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Characterization of the Endosymbiont of a Deep-Sea Bivalve, *Calyptogena soyoae*

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We have purified DNA from gill tissue of a marine bivalve, *Calyptogena soyoae*, collected from the deep-sea cold seep communities in Sagami Bay, Japan. An rRNA gene was amplified, cloned, and sequenced. In situ hybridization revealed that the sequence is that of a bacterial endosymbiont within the gill of *C. soyoae*.

The existence of symbiotic bacteria has been well established for several invertebrates found in hydrothermal vent areas (for reviews, see references 18 and 20). These symbiotic bacteria are thought to provide the marine bivalve with autotrophically fixed carbon. The source of energy for these symbioses appears to be oxidation of reduced inorganic substrates such as sulfide and thiosulfate. Such bacterial symbionts form the primary base of the food chain of the dense populations of invertebrates at the deep-sea hydrothermal vents and cold seeps. Supporting evidence comes from carbon isotope fractionation (3, 23, 25) and the presence of bacterial autotrophic and sulfur-related enzymes (5, 13) in the gills of marine bivalves.

Symbionts in the gill tissue of *Calyptogena* spp. and other marine mollusks resemble gram-negative prokaryotic cells when observed by electron microscopy (4, 10–12, 14, 27). The symbionts have not yet been cultured and little is known about their metabolic capabilities. A sulfur-oxidizing bacterium was reported to be isolated from the gill tissue of a marine bivalve, *Thyasira flexuosa* (29); however, the isolate was unrelated to the symbiont (8).

Recently, rRNA sequences of symbiotic bacteria of marine invertebrates have been determined directly from rRNA or indirectly from rRNA genes by PCR (7–9). These symbionts were shown to be closely related to each other and fell within a limited domain of the gamma subdivision of the purple bacteria (7–9). The symbiotic species are unique to and invariant within their respective host species.

Ohta and Laubier (22) have reported the occurrence of communities dominated by deep-sea giant clams of the genus *Calyptogena* (15, 22). The communities are thought to be sustained by chemosynthetic energy sources present at connate water seepage in subduction zones (15, 22).

This paper reports the partial 16S rRNA sequence of a symbiont within *Calyptogena soyoae* and in situ hybridization of symbionts in gill tissue of the clams obtained from Sagami Bay in Japan.

Samples. The deep-sea marine bivalve *C. soyoae* was collected at a depth of 1,100 m in Sagami Bay by a deep-sea submarine, Shinkai 2000 (15), and stored frozen until use. The frozen specimen was thawed, and the gill tissue was removed with a sterilized knife.

DNA preparation. Tissue blocks of 0.1 to 0.2 g were immersed in extraction buffer (10 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.1% dithiothreitol, 0.1% sodium dodecyl sulfate [SDS], 0.5 mg of proteinase K per ml) and incubated at 37°C overnight. The mixture was centrifuged at 1,000 rpm for 2 min, and the pellet was suspended in 2 ml of the extraction buffer and frozen and thawed twice. The mixture was treated with 0.5 mg of proteinase K per ml and extracted with phenol and then with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol.

Alternatively, 20 mg of tissue was frozen and thawed three times in 0.1 ml of PCR buffer (20 mM Tris-HCl [pH 8.3], 4.8 mM $MgCl_2$, 25 mM KCl, 0.5% Tween 20). Proteinase K (0.1 mg/ml) digestion was performed overnight at 57°C, and the enzyme was inactivated by heat treatment at 95°C for 10 min; the sample was stored at −20°C.

DNA amplification and sequence analysis. A partial sequence of the 16S rRNA gene was amplified by PCR in PCR buffer (17). Primers 910f (dAAACTCAAATGAATTG) and 1400r (dACGGGCGGTGTGTRC) were used; they were designed from universally conserved sequences (19). Forty cycles of the following thermal program were used: 94°C for 20 s, 40°C for 30 s, and 72°C for 1.5 min.

The amplified DNA was recovered from a low-melting-point agarose gel (0.8%) and ligated with a ddT-tailed vector as described by Horton and Graham (16) with modifications. Replicative form DNA of bacteriophage M13mp18 (21) was digested with *Sma*I and ddT tailed with 0.1 mM ddTTP by terminal transferase. The nucleotide sequence was determined by the dideoxy chain termination method with a DNA sequencer (ABI 370A).

Section preparation. Fixation, embedding, and sectioning of gill tissues were done as described by Wilkinson (28). A block of the gill tissue was prefixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight, incubated with 0.85% saline for 30 min twice, dehydrated with an ethanol series, and then embedded in paraffin (2). The paraffin blocks were sectioned to a 4-μm thickness. The thin section was fixed on slides coated with 3-aminopropyltriethoxysilane (24). Paraffin was removed by passing the slides through xylene, and the sections were rehydrated through an ethanol series and postfixed with 4% paraformaldehyde in PBS. They were rinsed with PBS and treated with 0.25% acetic anhydride in 0.1 M triethanolamine. The acetylated slides were dehydrated through an ethanol series and air dried. The sections were stored at room temperature until use.

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  1 TTGGGTTTAAGTCCCCGTAACGAGCGCAACCCTTTATCCTTATTTGCAAGTACATAATGG
    ******* **** ****************** ** ****** ** * ** ** *   **
  1 TTGGGTT AAGT CCCGTAACGAGCGCAACCC TTGTCCTTAGTTACCAGCACCT CGGG
                                     1110r
 61 TGGGAACTATAAGGAGACTGCCGGTGATAAACCGGAGGAAGGCGGGGACGACGTCAAGTC
    **** *** ***************** ************* ***** ***********
 57 TGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC
                                         1234r
121 ATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGGAAGGACAAAGGGTCGCT
    *************** * ***********************   * ********* **
117 ATCATGGCCCTTACGGCNAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCG
                              1284r      1229r
181 AAGCCGTGAGGTGGTGCTAATCTCACAAATCTTTTCGTAGTCCGGATCGGAGTCTGCAAC
    ****** ******* ******* ** *** *   *************** **********
177 AAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC

241 TCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGT
    ****** **************************   *********   *************
237 TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGT

301 TCCCGGGTCTTGCACACACCGCCCGT
    ******* **** *************
297 TCCCGGGCCTTGTACACACCGCCCGT
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FIG. 1. Comparison of rRNA sequences of *C. soyoae* endosymbiont (first row) and *P. aeruginosa* (second row). Asterisks indicate matched bases. Fluorescent probes were designed complementary to the portions of the sequences underlined.

Oligonucleotide probes. Oligodeoxynucleotide probes were synthesized with a DNA synthesizer (ABI 381A). An aminohexyl linker (Amino Link 2; ABI) was attached to the 5′ end of each oligonucleotide. Crude oligonucleotides were labeled with fluorescein isothiocyanate (Wako Chemicals), as described by Delong et al. (6). Oligonucleotides were separated from unreacted dye by passing the solution through a Sephadex G-50 column equilibrated with 20 mM tetraethylamine bicarbonate, pH 8.0. The eluate was dried by centrifugal evaporation and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

The following six probes were used: universal probes 910r d(CAATTCATTTGAGTTT) and 1400r d(ACGGGCGGTGTGTAC), symbiont-specific probes 1234r d(CCTTTGTCCTTCCCATTGTA) and 1284r d(GGACTACGAAAAGATTTGT), and *Pseudomonas aeruginosa*-specific probes 1110r d(AGGTGCTGGTAACTAAGGACA) and 1229r d(CCTTTGTACCGACCATTGTA).

In situ hybridization. The slides were hybridized with probes as described by Amann et al. (1). Nine microliters of hybridization solution was applied to each of the sections fixed on the slide, and the slides were incubated at 37°C for 30 min. Fluorescent oligonucleotide, 50 ng in 1 μl, was added to the mixture, which was incubated at 37°C for another 4 h. The hybridization mixture was removed by flushing the slide with 3 ml of washing solution (0.9 M NaCl, 50 mM sodium phosphate [pH 7.0], and 0.1% SDS), and the slides were immersed in washing solution at 37°C for 20 min. They were then rinsed with distilled water and air dried. The slides were mounted in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution and observed with an epifluorescence microscope (Nikon Optical Co., Tokyo, Japan) equipped with a fluorite flat-field ×40 objective and a blue and green filter set. Photomicrographs were taken with Kodak ASA400 films. Exposure times were 0.4 s for phase-contrast micrographs and 4 s for epifluorescence micrographs.

rRNA sequence. After PCR, a DNA fragment of the expected size, approximately 490 bp, was detected from DNA samples from gills of marine bivalves, prepared by using two

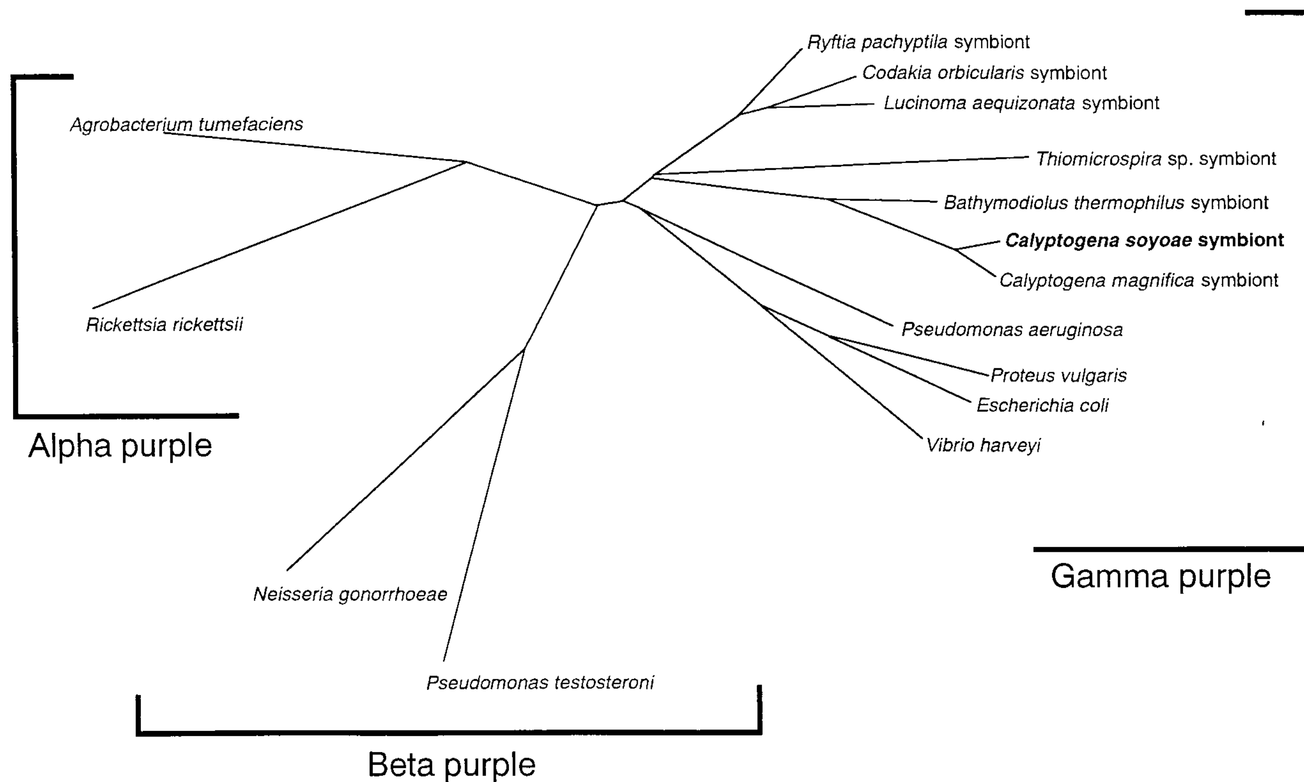

FIG. 2. Phylogenetic tree based on 16S rRNA sequences from the *C. soyoae* symbiont (this work) and selected free-living and chemoautotrophic symbiotic bacteria (7–9).

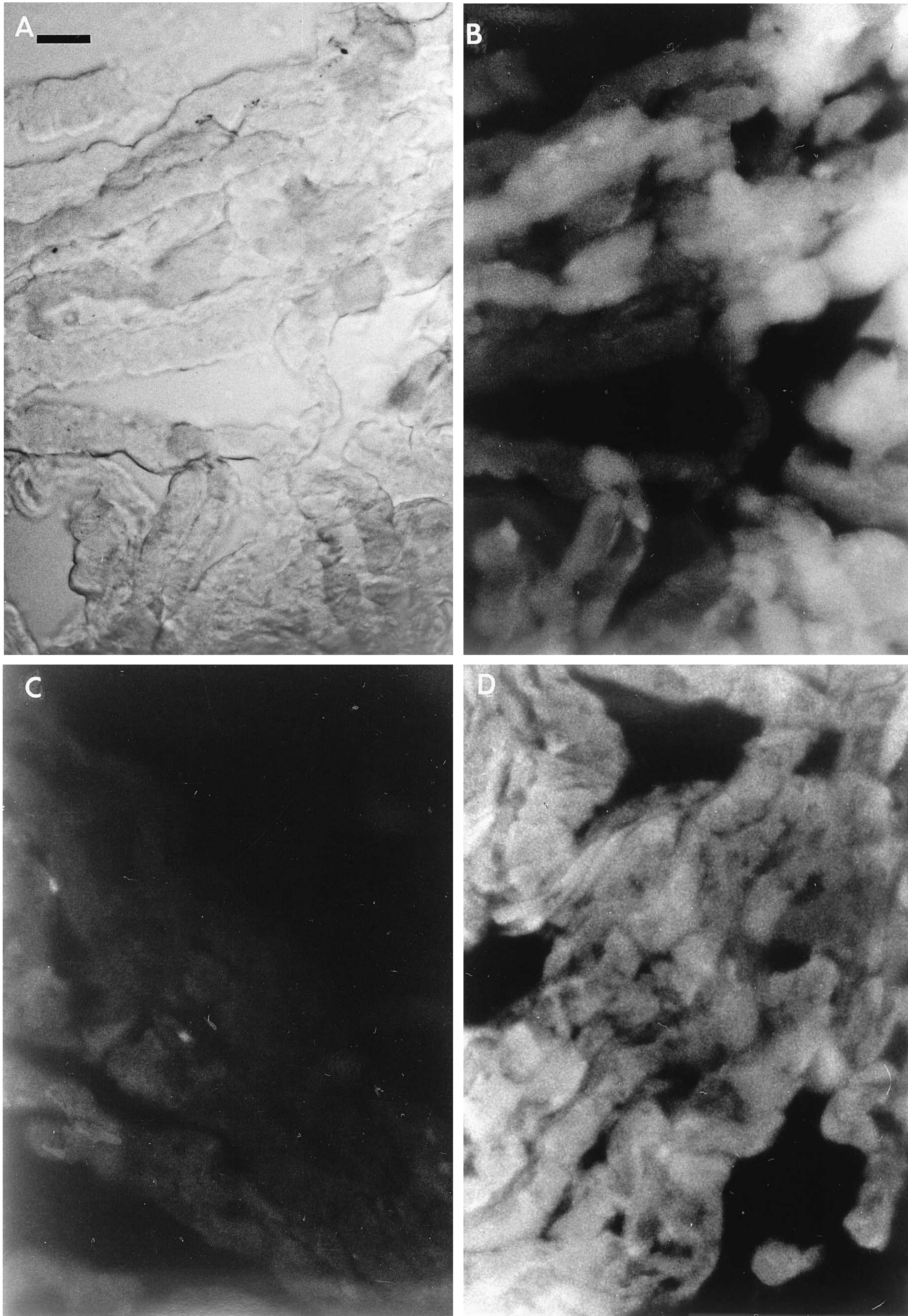

FIG. 3. Pictures of gill tissue of *C. soyoae*. (A) Phase-contrast photomicrograph of a tissue section. Bar, 20 μm. (B) Epifluorescence photomicrograph of the same field as panel A. The section was hybridized with symbiont-specific probes (1234r plus 1284r). (C) Epifluorescence photomicrograph of a section hybridized with *P. aeruginosa*-specific probes (1110r plus 1229r). (D) Epifluorescence photomicrograph of a section hybridized with universal probes (910r plus 1400r).

different procedures. The DNA fragments were cloned into an M13 bacteriophage vector. Three clones were isolated from the fragments amplified in three independent reaction mixtures. The sequences determined from the three isolates were identical and are shown in Fig. 1.

Our results indicate that the sequence represents a single bacterial species and that the species is at least predominant in the gill tissues, though we cannot dismiss the possibility that the same fragment was cloned because of preferential lysis or amplification of the fragment. The sequence was most similar (97.2%) to that of the *Calyptogena magnifica* symbiont reported by Distel et al. (7) and showed 86.7% similarity to that of *P. aeruginosa* among nonsymbiotic organisms.

A phylogenetic tree based on 16S rRNA of selected symbiotic and free-living bacteria was calculated by the N-J method with the software package ODEN in the National Institute of Genetics, Mishima, Japan (Fig. 2). Distel et al. have reported a phylogenetic tree derived from 16S rRNA sequences of invertebrate-associated symbiotic bacteria (7, 8). The symbionts make up two distinct phylogenetic clusters. The first cluster contains the symbionts of lucinoid clams and of the tube worm, and the second contains the symbionts of the hydrothermal vent bivalves *C. magnifica* and *Bathymodiolus thermophilus* (7–9). The symbiont of *C. soyoae* was included in the second group.

Distel et al. also reported that the presence of a particular symbiont population in a given host does not reflect the geographical location or environment in which the host animal is found (7). The gill tissue of *C. soyoae* collected from an area of cold seepage in subduction zones (15) contained symbiotic bacteria closely related to the symbiotic bacteria of *C. magnifica* collected at hydrothermal vent sites (Fig. 2). The results are compatible with the findings of Distel et al. (7).

In situ hybridization and microscopic observations. Portions of the sequence used for hybridization probes are indicated in Fig. 1. The sequences of the symbiont-specific probes 1234r and 1284r differ by at least 4 bases from the corresponding sequences of *Escherichia coli*, *P. aeruginosa*, *Pseudomonas putida*, and the *Lucinoma annulata* symbiont (7, 8).

Figure 3A shows the section observed under a phase-contrast microscope. The panel shows a cross section of multilayered cells of gill tissue. Some cells are optically more dense than others. Panel B shows the same field observed under a fluorescence microscope. Cells are stained with the symbiont-specific fluorescence probes. It is clear that the symbiont is present inside the cells of the gill tissue. The darker cells under phase-contrast conditions fluoresced brighter than the others.

Panel C shows a section hybridized with the probe specific to *P. aeruginosa*, a closely related nonsymbiotic bacterium, as a negative control. Fluorescent points are visible. Similar bright points were also observed in the sections without probes. Although it may show epifluorescent bacteria, the region is restricted to a small area.

Panel D shows the result of hybridization with universal probes, sequences of which are common in all known organisms including eubacteria and eukaryotes. Cells are homogeneously stained with the probes. It is possible that the probes hybridized not only with bacterial rRNA but also with rRNA of the host gill cells.

The results clearly prove that the sequence of bacterial rRNA determined exists within the cells of gill tissue. The universal probes stained the section homogeneously, while the symbiont-specific probes stained it nonhomogeneously. Probably about half of the cells of the tissue tested contain symbiotic bacteria.

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