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Bacterial host specificity of Lucinacea endosymbionts: Interspecific variation in 16S rRNA sequences

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Abstract

Three tropical lucinid clams (*Codakia orbiculata*, *Codakia pectinella* and *Lucina nassula*) from a shallow coastal environment have been studied regarding to their thioautotrophic bacterial endosymbionts. The 16S rRNA genes (rDNA) from these three endosymbionts were amplified using PCR. Phylogenetic analysis by distance matrix and parsimony methods always placed the newly examined symbionts within the monophyletic group composed of symbionts of the bivalve superfamily Lucinacea. A same single 16S rRNA sequence was found in *C. orbiculata* and *C. pectinella* and was identical to that found in *C. orbicularis* and *Linga pensylvanica*, two other lucinids living in the same type of environment. These data indicate that a same symbiont species may be associated with different host species. *Lucina nassula* hosts a symbiont with a distinct 16S rDNA sequence, but very closely related to the former.

Keywords: Thioautotrophic bacteria; Lucinacea; Symbiosis; 16S rDNA; Phylogeny

1. Introduction

Symbiotic thioautotrophs are known to be distributed across a broad range of host taxa in marine environment (Annelida, Mollusca, Pogonophora and Vestimentifera) [1–4]. The greatest diversity of intracellular thioautotrophic endosymbioses is found among bivalve mollusks, particularly in the superfamily Lucinacea [5]. Thioautotrophic symbionts have not been cultured from their hosts, nor has a free-living life stage of the symbionts been isolated from the environment. Comparisons of rRNA sequences have become particularly useful in characterizing symbionts, because these sequences can be obtained without isolating the bacteria from their habitat or host.

Chemoautotrophic sulfur oxidation is extremely widespread in bacteria [6]. In the Proteobacteria alone, it is in at least four of the five subdivision (alpha, beta, gamma and epsilon). However, all of the thioautotrophic symbionts examined to date unambiguously belong to the gamma subdivision. Symbionts of the bivalve superfamily Lucinacea form a distinct monophyletic lineage specifically associated with the host family [5].

Previous investigations of thioautotrophic symbiont phylogeny based on 16S rRNA sequences have shown that the symbionts found in each bivalve species were unique and invariant within that host species [5]. However, the concept of monospecific

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association has recently been revised with the demonstration of the coexistence of methano- and thioautotrophic bacteria in the hydrothermal vent mussel [7]. It has also been observed that the specificity of the association between host and symbiont was unique to each host species [5,8]. However, we recently have found the same 16S rDNA sequence in two different tropical bivalve species, *Codakia orbicularis* and *Linga pensylvanica* [9].

In this investigation, we sought to establish the phylogenetic relationships of the symbionts populating the gills of Lucinacea bivalves living in seagrass beds off the French West Indies, and the specificity of these associations.

2. Materials and methods

2.1. Organisms

Adult specimens of *Codakia orbiculata*, *Codakia pectinella* and *Lucina nassula* were collected by hand from a shallow water seagrass bed (*Thalassia testudinium* environment), off Guadeloupe in the Caribbean area. This zone was already sampled for *C. orbicularis* [9]. Live specimens were processed for dissection and DNA extraction within 24 h.

2.2. DNA extraction, PCR amplification and sequencing

Nucleic acids from symbiont-containing gills and symbiont-free foot tissues (negative controls) were prepared as described elsewhere [9]. Samples of DNA were independently obtained from two specimens of each host species, with the exception of *Lucina nassula*, of which only one specimen was available. The 16S rRNA genes from each of these symbiont DNA samples was then amplified with bacterial primers [10] and sequenced independently using methods described previously [9].

2.3. Phylogenetic analysis

Sequences were manually aligned with published sequences from previously described thioautotrophic symbionts by using the ae2 sequence editor [11]. Nucleotide positions which were undetermined or of ambiguous identity in at least one sequence of the database used for the phylogenetic analysis and sequence regions which could not be aligned with certainty were eliminated from consideration. A total of 1137 nucleotide positions were utilized in this analysis. Phylogenetic analyses were performed by using the following programs, contained in the Phylip 3.5 package [12].

Evolutionary distances were estimated by using DNADIST with Jukes and Cantor correction. Phylogenetic trees were constructed using NEIGHBOR. Maximum parsimony analysis was performed using DNAPARS. Bootstrap values based on the analysis of 100 trees were calculated using the programs SEQBOOT and CONSENSE. Bootstrap values greater than 50% are given but are considered to support the grouping of organisms in an associated node only at values greater than 75% [13].

2.4. Nucleotide sequence accession number

The sequence data have been deposited in the European Bioinformatics Institution database under the accession numbers X84979 and X95229.

3. Results and discussion

No amplification products were detected using DNA from control tissues (symbiont-free foot tissues). PCR amplification performed on each gill tissue produced a single DNA fragment of the expected size ($\sim$ 1.5 kb). Direct sequence analysis indicated that PCR products from the gill tissues of each host species contained a single detectable sequence, invariant within the examined samples of that host species. This indicates that the symbiont population of each host species is composed entirely, or at least predominantly, of a single symbiont type.

Continuous nucleotide sequences were determined from the 16S rRNA gene of *C. orbiculata* (positions 42–1503 of the *Escherichia coli* structural model [14]), *C. pectinella* (positions 74–1510) as well as that from *Lucina nassula* (positions 29–1510). Sequences of *C. orbiculata* and *C. pectinella* symbionts were 100% identical at all nucleotide positions determined (1437 nucleotides), suggesting that these symbionts represent the same species [15].

Interestingly, these two bivalves co-occur in seagrass beds off Guadeloupe with *C. orbicularis* which hosts a symbiont with an identical 16S rRNA sequence. Moreover, the same symbiont was described in *Linga pensylvanica*, another lucinid clam living in seagrass beds off Martinique (but absent from Guadeloupe) [9], as well as in *C. orbicularis* collected in the Bahamas in the same habitat [8]. Hence, the same symbiotic bacterium is found in four different host species living in similar habitats, irrespective of their geographical location.

The *Lucina nassula* gill symbiont represents a new species closely related by sequence similarity to the symbionts of *C. orbicularis* (and hence *C. orbiculata*, *C. pectinella* and *Linga pensylvanica*, similarity of 98.3%), *Lucina floridana* (98.2%) and *C. costata* (97.8%), all members of the bivalve superfamily Lucinacea living in seagrass beds (Table 1).

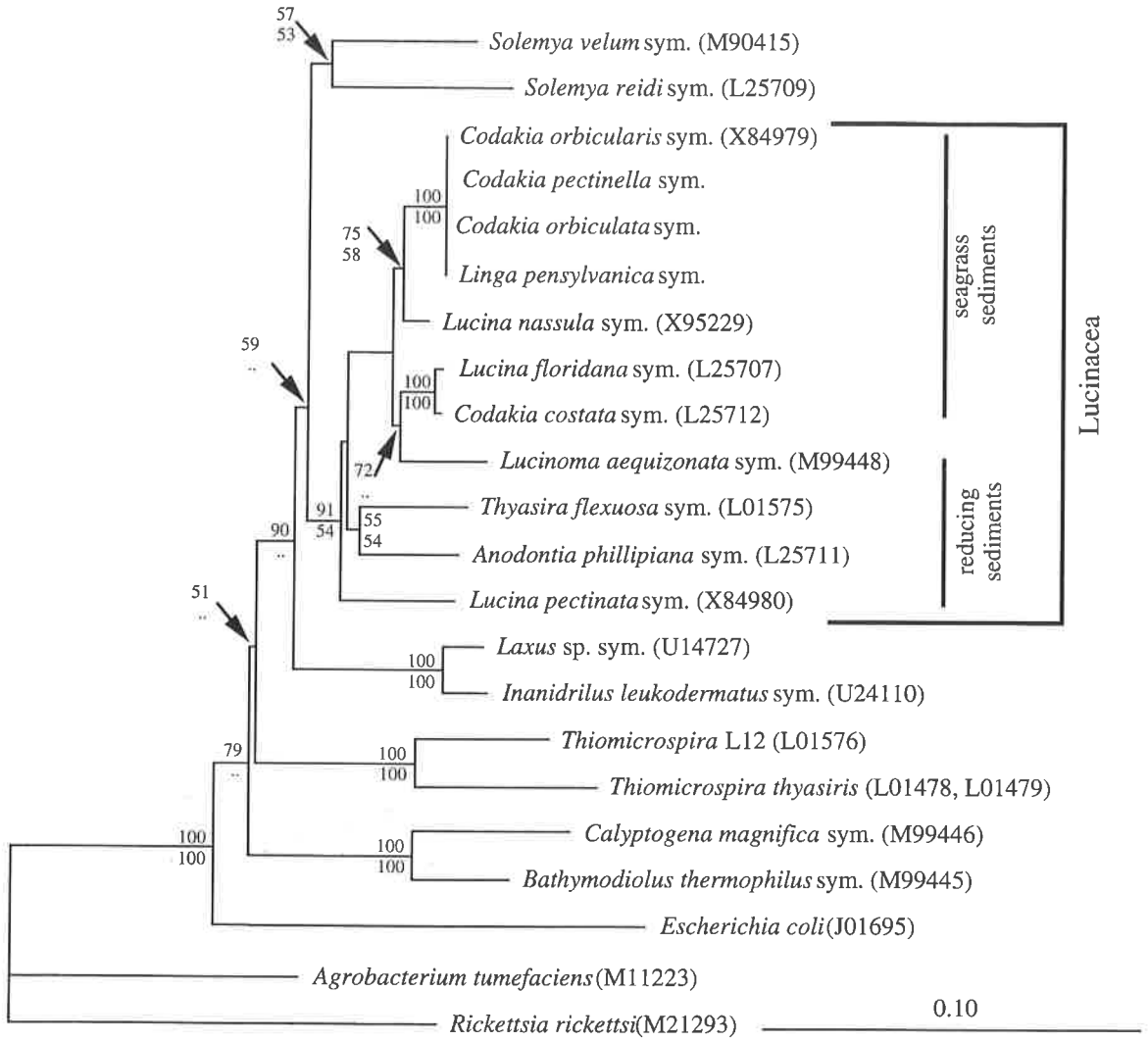

Fig. 1. Evolutionary distance tree (based on 16S rRNA sequences) from lucinid clam symbiotic bacteria and selected symbiotic and free-living representatives of the class Proteobacteria. Bootstrap values for selected nodes which are supported in more than 50 of 100 trees by distance analysis (upper numbers) and parsimony analysis (lower numbers) are shown. The scale bar indicates 0.1 nucleotide substitutions per sequence position.

Table 1
16S rRNA sequence similarity analysis of lucinid clam symbiotic bacteria and selected symbionts and free-living bacteria

	1–4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1: *Codakia orbicularis* sym. [a]	100.0															
2: *Codakia pectinella* sym. [a]	100.0															
3: *Codakia orbiculata* sym. [a]	100.0															
4: *Linga pensylvanica* sym. [a]	100.0															
5: *Lucina nassula* sym. [a]	98.3															
6: *Lucina floridana* sym. [a]	97.3	98.2														
7: *Codakia costata* sym. [a]	97.6	97.8	99.6													
8: *Lucinoma aequizonata* sym. [b]	96.2	96.4	96.7	97.0												
9: *Thyasira flexuosa* sym. [b]	94.3	95.1	95.0	95.0	94.1											
10: *Anodontia philipiana* sym. [b]	94.7	95.3	95.3	94.9	94.0	94.8										
11: *Lucina pectinata* sym. [b]	94.6	95.3	94.1	94.1	93.0	94.4	94.2									
12: *Solemya velum* sym.	92.8	92.8	92.4	92.1	91.4	92.1	92.8	92.2								
13: *Solemya reidi* sym.	91.5	91.8	92.0	91.6	90.9	90.8	91.0	90.7	91.9							
14: *Laxus* sym.	92.3	92.2	91.6	91.6	90.7	90.1	89.6	92.1	90.2	90.5						
15: *Inanidrilus leukodermatus* sym.	91.9	92.1	91.6	91.6	90.4	90.3	90.1	91.7	90.3	90.3	97.8					
16: *Thiomicrospira* L12	88.7	89.0	88.3	88.3	88.0	88.8	88.4	88.7	87.6	87.8	87.4	87.6				
17: *Calyptogena magnifica* sym.	88.0	87.3	87.8	88.1	87.4	88.1	86.6	87.4	85.8	86.5	86.9	87.1	85.0			
18: *Bathymodiolus thermophilus* sym.	89.2	89.4	90.1	89.7	88.9	89.4	89.4	88.0	87.8	88.0	87.1	87.2	87.0	93.7		
19: *Escherichia coli*	84.2	84.8	85.0	84.7	83.6	84.2	84.4	85.8	84.7	85.0	84.5	84.9	83.0	82.9	84.6	
20: *Rickettsia rickettsi*	80.1	80.1	80.3	80.5	80.8	81.5	80.0	80.7	80.7	81.0	80.4	80.0	79.0	80.1	79.7	77.3

Analysis was restricted to *E. coli* nucleotide positions 97–1367, excluding the following positions for which data were either missing or ambiguous in one or more sequences and positions at which alignments were uncertain: 185, 190, 200–207, 211–217, 232, 272, 342, 343, 420, 452–463, 467–479, 497–547, 573, 578, 579, 587, 620, 628, 642, 650, 651, 700, 771, 840–846, 861, 862, 907–909, 930, 935, 941, 942, 1121, 1129, 1131, 1134, 1155, 1175, 1184, 1207, 1265, 1285 and 1293.

[a] Lucinacea species living in seagrass beds.

[b] Lucinacea species living in reducing sediments.

Comparison with the symbionts of the Lucinacea bivalves living in highly reduced sediments showed lower similarity values of between 96.4% and 95.1% (Table 1).

Comparative 16S rDNA gene sequence analysis of the three symbionts with representative members of the Bacteria indicated that they were related to the gamma subdivision of the Proteobacteria. Both distance and parsimony algorithms produced phylogenetic trees with mostly identical topologies. More specifically, these sequences fall within the distinct cluster containing 16S rRNA sequences of all symbionts from bivalves of the superfamily Lucinacea (Fig. 1). Bootstrap resampling of the sequences for distance analysis revealed significant support (91%) for the cluster containing the Lucinacea symbionts. This phylogenetic congruence among host and symbionts has already been described [5].

A distinction between bivalves living in slightly reduced and heavily reduced sediments is highlighted by the raw similarity data. However, the phylogenetic relationships among the Lucinacea symbiont group were difficult to resolve in our analyses, because the branching orders were not supported by bootstrap data and were different depending on the method of phylogenetic analysis used. The close phylogenetic relationship among symbionts of the Lucinacea superfamily bivalves could be explained by the proximity and/or the similarity of the environments in which these hosts are found (seagrass beds, highly reduced sediments). However, the final arrangement within the Lucinacea group of symbiont requires more 16S rDNA sequences of symbionts belonging to these two types of environment for comparative analysis.

Previous investigations have shown that the specificity of the association between host bivalve and symbiont was unique to each host species [5]. However, we showed that at least four species of Lucinacea bivalve host the same endosymbiotic bacteria, results based on 100% 16S rDNA sequence similarity. Other associations between marine animal hosts and symbiotic microorganisms examined to date display this moderately specific level of association [16]. Partial 16S rRNA sequence analysis of bacterial isolates and in situ hybridization revealed the similarity between the bacterial symbionts found in the gills of four species of *Teredinidae* mollusks [17]. This is also the case for the luminous symbiose-competent *Vibrio fisheri* found in the light organs of fishes and squids [18]. In the case of the symbionts of the vestimentiferans *Ridgea piscesae* and *Riftia pachyptyla*, similarity of the symbionts has been suggested but not rigorously demonstrated [19].

The conclusions of Distel and collaborators [5], namely, that sequences from each host species were unique to and invariant within that host species, are not supported by the current data. These new results suggest that a same symbiont may establish symbiosis with many Lucinacea bivalve host species sharing the same biotope. Moreover, a recent study on *C. orbicularis* symbiont strongly suggested an environmental mechanism of symbiont transmission, where progeny is thought to acquire their symbiont by reinfection from a free-living stock of microorganism [20]. This conception is strengthened with the description of bivalves living in the same environment and hosting the same bacterial endosymbiont. Currently, we are examining the symbiont transmission mode in the other lucinid species living in the French West Indies. Attempts to identify the symbionts from free-living bacteria sampled in seagrass bed will help to elucidate this mechanism.

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