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# Rapid Method for Direct Extraction of mRNA from Seeded Soils

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**A protocol for direct extraction of mRNA from soil samples was developed. Soil samples (10 g) were washed twice with 120 mM phosphate buffer (pH 5.2). The lysis of cells, fixation of RNA, and hydrolysis of DNA were achieved by vigorously shaking the washed soil in a 4 M guanidine thiocyanate solution containing 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The pH of the homogenized mixture was adjusted with 2 M sodium acetate (pH 4.0); the mRNA was then extracted with phenol and chloroform. Total RNA was precipitated with isopropanol. This method extracts up to 17 μg of total RNA per g (wet weight) of soil containing $8.0 \times 10^8$ cells of *Pseudomonas aeruginosa* PU21, and mRNA has been detected in 160-ng total RNA fractions. This method has been used for the detection of mRNA transcribed from specific biodegradative genes, including the *nah* and *mer* operons, in contaminated soils. This extraction method can be completed within a few hours and has tremendous potential for ecological studies of in situ gene expression among soil microbiotas.**

Molecular biology techniques have recently been used by many microbial ecologists to study the particular bacterial genotypes occurring in natural systems, obviating the need for cell cultivation (1, 10, 17). The isolation of DNA from different environmental samples has been documented by several investigators using different extraction methods (6, 8, 11, 16). The utility of gene probes against purified microbial nucleic acids can be applied to (i) track the fate of genetically enhanced microorganisms (7), (ii) identify phylogenetic group-specific microorganisms (2, 12), and (iii) determine the diversity of bacterial communities in natural environments (13, 14). The presence of certain bacterial structural genes in extracts from soil and sediments has been shown by DNA-DNA hybridization techniques (8, 16). To understand whether the genes are actually functioning in native environments, positive detection of their expression products (mRNA or enzymes) in situ becomes necessary. It has been shown in pure culture studies that the levels of mRNA isolated from environmental bacteria were affected by temperature and trigger chemicals (15). However, because of the instability of mRNA and the presence of humic materials in natural soil or sediments, the detection of mRNA has been difficult until now. In this study, we present a method for direct extraction of mRNA from soils and sediments. The transcripts of *merA* and *nahAB* genes in these samples were demonstrated through DNA-RNA hybridization. This method is modified from a protocol for mRNA extraction of eukaryotic cells described by Chomczynski and Sacchi (3). In order to circumvent the loss of mRNA during purification steps, the crude total RNA extracts containing humic materials were used for hybridization.

## MATERIALS AND METHODS

**Sampling sites.** Subsurface soils from an old gas manufacturing site in southern California were used for RNA extraction. The contaminated soils contained high concentrations of polynuclear aromatic hydrocarbons (6.5 μg/g of soil) and toxic metals. The total CFU of heterotrophic bacteria in both samples were enumerated with plate count agar (PCA; Difco, Detroit, Mich.). The isolated mercury- or methyl mercury-resistant strains were able to grow on PCA amended with 25 μg of $Hg^{2+}$ (as $HgCl_2$) per ml or 3 μg of $CH_3$-$Hg^+$ [as $CH_3$(Hg)OH] per ml, respectively, and the naphthalene degraders were isolated from minimal medium (10) saturated with naphthalene vapor. All soil samples were measured in grams (wet weight) throughout the experiments unless otherwise indicated and were stored at −20°C before analysis. The water content of the southern California soil was determined to be 10%.

**Organisms.** Two mercury-resistant strains, including one laboratory strain, *Pseudomonas aeruginosa* PU21, and one environmental bacterial isolate, *P. putida* VNM43, were selected as seeding organisms. In addition to mercury resistance genes, *P. putida* VNM43 isolated from the southern California soil contains DNA sequences homologous to *nahAB* genes which encode the first two steps in the upper pathway of naphthalene degradation (18). Three naphthalene degraders used as seeding organisms were a laboratory strain, *P. putida* PpG7 (NAH7), and two southern California soil strains, *P. luteola* V55 and *P. fluorescens* V22. All bacterial isolates were maintained on PCA at room temperature and were identified with API Rapid NFT identification kits (Analytab Products, Plainview, N.Y.).

**Extraction of mRNA from soils and sediments.** Ten grams of southern California soils was mixed with 20 ml of 120 mM sodium phosphate buffer (pH 5.2) containing 1% diethyl pyrocarbonate, shaken at 150 rpm for 15 min, and centrifuged at 6,000 × *g* for 10 min. The pellets were washed once with phosphate buffer and mixed with 15 ml of denaturing solution (4 M guanidine thiocyanate [Fluka Chemical Corp., Ronkonkoma, N.Y.], 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) by shaking at 200 rpm for 1 min. This procedure was followed by the addition of 1.5 ml of 2 M sodium acetate (pH 4.0) to the mixture, from which mRNA was then extracted with 15 ml of phenol and 3 ml of chloroform-isoamyl alcohol (24:1), and the mixture was shaken vigorously to obtain a homogenous phase. The soil

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lysates were kept on ice for 15 min and then centrifuged at 10,000 × *g* for 20 min at 4°C. An aliquot (15 ml) of the top aqueous phase was extracted once with an equal volume of chloroform. After the chloroform extraction, a 10-ml aliquot from the top layer was mixed with an equal volume of cold isopropanol and stored at −20°C for 1 h to precipitate total RNA. The RNA-containing pellets were obtained by centrifugation at 10,000 × *g* for 20 min at 4°C and vacuum dried before resuspension in 100 μl of 0.5% sodium dodecyl sulfate. The pellets were yellowish because of the presence of humic materials.

The extracted mRNA from soils was validated by RNase (0.2 μg/ml) digestion and Northern (RNA) hybridization. Because of the interference of humic materials in UV spectrophotometry, the total RNA in the crude extract was quantified by ethidium bromide fluorescent methods (9) using a RNA marker (0.23- to 9.5-kb ladder; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as a standard. The total RNA from bacterial pure cultures was quantified spectrophotometrically at a wavelength of 260 nm. The sterile soil was seeded with known concentrations of bacteria for the determination of recovery efficiency. The sterile samples were obtained by autoclaving at 121°C for 30 min.

**$^{32}$P labeling of DNA probes and hybridization.** The narrow-spectrum (*merA*) mercury resistance gene fragment was kindly provided by S. Silver, and the *nahAB* fragment was a gift from B. Ensley. The *merA* encoding mercuric reductase originated from the plasmid pDU1358 (5). Samples (0.1 μg) of DNA fragments of *merA* (1 kb) and *nahAB* (6.4 kb) were labeled with [α-$^{32}$P]dCTP with a random priming kit (Bethesda Research Laboratories). The specific activities of the labeled probes were about $10^8$ dpm/μg of DNA. One-tenth of the RNA crude extracts was denatured in a solution containing 6× SSC (1× SSC is 0.15 M NaCl–0.015 M trisodium citrate, pH 7.0) and 7.4% formaldehyde at 65°C and filtered onto nitrocellulose membranes (0.2-μm pore size) with a slot blot apparatus (Minifold II; Schleicher & Schuell, Keene, N.H.). The positive RNA controls were obtained from pure bacterial cultures (15), including a mercury-resistant *P. aeruginosa* strain, PU21, and a naphthalene degrader, *P. putida* PpG7(NAH7), and the negative RNA control was from *Escherichia coli* DH5α. The membrane filters were then hybridized with the indicated DNA probes under high-stringency conditions as previously described (15). RNA gel electrophoresis was carried out in a 1% agarose gel containing 2% (wt/wt) formaldehyde, 1 μg of ethidium bromide per ml, and 0.2 M MOPS (morpholinepropanesulfonic acid)–10 mM disodium EDTA–10 mM sodium citrate (pH 7.0). RNA was transferred from the gel to a nitrocellulose membrane with a vacuum blotter (VacuBlot Transfer System, model VAC-1000; American Bionetics, Emeryville, Calif.) by the protocol suggested by Davis et al. (4).

**Applications.** Direct extraction of mRNA was applied to nonsterile soils with four different treatments: (i) Unseeded but amended with 100 mM salicylate, (ii) unseeded and not amended, (iii) seeded with two selected strains (NAH7 and *P. luteola* V55) and amended with 100 mM salicylate, and (iv) seeded but not amended. The mRNA extraction was performed after the samples were incubated at 23°C for 3 days. The seeding organisms were washed with 10 mM phosphate buffer (pH 7.0) before they were inoculated into the soil or sediments.

TABLE 1. Numbers of CFU of heterotrophic bacteria from southern California soils on different selective media

| Medium | No. of CFU/g (wet wt) of soil (mean ± SD) | % of PCA counts |
|---|---|---|
| PCA | $(4.1 \pm 0.1) \times 10^6$ | 100 |
| PCA with 25 μg of $Hg^{2+}$/ml | $(3.5 \pm 1.1) \times 10^5$ | 9 |
| PCA with 3 μg of $CH_3$-$Hg^+$/ml | $<10^2$ | <0.005 |
| MMN[a] | $(6.0 \pm 1.0) \times 10^5$ | 15 |

[a] MMN, Minimal medium saturated with naphthalene vapor.

## RESULTS AND DISCUSSION

Table 1 shows the numbers of CFU of heterotrophic bacteria from southern California soil. In the southern California soil, 9, <0.005, and 15% of the culturable bacteria were found to be phenotypically $Hg^{2+}$ resistant, methyl mercury resistant, and naphthalene degradative, respectively. All strains isolated from the southern California soil were identified as *Pseudomonas* spp. The detection of mercury-resistant strains in the southern California soil highly contaminated with polynuclear aromatic hydrocarbons could be attributed to the presence of trace metal contaminants in the original oil products which exerted a selective pressure for these strains.

When the nonsterile southern California soils were seeded with mercury-adapted *P. aeruginosa* PU21 at $8.0 \times 10^8$ cells per g, the *merA* transcripts were found as shown in Fig. 1. The *merA* transcripts from the seeded soil were detected at a minimal concentration of 160 ng of RNA fractions per g of soil. The total RNA extracted from sterile, seeded soil ($8.0 \times 10^8$ cells of *P. aeruginosa* PU21 per g) was 17 ± 2 μg/g (mean ± standard deviation), and the yields of nonsterile, unseeded samples were less than 1 μg of RNA per g. The unseeded southern California soil did not show much evidence of RNA after direct extraction of total nucleic acids from these

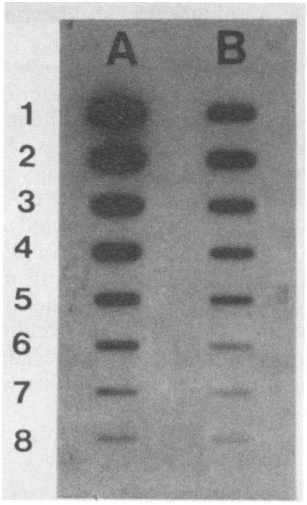


FIG. 1. Hybridization signals of *merA* transcripts extracted from southern California soil seeded with $8 \times 10^8$ CFU of *P. aeruginosa* PU21 per g (wet weight) after 3 h of incubation at room temperature (A) and a pure culture of *P. aeruginosa* PU21 (B). RNA quantities in slots: (A) 1, 20 μg; 2, 10 μg; 3, 5 μg; 4, 2.5 μg; 5, 1.25 μg; 6, 0.625 μg; 7, 0.31 μg; 8, 0.16 μg; (B) 1 and 2, 5 μg; 3, 2.5 μg; 4, 1.25 μg; 5, 0.62 μg; 6, 0.31 μg; 7, 0.16 μg; 8, 0.08 μg.

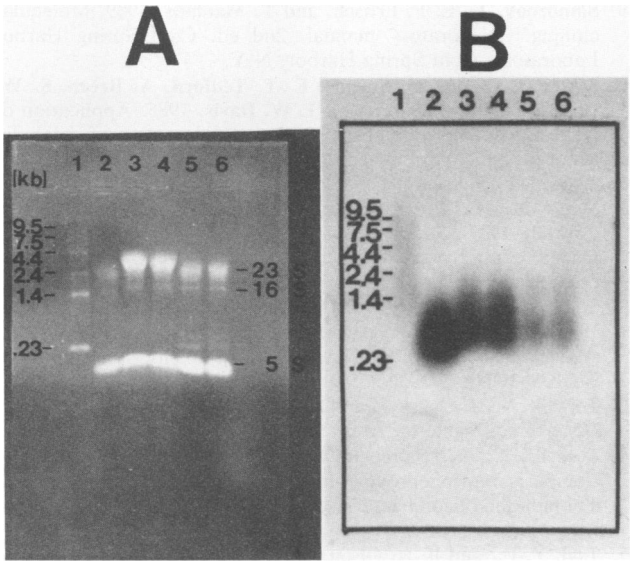


FIG. 2. (A) Total extracted RNA on a 1% ethidium bromide-stained agarose gel. (B) Northern hybridization signals of *merA* transcripts from panel A. Lanes: 1, RNA size marker (5 μg); 2, total RNA (30 days old at −20°C) from *P. aeruginosa* PU21; 3 and 4, total RNA from southern California soil seeded with *P. aeruginosa* ($8 \times 10^8$ cells per g [wet weight]); 5 and 6, total RNA (3 days old at −20°C) from *P. aeruginosa* PU21.

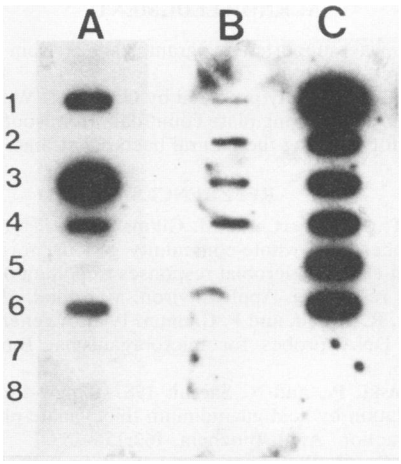


FIG. 3. Hybridization signals of *merA* (A) and *nahAB* (B) transcripts extracted from southern California soil and *nahAB* transcripts from bacterial cultures (C). Slots 3 to 8 in panel A and 1 to 4 in panel B illustrate the hybridization signals from 10 and 20% of extracted total RNA, respectively. Additionally, sterile and nonsterile soils were used for slots 3 to 8 in panel A and slots 1 to 4 in panel B, respectively. Slots: (A) 1, 2 μg of total RNA from $Hg^{2+}$ adapted *P. putida* VNM43; 2, RNase digestion of A1; 3, seeded with $1.6 \times 10^9$ cells of *P. putida* VNM43 per g; 4, seeded with $1.6 \times 10^8$ cells of *P. putida* VNM43 per g; 5, RNase digestion of A4; 6, seeded with $1.6 \times 10^7$ cells of *P. putida* VNM43 per g; 7, seeded with $1.6 \times 10^6$ cells of *P. putida* VNM43 per g; 8, sterile, unseeded soil; (B) 1, seeded with $5.0 \times 10^7$ cells of *P. putida* PpG7 (NAH7) per g; 2, seeded with $3.7 \times 10^6$ cells of *P. luteola* V55 per g; 3, 100 mM salicylate added to NAH7-seeded soil; 4, 100 mM salicylate added to *P. luteola* V55-seeded soil; (C) 1, 10 μg of RNA from *P. fluorescens* V22; 2, 5 μg of RNA from *P. fluorescens* V22; 3 and 4, 5 μg of RNA from NAH7; 5 and 6, 5 μg of RNA from *P. luteola* V55.

samples (16), suggesting degradation or less synthesis of RNA under natural starvation conditions. Therefore, low yields of RNA extracted from unseeded samples appeared reasonable. The recovery efficiency of total RNA from seeded ($1.6 \times 10^9$ cells of *P. putida* VNM43 per g), sterile southern California soil was 60% ± 5% (mean ± standard deviation) compared with RNA extraction from the same cell density of pure cultures. This indicates that approximately 40% of RNA was degraded or not extractable during the extraction procedures. rRNA (23S, 16S, and 5S) from both *P. aeruginosa* PU21-seeded, nonsterile soil and pure culture was clearly evidenced in the ethidium bromide-stained gel (Fig. 2A, lanes 3 to 6). Degradation of 23S and 16S rRNA at −20°C was manifested in a 30-day-old RNA of *P. aeruginosa* PU21 (lane 2). Northern hybridization signals of *merA* transcripts transferred from the agarose gel are shown in Fig. 2B, indicating that the sizes of most *merA* transcripts were between 0.23 and 1.4 kb. No distinct bands of *merA* mRNA were observed, suggesting that shearing of mRNA occurred during the extraction process.

The extracted RNA from sterile, seeded soil and bacterial pure culture was subjected to RNase digestion, and no hybridization signals were found (Fig. 3A, slots 2 and 5). Because the extraction was conducted under acidic conditions which caused hydrolysis of DNA, the hybridization signals found in the extracts were indeed due to the presence of mRNA. The acid-guanidine thiocyanate method has been used in extraction of mRNA from eukaryotic cells (3) and from prokaryotic cells (15), and no detectable DNA was found in the RNA extracts. The DNA-mRNA hybridization signals were detected when the sterile soil was seeded with a bacterial concentration as low as $1.6 \times 10^7$ cells per g. In a separate experiment, the production of *nahAB* transcripts was detected when the nonsterile soil was seeded with $5.0 \times 10^7$ cells of NAH7 per g or $3.7 \times 10^6$ cells of *P. luteola* V55 per g (Fig. 3B, slots 1 to 4). A noticeable increase in *nahAB* transcripts was observed after 3 days of incubation with 100 mM salicylate in soil seeded with the same number of bacteria, suggesting that salicylate, an intermediate metabolite in naphthalene degradation, induced the in situ production of *nahAB* transcripts in seeded samples. No *nahAB* transcripts were detected in either unseeded and unamended or unseeded and salicylate-amended southern California soils (data not shown). This observation could result from the low RNA content in the starved cells in situ, or the toxicity of salicylate at 100 mM could have decreased the cell density from $4.1 \times 10^6$ to $1.6 \times 10^6$ CFU/g, which was beyond the detection limit for mRNA. The RNA extraction data from three naphthalene-grown bacteria showed that *P. luteola* V55 produced the highest level of *nahAB* mRNA per 5 μg of total RNA among these bacteria (Fig. 3C, slots 2 to 6).

In conclusion, the extraction method described in this article has the advantages that it can (i) be completed in a relatively short period, (ii) analyze a large number of samples simultaneously, and (iii) obtain 60% recovery efficiency from seeded samples; therefore, this method has tremendous potential for studying in situ gene expression. However, if high-cation-exchange-capacity substrates such as sediments are used for extraction, RNA may precipitate with humic materials after isopropanol extraction, which could reduce the recovery efficiency.

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## REFERENCES

1. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and $Hg^{2+}$ resistance. Appl. Environ. Microbiol. **55:**1574–1577.
2. **Barry, T., R. Powell, and F. Gannon.** 1990. A general method to generate DNA probes for microorganisms. Bio/Technology **8:**233–236.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Davis, L. G., M. D. Dibner, and J. F. Battey (ed.).** 1986. Basic methods in molecular biology, p. 143–146. Elsevier Science Publishing, New York.
5. **Griffin, H. G., T. J. Foster, S. Silver, and T. K. Misra.** 1987. Cloning and DNA sequence of the mercuric- and organomercurial-resistance determinants of plasmid pDU1358. Proc. Natl. Acad. Sci. USA **84:**3112–3116.
6. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54:**703–711.
7. **Jansson, J. K., W. E. Holben, and J. M. Tiedje.** 1989. Detection in soil of a deletion in an engineered DNA sequence by using DNA probes. Appl. Environ. Microbiol. **55:**3022–3025.
8. **Ogram, A., G. S. Sayler, and T. Barkay.** 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods **7:**57–66.
9. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Sayler, G. S., M. S. Shields, E. T. Tedford, A. Breen, S. W. Hooper, K. M. Sirotkin, and J. W. Davis.** 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. **49:**1295–1303.
11. **Somerville, C. C., I. T. Knight, W. L. Straube, and R. R. Colwell.** 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. Environ. Microbiol. **55:**548–554.
12. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54:**1079–1084.
13. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56:**782–787.
14. **Torsvik, V., K. Salte, R. Sørheim, and J. Goksøyr.** 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Appl. Environ. Microbiol. **56:**776–781.
15. **Tsai, Y.-L., and B. H. Olson.** 1990. Effects of $Hg^{2+}$, $CH_3$-$Hg^+$, and temperature on the expression of mercury resistance genes in environmental bacteria. Appl. Environ. Microbiol. **56:**3266–3272.
16. **Tsai, Y.-L., and B. H. Olson.** Appl. Environ. Microbiol., in press.
17. **Walia, S., A. Khan, and N. Rosenthal.** 1990. Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl. Environ. Microbiol. **56:**254–259.
18. **Yen, K.-M., and I. C. Gunsalus.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA **79:**874–878.