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Isolation and Characterization of RNA from Low-Biomass Deep-Subsurface Sediments

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Three methods for the isolation of microbial RNA from low-biomass deep-subsurface sediments have been developed and evaluated. RNA was isolated from samples taken from depths ranging from 173 to 217 m, and samples represented a variety of lithologies, including lacustrine, fluvial sand, and paleosol sediments. Cell numbers in these samples were estimated to be between log 4.0 and log 5.1/g on the basis of phospholipid fatty acid analysis. The most efficient method examined is based on the direct lysis of microbial cells followed by the extraction of RNA with alkaline phosphate buffers and subsequent inactivation of nucleases by extraction with guanidinium isothiocyanate. Estimated recoveries of mRNA for this method are approximately 26%. The recovered RNA included both mRNA and rRNA, as evidenced by the detection of sequences homologous to transcripts from the toluene-4-monooxygenase gene of *Pseudomonas mendocina* KR1 and bacterial, archaeal, and eukaryotic rRNA. An unexpectedly high relative concentration of archaeal rRNA (22 to 40%) was observed for these samples.

The use of RNA in studying the structures and activities of microbial communities has become of great interest in recent years. The analysis of rRNA has proven to be particularly powerful in studies of the distribution and relative activities of specific phylogenetic groups in sediments (8) and aquatic samples (6, 13, 15), and the use of mRNA in determining the in situ activities of genes of ecological (27) and environmental (10) relevance is currently being investigated in a number of laboratories. A requirement of these studies is that sufficient amounts of RNA be isolated for analysis, and when measurement of specific activities of genes or organisms is of interest (7, 10, 17), the efficiency of extraction of RNA relative to that of DNA must be known.

We are particularly interested in the ecology of low-biomass deep-subsurface sediments because of their potential role in degrading contaminants associated with groundwaters (1, 4, 5, 14, 19, 20, 26). Little is known of the phylogenetic affiliations of microorganisms indigenous to deep-subsurface sediments or of the presence and activity of genes involved in the degradation of common groundwater contaminants. Analysis of rRNA and specific mRNA is a particularly inviting strategy for addressing these questions, but the efficient extraction of nucleic acids from these samples may be difficult because the amounts of biomass present are small. Many deep-subsurface sediments exhibit cell numbers that are below log 5/g (30). Most current methods for the isolation of RNA from environmental samples are either for matrices such as surface sediments that contain relatively large amounts of RNA (10, 22, 31) or for aquatic samples that are not complicated by the presence of particulates or significant amounts of organic carbon that must be separated from the nucleic acid (16, 27). While existing methods may be appropriate for the matrices they were designed for, they may not be suitable for the efficient extraction of nucleic acids from very-low-biomass sediments. Reevaluation, modification, and optimization of existing procedures may be required for obtaining sufficient amounts of RNA from deep-subsurface sediments.

RNA isolation by most of these methods may be divided into four general phases: (i) cell lysis, (ii) inactivation of nucleases, (iii) extraction of RNA from the environmental matrix, and (iv) purification. The crucial phases in any RNA isolation method are the lysis and nuclease inactivation steps, and the most important differences between reported methods are in these two steps. Efficient lysis of the target microorganisms is required for intracellular RNA to be released, and a high degree of efficiency is necessary to prevent bias due to preferential lysis of certain organisms. A number of lysis procedures have been described for use in RNA isolation, including lysis by solubilization of cell membranes by detergent (both boiling and at room temperature [16]), ballistic disintegration by mixing with glass or zirconium beads (16, 23, 25, 27), and enzymatic degradation of membranes coupled with osmotic shock (22). The relative lysis efficiencies of these procedures are not known at this time, and the optimum procedure is likely to be dependent upon the matrix of interest.

Inactivation of nucleases is essential because of the lability of RNA and the widespread distribution of RNases; enzymatic degradation of RNA is probably the greatest single source of loss of RNA encountered during any of these procedures. Protein denaturants such as diethylpyrocarbonate (DEPC), guanidinium isothiocyanate, phenol, and detergents such as sodium dodecyl sulfate (SDS) are typically employed to inactivate RNases. Most RNA isolation procedures incorporate at least some protein denaturants during lysis to protect the released nucleic acid from environmental nucleases, and most of the denaturants listed above also aid in lysis.

Extraction of RNA from the environmental matrix after lysis is usually assumed to be accomplished by the lysis-denaturing solution. The adsorption of nucleic acids to soils and sediments may be significant (24) and may significantly decrease recoveries of RNA, depending on the sediment. Most lysis-denaturant solutions may not be efficient extractants for nucleic acids from soils and sediments, although their efficiency is highly dependent on the nature of the environmental matrix. In gen-

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TABLE 1. Characteristics of sediment samples used in this study

Sample no.	Lithology	Depth (m)	% Organic carbon	TKN (%)[a]	Log cells/g[b]	pH[c]	Moisture[d] content (%)
1	Lacustrine	173.3–173.5	0.03	0.01	4.7	7.8	27.76
2	Lacustrine	174.0–174.2	0.031	<0.01	5.1	8.0	25.12
27	Fluvial sand	193.3–193.6	0.025	0.01	4.2	6.6	18.40
28	Fluvial sand	194.0–194.3	0.025	0.01	4.0	7.3	21.47
29	Fluvial sand	195.2–195.6	0.023	0.01	3.7	7.5	17.98
34	Paleosol	217.5–217.7	ND[e]	ND	4.7	ND	20.00

[a] TKN, total Kjeldahl nitrogen.
[b] Estimated log of number of cells per gram of sediment by phospholipid fatty acid analysis (28).
[c] In expressed porewater.
[d] Determined gravimetrically (wt/wt).
[e] ND, not determined.

eral, alkaline extractants with at least 0.1 M phosphate are the most efficient extractants (25), but most lysis-denaturant solutions contain little phosphate and are slightly acidic (pH 5.2) to prevent the hydrolysis of RNA under alkaline conditions.

The final step in most isolation procedures for soils and sediments is purification of the RNA of organic contaminants that coextract with the nucleic acids. Partial purification is frequently affected by extractions with phenol and chloroform during denaturation, but many humic compounds copurify with nucleic acids. These contaminants must be at least partially removed to prevent interference with hybridization, quantification, and enzymatic manipulation. Loss of both RNA and DNA may be significant during purification and is problematic in samples containing significant amounts of organic carbon and low biomass concentrations.

To overcome some of the limitations incurred during isolation of RNA from low-biomass sediments, we have developed methods for isolating RNA from low-biomass deep-subsurface sediments and have evaluated the relative recoveries for three methods. The three different approaches evaluated in these studies were as follows: method I, lysis by SDS and freeze-thaw cycles, extraction with an alkaline phosphate buffer, and removal of salts by dialysis; method II, lysis and extraction by boiling in phosphate buffer and SDS plus a freeze-thaw cycle, followed by extraction in an alkaline phosphate buffer and dialysis; and method III, lysis by boiling in SDS followed by extraction with alkaline phosphate buffer and denaturation of nucleases by guanidinium isothiocyanate and phenol. Nucleases are assumed to be denatured by heating in SDS in the first two approaches. These protocols were designed to minimize the number of steps involved in order to decrease sample manipulation that might lead to the degradation of RNA. Biomass and organic carbon contents were low in these samples, and it was believed that extensive treatments to denature RNases would not be necessary.

The samples used in this study were taken from pristine sediments at depths between 173 and 217 m and exhibit biomass concentrations corresponding to approximately log 4.0 to log 5.1 cell equivalents per g on the basis of estimates obtained by phospholipid fatty acid analysis (28). We have used these samples to develop and evaluate the different RNA isolation procedures described above, and the utility of the recovered RNA was demonstrated by estimating the relative abundances of rRNA from the three domains (*Archaea*, *Bacteria*, and *Eucarya*) and by screening the samples for the presence of mRNA transcribed from a gene involved in the degradation of toluene and trichloroethylene.

MATERIALS AND METHODS

Collection of sediment samples. Subsurface samples were collected during August 1992 from the Yakima Barricade borehole on the U.S. Department of Energy's Hanford site in south-central Washington State. The borehole is located hydrologically upgradient from areas of the site influenced by disposal of organic and inorganic chemicals. Samples were collected from the Ringold Formation, which is composed of lacustrine, paleosol, and fluvial sediments (Table 1) ranging in age from 4 to 10.5 million years (late Miocene to Pliocene). Cores were obtained by using cable tool percussion drilling and a split-spoon core barrel containing a sterile Lexan liner. Methods for disinfection of sampling tools and processing of samples were similar to those described in previous reports (11). Bromide (solute) tracers and fluorescent microsphere (particulate) tracers were introduced into different locations of the core barrel to assess the degree to which cores were contaminated during drilling and core recovery operations (11).

On core recovery, the Lexan liner was removed from the split-spoon core barrel, the ends were capped, and the cores were immediately transferred to an on-site anaerobic glove bag containing argon gas. The Lexan liner was sawed open, the outer 1 cm of sediment was removed with sterile tools, and the sediment was sampled for microbiological, chemical, physical, and tracer analyses. Sediment samples for microbiological and tracer analyses were homogenized, sealed in sterile canning jars, and refrigerated for 1 to 5 h prior to transport to the laboratory on blue ice. Samples intended for nucleic acid extractions were placed immediately into a −20°C freezer upon removal from the glove bag on site until they were transported, on ice, to Pacific Northwest Laboratory. All samples were frozen completely within 16 h after coring. On arrival at Pacific Northwest Laboratory, samples were removed from the jars and immediately either shipped on dry ice by overnight mail or frozen at −70°C for later shipment on dry ice. On arrival at Washington State University, and within 24 h of shipment, samples were frozen at −20°C.

Microspheres were enumerated by epifluorescent microscopy, with a detection limit of log 3.3 microspheres per g. Bromide concentrations in the cores were 2 to 3 orders of magnitude lower than in slurry recovered from the core barrel (21). Microsphere concentrations were either below the detection limit or 4 to 6 orders of magnitude lower in the cores than in nonsample water and slurry recovered from the core barrel.

Cell equivalents presented in Table 1 were estimated by using values of 1.72×10^{-13} g per cell (on a dry-weight basis) based on cells observed in shallow-subsurface aquifer sediments (2) and 50 μmol of phospholipid fatty acid per g of dry cells (32).

RNA isolation from sediments. (i) Method I. All glassware, plasticware, and solutions were autoclaved, and all solutions were treated with 0.1% DEPC and then autoclaved to inactivate nucleases. Frozen samples were thawed for 10 to 20 min at room temperature immediately prior to processing. Between 60 and 120 g of sediment was divided into aliquots of 4 g each, placed in 50-ml Oak Ridge tubes, and mixed with 10 ml of extraction buffer (0.2 M sodium phosphate buffer [pH 8.0], 0.1 M EDTA). Direct lysis of cells was effected by the addition of 1.5 ml of 10% SDS and incubation in a 65°C water bath for 2 h with occasional gentle mixing. The lysis step was completed by freezing at −80°C for 30 min or at −20°C for 2 h and then thawing in a 65°C water bath for 15 min.

Nucleic acids were extracted from the sediment by centrifuging the lysate at 12,000 × *g* at 10°C for 15 min, and the supernatant was transferred to a fresh tube. The pellet was extracted with 12 ml of extraction buffer and mixed well, and the mixture was incubated at 65°C for 15 min. This mixture was centrifuged as described before, and the supernatants were pooled. The pellet was then extracted a second time, and the supernatants from all replicates were pooled.

Low-molecular-weight contaminants (such as SDS and salts) were removed by exhaustive dialysis. The pooled supernatants were transferred to dialysis tubing (Spectrapor; molecular weight cutoff, 12,000 to 14,000; diameter, 28.6 mm; Spec-

trum Medical Industries, Inc., Los Angeles, Calif.) and dialyzed against sterile distilled water or sterile TE (0.010 M Tris-HCl, 0.001 M EDTA, pH 8) at room temperature. The dialysis buffer was changed hourly over the next 5 to 6 h, and a final dialysis was continued overnight.

The volumes of the final dialysate were measured, and the dialysate was transferred to 250-ml plastic centrifuge bottles. Nucleic acids were precipitated from the dialysate by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The samples were mixed several times by inversion and were kept at room temperature overnight. Precipitated nucleic acids were recovered by centrifugation at 10,000 × *g* at 4°C for 20 min, and the pellet was washed with 70% ethanol to remove salts. The washed pellet was centrifuged once more and briefly dried under a vacuum at room temperature. Care was taken not to dry the pellet completely to simplify resuspension of the nucleic acids. The partially dried pellet was then resuspended in 100 μl of sterile H_2O or TE per 15 g of starting sediment. DNA was removed by digestion with RNase-free DNase I (Sigma, Inc., St. Louis, Mo.), and the DNase I was removed by extraction with phenol and chloroform, followed by precipitation of the RNA with ethanol and resuspension in TE. This degree of purification was sufficient for these samples, as determined by successful reverse transcription with bacterial domain-specific primers (data not shown).

(ii) Method II. Method II is essentially the same procedure as method I, the only difference being in the lysis step. Between 60 and 120 g of sediment was divided into aliquots of 4 g each, placed in 50-ml Oak Ridge tubes, and mixed with 8 ml of extraction buffer (0.2 M sodium phosphate buffer [pH 8.0], 0.1 M EDTA) and 1.5 ml of 10% SDS. The mixture was placed in a boiling-water bath for 5 min. The boiled samples were frozen at −80°C for 30 min or at −20°C for 2 h and thawed in a 65°C water bath for 15 min. Nucleic acids were extracted from the sediment by centrifuging the lysate at 12,000 × *g* at 10°C for 15 min, and the supernatant was transferred to a fresh tube. The pellet was resuspended in 5 ml of lysis buffer, and a second round of boiling was conducted. Freezing and thawing were not repeated. The boiled mixture was centrifuged as described above, the supernatants were pooled, and the pellets were discarded. All subsequent steps were exactly as described above for method I.

(iii) Method III. Samples were lysed by two cycles of boiling and one freeze-thaw cycle, as described for method II. Nucleases remaining in the pooled supernatants were inactivated by extraction with 11.2 ml of guanidinium isothiocyanate-phenol-Sarkosyl solution (GIPS) (16, 27), containing 4 M guanidinium isothiocyanate, 0.5% Sarkosyl, 25 mM sodium citrate (pH 7.0), 11.2 ml of phenol, and 6.4 ml of $CHCl_3$-isoamyl alcohol (24:1). The mixture was centrifuged at 6,000 × *g* at 4°C for 10 min, and the aqueous upper phase was transferred to a clean Oak Ridge tube. Nucleic acids were precipitated by the addition of 2 volumes of ethanol. Nucleic acids were recovered, and DNA was digested with DNase I as described for method I.

Determination of extraction efficiency. Extraction efficiencies for all three methods were determined by using sample 29. Sample 29 was chosen as the model sediment for these studies because of the greater availability of these samples and their similarity to the other samples with respect to organic carbon content, biomass, pH, nitrogen content, and water content (Table 1). *tmoABCDE* that had been subcloned into a Bluescript vector (Strategene Cloning Systems, La Jolla, Calif.) to produce pMR403 (supplied by M. Romine, Pacific Northwest Laboratory) was used to generate the tracer. mRNA tracer consisting of a 900-base mRNA molecule corresponding to toluene monooxygenase *tmoAB CDE* (30, 34) was generated by an in vitro transcription system as recommended by the vendor (Stratagene Cloning Systems) by using [α-^{32}P]UTP (specific activity, 800 Ci/mmol; New England Nuclear, Wilmington, Del.) and purified of unincorporated nucleotides by centrifugation in Centricon 100 microconcentrator tubes (Amicon, Inc., Beverly, Mass.). The radiolabeled tracer was spiked into three replicates immediately prior to lysis at a rate of approximately 2.5 × 10^5 cpm/g, and loss of the label was determined following each step in the procedure by removal of an aliquot from the total and analysis by scintillation counting.

Detection of *tmoA* and *todC1* mRNA by reverse transcriptase PCR (RT-PCR) and hybridization. RNA from the samples was converted to cDNA by Moloney murine leukemia virus reverse transcriptase (BRL Gibco, Bethesda, Md.) and primers specific to genes for toluene monooxygenase and toluene dioxygenase in separate reactions. mRNA annealing to primers based on the cloned toluene-4-monooxygenase gene *tmoA* of *Pseudomonas mendocina* KR1 (34) was reverse transcribed with a synthetic oligonucleotide primer with the following base composition: GGA ATA GAT CCC AGT ACC AGG. Similarly, mRNA homologous to the toluene dioxygenase gene *todC1* of *Pseudomonas putida* F1 (35) was reverse transcribed by the synthetic oligonucleotide primer GTA TTG ATA CCT GGG AGG AAG.

cDNA from these reactions was added to PCR mixtures by using the following pairs of primers: primers specific to *tmoA*, GCT ATG TTA CCG AAG AGC AGC and GGA ATA GAT CCC AGT ACC AGG, resulting in the production of a 900-bp product; and primers specific to *todC1*, GCG AGA TAG AAG CGC TCT TTG and GTA TTG ATA CCT GGG AGG AAG, resulting in the production of a 924-bp product. PCRs were conducted with 100-μl volumes containing 0.5 pmol of each primer per μl, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 5 U of *Taq* polymerase (Promega Corp., Madison, Wis.). Amplifications were conducted with a model 480 Thermocycler (Perkin-Elmer, Norwalk, Conn.) by performing one cycle at 94°C for 2 min and then 40 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min. A final extension was carried out during the last cycle at 72°C for 5 min. pMR403 and pMR601 containing *tmoABCDE* and *todC1C2BA*, respectively, were supplied by M. Romine (Pacific Northwest Laboratory) and used as positive amplification controls.

TABLE 2. Percent recovery efficiencies of mRNA from sample 29[a]

Method	% Recovery following extraction[b]	% Final recovery[c]
I	93 (7.1)	6.0 (4)
II	82.8 (8.3)	6.9 (1.8)
III	86.7 (4.4)	26.4 (4.8)

[a] Values in parentheses are percent coefficients of variation based on three replicates.
[b] Recovery of the label following lysis and extraction from the sediment.
[c] Recovery of starting label in the final precipitate.

tmoA amplification products were confirmed by hybridization of Southern blots with a cloned version of this gene. Radiolabeled mRNA from *tmoABCDE* was generated as described above for the mRNA tracer used in the determination of extraction efficiency. Electrophoretic separation of the amplification products and Southern blotting to Nytran membrane (Schleicher and Schuell, Keene, N.H.) were done by standard procedures (29). DNA was fixed to the filters by baking in a vacuum oven at 80°C for 2 h. Membranes were prehybridized for 6 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–200 μg of denatured salmon testis DNA (Sigma) per ml–1× PE (5× PE is 0.25 M Tris-HCl [pH 7.5], 0.5% [wt/vol] sodium PP_i, 5% SDS, and 1% polyvinylpyrrolidone [molecular weight, 40,000], 1% Ficoll, 0.025 M EDTA, and 1% bovine serum albumin). Following prehybridization for 6 h, the prehybridization buffer was removed and replaced with fresh hybridization buffer, consisting of prehybridization buffer containing approximately 3 × 10^6 cpm of labeled probe per ml, and the membranes were hybridized for 16 h at 42°C. All prehybridizations and hybridizations were conducted in Hybrid-Ease chambers (Hoeffer Scientific Instrument Co., San Francisco, Calif.). Membranes were washed twice in 2× SSC–0.1% SDS at 65°C for 15 min and twice in 0.1× SSC–0.1% SDS at 65°C for 15 min and then exposed to X-ray film at −70°C for 20 h.

Samples were also screened for the presence of mRNA from *tmoA* and *tdoC1* by direct hybridization. RNA taken from samples was applied to Nytran membrane (Schleicher & Schuell) by a standard dot bot technique (29, 30) and hybridized to the appropriate probes as described above. Negative controls consisted of samples that had been treated with RNase A.

Hybridization probes. The contributions of the three phylogenetic domains (*Archaea*, *Eucarya*, and *Bacteria*) to the recovered rRNA were determined by hybridization with domain-specific oligonucleotide probes suggested by DeLong (6) and Giovannoni et al. (15). Sequences of these probes were as follows: universal, ACG GGC GGT GTG TRC; archaea, GCG CCT GST GCS CCC CGT AGG GCC; bacteria, GCT GCC TCC CGT AGG AGT; eucarya, GGG CAT CAC AGA CCT G. Standard species were as follows: archaea, *Haloferax volcanii* and *Sulfolobus solfataricus*; bacteria, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, and *Escherichia coli*; eucarya, *Rhizoctonia solani*, *Pythium irregulate*, *Gaeumannomyces graminis* var. tritici, and *Magnaporthe grisea.*

Oligonucleotide probes were end labeled by T4 polynucleotide kinase (Promega) with [γ-^{32}P]ATP (>5,000 Ci/mmol) and purified of unincorporated nucleotides by centrifugation in Centricon 3 microconcentrator tubes.

RNA from samples and standards was denatured in 0.5% glutaraldehyde applied to Nytran membrane. Membranes were prehybridized at 45°C for 1 h in 0.9 M NaCl–50 mM NaH_2PO_4 (pH 7)–5 mM EDTA–0.5% SDS–10× Denhardt's solution–0.5 mg of poly(A) per ml. Hybridizations were conducted at 45°C for 12 to 16 h with the same solution as that used for prehybridization with the addition of approximately 10^6 cpm of probe per ml. Membranes were first washed at room temperature for 30 min in 1× SET (150 mM NaCl, 20 mM Tris-HCl [pH 7.8], 1 mM EDTA)–0.5% SDS. Final washes were in 1× SET–0.5% SDS for the following times and temperatures: universal and bacterial probes, 45°C for 45 min; eucaryal probe, 40°C for 30 min; archaeal probe, 69°C for 30 min. Relative compositions were determined by autoradiography and analysis by scanning densitometry and normalization of domain-specific signals to the signals from the universal probe, as described by Giovannoni et al. (15).

RESULTS

Extraction efficiency. Three RNA isolation techniques were evaluated with sediment sample 29, a fluvial sand sample containing approximately log 4.2 cells per g and 0.02% organic carbon (Table 1). Loss of the RNA was determined at two stages, and the results are presented in Table 2.

Analysis of extracted RNA. RNA isolated by method I was characterized as to size and composition. Two major electrophoretic bands were observed in the recovered RNA and are

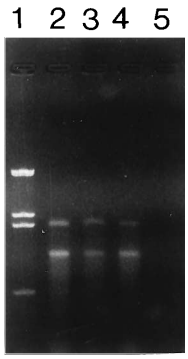

FIG. 1. Agarose gel of RNA extracted by method I. Lanes: 1, pBR322 digested with *Bst*NI (molecular weight marker); 2, sample 34, equivalent of 8 g of sediment, treated with DNase I; 3, sample 28, equivalent of 8 g of sediment, treated with DNase I; 4, sample 27, equivalent of 8 g of sediment, treated with DNase I; 5, sample 34, equivalent of 8 g of sediment, treated with DNase I and RNase A.

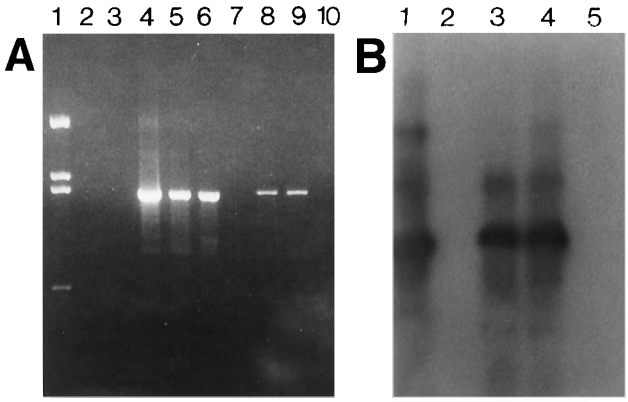

FIG. 2. RT-PCR of *tmoA*. (A) Agarose gel electrophoresis of PCR products. Lanes: 1, pBR322 digested with *Bst*NI (molecular weight marker); 2, water (no-template control); 3, SeaPlaque agarose (no-template control); 4 through 6, pMR403 (positive control) at 1 ng, 10 pg, and 1 pg, respectively; 7, sample 27; 8, sample 28; 9, sample 34; 10, sample 28 treated with DNase I and RNase A. (B) Autoradiogram of Southern blot hybridized with *tmoA*. Lanes: 1, pMR403 (positive control); 2, sample 27; 3, sample 28; 4, sample 34; 5, sample 28 treated with RNase A prior to reverse transcription.

assumed to be due to small- and large-subunit rRNA molecules (Fig. 1).

The distribution of rRNA among the three domains (Table 3) was estimated by the method suggested by Giovannoni et al. (15). Most of the rRNA originated from bacteria, although significant amounts of rRNA from the archaea and the eucarya were also isolated. The relative amounts of bacterial, archaeal, and eucaryal rRNA isolated by this method may reflect the lysis procedure used as well as the true proportions of rRNA from the different groups in the sediment, because all three lysis procedures were probably more efficient at lysing gram-negative bacteria than at lysing either gram-positive bacteria or eukaryotes (23). The sum of the percentages of the three domains within each sample is greater than 100% for all but one sample (sample 34), an observation that has been reported before (6) and may be attributed to partial degradation of sites for universal-probe annealing in some samples.

RNA from all samples was screened for sequences similar to those encoding the toluene-4-monooxygenase (*tmoA*) of *P. mendocina* KR1 (34) and the toluene dioxygenase (*todC1*) of *P. putida* F1 (35). No transcripts from *todC1* were detected in any sample by either RT-PCR or direct hybridization of dot blots. Transcripts from *tmoA* were detected in samples 28 and 34 by RT-PCR (Fig. 2) and by direct hybridization in dot blots (data not shown). Samples 28 and 34 were taken from the fluvial sand and the paleosol lithofacies, respectively. Dissolved oxygen contents could not be determined, but the expressed pore waters for sample 28 exhibited an E_h value of 290 mV (Table 1) and a nitrate content of 2.8 mg/liter (data not shown), indicating that the predominant microbial respiratory process would have been denitrification. Nitrate and E_h values for sample 34 were not measured, but it is likely they would have been similar to those observed for sample 28.

TABLE 3. Composition of isolated rRNA[a]

Sample	% rRNA from domain:		
	Archaea	*Bacteria*	*Eucarya*
1	26.5	70.4	15.6
2	27.0	74.9	26.1
27	27.6	79.4	20.2
28	40.2	58.7	15.7
34	22.1	71.1	6.3

[a] Values are percentages of total rRNA. Hybridizations were conducted with RNA extracted from 20-g samples.

DISCUSSION

Three methods for the extraction of RNA from low-biomass sediments were developed and evaluated. The alkaline phosphate buffer efficiently extracted the radiolabeled tracer by all three methods, and the moderate alkalinity (pH 8) of the buffer did not significantly damage the RNA, as indicated by the successful RT-PCR of the recovered RNA. Extraction of adsorbed RNA is required for quantitative separation of the nucleic acid from the sediment, as only 76.6% extraction efficiency was observed when one-half of the extractant volumes reported here were used (data not shown). Alkaline phosphate is a more efficient extractant than saline TE and SDS, as boiling and extraction in saline TE and SDS resulted in only 43.8% extraction efficiency (data not shown).

The final recoveries by methods I and II were similar (6.0 and 6.9%), indicating that the boiling step added in method II did not inactivate significantly more nuclease than did lysis by SDS and a freeze-thaw cycle (method I). Significantly more RNA was recovered by method III (26.4%) than by either of the other two methods, indicating that nucleases not inactivated by methods I and II were at least partially inactivated by extraction with GIPS in method III. RNA may also have been lost by the inefficient precipitation of low concentrations of nucleic acids by ethanol even with the addition of glycogen (16).

rRNA recovered by method I was of suitable quality for amplification by RT-PCR, and sequences specific to bacterial 16S rRNA were amplified and the amplification products were subsequently cloned. These clones will be subjected to further phylogenetic analysis. The composition of the recovered rRNA was determined by quantitative hybridization with domain-specific oligonucleotides, and all three domains (bacteria, eucarya, and archaea) were represented in the samples tested. Bacteria constituted the most abundant fraction (58.7 to 79.4%), followed by archaea (22.1 to 40.2%) and eucarya (6.3 to 26.1%). As discussed above, the relative proportions of the three domains in the recovered rRNA are partially dependent on the lysis procedure, which may have resulted in the under-

representation of eucarya in the recovered rRNA. A comparison of the compositions of rRNA recovered by the three different methods would have been of value in determining the dependence of the observed contributions of the three domains on the lysis procedure, but because of the limited availability of these samples, this was not possible. The relative dominance of bacteria in these samples is to be expected, both because of a possible bias in the lysis procedure and because bacteria are generally considered to be the dominant organisms in pristine low-biomass deep-subsurface sediments (20). It should be noted that since the degree of background hybridization was not determined with an antisense oligonucleotide probe in these experiments, it is not known at this time if the 6.3% eucaryal contribution observed for sample 34 is above the background hybridization level. There was, however, no detectable hybridization between different probes and standards (e.g., the eucaryal probe and archaeal or bacterial standards), indicating that the level of background hybridization was low.

The relative abundance of archaeal rRNA in these sediments is of note, as this is the first estimation of the relative abundance and activities of archaea in deep-subsurface sediments by using molecular approaches. These results indicate that archaea may be very active members of deep-subsurface communities. Most archaea fall into three distinct groups: the obligately anaerobic methanogens, the extreme halophiles, and the extreme thermophiles; however, new and as yet incompletely described groups have recently been found in oxic marine environments (6, 13). It is not currently known which of these groups were responsible for the archaeal rRNA in these samples, but it is unlikely that significant amounts of either extreme thermophiles or extreme halophiles would have been present in these sediments. It is more likely that the archaeal rRNA originated from either methanogens or a new and possibly undescribed group. These sediments are predominantly anaerobic, as evidenced by the much greater numbers of culturable fermenting and iron-reducing bacteria than of culturable aerobic heterotrophs found in these samples (21). It is therefore possible for strict anaerobes such as methanogens to be active in these sediments. Methanogens are found in a wide variety of environments, including those that are not generally thought to be anaerobic, such as surface soils (3). Anaerobic pockets that could support methanogenesis may exist within soil or sediment aggregates (3). Even though approximately 2 kg of sediment was homogenized prior to subsampling for microbiological assays, the subsamples from which the rRNA was extracted (20 g) were much larger than the 1-g subsamples used for methanogen enrichments. It is possible that significant regions of heterogeneity that would have been masked by the mixing of this quantity of sediment were present in the samples. Culturable methanogens in these samples were not detected (21), but these methods may not have detected methanogens because of a possible inability to culture them. Questions concerning the phylogenetic origin of the archaeal rRNA found in these sediments will not be answered until the archaeal sequences are amplified, cloned, and sequenced (6, 18).

mRNA was also recovered from these samples, as evidenced by the detection of transcripts from samples 28 and 34 that hybridized to the gene encoding toluene-4-monooxygenase (*tmoA*) in *P. mendocina* KR1 (34). The detection of *tmoA* mRNA, indicating that the gene is induced in these samples, was not expected. The samples were taken from a pristine site, and the organic carbon contents were low (0.025%), indicating that the concentration of inducing substrate would have been quite low. As mentioned above, these sediments are thought to be predominantly anaerobic, with nitrification being the dominant form of respiration. The host originally described for *tmoA*, *P. mendocina* KR1, has not been shown to grow on toluene under denitrifying conditions (12). Microorganisms capable of growth on toluene under denitrifying conditions have been recovered from a variety of habitats (12), and it is possible that the host of the genes we have detected is not *P. mendocina* KR1 and metabolizes substrates under denitrifying conditions. The genes responsible for metabolism of toluene in *P. mendocina* KR1 are thought to have been acquired by horizontal gene transfer (34), and interspecies transfer of these genes has recently been demonstrated in a laboratory (33). In addition, the geochemistry of these sediments is likely to be spatially heterogeneous (21), and although the overall oxygen and carbon contents of the samples were low, there may have been microniches containing sufficient oxygen and carbon to support transcription of *tmoA*-like genes.

The finding of *tmoA* genes being actively transcribed in these sediments was not expected, and several possible explanations must be considered. One source of ambiguity may be cross-hybridization with other genes related to, but not identical to, *tmoA*. Many oxygenases share regions of homology, and it may be that the sequences detected were not actually *tmoA*, but rather some related oxygenase. For the amplification of the expected 900-bp fragment with primers directed toward *tmoA* to occur and for the amplification product to hybridize at moderate stringency to *tmoA*, the related oxygenase would have to share a high degree of similarity with the *tmoA* product. We cannot state conclusively that we have detected *tmoA* transcripts in these sediments, but we can be certain that we have detected transcripts that are from a gene that encodes an enzyme that is at least very closely related to the *tmoA* product and is most likely an oxygenase.

Another possible explanation for the presence of *tmoA* transcripts may be found in sampling or in the processing of samples. Exposure to oxygen during or after sample collection may have induced the activity of the resident microorganisms. Utmost care was taken during sample collection and the initial sample processing, with all manipulations being conducted in an Ar-filled glove bag. To avoid stimulation due to exposure to H_2, no O_2 scrubbing system was used during the initial processing. Therefore, low concentrations of O_2 would have been present in the glove bag during processing of the cores. Sample temperatures were 4°C or less within 2 h after sectioning of the core, and the maximum time between retrieval of the core and complete freezing was 16 h. Samples remained frozen until immediately prior to extraction of the nucleic acids, and the time between thawing of the samples and cell lysis was less than 30 min.

Why *tmoA* mRNA was detected in sample 28 and not in the almost identical sample 27 is not known. Both samples were taken from approximately adjacent depths (separated by approximately 1 ft. [30.48 cm]), both were taken from the same sediment type (fluvial sand), and the two samples exhibited almost identical chemical and physical characteristics. An indication that the resident microbial communities in these samples were different is shown by the relative proportions of the three domains in samples 27 and 28. Approximately 79.4% of the rRNA in sample 27 and only 58.7% of that in sample 28 were of bacterial origin. The only known host of *tmoA* is bacterial; this would again lead one to predict that sample 27 might be more likely to contain more *tmoA* than would sample 28. It is most likely that differences between these samples that influenced the rates of transcription (and activity of the hosts) but were not reflected in the geochemical parameters measured were present.

The observation that *tmoA* transcripts were detected in two

samples but that transcripts of *todC1*, a gene encoding the toluene dioxygenase of *P. putida* F1, were not detected in any samples may be related to the relative competitiveness of the two enzymes under substrate and oxygen limitations. By chemostat studies of competition between toluene-degrading strains under both oxygen- and toluene-limiting conditions, Duetz et al. (9) showed that strains such as *P. mendocina* KR1 that harbor toluene monooxygenases inhibited by competition strains such as *P. putida* F1 that possess toluene dioxygenases. These results may explain why sequences homologous to both the toluene dioxygenase of *P. putida* F1 and toluene monooxygenase of *P. mendocina* KR1 were detected in DNA from sample 28 in a parallel study (30) but only *tmoA* transcripts were detected in the present study.

The techniques developed in this study are suitable for use in molecular ecology studies of low-carbon, low-biomass sediments. One of the more significant limitations of the techniques presented here is that the efficiency of lysis is unknown at this time. It may be possible to obtain a less biased sample by using a more rigorous lysis method such as that developed by More et al. (23).

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