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The detection of Gram-negative bacterial mRNA from soil by RT-PCR

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Abstract

The method described extracts high quality RNA from a clay-rich soil for which other published extraction protocols have failed. Transcript encoding the enzyme β-glucuronidase could be reliably detected by reverse transcriptase polymerase chain reaction in soil seeded with a genetically modified *Rhizobium leguminosarum* containing the *uidA* gene.

Keywords: Bacterial mRNA; Reverse transcriptase polymerase chain reaction; Soil; *Rhizobium leguminosarum*

1. Introduction

Soil contains a highly diverse and complex microbial community, the activity of which is essential for all major soil processes. The detection of specific DNA sequences directly from this soil community is proving to be a useful tool with which to improve our understanding of these soil processes. However, soil function depends not on the genes present in the soil microbial community, but on the expression of those genes. Thus, to further investigate the activity of soil micro-organisms it would be extremely useful to be able to detect gene expression directly. One approach is to extract and detect mRNA, the primary product of gene transcription, directly from soil. However, the extraction of RNA from soil is technically difficult. The extreme heterogeneity of soil; the matrix of surfaces capable of binding nucleic acids, mostly clays and organic materials [1]; a range of enzymatic inhibitors, mostly humic materials [1,2]; and ubiquitous RNases, all present technical problems. Despite these difficulties, methods have been reported describing the extraction of RNA from mainly sandy soils [1,3–8]. Specific sequences were detected in these extracts by hybridisation to Northern blots [1,5,8], by ribonuclease protection assays [4] and by reverse transcriptase polymerase chain reaction (RT-PCR) [3,7,9]. The latter, with its versatility and extreme sensitivity, has the greatest potential for use with environmental samples. Eukaryotic mRNAs (which are less transient than prokaryotic transcripts) have been extracted from soil using oligoT based purification methods [3,9]. Bacterial transcripts, which lack polyA tails, are not applicable to these methods. To date, only Selenska

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and Klingmüller [7] have reported the detection of bacterial mRNA from soil by RT-PCR.

The aim of this study was to develop a quick and reliable technique to extract RNA of a quality suitable for RT-PCR from a clay-rich soil. To determine the effectiveness of this technique, soil seeded with the genetically modified *Rhizobium leguminosarum* CT0370 [10] was used as a model system. This strain was released into the field in 1994 and remains present at approximately 10^4 cfu g^{-1} soil. Although it can be detected by plate counts and by PCR [11] nothing is known of its in situ metabolic activity.

2. Materials and methods

2.1. Soil and bacterial strains

The soil is a silty clay loam (32% clay, pH 7.5, 1.5% carbon, 0.16% nitrogen, analysed by Leco combustion method, Leco Corporation, St. Joseph, MI, USA) on which cereals have been grown for the last 10 years. Cultures of *R. leguminosarum* bv. *viciae* CT0370, a strain which contains a chromosomally integrated *uidA* gene with an *nptII* promoter (Fig. 1), were grown in TY (5 g tryptone, 3 g yeast extract, 1.3 g $CaCl_2 \cdot 6H_2O$) broth to late exponential phase (6.43×10^8 cfu ml^{-1}). The culture was pelleted at $8000 \times g$ for 15 min at 4°C, resuspended in H_2O and inoculated into fresh, sieved, soil at the rate of 1 ml culture g^{-1} soil. The mix was vortexed and RNA extraction begun immediately.

2.2. Assay of β-glucuronidase activity

This assay is essentially as described by Wilson et al. [12]. A 1-ml aliquot of CT0370 culture was pelleted and resuspended in 100 μl 50 mM sodium phosphate, pH 7, 1 mM EDTA. A 10-μl drop of 0.1% SDS and 10 μl of chloroform were added and the mix vortexed. A 50-μl aliquot of the upper layer was added to 450 μl 50 mM sodium phosphate pH 7, 1 mM EDTA, 10 mM *p*-nitrophenyl glucuronide and incubated at 37°C. Aliquots of 50 μl were removed at intervals, and added to 40 μl 0.4 M Na_2CO_3 to stop the reaction. The mix was centrifuged for 1 min and the supernatants' absorbance measured at 420 nm. The rate of change in absorbance was compared to that obtained with reactions containing known amounts of β-glucuronidase (NBS Biologicals, Huntingdon, UK).

2.3. Extraction of RNA

All H_2O and solutions were treated overnight with 0.1% diethyl pyrocarbonate and autoclaved. All glassware was baked for 12 h at 180°C. All plasticware was soaked for 12 h in 3% H_2O_2 and rinsed with H_2O. RNA was extracted using a modified version of the protocol described by Lamar et al. [9]. Samples were wrapped in muslin, submerged in liquid N_2 and freeze dried overnight. The dried samples were ground with a mortar and pestle which was cooled on dry ice and 2 ml g^{-1} soil lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 2% sarkosyl, 120 mM sodium phosphate, pH 8) added. The slurry was allowed to melt and 2 ml g^{-1} soil phenol mix (25 parts phenol, pH 6.9: 24 parts chloroform:1 part isoamyl alcohol) added, the mix shaken and centrifuged at $18\,000 \times g$ for 15 min at 4°C. To the supernatant were added 0.2 volume 1 M acetic acid and 0.7 volume ethanol. The mix was vortexed, incubated at −20°C for 1 h, centrifuged at $18\,000 \times g$ for 15 min at 4°C and the pellet resuspended in 200 μl H_2O.

2.4. Purification of RNA

Samples were purified using a modified version of the DNA purification protocol described by Cullen et al. [13]. Columns were prepared by loading 1 ml of Sephadex G-75 suspension (Pharmacia Biotech, St. Albans, UK) [14] or 0.5 ml of polyvinylpolypyrrolidone (PVPP) (Sigma, Poole, UK) suspended in H_2O into Bio-spin® columns (Bio-Rad, Hemel Hempstead, UK) and centrifuging at $1200 \times g$ for 3 min at 4°C in a swing-out rotor. Samples were loaded onto Sephadex columns and centrifuged as before. The eluent was purified on a QiaEasy® column (Qiagen, Crawley, UK) as recommended by the manufacturer and finally loaded onto a PVPP column and centrifuged as before. If the eluent remained brown, indicating contamination by humic materials, it was passed through fresh Sephadex and PVPP columns. The RNA was precipitated with an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate,

incubated for 1 h at −20°C, centrifuged at 12 000 × *g*, washed in 70% ethanol and resuspended in 50 μl H_2O. The RNA preparations were prestained and examined by electrophoresis on 1% agarose denaturing formaldehyde gels [14]. This was the most practical method for determining both the quality and quantity of RNA as contaminating humics make spectrophotometric readings unreliable.

2.5. Amplification by RT-PCR

Aliquots of 15 μl of RNA preparations, equivalent to 1.5 g soil, were incubated at room temperature with either 2.5 μl DNase (Boehringer Mannheim, Lewes, UK) and 2.5 μl RNasin™ (5′-3′, Bishop Stortford, UK) or 2.5 μl DNase and 2.5 μl RNase (5′-3′). After 15 min, 2 μl of 25 mM EDTA was added and the mix heated to 70°C for 10 min. The RT-PCR reaction was essentially as described by Selenska-Pobell [15], using primers G3 (5′-TTTAACTATGCCGGGATCCATCGC-3′) and G4 (5′-CCAGTCGAGCATCTCTTCAGCGTA-3′), which bind to an internal portion of the *uidA* gene (Fig. 1). To 14.6 μl of reverse transcriptase mix (10 mM Tris pH 8.3, 90 mM KCl, 0.9 mM $MnCl_2$, 0.2 mM dNTPs, 0.2 μM primer G4) was added 0.4 μl (2 U) rTth (Boehringer Mannheim) and 5 μl of enzyme-treated RNA preparation, the mix was incubated at 70°C for 15 min. A 5-μl aliquot was added to 15 μl of PCR mix (10 mM Tris pH 8.3, 100 mM KCl, 2.5 mM $MgCl_2$, 0.75 mM EGTA, 5% glycerol; 0.05% Tween, 0.3 mM dNTP mix, 0.2 μM primer G3 and 0.2 μM primer G4). Cycle conditions were 95°C for 2 min; then 55 cycles of 94°C for 1 min; 50°C for 2 min; 72°C for 1 min and finally 72°C for 5 min, using a Biometra, Trio TB-1 thermocycler. The PCR product was electrophoresed on a 3% agarose gel and stained with ethidium bromide. Southern blots [14] were probed with a gel purified *Bam*HI fragment of pSM26 [10] DIG labelled using a nick translation labelling kit (Boehringer Mannheim) and developed as recommended by the manufacturer.

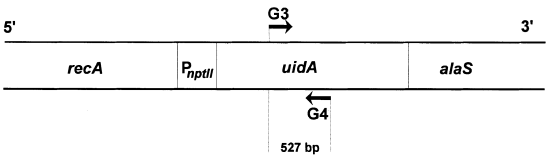

Fig. 1. The insertion site of the *uidA* gene in the chromosome of CT0370.

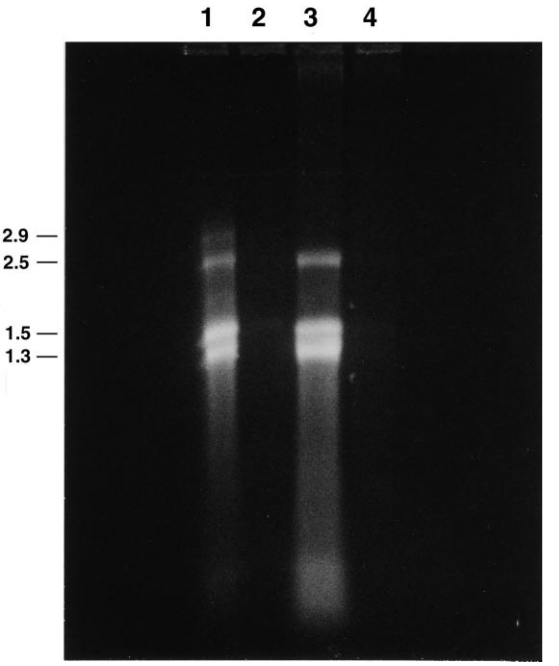

Fig. 2. A comparison of soil RNA extraction methods. Lane 1: RNA from 0.5 g seeded soil extracted using the method described here. Lane 2: RNA from 0.5 g seeded soil [7]. Lane 3: RNA from CT0370 extracted using the method described here. Lane 4: RNA from CT0370 [7]. *Unusual fragmentation of large rRNA subunit found in CT0370 [17].

3. Results and discussion

This RNA extraction technique consistently gave yields of approximately 5 μg RNA g^{-1} soil with clearly defined rRNA bands and of a quality suitable for RT-PCR of mRNA. Several other published extraction techniques [4,5,7,9] were attempted, with and without modifications, but did not give discernible rRNA bands, or gave low yields from the clay-rich soils used. For example, that of Selenska and Klingmüller [7] gave intact 16S (1.5-kb) and 23S (2.9-kb) bands but with considerable lower yields (Fig. 2).

RNA extractions are conventionally carried out under acid conditions, in this study optimum yields were obtained with extractions buffered at pH 8.

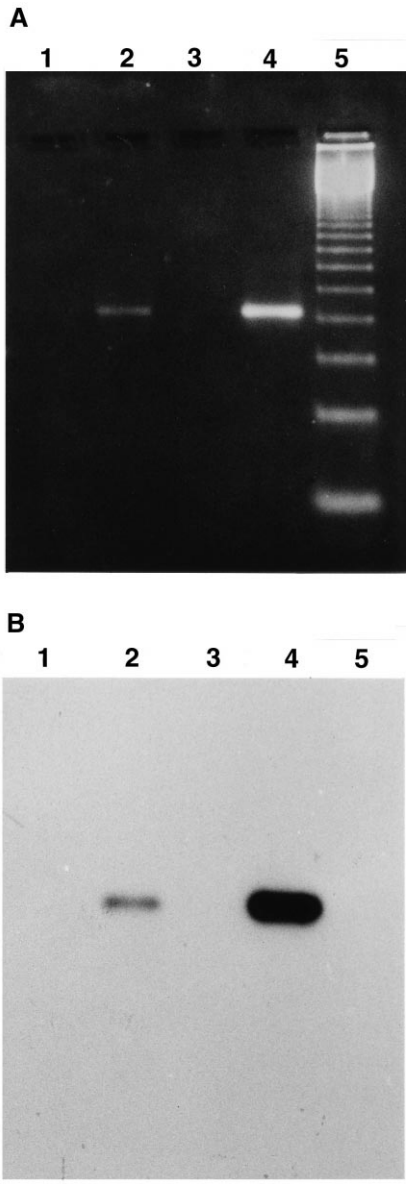

Fig. 3. A: RT-PCR signal from β-glucuronidase mRNA extracted from pure culture of CT0370 and from soil seeded with 6.43×10^{8} CT0370 g^{-1} soil. B: Southern blot of A probed with a fragment containing the *uidA* gene. Lane 1: DNase and RNase treated RNA from seeded soil. Lane 2: DNase treated RNA from seeded soil. Lane 3: DNase and RNase treated CT0370 RNA. Lane 4: DNase treated CT0370 RNA. Lane 5: 123 bp ladder (Gibco, Paisley, UK).

Losses observed during acid extractions may be due to clays, which bind nucleic acids, particularly under acid conditions [16]. Initial Sephadex G-75 and QiaEasy® purifications were essential to remove ionic contaminants, which otherwise prevented the PVPP columns binding humic materials. The PVPP columns did remove some RNA. If excessively long columns were used, or if preparations were at lower pHs all the RNA remained bound to the column. The hydrogen bonds through which PVPP binds phenolics are reported to become unfavourable at high ionic concentrations and to be favoured under acid conditions. PVPP has been used to effectively purify DNA without appreciable loss [13]. Presumably the double helical structure of DNA prevents the nucleotide bases from hydrogen bonding with the PVPP, while those of RNA remain exposed.

The CT0370 cultures expressed the *uidA* gene at a rate equivalent to 47.1 ± 19.1 nU cfu^{-1}. Transcript encoding the β-glucuronidase gene could be reliably detected from soil seeded with the equivalent of 2.19×10^{7} cfu $reaction^{-1}$, the identity of the product was confirmed by hybridisation (Fig. 3). The method reported here is the only one that yielded RNA from these clay-rich soils of a quality or in sufficient quantity to be suitable for RT-PCR. The protocol is relatively quick, taking approximately 4 h from freeze dried soil to finished extract. This method works well in this model system, however the protocol will have to be optimised further to detect the lower levels of mRNA likely to be observed in the field. The ability to detect prokaryotic mRNA directly from arable soils will be an extremely useful tool with which to further our understanding of soil ecology and this method is an important step towards that goal.

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