Biol Fertil Soils (1997) 25:159–162

ORIGINAL PAPER

N. Teaumroong · Ch. Schwarzer · B. Auer
K. Haselwandter

A non-radioactive DNA probe for detecting dicyandiamide-degrading soil bacteria

Received: 15 July 1996

Abstract Some soil bacteria are capable of degrading the nitrification inhibitor dicyandiamide (DCD). One of the most efficient isolates is strain EK1 of *Mycobacterium* sp. For detecting this and closely related DCD-degrading bacteria in soil we developed a non-radioactive DNA probe. A 1.7-kb EK1 DNA fragment was selected from a genomic library and labelled with digoxigenin. The probe was highly specific for EK1 and closely related or identical species of soil bacteria. A method for direct detection of DNA from soil was developed. The sensitivity of this methodology allowed detection of 2×10^3 EK1 cells g^{-1} soil.

Key words Nitrification inhibitor · Dicyandiamide · *Mycobacterium* sp. · Non-radioactive DNA probe · DNA hybridization

Introduction

Dicyandiamide (DCD) is applied in agriculture and horticulture as a nitrification inhibitor or nitrogen stabilizer (Hauck 1984; Vilsmeier et al. 1987). An interaction with metal oxides has been reported to initiate the inorganic degradation of DCD in soil. This mainly depends on soil temperature, pH, moisture and clay contents (Vilsmeier et al. 1987). An entirely microbial degradation of DCD by a soil bacterium (*Mycobacterium* sp., strain EK1) which used this compound as single N-source under pure culture conditions has been demonstrated (Hauser and Haselwandter 1990). It has been shown that this mineralization is indeed due to the enzymatic activity of EK1 as determined by its Michaelis-Menten kinetics (Schwarzer and Haselwandter 1991). The microbial degradation of DCD has been confirmed by showing that there are at least two different catabolic pathways in two isolates of soil bacteria (presumably *Pseudomonas* sp. and *Rhodococcus* sp.); both pathways seem to be different from the inorganic catalytic DCD breakdown involving metal oxides (Hallinger et al. 1990). Rapid microbial degradation of DCD was also demonstrated clearly by Rajbanshi et al. (1992).

The objective of this study was to develop a non-radioactive DNA probe for detecting DCD-degrading soil bacteria. The non-radioactive DNA probe is shown to be sufficiently sensitive and specific for a study of the epidemiology of DCD-degrading bacteria such as EK1 in soil.

N. Teaumroong
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon-Ratchasima 30000, Thailand

Ch. Schwarzer · K. Haselwandter (✉)
Department of Microbiology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria
Tel.: 0043/512507 6003; Fax: 0043/512507 2928;
e-mail: kurt.haselwandter@uibk.ac.at

B. Auer
Department of Biochemistry, Peter-Mayrstrasse 1A, University of Innsbruck, A-6020 Innsbruck, Austria

Materials and methods

Strains and media

The following strains of DCD-degrading soil bacteria were used: *Mycobacterium* sp. strains EK1, EK3–14 and EK3–17, a mixed culture of *Xanthomonas maltophila* and *Micrococcus* sp. (GART-1), a mixed culture of *X. maltophila* and *Agrobacterium radiobacter* (GART-2), and unidentified strains of soil bacteria (St-1, St-3, St-6 and EK3–10). All these cultures were isolated in a previous study (Hauser 1988). The bacteria were cultivated and maintained in minimal medium containing DCD (1.60 g l^{-1}) as single N-source, glucose 5.5 g, $Na_2HPO_4.12H_2O$ 10.25 g, KH_2PO_4 5.20 g, $MgSO_4.7H_2O$ 0.40 g, $ZnCl_2$ 0.01 mg, $FeSO_4$ 0.01 mg, $MnSO_4$ 0.005 mg, $CoCl_2$ 0.005 mg, $MoPO_4$ 0.005 mg and $CuSO_4$ 0.001 mg l^{-1} distilled water at pH 7.0 (Hauser and Haselwandter 1990). Liquid cultures were shaken at 100 rpm and 30°C (Psycrotherm, New Brunswick Inc.).

As a host for plasmid preparation and construction of a genomic library of EK1, *Escherichia coli* strain JM 105 was used. Transformants were cultivated on tryptone-yeast extract medium (TY) containing 50 μg ampicillin ml^{-1} (Maniatis et al. 1982).

Isolation of genomic DNA and preparation of the genomic library

Genomic DNA was isolated from EK1 and other DCD-degrading soil bacteria using a modified version of the Birnboim and Doly (1979) method. After the first cell washing step, NaOH and subsequently sodium dodecyl sulfate (SDS, final concentration 0.2 *N* and 1.0%, respectively) were added to remove the rigid cell walls of these bacteria. Genomic DNA of EK1 was partially digested with the restriction enzyme *Mbo*I, ligated into *Bam*HI-treated plasmid pBluescript KSII+ and these constructs transformed into competent cells of *E. coli* strain JM 105 according to the method of Hanahan (1983).

Probe preparation

One clone was selected for extracting a recombinant plasmid (p9) which contained an EK1 DNA fragment. This plasmid was extracted by the alkaline-lysis method (Maniatis et al. 1982) and digested with the restriction enzymes *Eco*RI and *Xba*I. The 1.7-kb DNA fragment was recovered from the electrophoresis gel by using diethylaminoethanol (DEAE) paper, from which it was eluted in a spun-tube with elution buffer (20 m*M* TRIS-HCl, 1 m*M* ethylenediaminetetraacetate (EDTA), and 1.5 *M* NaCl at pH 8.0) (Dretzen et al. 1981).

For further purification the fragment was extracted with phenol-chloroform, re-precipitated with 96% ethanol, washed with 70% ethanol, dried under vacuum and dissolved in TE buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0). This fragment was labelled by the random primed labelling technique with the digoxigenin non-radioactive DNA-labelling kit (Boehringer Mannheim, Cat. no. 1175 033).

Direct extraction of DNA from soil

Aliquots of 1.0 g of an agricultural soil from the Innsbruck area (gleyic fluvisol; 3.5% organic matter, pH 7.4, 15% $CaCO_3$, 16 mg phosphoric acid 100 g^{-1} soil, 14 mg potassium 100 g^{-1} soil, 12 mg kg^{-1} copper, 4 mg kg^{-1} zinc, 190 mg kg^{-1} manganese, 250 mg kg^{-1} iron, 0.30% total nitrogen and 4.40% total carbon) were mixed with 2.5 ml 0.12 *M* phosphate buffer (pH 8.0) on an end-over-end shaker for 15 min at room temperature. The slurry was pelleted by centrifugation at 10 000×*g* and 4 °C for 20 min. The pellet was washed again with the same buffer (5 ml). NaOH and polyoxyethylene-20-cethylether (Brij 58) were added to give final concentrations of 0.2 *N* NaOH and 1.0% Brij 58 for initiating the disintegration of rigid bacterial cell walls and extracting humic substances from soil by end-over-end shaking for 15 min. The non-ionic detergent Brij 58 was used to solubilize some cell components prior to cell lysis with lysozyme. After centrifugation at 18 000×*g* and 4 °C for 20 min the dark-brown supernatant was discarded. The pellet was extracted 3 times by adding 2.5 ml 0.12 *M* phosphate buffer (pH 8.0), and centrifugation at 2000×*g* and 4 °C for 5 min (Selenska and Klingmüller 1991). The supernatants from all three extractions were collected in one tube, which contained 15 mg lysozyme ml^{-1}, incubated in a 37 °C water bath for 2 h with 60 rpm agitation (Tsai and Olson 1991). Subsequently, 2.0 ml SDS (10%) containing proteinase-K (2.5 mg ml^{-1}) was added. Three cycles of freezing in liquid nitrogen at –196 °C and thawing in a 70 °C water bath were conducted to break bacterial cells (Bollet et al. 1991). After freezing and thawing, the solution was extracted twice with phenol-chloroform (Maniatis et al. 1982). DNA from the aqueous phase was precipitated with 96% ethanol; the pellet was washed again with 70% ethanol before it was dried under vacuum and re-suspended in 0.5 ml TE buffer containing 100 µg ml^{-1} RNase A. For further purification, the nucleic acids were adsorbed onto a Nensorb column 20 (NEN Research Products, Biotechnology System Division, Du Pont). DNA was recovered from the column as suggested by the manufacturer. DNA concentration was estimated on the basis of the relative fluorescence compared with DNA standards that were stained with ethidium bromide. For determination of viable soil bacteria at each step of the DNA extraction procedure, 0.1 ml suspension was plated (three replicates) on TY medium (bacteriological peptone 8.0 g, yeast extract 5.0 g and NaCl 2.5 g in 1.0 l deionized water). The sensitivity of the EK1 DNA probe was determined as follows: between 2×10^7 and 2×10^1 cells of EK1 ml^{-1} were added to aliquots of 1.0 g soil. Subsequently DNA was extracted from soil as described above.

The recovery rate was determined by adding 2×10^7 EK1 cells suspended in 2.5 ml 0.12 *M* phosphate buffer to 1.0 g autoclaved soil. This was followed by shaking on an orbital shaker at 350 rpm at room temperature for 1 h to equilibrate and allow for binding of EK1 cells to soil colloids (Pillai et al. 1991). The number of viable EK1 cells was determined by plating (three replicates) 0.1 ml suspension of TY medium.

Gel electrophoresis and Southern blot hybridization

DNA which was extracted and purified from soil was digested with restriction enzymes (*Pst*I, *Bam*HI, *Eco*RI or *Hind*III) applying standard procedures (Maniatis et al. 1982). Gel electrophoresis was carried out in 1.0% agarose at 70 V for 2.5 h, followed by capillary transfer of the DNA from agarose gel to nitrocellulose membranes (BA85; Schleicher & Schuell; see Dalgleish 1987). A non-radioactive digoxigenin-labelled 1.7-kb fragment of EK1 DNA was used as probe. DNA hybridization was carried out at 68 °C for 24 h in the presence of 5×SSC (1×SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate). The membrane was washed twice for 5 min at 68 °C with 0.1×SSC and 0.1% (w/v) SDS. The hybridized DNA was detected on the membranes with an anti-digoxigenin-alkaline phosphatase conjugate and a colour reaction specific for alkaline phosphatase (Boehringer Mannheim, Cat. no. 1093 657).

Results and discussion

Probe specificity for DCD-degrading bacteria

A genomic library of EK1 was constructed by limited digestion of EK1 DNA with the restriction enzyme *Mbo*I and ligation of the fragments into pBluescript KSII+. From 1.3×10^5 colonies, one clone containing a plasmid with an insert of 1.7 kb (p9) was isolated and labelled with digoxigenin. By Southern blot analysis of EK1 DNA digested with the restriction enzymes *Pst*I, *Bam*HI, *Eco*RI and *Hind*III with the 1.7-kb DNA fragment, a specific hybridization pattern for each restriction enzyme was found (data not shown). Applying this technique the DNA probe proved to be highly specific for EK1 and closely related strains such as EK3–14 and EK3–17, and discriminated other DCD-degrading strains of soil bacteria.

To detect the specificity of the 1.7-kb EK1 DNA probe, the genomic DNAs from other DCD-degrading soil bacteria and *E. coli* JM105 as a representative for non-DCD-degrading bacteria were isolated and digested with the restriction enzyme *Pst*I. Figure 1 shows the Southern blot after hybridization and colour reaction. DNA digests from the DCD-degrading bacterial strains EK1, EK3–14 and EK3–17 show the same specific hybridization pattern, whereas in DNA digests from other DCD-degrading soil bacteria and *E. coli* JM105 no specific fragment was detected. This suggests a close relationship of EK1 with EK3–14 and EK3–17 but not with the other DCD-degrading strains of soil bacteria.

To investigate a possible inhibition of specific hybridization by excess of *E. coli* JM105 or DNA from other non-related soil bacteria, mixtures of EK1 DNA with a

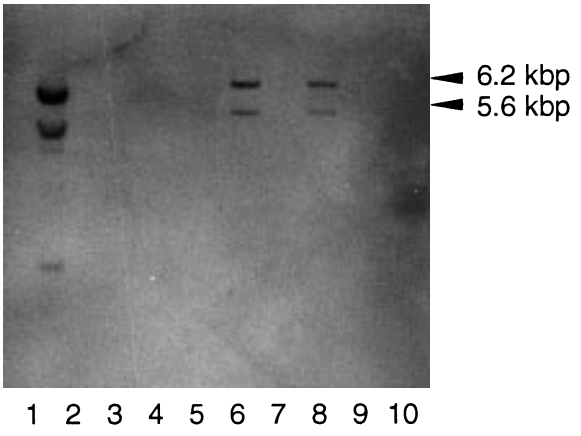

Fig. 1 Hybridization of 1.0 µg genomic DNA from DCD-degrading soil bacteria and *E. coli* JM105. The genomic DNA was digested with the restriction enzyme *Pst*I and hybridized with 1.7-kb EK1 DNA probe. Assignments: *lane 1* EK1, *2* GART-1, *3* EK3–10, *4* ST-3, *5* St-6, *6* EK3–14, *7* St-1, *8* EK3–17, *9* GART-2 and *10 E. coli* JM105. Brief characterization of strains is given in "Materials and methods"

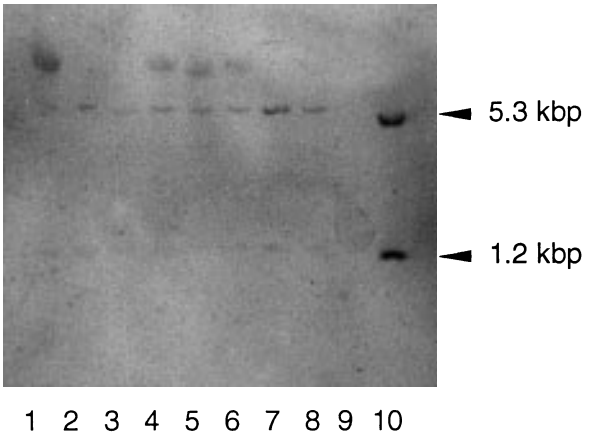

Fig. 2 Hybridization of mixtures of genomic DNA, digested with the restriction enzyme *Bam*HI, with the 1.7-kb EK1 DNA fragment. Assignment of lanes as follows:

Lane	Strains (µg)				
	EK1	*E. coli* JM105	GART-1	St-6	EK3–14
1	0.1	1.0	–	–	–
2	0.1	–	1.0	–	–
3	0.1	–	–	1.0	–
4	0.1	0.5	0.5	–	–
5	0.1	0.5	–	0.5	–
6	0.1	0.33	0.33	0.33	–
7	0.1	–	–	–	1.0
8	0.1	0.25	0.25	0.25	0.25
9	–	–	–	–	–
10	1.1	–	–	–	–

tenfold amount of competitive DNA were analyzed. Strains GART-1 and St-6 were used as they represent different genera of soil bacteria which are also able to degrade DCD; their DNA, however, did not show any signal

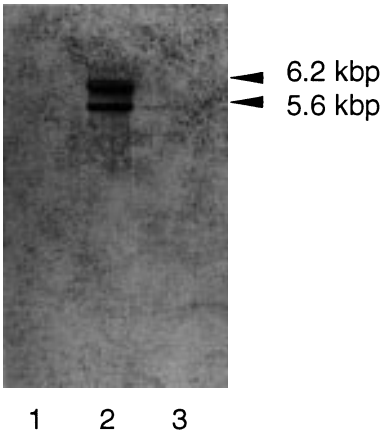

Fig. 3 Hybridization of DNA extracted directly from 1.0 g soil, digested with the restriction enzyme *Eco*RI with the 1.7-kb EK1 DNA probe. *Lane 1* control (uninoculated soil), *2* soil inoculated with 2×10^7 EK1 cells, *3* inoculated with 2×10^3 EK1 cells

with the probe. As depicted in Fig. 2, a specific restriction fragment could be detected in EK1 DNA digested with the restriction enzyme *Bam*HI by the 1.7-kb DNA probe even when mixed with DNA from other soil bacteria and *E. coli* JM105. Addition of DNA extracted from EK3–14, which is closely related to EK1, enhanced the EK1-specific signal (Fig. 2, lane 7). In all the lanes containing *E. coli* JM105 DNA, a signal in the high molecular weight range was observed, indicating cross-hybridization of the 1.7-kb DNA probe with *E. coli* DNA. However, this did not interfere with the specific detection of EK1 DNA by Southern blot analysis. Furthermore, DNA extracted from soil containing approximately 8.35×10^{10} viable bacterial cells g^{-1} neither showed any signal nor interfered with the EK1-specific signal (see Fig. 3).

Efficiency of bacterial recovery from soil and sensitivity of the detection system

When 2×10^7 EK1 cells were added to 1.0 g autoclaved soil and treated with 0.2 *N* NaOH solution, about 4.7×10^6 viable cells (25%) were recovered. This recovery rate is somewhat lower than that reported in previous studies for recovery of viable soil bacteria. The reported rates were 34.9% and 27.8%, respectively (Holben et al. 1988; Steffan et al. 1988). The main purpose for including an NaOH treatment in the extraction protocol was to initiate the disintegration of the rigid cell wall of EK1. Another advantage of the alkaline treatment was that some humic substances could be extracted from the soil sample (Wand et al. 1967). Elimination of humic acids is a prerequisite for further purification of the extracted DNA and restriction enzyme analysis (Holben and Tiedje 1988; Trevors and van Elsas 1989; Sayler and Layton 1990).

In the present study a Nensorb column 20 was used for final DNA purification. DNA purified by means of glassmilk (Geneclean II kit, Bio 101), Wizard DNA clean-up system (Promega) (van Elsas and Smalla 1995) or the gel

purification method (Myrold et al. 1995) gave results similar to those obtained with Nensorb column 20.

By the procedure developed here, approximately 25 μg DNA could be recovered from 1.0 g soil. This is approximately 30% assuming a mean DNA content of a bacterial cell of 6 fg (Ingraham et al. 1983), and a DNA content of an *E. coli* cell of 9 fg (McCoy and Olson 1985). This amount of DNA recovery was somewhat higher than that estimated from direct extraction using lysozyme (10%) (Hilger and Myrold 1991) and the direct lysis method (22%) (Holben et al. 1988). On the basis of total DNA recovered (25 μg g^{-1} soil), the recovery rate achieved in this study was intermediate to that reported by Selenska and Klingmüller (1991; 50 μg g^{-1} soil) and van Elsas and Smalla (1995; 15–20 μg g^{-1} soil) or Saano and Lindström (1995; 10 μg g^{-1} soil).

The detection limit of this method was estimated by adding EK1 cells to a soil sample containing a total number of viable bacteria (estimated by plating on TY agar) of about 8.35×10^{10} cells g^{-1}, none of which were able to grow on DCD minimal medium. DNA extracted from 1.0 g of such a soil showed no specific signal after *Eco*RI digestion, electrophoresis and transfer to nitrocellulose membrane. The detection limit of this probe by using non-radioactive labelled DNA was approximately 2×10^3 cells g^{-1} soil (Fig. 3, lane 3), thus rendering this method useful for detecting DCD-degrading soil bacteria in enrichment cultures.

The detection limit in the present study was almost in the same range as the studies employing PCR-based techniques for specific detection of soil microorganisms. For genetically modified *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens*, the detection limit was at 10^3 cells g^{-1} soil, whereas for *Mycobacterium chlorophenolicum* it was 10^2 cells g^{-1} soil when a 16S ribosomal RNA-based PCR amplification system was applied (van Elsas and Smalla 1995). For a further increase in sensitivity, linking DNA hybridization with the most probable number method (MPN) could be envisaged. In previous studies this approach has allowed the enumeration of bacteria present at population densities of 10–100 cells g^{-1} soil (Fredrickson et al. 1988; Sayler and Layton 1990).

Acknowledgements: We thank Dr. M. Ebner of our Institute for the photographs and the Bund-Bundesländerkooperation of Austria for financial support.

References

Biochemica Boehringer Mannheim (1989) DNA labeling and detection kit. Non-radioactive. Application manual. Mannheim, Germany

Birnboim, HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res 7:1513–1523

Bollet C, Gevaudan MJ, Lamballerie de X, Zandotti C, Mico de P (1991) A simple method for the isolation of chromosomal DNA from gram positive or acid-fast bacteria. Nucl Acids Res 19:19–55

Dalgleish R (1987) Southern blotting. In: Boulnois GJ (ed) Gene cloning and analysis, a laboratory guide. Blackwell Scientific, Oxford, pp 49–51

Dretzen G, Bellard M, Sassone-Corsi P, Chambon P (1981) A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal Biochem 112:259–298

Elsas van JD, Smalla K (1995) Extraction of microbial community DNA from soils. In: Akkermans ADL, Elsas van JD, Bruijn de FL (eds) Molecular microbial ecology manual, Chap. 1.3.3. Kluwer, Dordrecht, pp 1–11

Fredrickson JK, Bezdicek DF, Brockman JF, Li SW (1988) Enumeration of Tn5 mutant bacteria in soil by using a most-probable-number-DNA hybridization procedure and antibiotic resistance. Appl Environ Microbiol 54:446–453

Hallinger S, Wallnöfer PR, Goldbach H, Amberger A (1990) Several aspects of bacterial dicyandiamide degradation. Naturwissenschaften 77:332–334

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Hauck RD (1984) Nitrogen management in the no-till system. In: Hauck RD (ed) Nitrogen in crop production. American Society for Agronomy, Madison, pp 551–560

Hauser M (1988) Untersuchungen zur Mineralisation von Dicyandiamid durch Bodenbakterien. MSc Thesis, University of Innsbruck

Hauser M, Haselwandter K (1990) Degradation of dicyandiamide by soil bacteria. Soil Biol Biochem 22:113–114

Hilger AB, Myrold DD (1991) Method for extraction of *Frankia* DNA from soil. Agric Ecosystems Environ 34:107–113

Holben WE, Tiedje JM (1988) Applications of nucleic acid hybridization in microbial ecology. Ecology 69:561–568

Holben WE, Jansson JK, Chelm BK, Tiedje JM (1988) DNA probe method for detection of specific microorganisms in the soil bacterial community. Appl Environ Microbiol 54:703–711

Ingraham JL, Maaloe O, Neidhardt FC (1983) Growth of the bacterial cell. Sinauer Associates Inc., Sunderland, Mass., pp 1–48

Maniatis T, Fritsch EF; Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

McCoy WF, Olson B (1985) Fluorometric determination of the DNA concentration in municipal drinking water. Appl Environ Microbiol 49:811–817

Myrold DD, Martin KJ, Ritchie JN (1995) Gel purification of soil DNA extracts. In: Akkermans ADL, Elsas van JD, Bruijn de FL (eds) Molecular microbial ecology manual, Chap. 1.3.5. Kluwer, Dordrecht, pp 1–9

Pillai SD, Josephson KL, Bailey RL, Gerba CP, Peper IL (1991) Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. Appl Environ Microbiol 57:2283–2286

Rajbanshi SS, Benckiser G, Ottow JCG (1992) Mineralization kinetics and utilization as a N source of dicyandiamide (DCD) in soil. Naturwissenschaften 79:26–27

Saano A, Lindström K (1995) Small scale extraction of DNA from soil with spun column cleanup. In: Akkermans ADL, Elsas van JD, Brujin de FL (eds) Molecular microbial ecology manual, Chap. 1.3.4. Kluwer, Dordrecht, pp 1–6

Sayler SG, Layton AC (1990) Environmental application of nucleic acid hybridization. Annu Rev Microbiol 44:625–648

Schwarzer Ch, Haselwandter K (1991) Enzymatic degradation of the nitrification inhibitor dicyandiamide by a soil bacterium. Soil Biol Biochem 23:309–310

Selenska S, Klingmüller W (1991) DNA recovery and direct detection of Tn5 sequences from soil. Lett Appl Microbiol 13:21–24

Steffan RJ, Goksoyr J, Bej AK, Atlas RM (1988) Recovery of DNA from soils and sediments. Appl Environ Microbiol 54:2908–2915

Trevors JT, Elsas van JD (1989) A review of selected methods in environmental microbial genetics. Can J Microbiol 35:895–902

Tsai YL, Olson BH (1991) Rapid method for direct extraction of DNA from soil and sediments. Appl Environ Microbiol 57:1070–1074

Vilsmeier K, Bornemisza E, Amberger A (1987) Urea, ammonium sulfate and dicyandiamide transformations in Costa Rican soils. Fert Res 12:255–261

Wand TSC, Cheng SY, Tung H (1967) Extraction and analysis of soil organic acids. Soil Sci 103:360–366