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Magnetic capture–hybridization method for purification and probing of mRNA for neutral protease of *Bacillus cereus*

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Abstract

A magnetic capture–hybridization method was assessed for the isolation of prokaryotic mRNA for the neutral protease of *B. cereus* from liquid culture. A biotin-labeled specific probe was hybridized to the mRNA transcripts and subsequently captured by streptavidin-coated paramagnetic beads. mRNA was detected by dot–blot hybridization with a ds DIG-labeled DNA-probe. The magnetic capture hybridization is a rapid and simple method and has a promising potential for gene expression studies in complex samples. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bacillus cereus; Magnetic capture–hybridization; Neutral protease; Prokaryotic mRNA.

1. Introduction

The investigation of gene expression at the transcriptional level in natural environments is of basic interest in microbial ecology studies. This type of study bridges the gap between structural and functional diversity of microbial communities, since specific gene expression of certain microorganisms under different conditions can be demonstrated. The isolation of prokaryotic mRNA from complex habitats like soil has been performed successfully by several authors (Pichard and Paul, 1991; Tsai et al., 1991; Selenska and Klingmüller, 1992). However, problems may still exist with the detection of low copy number mRNA especially from natural environments. Prokaryotic mRNA can be rapidly degraded by cell indigenous or exogenous RNases during purification. Steps like washing the cells, phenol-chloroform extractions, precipitation of RNA and CsCl density gradient centrifugation for the elimination of inhibiting compounds like humic acids may diminish the yields considerably.

The use of the magnetic capture–hybridization method can reduce the number of manipulations in comparison to common procedures. This is important for a more sensitive detection of mRNA target molecules. For the isolation of eukaryotic mRNA from complex matrices the magnetic capture–hybridization method is an established tool for gene expression studies especially in clinical microbiology. The hybridization of poly-A mRNA molecules to oligo (dT) probes allows direct extraction of purified mRNA from crude lysates and has been performed for complex matrices such as mouse liver lysate (Oaklander and Ekenberg, 1994) and soil (Tebbe et al., 1995). Since hybridization is less

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influenced by humic acids than enzymatic treatments like PCR, this method was used for the isolation of bacterial DNA from soil in order to overcome the inhibitory effect of coextracted humic acids during PCR (Jacobsen, 1995). Applications with complex matrices (Oaklander and Ekenberg, 1994; Tebbe et al., 1995; Muir et al., 1993; Spurkland, 1992; Chen et al., 1998) indicate, that mRNA or DNA isolated by magnetic capture–hybridization is free of any inhibitory compounds and can be directly used for RT–PCR or PCR without any further treatments.

In the present study we demonstrated that this method is also suitable for the isolation of prokaryotic mRNA, using a specific biotin-labeled DNA-probe for the capture of neutral protease mRNA of *Bacillus cereus*. mRNA was subsequently fixed to streptavidin-coated paramagnetic beads and the mRNA was detected by dot–blot hybridization with a specific DIG-labeled functional ds DNA-probe.

2. Material and methods

2.1. Strains and culture conditions

B. cereus DSM 3101^T was grown at 30°C in 300 ml of nutrient broth medium (Merck, Darmstadt, Germany) with shaking at 130 rpm. Growth was followed by measuring the optical density at 546 nm. The *Bacillus cereus* culture was obtained from the German Culture Collection of Microorganisms and Cell Cultures (DSZM).

2.2. Enzyme assay

The activity of neutral protease was determined by using azocasein (Sigma, Deisenhofen, Germany) as the protease substrate (Kühn and Fortnagel, 1993). One ml of cell suspension was pelleted by centrifugation at 12 000 × *g* for 2 min at 6°C. To 200 μl of supernatant, 20 μl of 10 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, Deisenhofen, Germany, in 98% ethanol) and 200 μl of azocasein solution (100 mg/ml in 100 mM Tris/HCl, pH 7.4) were added. After incubation for 1 h at 37°C, 600 μl of 15% (w/v) trichloroacetic acid were added and a further incubation at 37°C for 10 min was performed. After centrifugation at 12 000 × *g* for 2 min at room temperature the absorbance at 400 nm was determined.

2.3. Preparation of the beads

A volume of 200 μl of Dynabeads M-280 Streptavidin (Dynal, Hamburg, Germany) was transfered into a sterile 1.5-ml Eppendorf tube placed in a Magnetic Particle Concentrator (MPC, Dynal, Hamburg, Germany). After 30 s the supernatant was removed. The particles were washed three times with 0.5 × SSC (RNase free, Promega, Madison, USA) each time removing the tube from the MPC, resuspending in washing buffer and subsequent concentration of the beads.

2.4. Biotin-labeled capture probe

As a capture probe we used a 5′biotinylated oligonucleotide with the sequence 5′ B-GTAACAGGAACGAATAAAGTAG-3′, which is complementary to the mRNA transcript and is situated at the beginning of the sequence coding for the mature neutral protease of *Bacillus cereus* (Wetmore et al., 1992).

2.5. Cell lysis and mRNA extraction

One ml of *B. cereus* cell suspension was pelleted by centrifugation at 12 000 × *g* for 2 min at 4°C and the cell-free supernatant was discarded. The pellet was rapidly frozen in liquid nitrogen and stored at − 70°C or it was directly used in the mRNA isolation procedure. For lysis the cells were resuspended in 1 ml of GTC extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.1) and rapidly introduced into a precooled 12-ml glass tube containing 2 g of 0.17–0.18 μm diameter glassbeads (Braun, Melsungen, Germany). A volume of 20 μl of β-mercaptoethanol was added. Cell lysis was performed by homogenization in a bead beater (Braun, Melsungen, Germany) for 2 min at 2000 rpm at room temperature. For the magnetic capture–hybridization 2 ml of prewarmed (70°C) dilution buffer (6 × SSC, 10 mM Tris–HCI, pH 7.4; 1 mM EDTA, 0.25% SDS), 20 μl of β-mercaptoethanol and the biotinylated oligonucleotide (250 pmol/mg

of particles) were added to the lysate and mixed by inversion several times. The mixture was incubated at room temperature for 5 min. The suspension was transferred into two sterile Eppendorf tubes and centrifuged at room temperature for 10 min at $12\,000 \times g$. The supernatant containing the RNA–DNA-probe hybrids was transferred into two sterile Eppendorf tubes each containing the prepared beads. To minimize nonspecific binding of DNA, tubes containing the beads were preliminary treated with 0.1% blocking reagent (Boehringer, Mannheim, Germany) in 500 µl of $0.5 \times$ SSC for 1 h. After attachment of the hybrids to the beads the tubes were placed into the MPC. The beads were washed three times with $0.5 \times$ SSC.

After the first concentration step, the two samples were pooled into one tube. For the elution of the mRNA, the beads were resuspended in diethylpyrocarbonate (DEPC)-treated water and incubated at 65°C for 2 min. After concentration of the magnetic particles the mRNA-containing solution was transferred into a new sterile Eppendorf tube.

2.6. Probe for detection

For detection of protease specific mRNA a DIG-labeled double stranded DNA-probe was prepared by incorporation of Digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) during PCR as described by the manufacturer. The 951 nucleotide sequence of the whole mature peptide (Wetmore et al., 1992) was used as the probe sequence.

2.7. RNase digestion

The DNA fragment coding for the mature peptide was amplified by PCR and the 951 bp product was used as negative control for digestion assay. The product was diluted in sterile distilled water and denatured at 94°C for 10 min. After addition of the capture probe, SSC was added to a final concentration of $6 \times$ SSC and the probe was allowed to hybridize to the target DNA strand. Isolation of the complex was performed as described above for mRNA.

For the RNase digestion assay, beads bound mRNA and DNA samples were divided into two equal aliquots, one untreated and one treated with RNase. For RNase treatment the beads were resuspended in 500 µl of RNase digestion buffer (10 mM Tris–HCl, 300 mM NaCl, 5 mM EDTA). After addition of 2.5 µl of RNase, DNase-free (Boehringer, Mannheim, Germany) incubation was performed at 37°C for 30 min. Washing and elution of the nucleic acid preparations were performed as described above.

2.8. Dot–blot hybridization

The aqueous nucleic acid solutions were mixed with $20 \times$ SSC and formaldehyde in the ratio of 5:3:2 respectively, incubated at 65°C for 10 min and chilled on ice for 5 min. The mixtures were hand blotted on a positively charged nylon membrane (Boehringer, Mannheim, Germany) and fixed by baking at 120°C for 30 min. Hybridization and chemiluminescent detection was performed according to Rost (Rost, 1995): The membrane was incubated with prewarmed prehybridization solution (0.25 mol Na_2HPO_4, pH 7.2; 1 mmol EDTA; 20% SDS; 0.5% blocking solution) at 68°C for 1 h. Hybridization was performed at 68°C overnight with denatured probe (1 µl of DIG-labeled PCR product in 2.5 ml of prehybridization solution). The membrane was washed for 3×20 min at 65°C in washing solution (20 mmol Na_2HPO_4, pH 7.2; 1 mmol EDTA; 1% SDS). Detection with CSPD was performed with the DIG detection kit (Boehringer, Mannheim, Germany) as described by the manufacturer. Chemiluminescence was detected by autoradiography.

3. Results and discussion

The growth of *B. cereus* and the activity of the neutral protease in the supernatant fluid is shown in Fig. 1. Proteolytic activity was detected after 7 h of growth, in the late exponential growth phase. Samples for mRNA preparation were taken after 8–9 h of growth.

Dot–blot hybridization of the ds DNA-probe to mRNA of three parallel extractions from each 5×10^9 exponentially growing cells is shown in Fig. 2. Identical size and intensity of the blots indicate the reliability of the procedure.

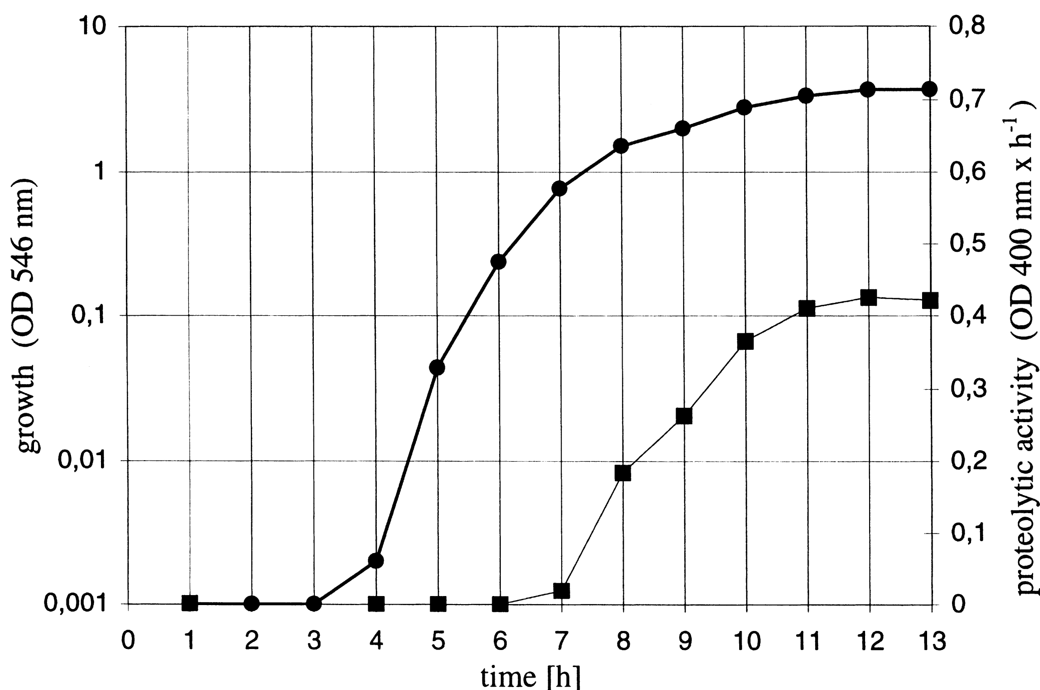

Fig. 1. Growth of *B. cereus* (●) and proteolytic activity (■) in liquid culture.

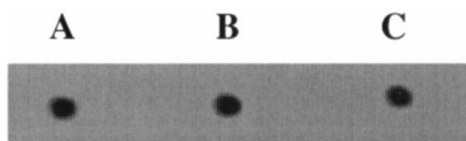

Fig. 2. Reproducibility of the mRNA isolation procedure. Dot–blot hybridization of the ds DNA-probe to mRNA preparations. A, B, C: Three repetitions, each 5×10^9 *B. cereus cells*; 0.3 mg of beads; time for binding of the mRNA-capture probe hybrid to paramagnetic particles was 5 min.

Complete cell lysis (as checked microscopically at 1000 × magnification) and effective destruction of RNases by GTC extraction buffer was completed in 5 min after sample withdrawal. The glass bead treatment was more effective in cell lysis than enzyme incubations (data not shown) and considerably reduces the time between sample withdrawal and denaturation of RNases in comparison to the common protocol for Gram-negative bacteria by lysozyme digestion (Kühn and Fortnagel, 1993). In addition, there is no need for washing the cells prior to lysis, which means a further reduction in time. Moreover the bead beater protocol is suitable for cell lysis in complex media like soil (Smalla et al., 1993). SDS containing buffers for which the Dynal capture system has been developed were not able to protect the prokaryotic mRNA against RNase activity. No mRNA could be detected by using SDS based denaturation buffers (data not shown). The optimal quantity of beads was tested while maintaining the capture probe concentration at 250 pmol/mg of particles (Hornes and Korsnes, 1990). The amount of 0.5 mg of paramagnetic particles was found to be sufficient for the isolation of mRNA from 5×10^8 cells in the 3-ml capture volume. Further addition of beads did not result in higher mRNA yields (Fig. 3).

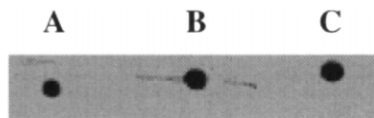

Fig. 3. mRNA yields in relation to the amount of paramagnetic particles for the preparation of 1×10^8 *B. cereus* cells. Dot–blot hybridization of the ds DNA-probe to mRNA. A: 0.3 mg; B: 0.5 mg; C: 0.7 mg of beads. Time for binding of the mRNA-capture probe hybrid to paramagnetic particles was 5 min.

Variations in incubation temperature (room temperature, 40°C and 70°C) for denaturation of potential mRNA secondary structures prior to capture–hybridization did not have any effect on mRNA yield for this special mRNA species (data not shown). However, the target region for the capture probe could be involved in secondary structures and the effect of prior denaturation should be investigated when working with another target region or mRNA species. Time for binding of the mRNA-capture probe hybrid to the magnetic particles has also been tested and was found to be optimal at 30 min with shaking at 100 rpm (data not shown).

RNase treatment revealed, that some DNA is coextracted by the procedure (Fig. 4) so that DNase digestion has to be performed prior to RT–PCR amplification of the transcripts. We assume that the target sequence of the DNA double strand was also accessible to the capture probe as a consequence of sharing and denaturation during the bead beating procedure.

In comparison to common protocols, washing of the cell pellet and enzyme digestion for cell lysis (up to 20 min in total (Hönerlage et al., 1995)) are not necessary. In addition, phenol–chloroform–extractions (commonly three times and more) and precipitation steps which account for loss of target molecules are avoided. The extraction of the pure target molecule is performed in less than 1 h and the mRNA can be eluted from the beads directly into the buffer of choice depending on subsequent treatment. As washing with $0.5 \times$ SSC allows complete elimination of humic compounds (Tebbe et al., 1995) which are known to inhibit Taq polymerase (Tsai et al., 1991; Tebbe and Vahjen, 1993), we expect that this procedure may also be successfully applied for gene expression studies of bacteria in complex microbial habitats.

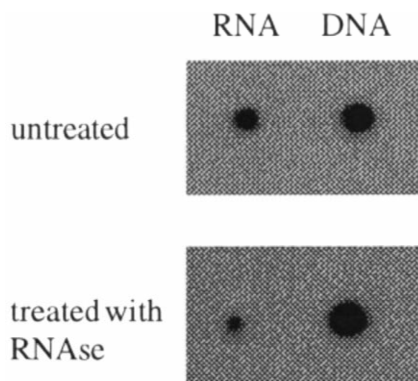

Fig. 4. Coextraction of DNA by the magnetic capture–hybridization method. Captured nucleic acids from 3.6×10^8 *B. cereus cells* (RNA) and captured PCR protease gene amplicons (DNA) were divided into two aliquots, one treated and one untreated with RNase. 0.5 mg of beads; time for binding of the mRNA-capture probe hybrid to paramagnetic particles was 30 min.

4. Conclusion

The magnetic capture–hybridization procedure was easily adaptable to the isolation of prokaryotic mRNA by using sequence-specific biotinylated oligonucleotides. By this procedure only the single mRNA species of interest is obtained. Transcripts of bacterial neutral protease mRNA can be isolated by direct capturing from crude lysate avoiding many working steps which affect mRNA yields considerably.

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