stituted for T4 DNA ligase. Sections were covered with glass coverslips and placed in a humidified box for 16 h at room temperature (23°C). In preliminary experiments, the time and temperature were shown to be optimal for in situ ligation. Sections were then washed in water (3× for 10 min).

Avidin-fluorescein conjugate (4 µg/mL; Vector Laboratories, Burlingame, CA, USA) was added to the sections in 50 mM sodium bicarbonate, 15 mM CA, USA) was added to the sections in 50 mM sodium bicarbonate, pH 8.2, for 45 min. 50 mM sodium bicarbonate, 15 mM coculture, and 800 U/mL ter - (3), but used Texas Red as the label rather than biotin. After completion of the ligation reaction and three 10 min washes in water, a mixture comprised of 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 mM dithiothreitol (DTT), 8 µM Texas Red dUTP (Molecular Probes, Eugene, OR, USA), and 800 U/mL terminal transferase (Roche Molecular Biochemicals) (20 µL per section) was added to the sections. The sections were incubated for 1 h at 37°C. After washing in water (3× for 10 min), the sections were mounted in Vectashield and observed by fluorescent microscopy.

For the detection of free DNA 3’ hydroxyls with terminal transferase, we used the procedure previously published (3), but used Texas Red® as the label. After completion of the ligation reaction and three 10 min washes in water, a mixture comprised of 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 mM dithiothreitol (DTT), 8 µM Texas Red dUTP (Molecular Probes, Eugene, OR, USA), and 800 U/mL terminal transferase (Roche Molecular Biochemicals) (20 µL per section) was added to the sections. The sections were incubated for 1 h at 37°C. After washing in water (3× for 10 min), the sections were mounted in Vectashield and observed by fluorescent microscopy.

Figure 2 is a composite micrograph showing background staining in apoptotic cells at high magnification using fluorescent detection. It demonstrates that the looped probe has higher background, caused by ligation-independent attachment to both cytoplasmic and nuclear compartments. Staining with the loopless probe shows localization of the signal in the areas of apoptotic chromatin condensation on the nuclear membrane. The double-staining procedure with visualization of both 3’ hydroxyls and full double-strand breaks demonstrates co-localization of both ligation and terminal transferase-based signals in the same nuclei with more intense staining of chromatin precipitated on the nuclear membrane by in situ ligat -

This data is consistent with our previous results (1,2).

The new probe design, analyzed by fluorescent microscopy, has resulted in a substantial reduction of nonspecific background, with no need for long stringent washes. It has also made the assay more cost effective by using a much shorter probe and reducing the number of biotins in the probe without loss of sensitivity.

In conclusion, our findings indicate that ligase-mediated apoptosis detection in tissue sections can be enhanced and simplified by using a newly designed loopless hairpin oligonucleotide probe. The reproducibility of the probe preparation, the economy of the reagents, the possibility of double-staining with terminal transferase and the simplicity of the assay are factors that make the method attractive for use by other laboratories.

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Extraction of Intact Ribosomal RNA from Anaerobic Bioreactor Samples for Molecular Ecological Studies

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Anaerobic bioreactors are considered an efficient means for the reduction of pollution loads from waste with concurrent production of bioenergy in the form of methane (1,7). A wide range of solid wastes such as sewage sludge and different forms of industrial wastes have been used, separately or in combination, as substrates in biogas reactors (2,7,9). Furthermore, anaerobic immobilized processes such as the upflow anaerobic sludge blanket (UASB) have been exploited for treatment of industrial effluents (14). With the increased use of anaerobic treatment of different environmental wastes, it is of major importance to understand the dynamics of the anaerobic biological activities involved in these processes.

Ribosomal RNA genes, especially 16S rRNA, are now considered one of the golden standards in studying population dynamics in different environments including biological reactors (13,15). The continual increase in comparative rDNA sequence information has facilitated the development of a wide variety of oligonucleotide primers and probes with specificities that range
from the subspecies to the domain level (3,8). These probes have been exploited in quantitative hybridization experiments to calculate rRNA concentrations of different microbial species in relation to the total rRNA available (5). An important factor in achieving such studies is to obtain pure and intact rRNA genes suitable for gene probing or even for reverse transcription (RT) to study microbial diversity in a given community.

However, published techniques for rRNA extraction, particularly from environmental samples (6,10), entail the use of a wide array of reagents and can be very labor intensive. Moreover, almost all published techniques require long incubations at -20°C or even -80°C from a few hours to overnight. Yet, little is published regarding ribosomal RNA extraction from manure, either fresh or digested, or granular sludge (12). The integrity and purity of the extracted rRNA genes, which are substantially affected during extraction procedures (11), are crucial parameters for subsequent manipulations in molecular studies. Still, most reported protocols verify the integrity of the extract by oligonucleotide probe hybridization, an insufficient criterion to indicate the presence of fully intact rRNA genes. Partial degradation of the ribosomal RNA would still be enough for tangible hybridization signals.

In this communication, we demonstrate a simple and straightforward protocol suitable for rRNA extraction from different forms of manure, granular sludge or just pure and/or mixed microbial cultures. The whole extraction procedure can be completed in 4 h with a beadbeater and a bench top centrifuge as the major instruments. We also describe a clear strategy to avoid any discernible degradation of 16S or 23S rRNA species by using three readily available reagents; namely sodium citrate, mercaptoethanol and chloroform. Equally important, we report for the first time a fast and reproducible methodology for a one-step RT-PCR amplification of almost the complete 16S and 23S rRNA genes.

Fresh manure samples (50 mL) were obtained from a full-scale biogas plant in Snertinge, Denmark. Aliquots of 1 mL were dispensed in 67 × 16 mm polypropylene tubes (Hounisens Laboraerieudsty, Risskov, Denmark) and kept at -80°C until needed.

Anaerobic granular sludge was from the active core of the upflow anaerobic sludge bed (UASB) reactors. Samples (50 mL) of granular sludge were obtained from a UASB full-scale bioreactor treating waste water from a paper pulp mill.

Along with the study, two reference strains from our collection, *Thermoaerobacter thermohydrosulphuricus* (AOR strain) and *Methanosarcina thermophila* (TM1), were used as controls for optimization of the protocol. The preparation of fresh culture of each strain was as published previously (4).

The integrity of the extracted RNA was verified by electrophoresis using 1.2% formaldehyde agarose gel. A standard 16S–23S rRNA marker (Roche Molecular Biochemicals, Kvistgård, Denmark) was also loaded along the samples to approximate extracted rRNA concentration. For quantitative slot blot hybridization, the concentration was determined by a combination of spectrophotometric reading at A$_{260}$ and formaldehyde agarose gel electrophoresis.

For slot blot hybridization, RNA samples (50 µL containing ca. 90 ng of rRNA) were denatured in 20× standard saline citrate (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (30 µL) and formaldehyde (20 µL) at 60°C for 15 min and immediately stored on ice. Each sample was slot-blotted in triplicate (ca. 30 ng of rRNA) on nylon membranes (Roche Molecular Biochemicals) using a Minifold® II Slot Bloter (Schleicher & Schuell, Fredriksberg, Denmark). Membranes were cross-linked in a Hoefer® UVC 500 UV Crosslinker (Amersham Pharmacia Biotech, Allerød, Denmark) at 254 nm for 2 min. Membranes were hybridized with the following probes (Escherichia coli 16S rRNA numbering system): (i) Bacterial domain-specific probe 5′-GCTGCCTCCGTTAGGAG-T-3′ (338–355) and (ii) Archeal domain-specific probe 5′-GTGCTCCCGCGCCAATTCCT-3′ (915–934) (8). Hybridization conditions, labeling and
Benchmarks

Table 1. Extraction of Ribosomal RNA

Before starting the extraction process, samples are washed 2–3× in low pH-buffered sodium citrate.

1. Prepare the required number of screw-capped 2 mL tubes (Biospec Products, Bartlesville, OK, USA) for beadbeating.

2. To each tube add the following in the order given: 0.5 g of 0.1 mm zirconium beads (Biospec Products), 0.5 mL of phenol (pH 4.5), 1 mL of sample (culture, manure or granular sludge) suspended in 50 mM of sodium citrate buffer (pH 5.5), 200 μL of SDS 10% (pH 7.0) and 25 μL of β-mercaptoethanol.

3. Start beadbeating immediately in a mini-beadbeater (Biospec Products) at maximal speed for 2 min.

4. Spin down in a bench top centrifuge at 5000×g for 10 min (avoid high speed, as zirconium beads may cause rupture of the tubes).

5. Transfer the aqueous phase into a clean 67 × 17 mm polypropylene tube containing 3 mL of chloroform.

6. Add a fresh batch of 1 mL sodium citrate, 200 μL of SDS, 25 μL of β-mercaptoethanol and repeat beadbeating, centrifuge at 5000×g for 10 min and transfer the aqueous phase to the respective tubes containing the beads.

7. Extract the samples once more with 1 mL of sodium citrate and collect the aqueous phase as above.

8. Extract the aqueous phase 3× with an equal volume of phenol (pH 4.5). Make sure to mix the two phases thoroughly each time before centrifugation.

9. Extract the resulting aqueous volume with an equal volume of chloroform.

10. Precipitate the RNA with 0.1 vol of 3M of sodium acetate (pH 5.2) and 3 vol of ice-cold absolute ethanol.

11. Mix thoroughly and immediately centrifuge at 20 000×g for 30 min.

12. Remove the supernatant and wash the pellet 2× with 75% ice-cold ethanol.

13. Aspirate the residual liquid and dry the pellet at 50°C for approximately 5 min. Aspirating most of the ethanol after the final wash will facilitate rapid drying of the pellet.

14. Add 20 U of ribonuclease inhibitor RNasin® (Promega, Madison, WI, USA) to the dry pellet and dissolve in 1 mL of DEPC-treated water.

15. Continue with subsequent manipulations or store the RNA at -80°C in 100 μL aliquots.

The following precautions should be observed: all chemicals and other reagents should be molecular biology grade. Water used in all preparations should be incubated with 0.1% DEPC at 37°C overnight, followed by autoclaving at 121°C for 30 min, and all reagents should be prepared in DEPC-treated water. All glassware and zirconium beads should be baked at 200°C for 2 h; disposable plasticware should be autoclaved at 121°C for 30 min. The beadbeater should be covered with two plastic bags of ice for 15 min before and during the beadbeating process. All manipulations, unless otherwise stated, should be carried out on ice and all centrifugation procedures at 4°C. The bench and instruments (e.g., the centrifuge, the blotting apparatus, pipets, etc.) should be sprayed with RNaseZap (Ambion, Austin, TX, USA) and wiped with paper towel. The extraction process should be completed without interruption.

washing temperatures will be published elsewhere. After air-drying, membranes were exposed to Biomax X-ray films (Eastman Kodak, Rochester, NY, USA) for qualitative examination or analyzed in a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA) for quantification.

The purity and integrity of the extracted RNA genes were further checked by RT. Although no visible DNA was co-extracted with RNA in the described protocol, it is of utmost importance to avoid any traces of DNA in the extract. To test for contaminating DNA, an aliquot of the extracted RNA (ca. 100 ng) was incubated with 1 U of RNase-free DNasel (Amersham Pharmacia Biotech) in the presence of 10 U of RNase inhibitor and 40 mM Tris-HCL (pH 7.5), 6 mM MgCl₂ at 37°C for 15 min. The sample was further incubated at 60°C for 15 min to inactivate the DNase. RT was carried out using the Titan One Tube RT-PCR System (Roche Molecular Biochemicals) with modifications as follows: in a 0.2 mL PCR tube, the following reagents were added in the same order (final concentration): 25 μL diethylpyrocarbonate (DEPC)-treated water, 4 μL dNTPs (200 μM), 1 μL each of forward and reverse primers (3′) (for 16S, forward and reverse primers were 5′-GAGTTTGA-TCCCTGCTCA-3′ and 5′-ACGGCTA-CCGTACTGACACCA-3′, respectively, and for 23S, forward and reverse primers were 5′-CCGAATGGGGVAAAG-GG-3′ and 5′-CCGGTCCTCCTCGTA-CT-3’, respectively) (0.2 μM), 10 μL 5× reaction buffer (1×), 2.5 μL dithiothreitol (DTT) (5 mM), 0.5 μL RNase inhibitor (10 U), 1 μL enzyme mixture and 5 μL RNA sample (ca. 20 ng). RT-PCR was started immediately as follows: 30 min at 55°C (one cycle) followed by 94°C for 2 min (one cycle) and finally 30 cycles of amplification with each cycle consisting of 94°C for 30 s, 55°C for 30 s and 68°C for 1 min (for 16S rRNA) or 2 min (for 23S rRNA). The program was terminated with a 7 min extension at 68°C and rapid cooling to 4°C. PCR products (5 μL aliquots) were examined by horizontal gel electrophoresis on a 1% ethidium bromide-stained gel.

To confirm that the PCR products (16S or 23S) are originating from rRNA by RT and not from any residual DNA,
a standard PCR was carried out to amplify 16S and 23S rDNA. Briefly, the following reagents were combined in a 0.2 mL PCR tube: 37 µL of double distilled water, 5 µL of 10× PCR buffer, 1 µL each of forward and reverse primers (0.2 uM), 1 µL of dNTPs (200 µM) and 5 µL of RNA sample (ca. 20 ng). The reactions were overlaid with 30 µL of mineral oil and transferred to a preheated (80°C) thermal cycler. One unit of Taq DNA Polymerase (0.2 µL, Amersham Pharmacia Biotech, Allerød, Denmark) was added to each tube, and thermal cycling was started immediately for a total of 30 cycles as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min (for 16S rDNA) and 2 min (for 23S rDNA). The program was terminated with a final extension at 72°C for 10 min followed by rapid cooling to 4°C. Aliquots of 10 µL from each reaction were examined by horizontal gel electrophoresis as described above.

Current methodologies dealing with rRNA extraction for microbial ecology studies comprise several lengthy procedures and a wide array of reagents (6,10,12). In our attempt to develop a simple and reproducible protocol, we first pursued analysis of the different requirements to obtain intact and pure rRNA extract.

Indigenous RNases, both free and cell bound, represent a substantial hurdle in obtaining good yields of intact rRNA, especially considering that many RNases do not need any cofactors to function. Equally important is the pH during the extraction procedure until rRNA is precipitated. It is well known that high pH would result in both degradation of rRNA and the preservation of significant amounts of DNA co-precipitating with rRNA (11). Keeping the pH at approximately 5.5 has the advantage of increasing the rRNA yield and simultaneously enhancing the degradation of DNA. Therefore, washing samples 2–3× before the lysis step should be performed with low pH sodium citrate buffer as described above. This removes any free RNases and reduces the pH of the sample to approximately 5.5. Figure 1 shows rRNA extracted from different samples including the two reference strains. Intact full-length 16S and 23S rRNA could be obtained with no visible co-precipitated DNA. The use of low pH phenol as the only means to provide low pH during the extraction process was found to be insufficient in providing good buffering conditions for sam-

Figure 1. Formaldehyde-agarose gel electrophoresis of rRNA extracted from different samples. Lanes 1–5: Thermoanaerobacter thermohydrosulphoricus (AOR strain), Methanosarcina thermophila (TM1 strain), fresh manure, digested manure and granular sludge.
samples like manure or sewage sludge. An additional advantage of washing samples before lysis is that it eliminates tangible amounts of background coloration of fresh and digested manure, thus reducing the number of phenol extraction steps necessary. This washing procedure is expected to be quite effective with other samples such as core sediments and waste water.

After lysis and beadbeating procedures, considerable amounts of RNases are expected in the aqueous phase. Immediate phenol extraction may result in only partial inactivation of these RNases. Receiving the fractions of the aqueous phase in chloroform with thorough shaking results in almost complete elimination of these degrading enzymes. Another significant factor during RNA extraction procedure is temperature. Extraction at high temperature using the hot phenol method results in a substantial fluctuation of temperature from incubation at $60^\circ C$ to storage on ice. This may result in a considerable activity of RNases leading to degradation of RNA genes. In our study, a much higher yield of rRNA was observed when the temperature was kept at approximately $4^\circ C$ during the whole extraction procedure. Cooling the beadbeater by the ice bags, handling all procedures strictly on ice and centrifugation in a cooling centrifuge at $4^\circ C$, substantially minimized elevation of the temperature over $10^\circ C$. This in turn minimized the activity of RNases.

A major technique in microbial ecology studies is oligonucleotide probe hybridization. Figure 2 shows a slot blot hybridization of extracted RNA using two oligonucleotide probes, one specific for bacterial 16S rRNA and the other for archael 16S rRNA. The intensity of the hybridization signal and the absence of any visible background confirmed the reliability of the current protocol for population dynamics studies. This protocol has now been used successfully for RNA extraction from a number of laboratory biogas reactors (Ibrahim and Ahring, unpublished data). However, the evaluation of the RNA quality by oligonucleotide hybridization is an insufficient criterion to determine the purity of RNA for further molecular manipulations such as molecular cloning or RT. The presence of any impurities would inhibit the polymerase activity. This is an overwhelming problem when it comes to RNA extraction not only environmentally but also clinically. Therefore, we resorted to a more sensitive test, RT-PCR, to confirm the purity and integrity of extracted rRNA genes. Almost the complete 16S rRNA and 23rRNA were reverse transcribed separately from reference strains, manure samples and granular sludge with subsequent amplification of 16S and 23S rDNA in a one-step RT-PCR (Figures 3 and 4). In addition to confirming the purity and integrity of the extract, the described RT-PCR technique is quite promising in further molecular ecology studies. The described protocol is well suited for the recovery of long RT-PCR products and therefore of potentially great use in studies relating to microbial diversity. To our knowledge, this is the first publication to describe the recovery of the complete 23S rDNA from environmental samples using RT-PCR.

In conclusion, we present a simple and straightforward protocol for RNA extraction from a variety of environmental sources suitable for different molecular manipulations. Moreover, the described assay for RT-PCR is expected to facilitate studies of microbial diversity, gene expression and differential microbial activities in a given community. Because sensitive and easily degradable genes like RNA have been successfully manipulated by the reported technique, it has the potential for handling other environmental genetic markers for different ecological studies. However, it is important to emphasize the need to evaluate the potential of this methodology for other complex matrices such as sediments and different types of soils.
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