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Novel major archaebacterial group from marine plankton

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MARINE bacteria often dominate the plankton biomass[1,2] and are responsible for much of the cycling of organic matter[3], but bacterial diversity is poorly understood because conventional identification methods (requiring culturing) miss about 99% of the organisms[4,5]. Recent advances permit characterization of microbial communities by analysis of 16S ribosomal RNA gene sequences directly from biomass without the need to culture the organisms[6]; such studies from surface ocean samples have found only eubacteria[7–10], not archaebacteria (or Archaea[11]), which are profoundly different[12]. Here we report 16S rRNA sequences obtained from Pacific Ocean bacterioplankton samples collected from depths of 100 m and 500 m. Among these we found sequences only distantly related to those of any organisms previously characterized by 16S rRNA sequences, with similarities to the nearest such relatives (extreme thermophiles) approximately the same as those between animals and plants. We suggest that these sequences are from a previously undescribed archaebacterial group that may have diverged from the ancestors of characterized organisms very early in evolution.

Our samples were collected from the western side of the California Current, roughly 350 miles west of San Diego. The area was oligotrophic at the time of sampling, as indicated by the low surface chlorophyll concentrations and a chlorophyll maximum layer at about 100 m depth, near the base of the euphotic zone and substantially below the surface mixed layer (Fig. 1). The samples for genetic analysis came from this biologically active layer (100 m depth) as well as from 500 m; the deeper sample was substantially below the euphotic zone and possessed a significantly lower total bacterial abundance and temperature (Fig. 1). Comparison of 16S rRNA gene sequences from these samples to those from similarly characterized organisms reveals that five of the clones from the 500-m sample and two from a 100-m sample form a cluster within the archaebacteria, yet this cluster is only distantly related (about 70% sequence identity with 16S rRNA) to any previously described archaebacterial group (Fig. 2). The sequences most similar to these clones come from extreme thermophiles, such as *Pyrodictium*. Measurable hybridization (under stringent conditions) of a probe made from one of the clones (NH49-8) to nucleic acid from the archaebacterium *Sulfolobus acidocaldarius*, but not that from the eubacterium *Escherichia coli* or eukaryotic calf thymus, was also consistent with the inferred archaebacterial origin of these clones.

A majority (5 out of 7) of our clones from 500 m were members of this group, as were a small minority (2 clones out of 10) from only 1 of 3 different 100-m samples collected a few days apart (>30 clones from 100 m were examined; information on other clones will be reported elsewhere). Because the distribution of clones in final polymerase chain reaction (PCR) products may not match the distribution of all 16S rRNA genes in the original unamplified DNA (owing to differences in amplification efficiency), we cannot yet say how prevalent these organisms are in sea water. This may best be determined by hybridization of specific oligonucleotide probes to RNA isolated from the natural communities[7] (not possible with these samples owing to the limited material remaining). But community DNA from the 500-m sample hybridized very strongly to DNA from 1,000 m (ref. 13), suggesting that the dominant organisms from 500 m may dominate a large depth range. It is therefore possible that this new group may be extremely abundant, and may be a signficant component of deep-sea metabolism.

At this point in our studies, we know nothing about these organisms other than their 16S rRNA sequences. Although the closest characterized apparent relatives are extreme thermophiles, the temperature of the water from which our samples came was 5–15 °C, and remote from any known hydrothermal sources. Also, the G+C content, which correlates with thermal stability, of our longest clones (NH49-8 and -9) was only ~51%, compared with 63% for *Sulfolobus* over the same region. Therefore, we think the sequences probably do not come from thermophiles. Phylogenetic statistical evaluation of the sequence data suggests we cannot at this time confidently place these clone sequences within the extreme thermophile branch of the archaebacteria. A bootstrap analysis, which involves creation of many phylogenetic trees from random resampling within the sequence[14], and with the eubacterium *Deinococcus radiodurans* as the outgroup, indicated that although the closest relative to our clones appears to be *Pyrodictium occultum*, the branch that leads to our clones does not consistently diverge with the extreme thermophiles; in 40% of 70 trees generated by the bootstrap analysis, the branch leading to our clones diverged within the

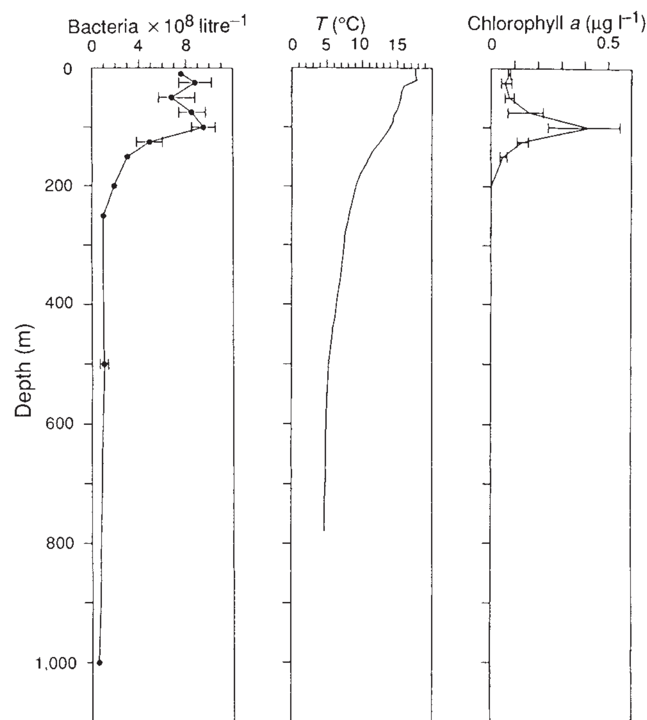

FIG. 1 Depth profiles of parameters at sampling location (within 2 km of 31° 50′ N 124° 6′ W). Error bars indicate range of values of the 7-day sampling period (21–27 April 1989) for the biological parameters, points without error bars were not replicated. Temperature profile was measured on 22 April 1989. Bacterial abundance was measured by epifluorescence microscopy with acridine orange stain[18], particulate chlorophyll *a* collected on 0.45-μm-pore-size filters was analysed by fluorometry[19] and temperature measured by an expendable bathythermograph.

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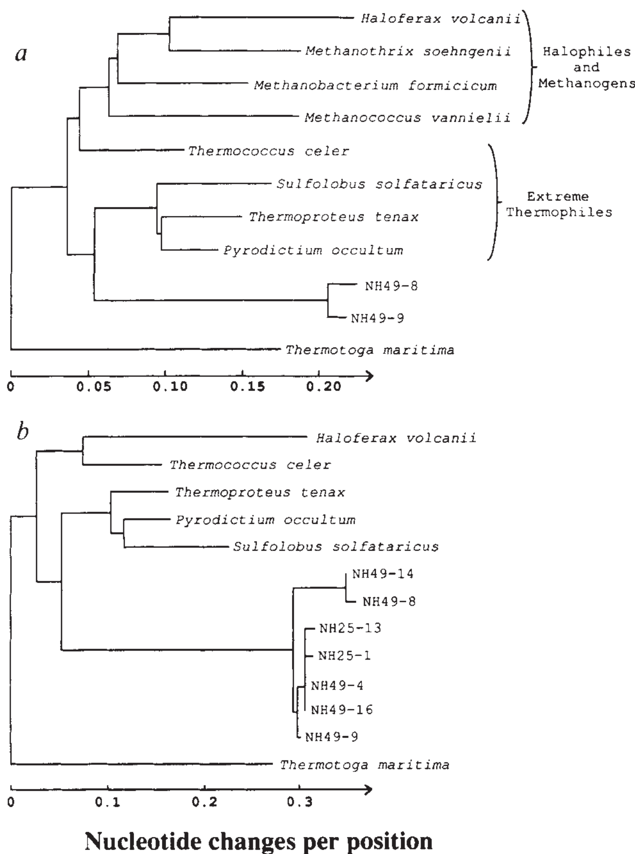

FIG. 2 Phylogenetic associations, based on 16S rRNA sequences, of clones and previously characterized organisms. All organisms shown are archaebacteria except for *Thermotoga maritima* (a eubacterium[12]). Clones beginning NH49- came from particulate DNA collected from 500 m depth on 27 April 1989, and those beginning NH25- from such DNA collected from 100 m depth on 23 April 1989; numbers after hyphens are arbitrary clone numbers. *a*, Representatives of known archaebacterial groups and clones NH49-8 and NH49-9, comparing 781 bases, essentially the full-length clone excluding regions of ambiguous alignment. *b*, Analysis of several marine clones, comparing 200 bases (both regions beginning at *E. coli* base number 537). Samples (100–200 litres) were collected in 30-litre Niskin bottles on a rosette sampler (General Oceanics, Miami). Microorganisms were filtered onto a 142-mm diameter 0.22-μm-pore-size Durapore filter (Millipore) after prefiltration through a Gelman A/E glass fibre filter that removed almost all eukaryotes and about 5–10% of the bacteria[20]. DNA was extracted with hot 1% sodium dodecyl sulphate and purified by precipitation with ethanol and extraction with phenol[21]. Partial genes encoding 16S rRNA were amplified from 1 ng DNA by PCR[22] with a GeneAmp Kit and recombinant *Taq* polymerase (Perkin Elmer–Cetus) and 1 μM each of the primers TTGAGCTCAAGCTTCAGCA/CGCCGCGGTAATA/TC AND TTTTGGATCCTCTAGAACGGGCGGTGTGTA/GC (these consist of linkers at the 5′ ends and 'universal' 16S rRNA sequences[23], located at *E. coli* position numbers 537 and 1,390, at the 3′ ends), over 30 cycles with the following temperature program: 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min. Negative controls (water instead of DNA) showed no amplification. Products of the proper size range (~950 base pairs) were cut out of a low-melting-agarose gel (SeaKem GTG), purified by extraction with phenol and precipitation with ethanol, digested with *Bam*H1 and *Hin*dIII, and cloned into M13 (ref. 24), then sequenced by the dideoxy chain termination method with Sequenase 2 (US Biochem.). Sequences were analysed by the distance matrix method[25]. Sequences from known organisms came from GenBank[26]. The analysis in *a* used the following base positions, numbered from the 5′ end of the aligned *Thermoproteus tenax* sequence: 496–498, 500–799, 803–806, 810–897, 899–906, 914–954, 1,002–1,071, 1,073–1,088, 1,090–1,091, 1,096–1,099, 1,104–1,245, 1,247–1,248, 1,250–1,354. Secondary structure analysis suggested that potential PCR artefacts did not affect the results significantly, as differences between clones usually included compensatory changes on opposite sides of base-paired regions[7,9]; also, a phylogenetic tree essentially the same as *a* was obtained with the first 400 bases or the last 400 bases of clones NH49-8 and NH49-9. Sequences from the clones reported here have been submitted to EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers Z11568–Z11573.

extreme thermophile lineage, in 36% the divergence occurred within the methanogen/halophile lineage, and in 24% the branch diverged closer to the eubacteria than the extreme thermophile split from the methanogen/halophile lineage. Therefore we cannot rule out that these organisms may be methanogens (common in anaerobic environments, so perhaps released from anaerobic guts of metazoa; the water is not anaerobic in this region), but the sequences are clearly distinct from all known groups of methanogens[11,12] (Fig. 2). Learning more about the organisms will probably require enrichment or isolation of such organisms. The sequences described here include several regions suitable for use as specific probes; therefore, many enrichment conditions can be tried and these can be screened with such probes to see which conditions, if any, enhance growth of this group. Given that other archaebacteria usually thrive under conditions in which there is little competition from eubacteria or eukaryotes (for example, high temperatures, low pH, high salt), it would be interesting to learn what niche these organisms fill in the sea. They may also be of interest phylogenetically because they appear to have diverged early in the evolution of life and have their closest characterized relatives within a broad group that until now was thought to consist only of extreme thermophiles[11].

Although it is unsatisfactory to have only a gene sequence, we now know there is something interesting to look for, and we have a probe to assist in the search. The approach we used to discover new microbial groups supplements traditional microbiological techniques[6–10,15–17], with this novel archaebacterial group appearing in the first few midwater plankton samples investigated. □

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