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RNA isolation from soil for bacterial community and functional analysis: evaluation of different extraction and soil conservation protocols

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Abstract

The impact of three different RNA isolation methods on the community analysis of metabolically active bacteria was determined by reverse transcription (RT) and PCR amplification of 16S rRNA genes and subsequent terminal restriction fragment length polymorphism (T-RFLP) analysis. Furthermore, soil samples were stored at different conditions in order to evaluate the effect of soil conservation methods on the outcome of the population analysis. The quality of mRNA was assessed by reverse transcription and PCR amplification of eubacterial glutamine synthetase genes. Our results indicated that the community composition as well as the abundance of individual members were affected by the kind of RNA isolation method. Furthermore, the extraction method influenced the recovery of mRNA. Lyophilization, storage at −20 °C as well as storage in glycerol stocks at −80 °C proved to be equally appropriate for the storage of soils and subsequent RNA isolation.

Keywords: RNA; Soil; 16S rRNA; T-RFLP analysis

1. Introduction

Traditional microbiological methods are based on the cultivation of microorganisms prior to analysis and suffer from various limitations such as the unculturability of many microbes and a lack of sensitivity (Hugenholtz et al., 1998). Molecular techniques using nucleic acids isolated directly from complex ecosystems, such as soil, provide a powerful alternative to classical approaches, and have been applied to study microbial communities (Borneman et al., 1996; Felske et al., 1998; Dunbar et al., 1999; Ramírez-Saad et al., 2000; Sessitsch et al., 2001). Most studies are based on the application of the 16S rRNA as a phylogenetic marker to describe the diversity, richness and structure of bacterial populations (Dunbar et al., 1999; Sessitsch et al., 2001; Smalla et al., 2001). In general, environmental DNA is used as template for PCR amplification of bacterial 16S rRNA genes and subsequent community analysis. However, DNA-based community analysis detects bacteria irrespective of their viability or metabolic activity. Furthermore, adsorption of naked DNA to soil particles (Lorenz and Wackernagel,

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1987) could result in biased population profiles. RNA-based community analysis is more suitable to describe the metabolically active members of a population, as the amount of rRNA produced by cells roughly correlates with the growth activity of bacteria (Wagner, 1994). However, due to the fact that RNA isolation from soil is laborious and that RNA is more prone to degradation by nucleases than DNA, only few studies have been based on RNA (Felske et al., 1997, 1998; Duarte et al., 1998; Lüdemann et al., 2000; Duineveld et al., 2001). In addition to the isolation of rRNA, the recovery of mRNA from environmental samples is essential for expression studies and microarray-based analyses.

Various methods have been reported for the isolation of RNA from soils (Moran et al., 1993; Duarte et al., 1998; Fleming et al., 1998; Griffiths et al., 2000), which usually apply similar lysis procedures to release bacterial cells from the soil matrix and to subsequently lyse them as for the extraction of DNA. Some protocols use acidic conditions in order to remove proteins, lipids and DNA by phase separation using phenol and chloroform leaving RNA in the liquid phase (Felske et al., 1996; Lüdemann et al., 2000). Alternatively, contaminants can be removed by enzymatic digestion (Fleming et al., 1998; Griffiths et al., 2000). A method for direct ribosome isolation has been developed by Felske et al. (1996) avoiding the extraction of free rRNA that has adsorbed to soil particles.

The choice of appropriate protocols for the isolation of DNA is essential for subsequent characterization of bacterial populations (Martin-Laurent et al., 2001; Niemi et al., 2001; Stach et al., 2001). Therefore, the objective of this study was to evaluate the effect of RNA extraction methods on the outcome of bacterial community analysis. Three protocols that work well in our laboratory have been used to extract RNA and ribosomes from soil. Reverse-transcription PCR (RT-PCR) followed by terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al., 1997; Sessitsch et al., 2001) of 16S rRNAs was applied to obtain community fingerprints of metabolically active bacteria. In addition, the quality of mRNA was tested by RT-PCR of eubacterial glutamine synthetase genes. We furthermore determined the effect of four different protocols for soil storage on the active bacterial populations.

2. Materials and methods

2.1. Soil sampling and preservation

A commercial soil substrate (Frux ED 63 not pasteurized soil substrate; Gebr. Patzer and Co., Sinntal-Jossa, Germany; 100–250 mg l^{-1} N, 100–250 mg l^{-1} potassium oxide, and 100–200 mg l^{-1} phosphorpentoxide, 85% peat, pH 5–6.5) was used to determine the effect of the choice of method for RNA extraction and soil preservation on the outcome of microbial community analysis. RNA extractions were either carried out immediately or samples were frozen at −20 °C after sample collection, lyophilized or mixed with glycerol solution (15% glycerol, 0.85% NaCl; 3 ml for 1 g soil) and subsequently stored at −75 to −80 °C for 4 weeks. Two replicate RNA extractions were performed with all samples.

2.2. Preparation of reagents and materials

For RNA extraction and handling, all glassware was treated with 0.1% diethylpyrocarbonate (DEPC), and nondisposable plasticware was cleaned with RNase Away (Gibco). Glassbeads were baked overnight at 180 °C. All solutions and water were treated with 0.1% (DEPC) overnight at 37 °C and autoclaved.

2.3. Isolation of RNA and ribosomes from soil

One RNA extraction protocol was based on a method that was originally developed for the isolation of RNA from pine trees (Chang et al., 1993) and will be referred to as the Chang method. To soil, (0.24 g fresh soil, 0.14 g lyophilized soil and soil stored at −20 and −80 °C) 1 ml extraction buffer (2% CTAB, 2% PVP K30, 100 mM Tris–HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5 g l^{-1} spermidine), which was freshly amended with 2% β-mercaptoethanol, was added. Samples were frozen in liquid nitrogen and ground in a mixer mill (Type MM2000, 220 V, 50 Hz, Retsch and Co., Haam, Germany) in the presence of two steel beeds (5 mm) at thawing. Then 0.3 g glassbeads (Sigma, 0.1 mm) were added and bead-beating was performed three times for 90 s in a mixer mill. Samples were extracted twice with chloroform followed by addition of 0.25 vol of 10 M LiCl to the aqueous phase. RNAs were precipitated at 4 °C

overnight and centrifuged at 9500 × *g* for 1 h at 4 °C. The resulting pellets were resuspended in 100 μl SSTE (1 M NaCl, 0.5% SDS, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) and RNA solutions of five isolations were pooled. Then, samples were extracted with chloroform, and RNAs were precipitated again with 2 vol ethanol at − 70 °C for 30 min. Pellets were obtained by centrifugation for 20 min at 10,000 rpm and subsequently washed with 70% ethanol, dried and resuspended in 45 μl RNase-free water. Contaminating DNA was removed by DNase digestion. For further purification, spin-columns were prepared using CL-6B (Pharmacia) and TE (pH 8.0). In general, passage through one or two spin-columns was required in order to remove RT-PCR inhibiting substances.

The second RNA extraction protocol used in this study was based on a method described by Fleming et al. (1998), which was slightly modified in our laboratory and will be subsequently referred to as the Fleming method. Soil (2.7 g fresh soil, 1.5 g lyophilized soil and 2 g soil stored at − 20 and at − 80 °C) and 4 g glassbeads (Sigma, 0.1 mm) were added to 20 ml H_2O. Bead-beating was performed in a bead-beater (Bead-beater™, Biospec Products) three times for 15 s. Extraction buffer (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA and 1% SDS), phenol and chloroform were heated to 60 °C and 5 ml of each solution was added to the soil suspension. Samples were incubated for 5 min at 60 °C and then shaken by mechanical action with a wrist action shaker for 5 min. Then tubes were centrifuged for 15 min at 13,000 × *g* at 4 °C. Supernatants were extracted with an equal volume of chloroform. RNAs were precipitated at − 20 °C overnight with 0.7 vol *iso*-propanol and pellets were obtained by centrifugation at 4 °C for 15 min at 13,000 rpm. Pellets were washed with 70% ethanol, air-dried and dissolved in 500 μl DEPC-treated water. RNAs were treated with DNase, extracted with phenol–chloroform, then again with chloroform and finally precipitated with ethanol. RNAs were dissolved in 200 to 400 μl water and purified with CL-6B spin-columns as described above.

Ribosomes were isolated by adding 10 g glassbeads (Sigma, 0.1 mm) and 10 ml TPM buffer (Felske et al., 1996) to 1.2 (lyophilized soil and soil stored at − 20 and − 80 °C) to 2 g (fresh) soil and by treating the suspension by bead-beating (Bead-beater™, Biospec Products) 2 × for 1 min in order to release bacterial ribosomes. The subsequent steps were carried out as described by Felske et al. (1996). Essentially, cell debris and soil particles were removed by centrifugation and ribosomes were precipitated by an ultra-high-speed centrifugation (2 h at 100,000 × *g*). Phenol extraction and ethanol precipitation were used to extract and purify rRNA, and DNAs were removed by DNAse digestion. RNA was dissolved in 35 μl water. Finally, passage through a CL-6B spin-column was needed for further purification.

RNA was quantified spectrophotometrically at 260 nm (Sambrook et al., 1989). RNA purity was estimated by calculating the ratio between the absorbances at 260 nm and 280 nm (A_{260}/A_{280}).

2.4. Reverse transcription and PCR amplification of 16S rRNA

RT-PCR was performed using the Superscript™ One-Step™ RT-PCR System (Gibco) according to the manufacturer's instructions. Reactions were performed with 0.15 μM each of the primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) labelled at the 5′ end with 6-carboxyfluorescein (6-Fam; MWG) and 926r (5′-CCGTCAATTCCTT-T(AG)AGTTT-3′) (Liu et al., 1997) and 0.1 to 1 μl extracted RNA. Reverse transcription was carried out for 30 min at 50 °C. Amplifications were carried out with a thermocycler (PTC-100™, MJ Research) using an initial denaturation step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min annealing at 52 °C and 2 min extension at 72 °C. The PCR reaction mixture (50 μl) used to check RNA for the presence of contaminating DNA contained 1 × reaction buffer (Gibco), 200 μM each dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$ and 2 U Taq DNA polymerase (Gibco), and 0.15 μM of the primer set 8f and 926r. RT-PCR and PCR amplification products were examined by gel electrophoresis on 1% agarose gels.

2.5. Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis was performed as described by Sessitsch et al. (2001) using RT-PCR products (10 μl, approximately 250 ng DNA) that were digested for 2

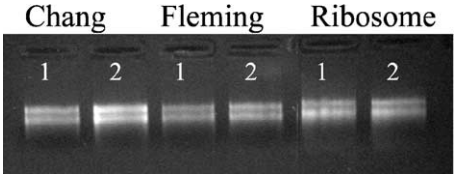

Fig. 1. Agarose gel electrophoresis of 3 μl aliquots of RNA extracted from lyophilized soil using the Chang method, the Fleming method and the ribosome isolation protocol. Numbers 1 and 2 indicate individual replicate extractions.

h in 20 μl with 10 U of the restriction enzyme *Alu*I (Gibco).

According to the range of the size marker, terminal restriction fragments (T-RFs) between 50 and 500 bp and peak heights of ≥50 fluorescence units were included in the analysis. Generally, the error for determining fragment sizes with our automated DNA sequencer was less than 1 bp, however, in some cases, a higher variation was found. Therefore, T-RFs that differed by less than 1.5 bp were clustered, unless individual peaks were detected in a reproducible manner. Sample profiles were normalized by standardizing the DNA quantity (i.e. total fluorescence) to the smallest quantity as suggested previously (Dunbar et al., 2001). Subsequently, representative sample profiles of replicate fingerprints were determined as shown by Dunbar et al. (2001).

In order to determine similarities between T-RFLP profiles, a binary matrix was generated recording the fluorescence intensities of peaks in normalized sample profiles. Cluster analysis (single linkage) was performed employing the STATISTICA software based on Pearson similarity values.

2.6. Reverse transcription and PCR amplification of glutamine synthetase (glnA) genes

RT-PCR was performed using the Superscript™ One-Step™ RT-PCR System (Gibco) according to the manufacturer's instructions. Reactions were performed with 0.15 μM each of the primers GS1β (5′-GATGCCGCCGATGTAGTA-3′) (Hurt et al., 2001) and GS2γ (5′-AAGACCGCGACCTTPATGCC-3′) (Hurt et al., 2001) and 0.1 to 1 μl extracted RNA. Reverse transcription was carried out at 50 °C for 30 min. Amplification was carried out with a thermocycler (PTC-100™, MJ Research) using an initial denaturation step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min annealing at 60 °C and 2-min extension at 72 °C. The PCR reaction mixture (50 μl) used to check RNA for the presence of contaminating DNA contained 1 × reaction buffer (Gibco), 200 μM each dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Gibco), and 0.15 μM of the primer set GS1β and GS2γ. RT-PCR and PCR amplification products were examined by gel electrophoresis on 1% agarose gels.

3. Results

3.1. RNA recovery

RNA could be isolated without nuclease contamination and all samples showed visible 16S and 23S rRNA bands on agarose gels (Fig. 1). However, 23S bands were not more intense than 16S bands indicating that minor degradation had occurred. Higher RNA

Table 1
Processing times, yields and purities of RNA samples

Extraction method	Time[a] (h)	RNA yield[b] (μg RNA g^{-1} dry weight soil)	A_{260}/A_{280} ratio
Ribosome isolation	17 + overnight precipitation		
Fresh		0.64 (0.37)	1.5–2.2
Lyophilized		1.49 (0.14)	1.6
−20 °C		1.15 (0.15)	1.6–1.7
−80 °C		1.99 (0.14)	1.7
Chang method	8 + overnight precipitation		
Fresh		1.43 (0.37)	1.5–1.6
Lyophilized		2.24 (0.00)	1.7–1.9
−20 °C		2.81 (0.05)	1.7–1.8
−80 °C		1.32 (0.01)	1.7
Fleming method	5 + overnight precipitation		
Fresh		3.08 (0.47)	1.3–1.4
Lyophilized		6.22 (3.63)	1.4
−20 °C		9.94 (1.41)	1.3–1.4
−80 °C		9.70 (0.80)	1.2–1.5

[a] Time required to isolate and purify RNA from eight samples.
[b] Values are means of two independently isolated samples with standard errors within parentheses.

quantities were obtained with lyophilized and frozen soil using the Fleming protocol than with other protocols (Table 1). The least laborious method, the Fleming protocol, resulted in highest yields, whereas lowest yields were obtained with the ribosome isolation protocol, which was also the most time-consuming RNA isolation method. In general, higher yields were achieved with lyophilized and with soils stored at −20 and −80 °C than with fresh soil. The purity of RNA isolated with the Chang and the ribosome isolation protocols showed comparable A_{260}/A_{280} values, whereas RNA recovered with the Fleming method showed lower purity (Table 1).

3.2. Impact of RNA isolation and soil conservation on bacterial community fingerprints

RNA isolated with different protocols was reverse transcribed and eubacterial 16S rRNA genes were amplified by PCR. T-RFLP analysis of PCR products was carried out in order to fingerprint the metabolically most active bacteria. Replicates showed a certain degree of variation, but grouped together when all profiles were compared. The total number of T-RFs found in all samples with fluorescence intensities above 50 was 27. However, as some fragments were only present in individual replicates or disappeared due to the normalization procedure, the total number of T-RFs found in representative sample profiles decreased to 19. The number of T-RFs in individual sample profiles ranged from 11 (fresh soil—Fleming method) to 15 (fresh soil—ribosome isolation). In general, application of the Fleming method resulted in a lower number of T-RFs, whereas the other protocols yielded similar numbers of terminal fragments (Table 1). Two T-RFs were only detected in fingerprints that were based on the ribosome isolation and comprised fragment sizes of 63 and 250 bp. Three fragments of 203, 206 and 208 bp showed higher intensities in profiles based on ribosome isolation than in profiles that derived from RNA isolated by the Chang and the Fleming methods. The 71-bp fragment

Table 2
Representative sample T-RFLP profiles based on RNA obtained with different isolation methods and from differently stored soil samples

T-RF	Ribsome isolation				Chang method				Fleming method			
(bp)	Fresh	Lyophil.	-20°C	-80°C	Fresh	Lyophil.	-20°C	-80°C	Fresh	Lyophil.	-20°C	-80°C
63	■	■	■	■								
71	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
141	■	▲	▲	▲	■	■	■	■	▲	▲	▲	▲
149	■	■	■	■	■	■	■	■	■	■	■	■
164					■			■			■	
172	■				■			■			■	■
198						■		■				
203	▲	▲	▲	▲	■	■	▲	■	■	■	■	■
204	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
206	■	■	■	■	■	■	■	■	■	■	■	■
208	▲	▲	▲	▲	▲	▲	▲	▲		■	■	
214						■						
232	■	■	■	■	■	■	■	■	■	■	■	■
238	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
246	▲	▲	▲	▲	▲	▲	■	■	▲	▲	▲	▲
250	■	■	■	■								
259								■				
273	■	■	■	■	■	■	■	■	■	■	■	■
280	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲

■, 50–99 fluorescence units; ■, 100–149 fluorescence units; ▲, 150–249 fluorescence units; ▲, >249 fluorescence units.

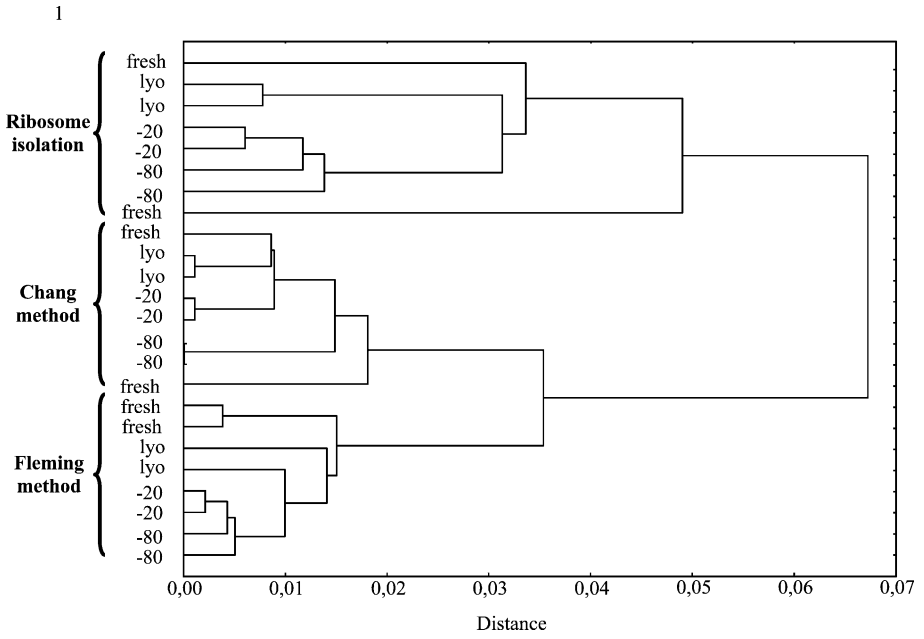

Fig. 2. Cluster analysis generated from representative sample T-RFLP profiles based on RNA isolated with different extraction methods and from differently stored soil samples.

was more abundant in RNA samples obtained with the Chang and the Fleming protocols than in those obtained by ribosome isolation (Table 2).

Although the three isolation protocols tested resulted in similar bacterial community structures, the kind of extraction protocol clearly influenced the outcome of community analysis, whereas the kind of conservation had only a minor impact (Fig. 2). Cluster analysis showed three clusters comprising profiles that have been obtained with RNA isolated with the Chang method, the Fleming method and the ribosome isolation protocol (Fig. 2). In general, replicate sample profiles showed high similarities. RNAs isolated with the Fleming and Chang protocols revealed highly similar populations, which showed some differences to those identified by ribosome isolation.

3.3. RT-PCR of eubacterial glutamine synthetase genes

The mRNA from all samples supported reverse transcription and PCR amplification of the eubacterial

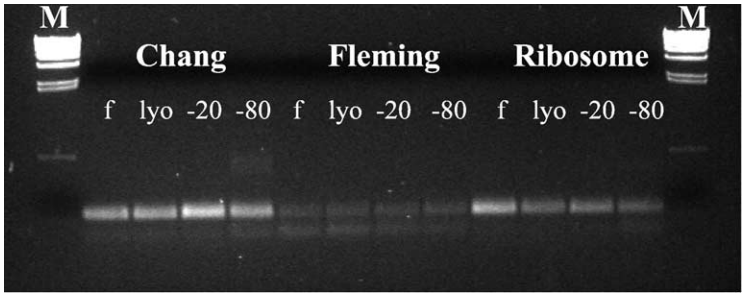

Fig. 3. Reverse transcription and PCR amplification of eubacterial glutamine synthetase genes based on RNA obtained with different isolation methods and from differently stored soil samples (f = fresh soil; lyo = lyophilized soil; −20 = soil stored at −20 °C; −80 = soil stored in glycerol stocks at −80 °C). *M* indicates the molecular weight standard λ/*Hind*III.

glutamine synthetase gene *glnA*. PCR fragments of the correct size (approximately 150 bp) were observed with all RNA samples, however, RNA isolated with the Fleming protocol yielded only very weak bands (Fig. 3). RNA that was not reverse transcribed did not result in a measurable *glnA* amplification.

4. Discussion

Bacteria belonging to different taxonomic groups and with varying cell envelopes are found in soil environments requiring efficient lysis methods in order to disrupt cells from all members of the bacterial consortium. In this study, three protocols for RNA isolation were compared regarding yield, purity, RNA-based community analysis and RT-PCR amplification of mRNA. It has been demonstrated that the isolation of ribosomes results in high-quality rRNA (Felske et al., 1997), however, the isolation procedure is rather laborious. Therefore, based on the results of various pre-experiments, two additional protocols were selected and tested. Our results showed that community analysis of metabolically active bacteria based on ribosome isolation yielded a higher number of T-RFs as well as higher fluorescence intensities of several peaks. This may be explained by a more efficient lysis of bacterial cells, particularly of Gram-positives and endospores, during this procedure, in which a higher ratio of soil and glassbeads was used than with other protocols. Bead-beating times of the ribosome isolation protocol were comparable to those applied for the Chang method, but were longer than those used for the Fleming protocol. Although a more efficient lysis was achieved with the ribosome isolation protocol, the RNA yield was lower as compared to other methods and isolation was time-consuming.

Hot detergent treatment proved to be ineffective in rupturing bacterial endospores (Kuske et al., 1998), and the use of bead-beating has been recommended for efficient cell lysis (Kuske et al., 1998; Miller et al., 1999). Therefore, a short bead-beating step has been included to the protocols published by Fleming et al. (1998), which was used for studying bacterial gene expression in soil. The protocol published by Chang et al. (1993) was originally designed for the isolation of RNA from pine trees, which is difficult because of the presence of high concentrations of polysaccharides, phenolic substances and RNases. For lysis of bacterial cells, a lysis step was included. Both protocols, the Fleming and the Chang methods, resulted in highly similar bacterial community structures, although a slightly lower number of bacterial species was detected by using RNA obtained with the Fleming method. Both protocols have the advantage of being simple and rapid. Higher yields have been obtained with the Fleming protocol, which may be due to the combination of bead-beating and a hot detergent treatment. Similarly, Kuske et al. (1998) achieved higher DNA yields by combining a hot detergent treatment with bead mill homogenization. Niemi et al. (2001) reported an improved efficiency of DNA isolation from soils due to the combined use of detergent, lysozyme treatment and bead-beating.

The extraction of nucleic acids from soil samples requires the separation of substances inhibiting subsequent enzyme reactions such as reverse transcription and PCR. Humic substances and heavy metals have been reported to interfere with DNA analyses (Wilson, 1997). Purification of RNA with Sepharose CL-6B spin microcolumns proved to be sufficient to remove substances inhibiting enzyme reactions as 16S rRNA genes could be reverse transcribed and amplified from all samples. Sepharose was also successfully applied for the purification of DNA isolated from environmental samples (Jackson et al., 1997). In addition, other systems such as Sephadex G200 microcolumns and the Wizard DNA Clean-Up System proved to be suitable for DNA purification, and may be also appropriate for the clean-up of RNA. However, some reports (Tsai and Olsen, 1991; Moran et al., 1993) indicated that the use of columns with a gel matrix is not suitable for the purification of RNA. RNA isolated by the Fleming protocol showed low A_{260}/A_{280} values as compared to RNA obtained with other protocols. In addition to the high quantities of RNA, large amounts of contaminating substances may have been extracted, which could not be completely removed by spin microcolumns. Felske et al. (1996) recommended direct ribosome isolation from soil because of the high purity of the extracted RNA. In this study, RNA isolated with the Chang method showed a comparable purity to RNA obtained by ribosome isolation.

Appropriate storage of soil samples for subsequent isolation of RNA is important as RNAs are rapidly

degraded by RNases. Our results demonstrate that all conservation methods tested are suitable as differently treated soil samples showed highly similar community fingerprints of active microbes. In general, higher RNA yields were obtained with lyophilized samples as well as with those stored at −20 or −80 °C. This may be at least partly due to the additional freeze–thaw cycle.

The functional analysis of bacteria, particularly in their natural environment, is gaining importance. Therefore, methods have to be developed or identified for the isolation of intact mRNA from environmental samples, which is particularly difficult because of the short half lives of bacterial transcripts. Bacterial glutamine synthetase genes could be successfully reverse transcribed and PCR amplified from RNA isolated with the Chang protocol and the ribosome isolation method. The major part of mRNA recovered with the Fleming protocol probably degraded during the isolation procedure as only weak RT-PCR bands were obtained. This is surprising as this method was originally used for the analysis of bacterial gene expression in soil. However, partial inhibition of the RT step can be excluded as 16S rRNA was successfully reverse transcribed and amplified. Although the expression of *glnA* genes was detected using RNA based on ribosome isolation, this method is not appropriate for the extraction of mRNA as cytoplasmatic mRNA is not isolated. In this study, the Chang method proved to be most suitable for gene expression studies, however, further improvement may be required to analyse expression of other genes than *glnA*.

Conclusively, the ribosome isolation protocol proved to be highly suitable for the analysis of metabolically active bacterial communities. However, also the Chang and the Fleming protocols may be appropriate for a rapid analysis of a large number of samples and may be further improved by altered bead-beating procedures. Among the protocols tested, the Chang isolation method was best suited for the recovery of bacterial mRNA from soil, but further testing is recommended. Lyophilization, storage at −20 °C as well as storage in glycerol stocks at −80 °C, is equally appropriate for the conservation of soils used for subsequent RNA extraction. Our studies will be extended to various soil types and further improvement will focus on the recovery of high-quality mRNA for functional analysis of soil microbial communities.

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