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A rapid and effective method of extracting fully intact RNA from thermophilic geobacilli that is suitable for gene expression analysis

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Abstract Extraction of intact RNA is essential for quantitative gene expression analysis. Isolating high quality RNA from gram-positive bacteria is known to be problematic particularly from organisms that have optimal growth temperatures greater than 45 °C. We report a novel extraction protocol for the rapid isolation of fully intact RNA from thermophilic *Geobacillus thermoleovorans* using a lysing matrix containing a mixture of ceramic and glass beads, triisopropylnaphthalene sulfonic acid (TNS), and *p*-4-aminosalicyclic acid (PAS). Combining both detergents, TNS and PAS, appeared to increase denaturation of RNases at thermophilic temperatures. Gel electrophoresis revealed that only RNA isolated using the TNS-PAS procedure demonstrated sharp, undegraded 23S, 16S, and 5S ribosomal RNA bands. RNA extracted from geobacilli using commercially available kits was extensively degraded and was not suitable for detecting gene expression. Total RNA yields extracted with the TNS-PAS protocol were greater than eightfold higher than those obtained with available kits. Critically, it was also shown that only RNA isolated with the TNS-PAS-based method was suitable for monitoring thermophile gene expression patterns using RT-PCR analysis.

Keywords Gene expression · *Geobacillus thermoleovorans* · RNA extraction · RT-PCR · *Thermus aquaticus*

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Introduction

The extraction of fully intact RNA is critical for many molecular biology techniques including RNA mapping, Northern blots, nuclease protection assays, RT-PCR, real-time PCR, and construction of cDNA libraries. However, because of the labile nature of RNA in comparison to DNA, its extraction can be problematic, depending on its source. This is due to the presence of the 2′-hydroxyl group of ribose, which makes the phosphodiester bonds of RNA substantially more susceptible to hydrolysis in comparison to those of DNA. Despite the availability of many commercially preprepared RNA extraction kits for bacteria, there have been reports in the literature describing problems in isolating RNA from gram-positive organisms and organisms that grow optimally at high temperatures (Zhong et al. 1997). This has been primarily due to difficulties in lysing complex cell walls and the inability of the detergent(s) to protect the nucleic acid sequence from extensive RNase cleavage. Both factors are particularly relevant when isolating RNA from thermophiles including *Geobacillus thermoleovorans* (Marchant et al. 2002) and *Thermus aquaticus*, which have optimum growth temperatures of around 70 °C. Hence the fidelity and rapidity of the extraction procedure is critical in bacterial gene expression studies, especially because of the short half-life of bacterial mRNAs (Belasco and Higgins 1988; Arraiano 1993).

There has been increasing interest in thermophilic geobacilli recently because of their ability to degrade alkanes. Thermophilic organisms possess substantial potential for the degradation of many classes of major environmental pollutants (Margesin and Schinner 2001). In addition, the alkane monooxygenase pathway is capable of carrying out a wide range of stereoselective and regio-selective oxidation reactions, giving it considerable commercial potential as a biocatalyst (Witholt et al. 1990). As a result much research has

focused on elucidating the organization and expression patterns of the genes involved in regulating this pathway. However, extraction of RNA from organisms that grow optimally at 70 °C has proved to be extremely difficult. This may be due to increased turnover rates of RNA in thermophilic organisms combined with the increased activity of RNases at elevated temperatures (Brown et al. 1993).

In this paper we present a method of rapidly extracting RNA from thermophilic geobacilli grown at temperatures up to 55 °C. To our knowledge no similar research has been reported for any hydrocarbon-degrading geobacilli. Despite the extensive use of guanidine isothiocyanate in RNA lysis buffers, it has been reported to be ineffective in denaturing RNases from thermophilic organisms (Zhong et al. 1997). Other methods commonly used to isolate RNA from bacteria include the use of kits from Ambion, Promega, Invitrogen, Qiagen, and Q-BIOgene. Detergents frequently used also include acid phenol, cesium chloride, and SDS, none of which have been validated for use in thermophilic organisms. Hence there is an immediate need for rapid RNA extraction procedures for organisms that have optimal growth temperatures greater than 45 °C. We report an extensive modification of the triisopropylnaphthalene sulfonic acid (TNS)–*p*-4-aminosalicyclic acid (PAS)-based protocol used by McGrath et al. (2000), that enables the extraction of fully intact RNA from thermophilic organisms grown at temperatures up to 55 °C. The modified protocol can be completed in less than 5 h, in comparison to the previously reported 2 days, with reduced reaction volumes and phenol chloroform steps, and delivers superior results compared to various commercially available kits in terms of quality and quantity of RNA.

Materials and methods

Bacterial strains and growth conditions

Geobacillus thermoleovorans strain T70 and *Thermus aquaticus* strain X1 were grown overnight in 20 ml nutrient broth flasks (Oxoid) over a range of temperatures from 50 to 70 °C. Cultures were shaken on a rotary shaker (200 rpm). Non-proteolytic *Clostridium botulinum* strain EVH was grown anaerobically overnight in 20 ml Wilkins Chalgren broth (Oxoid) at 33 °C and was used for comparative purposes.

Extraction of RNA using RNeasy and FastRNA kit, Blue

Using 200 µl from overnight cultures of strains T70, X1, and EVH, RNA was extracted using both kits according to the manufacturer's instructions. In the RNeasy protocol, cells were lysed and homogenized in the presence of a guanidium isothiocyanate-containing buffer. Ethanol was added to the lysate, and the samples applied to an RNeasy minispin column where the total RNA binds to a specialized membrane. Whereas in the FastRNA kit, cells were added to a specialized lysing matrix and homogenized using the FastPrep FP120 instrument. The manufacturer did not specify the contents of the chaotropic agent(s) used in the lysis buffer.

Extraction of RNA using the TNS-PAS protocol

Prior to the extraction procedure a lysing buffer was prepared by placing approximately 0.5 g of an equal mixture of 0.1 and 0.2 mm glass and ceramic beads in 1 ml prechilled lysozyme (50 mg ml^{-1}) containing TRIS EDTA buffer (Invitrogen). Overnight cultures (20 ml) of T70, X1, and EVH were cooled by placing in an ice bath for 15 min. Then 200 µl of each culture was added into the lysing buffer and vortexed vigorously for 40 s, followed by cooling in an ice bath. The mixture was centrifuged at 14,000 *g* for 8 min at 4 °C and the supernatant removed to a sterile microcentrifuge tube and placed on ice. Then 750 µl TNS buffer [1% TNS (sodium salt), 6% PAS (sodium salt), 200 mM TRIS-HCl, 25 mM EDTA, and 250 mM NaCl (pH 7.8)] was added to each sample, vortexed briefly, and left to incubate on ice for 25 min. Samples were centrifuged at 10,000 *g* for 8 min at 4 °C, and the supernatant collected. One volume of phenol:chloroform:isoamyl alcohol (Sigma) was added, and the samples were gently mixed, followed by centrifuging at 14,000 *g* for 10 min at room temperature. The RNA was precipitated with 2 vol ice-cold ethanol at −70 °C for 2–3 h. The RNA was then pelleted by centrifugation at 16,000 *g* for 25 min at 4 °C. The ethanol was decanted immediately, and the pellet air-dried while remaining on ice. The pellet was resuspended in 30 µl sterile RNase-free water, and stored at −70 °C until further use. The integrity of the ribosomal RNA extracted was analyzed on a 1.5% agarose gel (100 V for 60 min) containing 10 mg ml^{-1} ethidium bromide (Sigma). Gel electrophoresis analysis contained equal quantities of RNA from each extraction procedure to prevent overloading.

Removal of contaminating DNA from extracted RNA

Genomic DNA carried over from the extraction of RNA was removed with 10 U DNase I, RNase free (Roche), in a 13.25-µl reaction also containing 50 mM $MgCl_2$, and 10 µl template RNA. The reaction mix was incubated for 30 min at 37 °C. Effective DNase I inactivation was achieved by adding 1 µl of 20 mM EDTA to each sample and incubating the reaction mix at 37 °C for 1 min, followed by increasing the temperature to 65 °C for a further 10 min.

Quantification of RNA samples

RNA extracted using each of the described methodologies was quantified with the fluorescent nucleic acid probe RiboGreen (Molecular Probes). RNA concentrations were determined using both the high (20–1,000 ng ml^{-1}) and low range (1–50 ng ml^{-1}) protocols. The instructions were carried out according to the manufacturer's instructions with some minor modifications regarding the volumes of each reagent used. For the reverse transcription reaction, 200 ng DNase I-treated RNA was used as a template.

RT-PCR detection of *alkB* gene

The reverse transcription reaction was carried out using Thermoscript(Invitrogen) according to the manufacturer's recommendations. PCR conditions using degenerate primers (50 $pmol^{-1}$) for amplification of the *alkB* gene (forward TCTACGGSCAYTTCTACRTCGA and reverse CGGRTTCGCGTGGRTGRT, where R = G or A, Y = C or T, and S = G or C) were as follows: denaturation for 1 min at 94 °C, annealing for 30 s at 58.5 °C, and extension for 30 s at 72 °C for a total of 37 cycles, followed by a final extension cycle at 72 °C for 10 min. The RT-PCR conditions used to amplify a 250-bp fragment of toxin encoding mRNA from *C. botulinum* were the same as used by McGrath et al. (2000). Gene expression analysis was not carried out on *T. aquaticus*.

Table 1 Spectrofluorometric determination of DNase I-treated RNA concentrations using three different methods (readings are the average of duplicate assays). (*TNS* Triisopropylnaphthalene sulfonic acid, *PAS* *p*-4-aminosalicyclic acid)

RNA extraction technique	RNA yield (ng μl^{-1}) from *Geobacillus thermoleovorans* strain T70 at 50 °C	RNA yield (ng μl^{-1}) from *G. thermoleovorans* strain T70 at 55 °C	RNA yield (ng μl^{-1}) from *Clostridium botulinum* type E strain EVH	RNA yield (ng μl^{-1}) from *Thermus aquaticus* strain X1 at 50 °C	RNA yield (ng μl^{-1}) from *T. aquaticus* strain X1 at 55 °C
TNS-PAS	116.6	111.3	128.3	107.9	100.2
RNeasy kit (Qiagen)	23.2	12.8	38.8	14.1	12.5
FastRNA kit, Blue (Q-BIOgene)	31.2	–	36.5	46.5	–

Results and discussion

Table 1 shows that the TNS-PAS-based extraction procedure was superior in terms of RNA yields from all three organisms when compared to commonly used kits. The presence of fully intact ribosomal RNA bands using the TNS-PAS method was clearly visible from thermophilic *Geobacillus thermoleovorans* strain T70 and *Thermus aquaticus* strain X1 when grown at both 50 and 55 °C. This observation represents a significant increase in RNA extraction protocol flexibility, as it has been previously reported that the maximum cutoff point for successfully extracting RNA from thermophiles was 45 °C (Zhong et al. 1997). However, 45 °C is close to the minimum temperature for growth of strain T70 (Marchant et al. 2002) and is therefore of limited value for experimental work with extreme thermophiles. Furthermore the TNS-PAS method has the advantage of increased rapidity, reduced steps involving toxic chemicals such as phenol and chloroform, and there is no requirement for expensive equipment.

Contaminating genomic DNA carried over from each extraction procedure was routinely digested with DNase I. Figure 1a–c illustrates RNA isolations prior to DNase I treatment, as there is high molecular weight DNA present above the 23S ribosomal RNA bands. However, all traces of genomic DNA were completely removed prior to quantitation of RNA and RT-PCR analysis. In the current research all DNase I-treated RNA samples were quantitated with the RiboGreen probe, using both high and low range assays. The RiboGreen assay has become the method of choice for quantitating RNA in solution. The traditional $A_{260}/_{280}$ ratio is no longer considered an accurate measure of RNA quantity. RiboGreen allows enhanced sensitivity with less contaminant interference than with A_{260} nm absorbance methods (Jones et al. 1998). The major disadvantages of using A_{260} nm methods also include the contribution of proteins and free nucleotides to the absorbance signal. The standard curve plotted in Fig. 2 was generated from *Escherichia coli* rRNA standards using the high range assay and was used to determine the concentrations of the total RNA in the samples under investigation. Quantities of target RNA measured were determined per total RNA extracted from each organism (data for low range assay not shown).

Table 1 also shows five- and 3.7-fold increases in total RNA yields isolated from strain T70 using the TNS-PAS procedure at 50 °C, in comparison to the RNeasy and FastRNA techniques, respectively, while

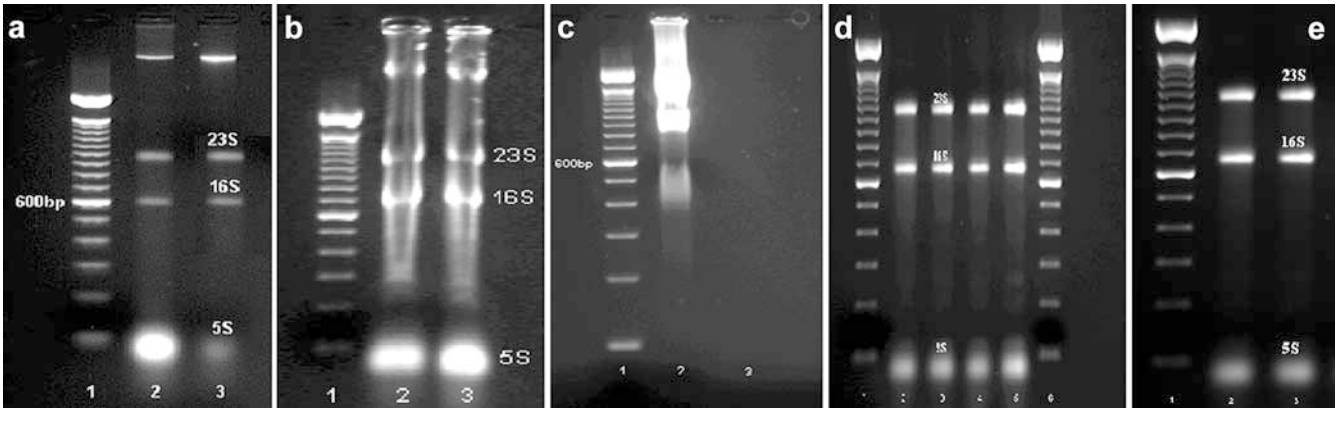

Fig. 1 a–c Extraction of RNA from *Geobacillus thermoleovorans* strain T70. *Lane 1* 100-bp DNA ladder. *Lane 2* Extraction of RNA at 50 °C. *Lane 3* Extraction of RNA at 55 °C. **a** Using triisopropylnaphthalene sulfonic acid (TNS)–*p*-4-aminosalicyclic acid (PAS). **b** Using the RNeasy method. **c** Using the FastRNA protocol. **d** Extraction of RNA from *Clostridium botulinum* strain EVH using the TNS-PAS method. *Lanes 1, 6* 100-bp DNA ladders. *Lanes 2–5* Isolation of RNA from *C. botulinum* at temperatures 33–36 °C. **e** Extraction of RNA from *Thermus aquaticus* strain X1 using TNS-PAS. *Lane 1* 100-bp DNA ladder. *Lane 2* Extraction of RNA at 50 °C. *Lane 3* Extraction of RNA at 55 °C

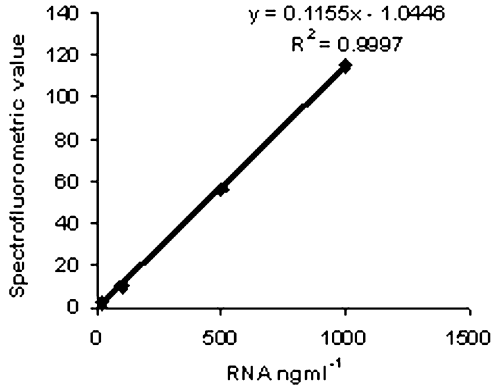

Fig. 2 Quantitation of RNA samples using high-range RiboGreen assay

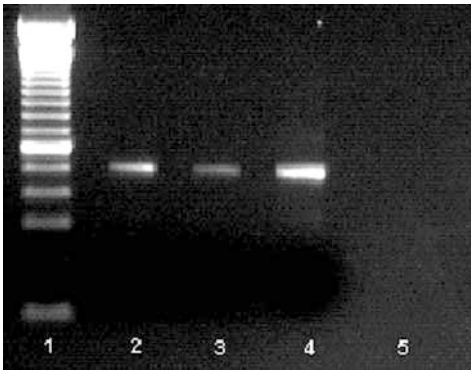

Fig. 3 RT-PCR detection of alkane monooxygenase (*alkB*) gene expression from *G. thermoleovorans* strain T70 under different conditions. *Lane 1* 100-bp DNA ladder. *Lane 2* Detection of *alkB* gene expression in strain T70 grown at 55 °C. *Lane 3* Detection of *alkB* gene expression in strain T70 grown at 50 °C. *Lane 4* Detection of *alkB* gene expression from soil. *Lane 5* Negative RT-PCR control

the RNA yields from strain T70 grown at 55 °C using the TNS-PAS extraction technique represent an 8.7-fold increase in comparison to the RNeasy kit. RNA was not detected with the FastRNA kit when T70 was grown at 55 °C (see Fig. 1c, lane 3). RNA yields isolated from *T. aquaticus* strain X1 were 7.7-fold and 2.3-fold higher with the TNS-PAS protocol at 50 °C when compared to the RNeasy and FastRNA kits, respectively. At 55 °C, total RNA yields were eightfold higher with the TNS-PAS method in comparison to the RNeasy technique. However, no RNA was could be detected and quantitated in solution at 55 °C with the FastRNA kit. Substantially greater quantities and quality of RNA were also extracted from *Clostridium botulinum* type E using the TNS-PAS-based protocol. These significant increases in RNA yields from the TNS-PAS methodology were reflected in the quality of the rRNA bands in the gel electrophoresis images. The integrity of the extraction procedure is clearly evident from the presence of sharp, undegraded 23S (approximately 1-kbp mark), 16S (approximately 650-bp mark), and 5S (approximately 100-bp mark) ribosomal RNA bands (see Fig. 1a, d, e). The relative intensity of the rRNA bands was taken as a ''general estimate'' of the quality and quantity of each respective extraction procedure. While the integrity of the ribosomal RNA cannot be assumed to be exactly correlated with mRNA quality and quantity, it does however provide a strong indication that the mRNA is intact and of sufficient quality for gene expression analysis studies. This expectation has been confirmed by our observations. In Fig. 1b (lanes 2 and 3) and c (lane 2), extensive smearing of rRNA bands using the RNeasy and FastRNA kits is highly indicative of RNA degradation from the thermophilic strain T70, and not due to overloading of the sample on the gel. Similar results were also observed for *T. aquaticus* and *C. botulinum* using both kits (images not shown). These observations were confirmed by the quantitative data which showed that only RNA isolated from strain T70 and *C. botulinum* strain EVH using the TNS-PAS method was suitable for detecting alkane monooxygenase (*alkB*) and neurotoxin gene expression, respectively, using RT-PCR analysis (*C. botulinum* images not shown). In RNA extractions where the ribosomal RNA was extensively degraded, no positive RT-PCR signal was detected. RNA extracted from soil samples known to contain sufficient numbers of thermophilic geobacilli using the TNS-PAS methodology was also successfully used as a template for RT-PCR analysis (see Fig. 3 lane 4), which further supports the use of this method for manipulation of environmental populations of thermophiles and other soil organisms. All extraction procedures and quantitation assays were carried out in duplicate at least.

Interestingly, the total RNA yields from all three techniques examined indicated that total RNA yields in T70 decreased as the growth temperature of the culture increased. When using the TNS-PAS method, total RNA yields from strain T70 decreased by only 4.5% as the growth temperature increased by 5 °C. In comparison a decrease of 45% in total RNA yields using the RNeasy kit was observed. This represents an approximate tenfold reduction in total RNA yields from strain T70 when using the RNeasy kit compared to the TNS-PAS method at 55 °C. Similar findings were determined with *T. aquaticus*. In the current study we were unable to isolate intact RNA from *G. thermoleovorans* strain T70 or *T. aquaticus* strain X1 at temperatures greater than 55 °C using any of the extraction techniques. It appears that the successful extraction of intact RNA from highly thermophilic geobacilli and *T. aquaticus* is highly dependent on both growth temperature and the detergent(s) used in the lysis buffer. This assumption is consistent with a previous finding, reporting the accelerated degradation of *Geobacillus stearothermophilus* (formerly known as *Bacillus stearothermophilus*) mRNA at temperatures of 45–55 °C (Stenesh and Madison 1979). Considering that the role of detergents is to protect RNAs from extensive RNase degradation, the choice of detergent

becomes critical when manipulating thermophilic RNAs. This is because the catalytic efficiency of specific RNases has been reported to be three to four times higher in thermophiles in comparison to *E. coli* (Brown et al. 1993). No research has been conducted as yet regarding the activities and stability of thermophilic RNases at dramatically lower temperatures, for example, 4 °C or lower. Increased thermostability of RNases in thermophilic organisms has been reported to be largely related to nucleotide sequence composition. Some of the factors involved in thermostability of thermophilic RNases include minimal irregularities in helical structures with increased hydrogen bonds in helices and increased base pairing at the bases of stem loops (Brown et al. 1993). The increased thermostability and activity of thermophilic RNases may also arise from reduced RNA secondary structures in thermophiles (Malcolm 1981), although there is no other direct evidence available to support this as yet. Secondary structures of RNA are known to confer resistance to RNase hydrolysis (Belasco and Higgins 1988; Arraiano 1993). As RNA secondary structures can be removed at elevated temperatures, it may be speculated that both increased RNase stability in thermophiles and reduced secondary structures within thermophilic RNAs may be intrinsically linked. Hence, the choice of detergent when extracting RNA from thermophiles is of immense importance. The modified TNS-PAS technique represents a novel breakthrough in RNA isolation procedures for thermophilic organisms, with much potential in future molecular work involving thermophilic gene manipulation and expression analysis studies.

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References

Arraiano CM (1993) Post-transcriptional control of gene expression: bacterial mRNA degradation. World J Microbiol Biotechnol 9:421–432

Belasco JG, Higgins CF (1988) Mechanisms of mRNA decay in bacteria: a perspective. Gene 72:15–23

Brown JW, Haas ES, Pace NR (1993) Characterisation of ribonuclease P RNAs from thermophilic bacteria. Nucleic Acids Res 21:671–679

Jones LJ, Yue ST, Cheung CY, Singer VL (1998) RNA quantitation by fluorescence-based solution assay: RiboGreen reagent characterisation. Anal Biochem 265:368–374

Malcolm ADB (1981) Thermophiles and RNA stability. J Theor Biol 92:479–481

Marchant R, Banat IM, Rahman TJ, Berzano M (2002) The frequency and characteristics of highly thermophilic bacteria in cool soil environments. Environ Microbiol 4:595–602

Margesin R, Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl Microbiol Biotechnol 56:650–663

McGrath S, Dooley JSG, Haylock RW (2000) Quantification of *Clostridium botulinum* toxin gene expression by competitive reverse transcription–PCR. Appl Environ Microbiol 66:1423–1428

Stenesh J, Madison JB (1979) Stability of bacterial messenger RNA in mesophiles and thermophiles. Biochim Biophys Acta 565:154–160

Witholt B, de Smet M-J, Kingma J, van Beilen JB, Kok M, Lageveen RG, Eggink G (1990) Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: background and economical potential. Trends Biotechnol 8:46–52

Zhong X, Luo J, Stevens E Jr (1997) Isolation of full length RNA from a thermophilic *Cyanobacterium*. Biotechniques 23:904–910