Intracellular Occurrence of ε-Proteobacterial 16S rDNA Sequences in the Vestimentiferan Trophosome

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Endosymbionts of vestimentiferan tubeworms are generally thought to be γ-Proteobacterial sulfur oxidizers. We have identified two novel 16S ribosomal RNA genes (rDNA), presumably of ε-Proteobacteria, from the trophosome content of a vestimentiferan tubeworm. Phylogenetic analysis suggested that the 16S rDNAs were closely related to those of Arcobacter, most of which are known to be associated with animal hosts but are non-chemosynthetic. Intracellular localizations of the 16S rDNAs were confirmed by in situ hybridization using their specific probes. This is the first report of ε-Proteobacterial 16S rDNAs associated with a vestimentiferan tubeworm.

Keywords: Vestimentifera, tubeworm, symbiotic, bacteria, Arcobacter, ε-Proteobacteria.

1. Introduction
Vestimentiferan tubeworms typically inhabit sulfide- and methane-rich environments such as hydrothermal vents and cold seeps. The worms have a unique strategy of life, in which they lack digestive organs but harbor chemoautotrophic (mainly thiotrophic) bacteria in a specialized tissue, the trophosome (Fisher, 1990). Reportedly more than 3 × 10^9 bacteria were packed in 1 g (wet) of trophosome of a tubeworm, Riftia pachyptila (Cavanaugh et al., 1981). Trophosome mass, in turn, accounts for 40 to 60% of total worm body mass (Felbeck and Childress, 1988), which makes the worm a chemoautotrophic animal.

Microscopic observations suggested more than one endosymbiont morphotype for vestimentiferan worms (Cavanaugh et al., 1981; de Burgh et al., 1989; Fisher, 1990) and a gutless annelid (Dubilier et al., 1995). However, morphotypes were often ascribed to pleomorphism of a single species. Ribosomal RNA analyses suggested that the trophosomal endosymbionts are monospecific, or at least 90% homogeneous, and related to chemoautotrophic sulfur-oxidizing bacteria (Stahl et al., 1984; Distel et al., 1988; Dubilier et al., 1995). In contrast, we have identified more than one 16S rDNA sequence from the trophosome content of the vestimentiferan tubeworm Lamellibrachia sp. This communication reports the intracellular localizations of the 16S rDNA sequences and their phylogenetic relation to Arcobacter, a member of the epsilon subdivision of Proteobacteria (ε-Proteobacteria).

2. Materials and Methods

2.1 Tubeworm collection and DNA extraction
Individuals of Lamellibrachia sp. were collected from a methane seep, 1167 to 1170 m deep, in Sagami Bay, Japan (Masuzawa et al., 1992), during the 722nd dive by the submersible Shinkai 2000 of the Japan Marine Science and Technology Center. Immediately after retrieval, fresh trophosome specimens were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), in preparation for in situ hybridization. After one week, fixed trophosomes were dehydrated in ethanol, and embedded in paraffin. Immediately after tubeworm collection, part of the specimens were frozen and stored at –80°C until DNA extraction. Trophosome content was aseptically extracted and centrifuged at 10,000 × g for 15 min. The pellets were treated with lysozyme and proteinase K. High molecular mass DNA was isolated from the lysate and purified by anion exchange column chromatography using ASAP Genomic DNA Isolation Kit (Boehringer-Mannheim), in preparation for DNA amplification by the polymerase chain reaction (PCR). Phenol/chloroform extraction resulted in DNA of poor quality, due to organic contaminants.

2.2 Amplification, cloning and sequencing of 16S rDNA
About 1.4 kb fragments of 16S rDNA were amplified by PCR with Taq polymerase using the primers of Eubac27F (AGAGTTTGATCCTGGCTCAG) and 1492R...
Fig. 1. Unrooted dendrogram showing the phylogenetic positions of the 16S rDNA sequences, TW-1 and TW-2, retrieved from the tissue of the vestimentiferan tubeworm *Lamellibrachia* sp. The source of other bacterial 16S rDNA sequences are shown in parentheses (EMBL/GenBank accession numbers).

Fig. 2. *In situ* hybridization to localize symbiotic 16S rDNAs in the cross-sections of the vestimentiferan tubeworm *Lamellibrachia* sp. Top left, a cross-section of trophosome stained with hematoxylin-eosin. Lobular infrastructure of the trophosome is shown. Top right, negative control with an antisense probe from the human prostate-specific antigen gene. Bottom left, hybrids of the symbiont-specific probe TW-1R localized in periphery of inner lobules. Bottom right, hybrids of the symbiont-specific probe TW-2R localized in periphery of inner and outer lobules. Scale bars, 50 μm.
ethanol, treated with proteinase K, and pre-heated to 100°C. Cross-sections were cut from the paraffin-embedded material of Cary et al. (1988) and other chemosynthetic symbionts (Fig. 1). A TW-1-specific probe (TW-1R) TTTCAGGGCCCGAGGC (antisense to the E. coli 16S rDNA position from 1042 to 1025) and a TW-2-specific probe (TW-2R) AGAAGCTTTAGTAACTA (antisense to the E. coli 16S rDNA position from 1041 to 1024) were synthesized on an Applied Biosystems automated DNA synthesizer. The oligonucleotide probes were then purified on 20% polyacrylamide gels and recovered by elution. In addition, a negative probe was designed from the human prostate-specific antigen gene (EMBL/GenBank X14810) that has been found only in primates. The sequence of the prostate-specific antigen gene (EMBL/GenBank X14810) was determined in the Ribosomal Database Project (RDP; Maidak et al., 1994). On the basis of the on-going trend, the amplified 16S rDNA fragments were cloned and sequenced. Two of a total of 20 clones were successfully sequenced and related to a novel bacterial group in association with vestimentiferan tubeworms. The other cloned sequences are being analysed, but they are unlikely to be in close relation with the two clones; the sequences of the other clones will be reported elsewhere.

Two 16S rDNA sequences in intracellular association with a vestimentifera were sequenced and designated as TW-1 and TW-2 (EMBL/GenBank accession numbers are D83060 and D83061). The matching between TW-1 and TW-2 was 95%, which suggests that the two belong to different species but the same genus. Both TW-1 and TW-2 were most closely related to Arcobacter nitrofigilis (87–88% matching), and to those of A. cryaerophilus and A. butleri (86–88% matching), all of which fell in the epsilon subgroup of the class Proteobacteria, or ε-Proteobacteria (Vandamme et al., 1991). An unrooted dendrogram based on the neighbor-joining method (Saitou and Nei, 1987) was constructed to position TW-1 and TW-2 in comparison with the Arcobacter-Campylobacter-Helicobacter cluster and other chemosynthetic symbionts (Fig. 1).

3. Results and Discussion

3.1 Analysis of 16S rDNA sequence

The 16S rDNA-based analysis has been widely accepted in bacterial taxonomy and phylogenetics (Woese, 1987), and a number of 16S ribosomal data are being integrated in the Ribosomal Database Project (RDP, Maidak et al., 1994). On the basis of the on-going trend, the amplified 16S rDNA fragments were cloned and sequenced. Two of a total of 20 clones were successfully sequenced and related to a novel bacterial group in association with vestimentiferan tubeworms. The other cloned sequences are being analysed, but they are unlikely to be in close relation with the two clones; the sequences of the other clones will be reported elsewhere.

Intracellular localization of a vestimentifera was sequenced and designated as TW-1 and TW-2 (EMBL/GenBank accession numbers are D83060 and D83061). The matching between TW-1 and TW-2 was 95%, which suggests that the two belong to different species but the same genus. Both TW-1 and TW-2 were most closely related to Arcobacter nitrofigilis (87–88% matching), and to those of A. cryaerophilus and A. butleri (86–88% matching), all of which fell in the epsilon subgroup of the class Proteobacteria, or ε-Proteobacteria (Vandamme et al., 1991). An unrooted dendrogram based on the neighbor-joining method (Saitou and Nei, 1987) was constructed to position TW-1 and TW-2 in comparison with the Arcobacter-Campylobacter-Helicobacter cluster and other chemosynthetic symbionts (Fig. 1).

3.2 In situ localization

Intracellular localization of ε-Proteobacterial 16S rDNA sequences, TW-1 and TW-2, was demonstrated by in situ hybridization, but it does not exclude the possibility of the presence of other true endosymbionts such as sulfur-oxidizing γ-Proteobacteria. Sulfur oxidizers would have been dominant as previously reported (Stahl et al., 1984; Distel et al., 1988; Dubilier et al., 1995); their occurrence in our vestimentiferans will be discussed elsewhere.

Vestimentiferan trophosome had lobular infrastructure, and TW-1R hybrid and TW-2R hybrid were detected in the periphery of the lobules (Fig. 2). TW-1R hybrid was localized in inner lobules, while TW-2R hybrid was detected in inner and outer lobules. The peripheral localization of symbiont-specific hybrids was previously reported with the vestimentiferan worm Riftia pachyptila (Cary et al., 1993). In the HE-stained section, several morphotypes (globules, ovoids, rods and threads; Naganuma et al., 1996) were observed, while the positive hybridization was restricted to globules and ovoids (Fig. 2). TW-1R hybrid was detected in slightly ovoid particles, while TW-2R hybrid was localized in association with the smaller globules (4 to 5 µm). Separate localization of hybrids to different morphotypes sug-
gests that the TW-1 and TW-2 were from different bacterial species though they were closely related to each other.

3.3 ε-Proteobacteria and tubeworms

Other than those from vestimentiferan worms, ε-Proteobacterial 16S rDNAs have recently been reported to predominate among the epibiotic microflora associated with rocks and animals at hydrothermal vents (Haddad et al., 1995; Moyer et al., 1995; Polz and Cavanaugh, 1995), including a tube-dwelling vent polychaete. Vent polychaetes are phylogenetically close to and often share an ecological niche with vestimentiferan worms. Thus, it does not seem surprising that ε-Proteobacteria were in intracellular association with a vestimentiferan worm, even though the association of ε-Proteobacteria and vent polychaetes was epibiotic, not necessarily endosymbiotic (Haddad et al., 1995).

Vestimentiferan worms are known to bear sulfur-oxidizing endosymbionts that are presumed to belong to γ-Proteobacteria (Distel et al., 1988). Sulfur-oxidation by ε-Proteobacteria is known for free-living Thiobulvum sp. (Wirsen and Jannasch, 1978). Some other ε-Proteobacterial species were reported to be capable of reducing sulfate and sulfur, i.e. sulfide production (Vandamme et al., 1991; Rainey et al., 1993). Intracellular association of ε-Proteobacterial 16S rDNAs with vestimentiferan trophosome suggests that ε-Proteobacteria are involved in multiple metabolic pathways regarding sulfur oxidation and/or reduction. This multiple metabolism may support the intracellular microbial production, as shown for a pogonophoran tubeworm and a vent mussel (Schmaljohann et al., 1990; Distel et al., 1995).

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References


