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Crenarchaeota in Lake Michigan Sediment†

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RNA from Lake Michigan sediment was hybridized with a DNA probe for archaeal 16S rRNA. There was a peak of archaeal rRNA abundance in the oxic zone and another immediately below it. Six contributing species were identified by PCR amplification of extracted DNA with primers specific for archaeal rDNA: two related to *Methanosarcina acetivorans* and four related to marine crenarchaeotal sequences. rRNA quantification using a DNA probe specific for this crenarchaeotal assemblage showed it is most abundant in the oxic zone, where it accounts for about 10% of total archaeal rRNA.

Introduction. Marine and freshwater sediments are major sinks of both natural and anthropogenic materials (5). Although microbial activity is recognized to control the transformation of these materials and influence processes in the overlaying water, relatively little is known about the contributing populations. This is mostly a consequence of the well-recognized limitations of pure-culture methodology. A more complete understanding of sediment processes and anthropogenic influence will require methods independent of prior growth in culture. For example, the direct recovery of rRNA sequences from the environment allows uncultured forms to be cataloged within a phylogenetic framework (28). With increasing use of this and other direct methods, the pure-culture collection is now recognized to comprise a very small fraction of the natural diversity in most environments (4, 16, 27). Sediments are no exception (9, 10, 20, 23).

We are employing a combination of molecular, microbiological, and chemical techniques to evaluate the contribution of different microbial groups to sediment processes at a site in Lake Michigan near Fox Point, Wis. For example, group-specific hybridization probes for the three domains (archaea, bacteria, and eucarya) are being used to measure temporal and vertical changes in abundance of the corresponding populations (18, 20).

We anticipated that most of the archaea in this environment would be anaerobic methanogens. However, there were characteristically two major peaks of archaeal 16S rRNA hybridization: one near the sediment surface in the oxic zone and one in the zone immediately below it. This unexpected distribution, and earlier studies showing that methanogen-specific probe hybridization could account for only a fraction of archaeal probe hybridization (1a), suggested the presence of novel archaea. We evaluated this hypothesis, using selective PCR amplification of archaeal 16S rDNA. We report here that four of the archaeal sequences recovered are closely related to the recently discovered pelagic marine crenarchaeota (7, 13, 14) but that these crenarchaeota accounted for only ~10% of the archaeal 16S rRNA in the near-surface sediments at the Fox Point study site.

Sample collection. The Fox Point sampling site is approximately 27 km northeast of Milwaukee, Wis. (latitude 43°11′40″N, longitude 87°40′11″W) at 101 m depth. The temperature is 1 to 4°C year-round. Organic carbon input is approximately 2 mol m^{-2} $year^{-1}$, making this a mesotrophic or oligotrophic site, and the sedimentation rate is 0.24 cm $year^{-1}$ (12). Sediment was collected on January 3, 1996, with a 30-cm^2 box corer from the R/V *Neeskay*. Cylindrical subcores (3-in. diameter) were taken for chemical analysis and nucleic acid extraction. The subcores were transported in an ice-water bath protected from the light, stored in the dark at 4°C, and processed within 24 h. Prior to sectioning a subcore for nucleic acid analysis, a disposable anaerobic glove bag (four-handed model; Sigma Chemical, St. Louis, Mo.) was taped over the end of the core liner. The glove bag atmosphere was exchanged three times and replaced with nitrogen, and slight positive pressure was maintained under continuous flow. The core was pushed up out of the liner with a water pressure-driven extruder and sectioned at 0.5-cm or greater intervals. Core slices were transferred to glass screw-cap vials and kept on ice under nitrogen until sectioning was completed and then were transferred to an anaerobic hood for the initial steps of RNA extraction. Sediment not used for RNA extraction was stored at 4°C for DNA extraction.

Oxygen measurement. Oxygen was measured with a Clark-style O_2 microelectrode (model 737GC; Diamond General Corp., Ann Arbor, Mich.) positioned with a micromanipulator and coupled to an analog chemical microsensor (Diamond General). Oxygen was depleted at a depth of approximately 2.5 cm in the sediment.

Oligonucleotide probes. Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, Calif.). The probes used, named by Oligonucleotide Probe Database conventions (2), were S-*-Univ-1390-a-A-18 (29), which targets most known forms of life; S-D-Arch-0915-a-A-20 (3), which targets the archaea; and S-K-Cren-0667-a-A-15 (8), which targets the recently discovered pelagic marine crenarchaeota.

S-K-Cren-0667-a-A-15 T_d determination. The dissociation temperature (T_d) for S-K-Cren-0667-a-A-15 was determined as described previously (19) with RNA transcribed in vitro from clone LMA226 (described below) as a reference. Studies in our laboratory with probes for different bacterial and archaeal rRNA transcripts have shown that identical T_ds are

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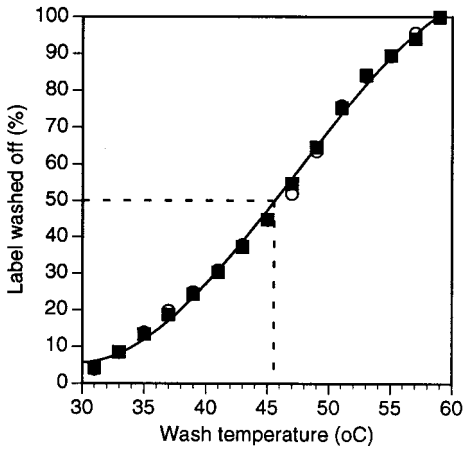

FIG. 1. S-K-Cren-0667-a-A-15 T_d determination. The dashed line shows the temperature (~46°C) at which 50% of the ^{32}P-labeled probe was washed off. The solid boxes and open circles represent replicate determinations.

determined with in vitro-transcribed RNA and RNA from pure culture (21a). pLMA226 was linearized with *Hin*dIII and transcribed with T7 RNA polymerase, and the integrity of the resulting RNA was checked on a polyacrylamide gel (data not shown). The T_d was determined to be 46°C (Fig. 1). In vitro-transcribed LMA226 RNA was also used as S-K-Cren-0667-a-A-15 reference RNA.

DNA extraction, amplification, and sequencing. The samples from depths of 0 to 0.5 cm, 0.5 to 1 cm, and 3.5 to 4 cm were selected for amplification because there were peaks of Arch-0915-a-A-20 hybridization at these depths (see Fig. 3). DNA was isolated by lysozyme and freeze-thaw treatments, phenol-chloroform extraction, and ethanol precipitation, essentially by the method of Tsai and Olson (26). DNA was separated from brown humic substances on a 1% agarose gel and purified from agarose by centrifugation in 0.2-μm-pore-size SpinX centrifuge filter units (Costar Inc., Cambridge, Mass.). DNA was amplified with the archaeon-specific primers S-D-Arch-0021-a-S-20 (7) and S-D-Arch-0915-a-A-20 (3) with a 1605 Air Thermo-Cycler (Idaho Technology, Idaho Falls, Idaho), according to the manufacturer's instructions. DNA was denatured (30 s, 94°C) and amplified for 30 cycles (92°C, 15 s; 42°C, 15 s; 72°C, 45 s), and products were extended (72°C, 1 min) to facilitate cloning. Amplification products were cloned into the TA vector (Invitrogen Corp., San Diego, Calif.), and isolated colonies were used to prepare plasmid DNA by an alkaline lysis miniprep procedure (23a). An automated DNA sequencer (model 4000L; LiCor Corp., Lincoln, Nebr.), a SequiTherm Long-Read Kit (LC) (Epicentre Technologies Inc., Madison, Wis.), and M13Fwd(-29) and M13Reverse IRD41-labeled primers (LiCor Corp.) were used for DNA sequencing. Clones were designated LMA (for Lake Michigan, archaeal amplification) followed by a number, and the corresponding plasmids are referred to as pLMA plasmids. Of 40 16S rDNA inserts sequenced, 4 were related to crenarchaeotal sequences, 4 were related to bacterial sequences, and the remainder were related to *Methanosarcina* sequences. Only the crenarchaeotal inserts and two of the *Methanosarcina*-like inserts were sequenced completely.

RNA extraction and membrane hybridization. Aliquots (0.2 g) of sediment were transferred with sterile 1-ml disposable syringes to screw-cap Eppendorf tubes containing low-pH buffer, buffer-equilibrated phenol (pH 5.1), sodium dodecyl sulfate, and 0.5 g of zirconium beads (24), shaken for 1 min with a Mini-Beadbeater (Biospec Products, Bartlesville, Okla.) under an anaerobic hood, and then transported on ice to our laboratory. RNA was isolated by bead beating, phenol-chloroform extraction, and ethanol precipitation as previously described (24), except that two bead-beating treatments of 3 (rather than 2) min each were used (23b). RNA was trans-

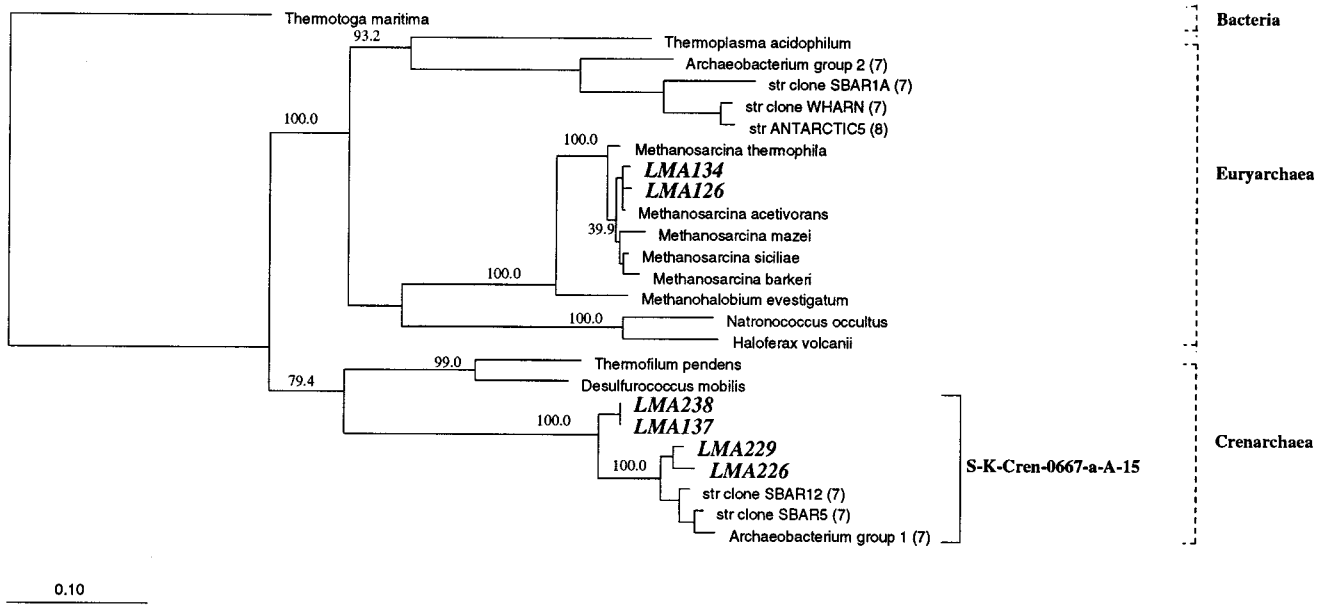

FIG. 2. Phylogenetic placement of crenarchaeotal and euryarchaeotal molecular isolates. A maximum-likelihood phylogenetic tree was constructed for the LMA isolates by using FastDnaML (11) and the ARB sequence analysis package (25). Bootstrap values were determined by using PHYLIP parsimony. The region of the 16S rRNA molecule between *E. coli* positions 21 and 915 was used in the analysis. Solid brackets show the sequences targeted by S-K-Cren-0667-a-A-15, dashed brackets show the classifications of the species included in the tree, numbers in parentheses are reference numbers, and the scale bar represents the estimated number of base changes per nucleotide.

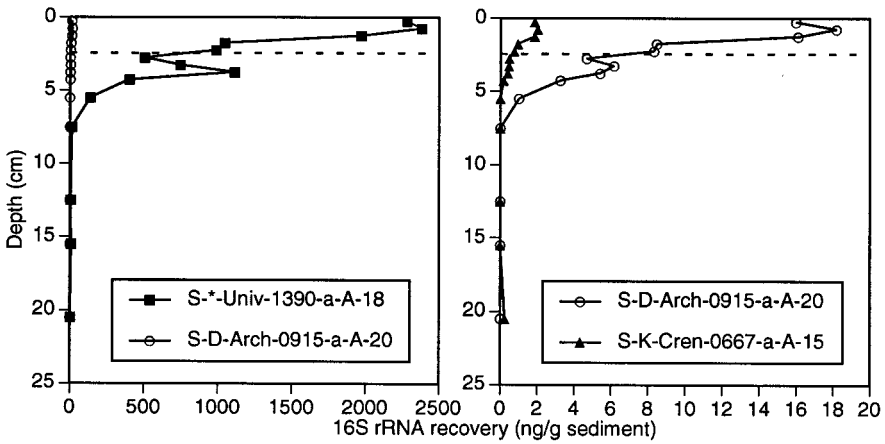

FIG. 3. Hybridization of sediment-extracted RNA with oligonucleotide probes for universal, archaeal, and crenarchaeotal small-subunit rRNA by depth in sediment. The dashed line shows the approximate depth of oxygen depletion.

ferred to nylon membranes in triplicate and probed with radiolabeled oligonucleotides as described previously (19). Membranes were prehybridized at 40°C and washed at 44°C (S-*-Univ-1390-a-A-18), 56°C (S-D-Arch-0915-a-A-20), or 46°C (S-K-Cren-0667-a-A-15). The in vitro-transcribed crenarchaeotal reference RNA, which does not include the S-*-Univ-1390-a-A-18 site, was quantified relative to archaeal reference RNA (from *Methanosarcina frisius*), which in turn was quantified relative to universal reference RNA (from *Escherichia coli*). Hybridization was measured with a PhosphorImager (model 400S; Molecular Dynamics Inc., Sunnyvale, Calif.).

Crenarchaeotal and euryarchaeotal molecular isolates. Four crenarchaeotal 16S rDNA molecular isolates (Fig. 2) were obtained from three different sediment depths: LMA137 and LMA226 (0 to 0.5 cm), LMA229 (0.5 to 1 cm), and LMA238 (3.5 to 4 cm). Their closest relatives are molecular isolates from the Pacific Ocean and Antarctica (7, 13, 14).

Molecular isolates LMA126 and LMA134 were obtained from the sediment sample from a depth of 0 to 0.5 cm and are most closely related to *Methanosarcina acetivorans*. Methanogens are unexpected in the oxic zone but can be found in anaerobic microniches in the sediment or in zooplankton digestive tracts (6). It is also possible that not all the DNA isolated came from living organisms: it has been shown that adsorption to sediment can protect DNA from nucleases (1, 17).

S-K-Cren-0667-a-A-15 hybridization by depth. S-K-Cren-0667-a-A-15 hybridization was approximately 10% of S-D-Arch-0915-a-A-20 hybridization or 0.1% of S-*-Univ-1390-a-A-18 hybridization (Fig. 3). Crenarchaeotal probe hybridization decreases with depth, becoming undetectable at 5 to 6 cm, except for a faint signal at 20 to 21 cm.

Discussion and conclusions. Our observation of "cold-water" crenarchaeota in Lake Michigan sediments further extends their known habitat range. No representatives of this group have yet been isolated in culture, and basic features of their physiology are unknown. Their isolation from the marine water column suggested an aerobic metabolism (8), and their greater abundance in the oxic region of the sediment is consistent with this suggestion. However, hybridization to the group-specific rRNA probe (S-*-Cren-0667-a-A-15) extended well below the region of oxygen depletion, indicating that members of this group may also be active in anaerobic habitats. Related crenarchaeota have been detected in marine holothurian midgut contents (21) and sponge tissues (22).

Although nucleic acid-based techniques are in principle more explicit than culture-based techniques, there are several caveats associated with data interpretation. The relationship between 16S rRNA recovery from sediments and total sediment population is not yet quantifiable: we do not know whether all organisms are lysed with equal efficiency or what proportion of the total rRNA is recovered. Specific controls are difficult to design for complex assemblages including many uncharacterized species, such as the sediment community. Thus, the comparison of independent direct measures will likely provide the most complete picture. To this end we are now comparing results of in situ hybridizations with fluorescently labeled 16S rRNA probes to our results with extracted RNA. Initial lipid analyses compare well with our hybridization results (27a).

The relative recovery efficiencies of RNA and DNA from different species in environmental samples are also unknown. Our DNA extraction procedure avoids a bead-beating step in order to avoid shearing chromosomal DNA. This less-harsh extraction could result in less-efficient recovery of DNA from cells with tough cell walls, which may explain why archaeal 16S rRNA sequences are difficult to amplify from our sediment samples. The nature of cold-water crenarchaeotal cell walls is unknown, so we cannot say whether DNA extraction from them might be favored relative to other archaea. Since primer design is based on existing sequence information, the primer pairs tested may not amplify the predominant populations. Attempts to increase amplification of archaeal sequences by using the universal primer S-*-Univ-1390-a-A-18 with an archaeon-specific 5′ primer resulted only in the amplification of bacterial sequences (1b).

This crenarchaeotal group did not contribute significantly to the suboxic archaeal peak and represents a relatively small fraction of archaeal rRNA at all depths, suggesting the presence of additional novel archaea. We are now more fully evaluating the diversity of the sediment archaeal populations and their distribution in the sediment and water column. Their role in these habitats may be inferred to some extent by relating their distribution to chemical processes at different locales and depths. For example, the suboxic archaeal peak coincides with the region of active iron and manganese reduction attributed to microbial dissimilatory reduction of the metal oxides. We are using this kind of information to define conditions for enrichments, which will be screened with DNA probes for the crenarchaeota as well as for archaea in general. Such an ap-

proach was previously used by us to isolate a novel sulfate-reducing bacterium (15).

Nucleotide sequence accession numbers. The LMA sequences discussed in this work are available through GenBank under the following accession numbers: U87515 to U87520.

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