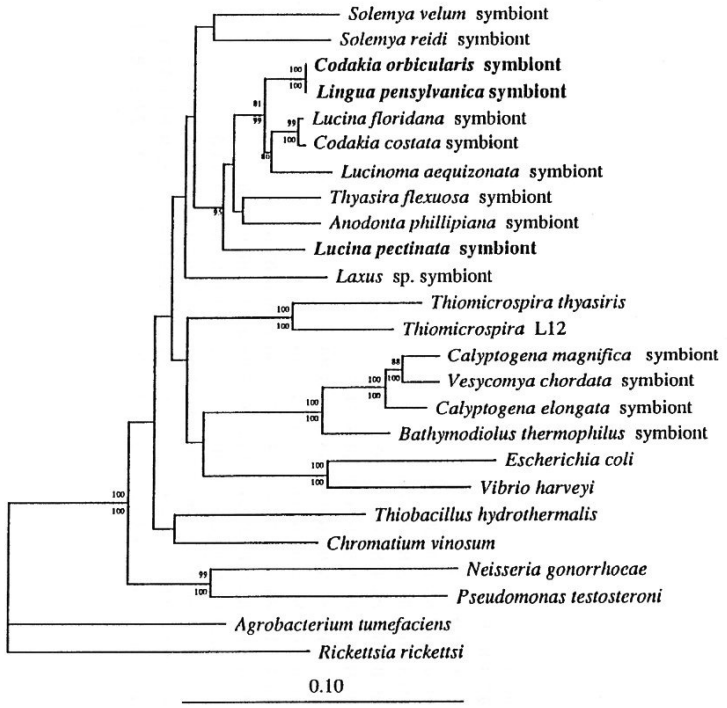

Figure 1. Phylogenetic tree showing partial sequences of the 16S rRNA gene aligned with the homologous sequences of diverse Lucinacea symbionts. This tree was constructed using distance values listed in Table 1. Scale bar represents 10 nucleotide substitutions per 100 nucleotide positions. Percentages of bootstrap resampling that support each topologic element are indicated (upper and lower values correspond to distance and parsimony analysis, respectively). This tree was rooted with two specimens of the α-Proteobacteria: *Agrobacterium tumefaciens* and *Rickettsia rickettsi*.

onts through an early infestation of eggs (Gustafson and Reid, 1988) has recently been shown to occur in deep-sea hydrothermal vent bivalves *Solemya reidi* (Cary, 1994) as well as in *Bathymodiolus thermophilus* (Cary et al., 1993) and in three species of the genus *Calyptogena* (Cary and Giovannoni, 1993). Horizontal transmission (bacteria transmitted to the new generation at some later stage of development through free-living symbiont forms [Le Pennec et al., 1988]) has been reported in *Riftia pachyptila* and *Ridgea piscesae* (Cary et al., 1993). For *Codakia orbicularis, Lingua pensylvanica,* and *Lucina pectinata,* we cannot support any of these hypotheses, but work is in progress to help elucidate the mode of transmission of their bacterial symbionts.

Experimental Procedures

Specimen collection

Specimens were collected by hand from shallow-water sea-grass beds, in Guadeloupe for *Codakia orbicularis,* and in Martinique for *Lingua pensylvanica. Lucina pectinata* was collected in Guadeloupe, from sulfide-rich black mud of mangrove swamps. Bivalves were kept alive until DNA extraction, which was performed within 24 hours.

DNA preparation

DNA was independently extracted from two specimens of each host species. Intact symbiont-containing gill tissue and symbiont-free foot tissue (as a negative control) were dissected from each specimen. Pieces of gills were carefully delaminated. To eliminate surface contaminants, samples of each specimen were washed in filtered (0.2 μm) seawater. Tissue samples were ground under liquid nitrogen, and the frozen powder was homogenized in 300 μl of lysis buffer (100 mmol/L NaCl, 100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0); 20 μg of RNase A was added to 180 μl of this lysate and incubated at 37°C for one hour. Then, 50 μl of TES-K (200 μg/ml proteinase K, 2% sodium dodecyl sulfate [SDS] in TE buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0]) were added, and the lysate was incubated overnight at 37°C. After digestion, the aqueous lysate was extracted by phenol-chloroform extraction (Sambrook et al., 1989). Nucleic acids were precipitated overnight with two volumes of 100% ethanol and 0.1 volume 3 M sodium acetate [pH, 5.3] at −20°C. The pelleted nucleic acids were

washed in 70% ethanol, dried, and resuspended in 100 μl of TE buffer.

PCR amplification and sequencing

We determined 16S rDNA sequences directly from PCR amplification products. PCR amplification was performed using 0.2 mmol/L each dNTP, 10 μl of 10× reaction buffer (Biotech International Ltd, Australia), 0.2 μg of each eubacterial 16S rRNA–specific primers (27f, 1492r) (Lane, 1991), 0.1 μg of template DNA, and 2.5 units of Tth plus DNA polymerase (Biotech International Ltd, Australia), in a total volume of 100 μl. Samples were amplified under the following regime: initial denaturation at 94°C for two minutes, followed by 30 cycles of denaturation at 94°C for one minute, annealing at 50°C for one minute, and extension at 72°C for two minutes, followed by a final elongation time of seven minutes at 72°C. Negative controls were always run as a precaution against laboratory-derived contamination. Thus, we amplified only the bacterial 16S rDNA contained in the mixture of prokaryotic and eukaryotic DNA extracted from the bivalve tissues.

The PCR products of three independent amplifications were combined for each sequencing reaction in order to reduce nucleotide misincorporation errors. Moreover, the authenticity of symbiont 16S rDNA sequence was determined from two specimens of each of the three host species.

PCR products were sequenced directly using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.), according to the manufacturer's protocol. The sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer.

Data analysis

Sequences were manually aligned with published sequences from representatives of the main sublines of descent of the Proteobacteria and with previously described symbiont sequences (Distel et al., 1988, 1994; Distel and Wood, 1992; Eisen et al., 1992; Lane et al., 1992). Because of the presence of nonsequenced 16S rDNA stretches and regions of alignment ambiguities with sequences of the databases, certain positions were excluded from the analysis. Therefore, a total of 1099 nucleotides was used.

Phylogenetic analyses were performed using the following programs, which are part of the Phylip 3.5 software package (Felsenstein, 1989) on a Sun SPARC station IPC. Distance analyses were performed using DNADIST with the Jukes and Cantor (1969) correction and a transition/transversion ratio of 2.0. Phylogenetic trees were constructed using NEIGHBOR (Saitou and Nei, 1987). Maximum parsimony analysis was performed using DNAPARS. Bootstrap values based on the analysis of 100 trees of 1099 polymorphic sites were calculated using the programs SEQBOOT and CONSENSE. Only the bootstrap values above 75% were considered to provide confidence limits for internal branching of the tree (Zharkikh and Li, 1992).

Nucleotide sequence accession number

The sequence data reported in this investigation have been deposited in the EMBL data library under accession numbers X84979 (*Codakia orbicularis* and *Lingua pensylvanica* symbiont) and X84980 (*Lucina pectinata* symbiont).

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