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Recovery and Phylogenetic Analysis of Novel Archaeal rRNA Sequences from a Deep-Sea Deposit Feeder

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In 1992, two independent reports based on small-subunit rRNA gene (SSU rDNA) cloning revealed the presence of novel *Archaea* among marine bacterioplankton. Here, we report the presence of further novel *Archaea* SSU rDNA sequences recovered from the midgut contents of a deep-sea marine holothurian. Phylogenetic analyses show that these abyssal *Archaea* are a paraphyletic component of a highly divergent clade that also includes some planktonic sequences. Our data confirm that this clade is a deep-branching lineage in the tree of life.

Holothurians, commonly known as sea cucumbers, are probably the most typical animal of the deep oceans and dominate, both numerically and by biomass (1), invertebrate megafauna in the abyss. Most are mobile epibenthic animals that wander over the sea floor feeding on the uppermost few millimeters of sediment. Their ecological dominance suggests that they play a critical role in modifying abyssal sediment and in structuring the communities that live within it. Little is known of the gut ecology of these organisms, although it is known that obligate barophilic bacteria are to be found therein (4). The presence of members of the *Archaea* in holothurian gut material has not previously been reported. *Oneirophanta mutabilis* (class *Holothuria*, order *Elasipodida*) is a mobile superficial sediment feeder typically found at the base of the continental slope at depths in the range of 2,850 to 4,832 m (1). In an attempt to characterize this environment, we created a gene library from midgut contents of an individual collected from a depth of 4,870 m at the Institute of Oceanographic Sciences Deacon Laboratory Deepseas Northern Site (48°51′N, 16°27′W).

The animal was dissected, and the gut contents were separated into those from the fore-, mid-, and hindsections, which were preserved at 4°C in a sterile container with 50% filtered absolute ethanol. Portions (500 μl) of the contents from the three separate parts of the gut were taken and washed by repeated suspension in 1 ml of distilled H_2O and centrifugation at 14,000 × *g* before final resuspension in water (1 ml). Hot 10% sodium dodecyl sulfate (100 μl) was added to the sample, which was then placed in a boiling-water bath for 2 min. The sample was centrifuged at 14,000 × *g* in a microcentrifuge for 2 min. The supernatant was removed and placed into a new microcentrifuge tube. Denatured protein was removed by the addition of an equal volume of phenol. Tubes were inverted by hand a number of times and centrifuged at 10,000 × *g* for 5 min, after which the top phase was removed and transferred into a new microcentrifuge tube, leaving behind the interface and bottom phase. The procedure was repeated with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) instead of phenol and repeated again with chloroform instead of the mixture to remove all traces of phenol. The nucleic acids were precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. After incubation overnight at −20°C, the nucleic acids were collected by centrifugation at 14,000 × *g* for 15 min and resuspended in water (100 μl). A further purification step was re-

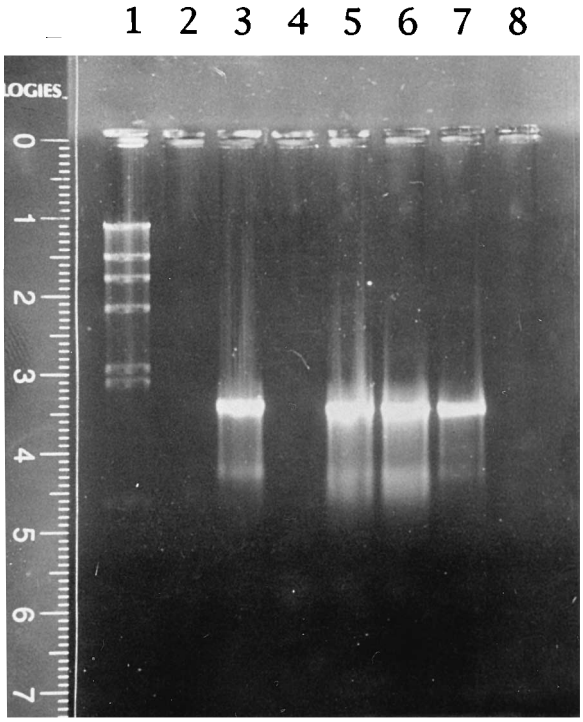

FIG. 1. Analysis of *O. mutabilis* gut contents with domain-specific primer combinations. Lane 1, λ *Hin*dIII-digested molecular weight marker (0.1 μg). Lanes 2 to 4, fore-, mid-, and hindgut content DNA analyzed with the UN-AB primer pair. Lanes 5 to 7, fore-, mid-, and hindgut content DNA tested with the UN-EB primer combination.

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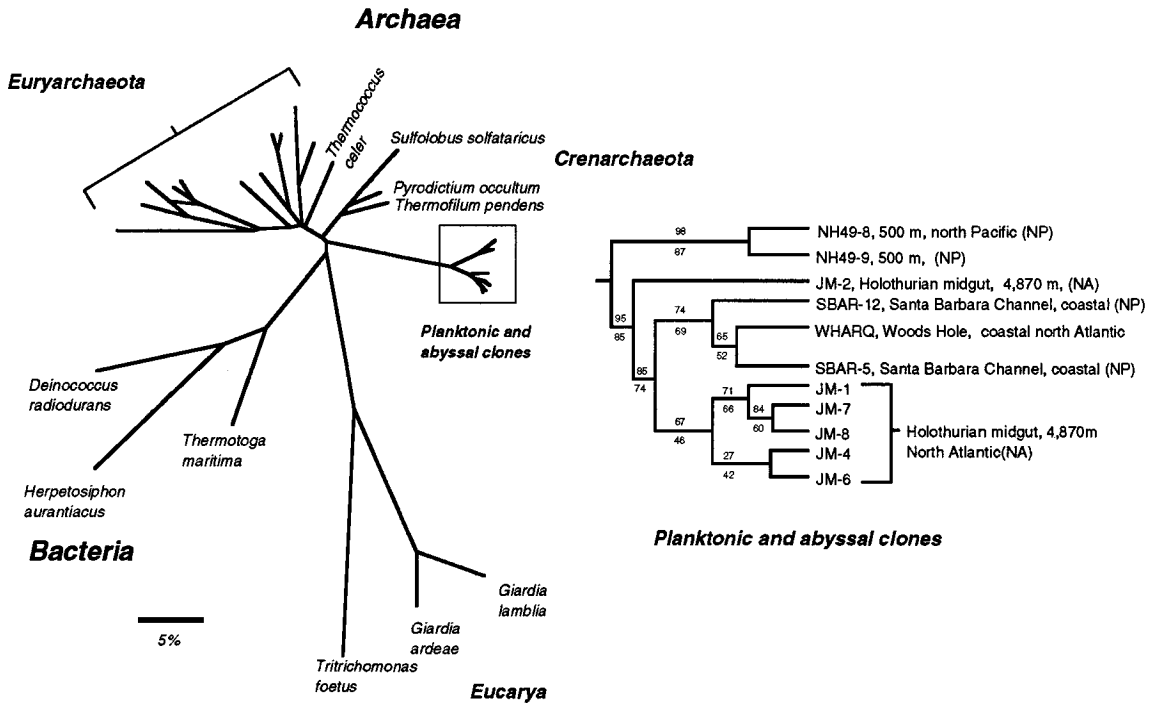

FIG. 2. Phylogenetic relationships of the novel abyssal sequences. A distance tree based on all substitutions from the 819-base alignment with (inset) a tree summarizing relationships within the abyssal-planktonic clade based upon parsimony and distance analyses of the 402-base alignment is shown. Distance analyses were made by the neighbor-joining method (13) with the Jukes and Cantor distance correction (9) implemented in PHYLIP 3.5c (6). The scale bar indicates 5 substitutions per 100 bases. Parsimony analyses were performed with PAUP 3.1.1 (14), with insertion-deletions treated as a fifth state. BPs for the distance and parsimony analyses are given below and to the left or right respectively of the internal nodes and are based on 100 bootstrap replicates.

quired to produce DNA of sufficient purity for PCR. The purification procedure employed was Sephadex gel filtration (10).

The designations and sequences of the primers and their reference positions on alignments of small-subunit rRNAs are EB (*Bacteria* specific), 5′-GAGTTTGATCCTGGCTCAG-3′ (8 to 27); AB (*Archaea* specific), 5′-TCCGGTTGATCCTGC CGG-3′ (3 to 21); and UN (universal), 5′-ACGGNWACCT TGTTACGAGTT-3′ (1423 to 1402) (standard International Union of Pure and Applied Chemistry nomenclature [N is G, A, T, or C; W is A or T; M is A or C]). Control reactions were performed to demonstrate the domain specificity of the EB-UN and AB-UN primer sets with the archaebacterial species *Haloferax volcanii* and *Methanosarcina barkerii* and the bacterial species *Escherichia coli* and *Bacillus subtilis* (data not shown). PCR were carried out under the following reaction conditions: 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A no-template negative control was also included. Agarose gel analysis of the products revealed successful amplification of bacterial genes coding for 16S rRNA (16S rDNA) sequences from all three gut samples (Fig. 1, lanes 5 to 7). Amplification of archaeal 16S rDNA sequences was apparent in the midgut sample (Fig. 1, lane 3). Note that we have performed a similar analysis on dissected gut contents of five individual *O. mutabilis* specimens. Successful amplification of archaeal 16S rDNA sequences from each individual occurred, but with no consistent location in the gut.

To facilitate cloning of these archaeal genes, a further round of amplification was carried out on a 1-μl aliquot, with PCR primers with restriction sites incorporated into the 5′ end. The sequences of these primers (restriction sites underlined) are 5′-TTTTGGATCCTCTAGAACGGGCGGTGTGTRC-3′ (516 to 537) and 5′-TGAGCTCAAGCTTCAGCMGTCC GCGGTAATWC-3′ (1390 to 1409). Two negative controls were employed for this round of amplification. One control contained a 1-μl aliquot of the negative control from the initial

TABLE 1. BPs based on 100 bootstrap replicates in four analyses of the 819-base alignment, for groupings with the abyssal-planktonic sequences

Relationship hypothesis supported	BPs under indicated conditions			
	All substitutions		Transversions only	
	Distance	Parsimony	Distance	Parsimony
Crenarchaeota–Euryarchaeota–abyssal-planktonic[a]	69	48	55	11
Crenarchaeota–abyssal-planktonic[b]	23	19	40	28
Eucarya–abyssal-planktonic[c]	29	39	28	50

[a] BPs supporting the monophyly of a *Crenarchaeota–Euryarchaeota*–abyssal-planktonic sequence clade.
[b] BPs supporting a specific relationship between the abyssal-planktonic sequences and the *Crenarchaeota* only.
[c] BPs uniting the abyssal-planktonic sequences and the *Eucarya*.

round of amplification, and the other was a no-template (distilled H_2O) control. Following amplification, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel and visualized with ethidium bromide. The PCR products were purified with a Magic PCR Prep kit (Promega Corp., Madison, Wis.) and digested with *Hin*dIII and *Bam*HI. The PCR products were ligated to the plasmid pBGS8 and introduced into *E. coli* by electroporation. Recombinant clones were detected by colony hybridization with the original PCR product. Partial sequences from six clones were phylogenetically analyzed, and we sought to elucidate the relationships of these clones to each other and to the previously reported novel archaea (2, 7).

An alignment was obtained from the ribosomal database project (11), and all new sequences were aligned to this by eye. Initially, an alignment of 402 bases, representing the extent of available small-subunit rDNA sequence information common to the novel abyssal sequences and previously reported planktonic sequences, was used (2, 7). These 11 sequences were included with 16 selected members of the *Crenarchaeota* and *Euryarchaeota*, with the eubacterium *Thermotoga maritima* included as an outgroup. Distance matrix analyses were carried out with PHYLIP 3.5c (6). Distances were calculated with the Jukes and Cantor (9) correction for superimposed substitutions. The neighbor-joining method (14) was used to construct phylogenetic hypotheses based upon these distances. All parsimony analyses were performed with PAUP 3.1.1 (15). Heuristic searches were performed with 10 random-addition sequence replicates and tree bisection reconnection branch swapping. PAUP was instructed to use parsimony-informative sites only. Distance and parsimony analyses placed the abyssal sequences in a major clade with two of the three groups of previously isolated planktonic sequences. Relationships within this clade (Fig. 2, inset) were supported by high bootstrap proportions (BPs), whereas relationships between this clade and other archaea were poorly supported and sensitive to the method of analysis. Interestingly, the abyssal sequences are split up by one of the groups of planktonic sequences and thus appear to be a phylogenetically diverse assemblage rather than a discrete monophyletic group associated with a specific environment.

Further investigation of the higher relationships of the abyssal-planktonic clade used an expanded alignment of 819 nucleotide positions, including only those planktonic and abyssal sequences for which these additional sequence data were available, leaving a total of seven archaeal sequences (two NH- and five JM- [Fig. 2, inset]). The taxonomic scope of the alignment was expanded to include basal eukaryotic and eubacterial taxa encompassing a range of G+C content values in an attempt to counter possible analytical bias due to thermophilic convergence (5). Parsimony and distance analyses, using all substitutions or transversions only, yielded three conflicting optimal hypotheses of a sister group relationship between the abyssal-planktonic clade and (i) *Archaea* (as in Fig. 1), (ii) *Crenarchaeota*, or (iii) *Eucarya* (with the *Crenarchaeota* also closer to the *Eucarya* than to the *Euryarchaeota* as in Rivera and Lake's controversial eocyte tree [13]). Some of the sequences in Fig. 2 demonstrate strongly opposed base-compositional biases, which limit the accuracy of inference under the currently available models. Bootstrap analyses (summarized in Table 1) and differences in parsimony (data not shown) also indicate that none of the competing hypotheses are well supported.

The incorporation of additional taxa into phylogenetic analyses may affect inferred relationships among other taxa (5). The limited sequence data available for the abyssal-planktonic clade is insufficient to resolve its relationships but demonstrates that it is a deep-branching clade with the potential to affect inferred relationships among other such groups. It has been suggested that these organisms may represent a third separate archaeal kingdom (12), and our results identify this supposition as one of three competing hypotheses. Thus, the acquisition of further sequence data for this clade may help to resolve fundamental phylogenetic relationships in the tree of life.

Identification of this novel abyssal-planktonic clade, known only from DNA sequences but which appears to have a cosmopolitan ecological distribution, further demonstrates the power of molecular methods to reveal microbial diversity to which traditional culturing may be selectively blind (8). The ultimate origin of the abyssal sequences, from symbionts of holothurians, from inhabitants of ingested abyssal sediments, or from organisms or DNA that has been deposited in the abyss, is not known. Molecular methods may also be the key to the isolation and further characterization of this enigmatic clade through the use of specific probes directed toward its rRNA (8). Recently, it has been demonstrated by molecular methods that uncultivated archaea represent up to 34% of the prokaryotic biomass in coastal Antarctic surface waters (3).

The sequences from this study have been deposited in the GenBank database and have been assigned accession numbers L24195 to L24201.

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