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Optimization of total RNA extraction from Gram-positive bacteria (coagulase-negative staphylococci) for expression studies

Optymalizacja metod izolacji całkowitego RNA z Gram-dodatnich bakterii (ze szczepów gronkowców koagulazo-ujemnych) do badań ekspresyjnych

The analysis of RNA provides a good reflection of an organism's gene expression profile. Compared to DNA, RNA is relatively unstable. This is largely due to the presence of ribonucleases (RNases), which break down RNA molecules. One of the key problems in expression studies and comparative transcript quantification is the time needed for bacterial cell wall lysis. Most commercial total RNA extraction kits assume 30 minutes' incubation of bacterial pellet with lysostaphin and/or proteinase K at 37°C. This time is long enough to change ratios of transcripts of interest. Rapidity of lysis is the most important parameter in RNA extraction as the half life of bacterial mRNA is extremely short [6].

The aim of this study was modification and optimization isolation procedures of RNA for successful stabilization, purification and analysis of RNA from Gram-positive bacteria.

MATERIAL AND METHODS

Two different bacterial cell wall disruption methods were compared: enzymatic digestion with lysostaphin [4, 10] and mechanical – vortexing with glass beads [7, 8]. Total RNA was extracted using the TRIzol Reagent [2, 3]. Residual DNA present in RNA preparations following purification was removed using RNase-free DNase I (Fermentas) treatment according to the manufacturer's instructions.

Enzymatic lysis. Overnight liquid culture of *Staphylococcus epidermidis* (3 ml) was centrifuged to collect bacteria. The strain was cultured in tryptic soy broth (TSB, Becton Dickinson). Cells pellet was incubated (37°C, 5 min) with different lysosthaphin (Sigma) concentration. Four water solutions of enzyme 14 U, 28 U, 42 U, 56 U of the total volume 50 µl were tested. Additionally, the influence of incubation time on total yield of RNA was investigated. Samples after 5, 10 and 15 min incubation with 56 U lysostaphin were treated with 1 ml of TRIzol Reagent. Total RNA was subsequently isolated according to the TRIzol Reagent manufacturer's instructions (Invitrogen).

Mechanical disruption. In parallel experiments 0.8 g of glass beads (425–600 µm in diameter, Sigma) were added to collected bacterial cells in 1 ml of TRIzol Reagent. The tubes were immediately processed in the shaker for 3 or 6 min at 3000 rpm and total RNA was subsequently isolated according to the TRIzol Reagent manufacturer's instructions (Invitrogen).

The quantity of RNA preparations were analyzed with BioPhotometr (Eppendorf). The integrity of RNA was confirmed by 2% agarose gel electrophoresis in TBE (1X) buffor.

For all studies a *Staphylococcus epidermidis* clinical strain was used. It is biofilm-producing strain (growth as black colonies on Congo red agar). Bacteria were isolated from the left nasal septum of a patient with lung cancer after thoracic surgery.

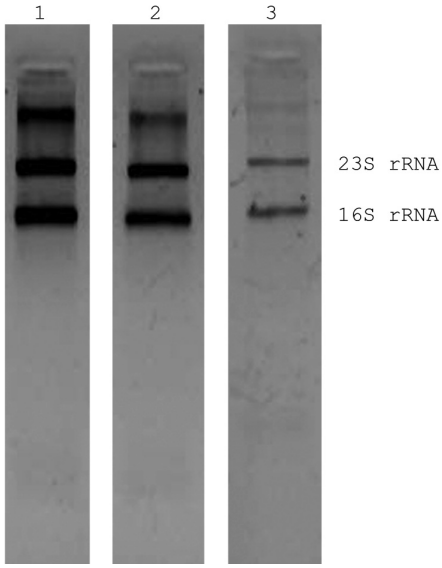

Fig. 1. Ethidium bromide stained agarose gel electrophoresis of total RNA preparations. Lane 1: 5 minute incubation with 56 U lysostaphin; lane 2: 5 minute incubation with 42 U lysostaphin; lane 3: 5 minute incubation with 14 U lysostaphin

RESULTS AND DISCUSSION

In this work we compared two methods of *Staphylococcus* cell wall disruption: enzymatic (lysostaphin incubation) [1, 4] and mechanical (high speed vortexing with glass beads) [7]. The best results (the highest RNA harvest) were obtained using the enzymatic method. Both the incubation time and the concentration of lysostaphin had influence on the result of RNA isolation (Table 1, 2). Mechanical vortexing of bacteria with glass beads gave less than half of the amount of total RNA obtained after lysostaphin incubation (Table 3).

Table 1. Changes in efficiency of RNA yield during 5 minute incubation of bacteria with lysostaphin

Lysostaphin unit per sample (U)	Yield of RNA (μg)
14	13.5
28	18.9
42	23.4
56	30

Some expression experiments require rapid and efficient methods of total RNA isolation [5, 9], especially when changes in amounts of transcripts during the time of bacteria growth are subject of

quantification analysis. Gram-positive bacteria like *Staphylococcus* have a rigid and thick cell wall and as much as 30 minutes of incubation with lyspstaphin (according to most of total RNA isolation protocols) is needed to remove this obstacle. During that time bacteria can actively change the ratio of transcripts [6]. We think the maximum shortening of cell wall disruption stage should be appreciated. The current study is focused on Gram-positive bacteria *Staphylococcus epidermidis.* Our modified isolation method makes the whole procedure much faster. As short as 5 min cell wall digestion with a high concentration of lysostaphin is enough to get a good yield of high quality RNA preparation.

Table 2. Yield of RNA in dependence on the time of incubation in the presence of 56 U of lysostaphin

Time (min)	Yield of RNA (μg)
5	30
10	29.1
15	25.8

Table 3. Yield of RNA in dependence on the time of mechanical disruption

Time (min)	Yield of RNA (μg)
3	13.68
6	13.62

CONCLUSIONS

In this paper we described the modified method of RNA extraction from Gram-positive bacteria. Easy and simple changes in protocol enable to isolate total quality RNA (not degraded, in correct proportion) in a short time and a sufficient quantity.

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SUMMARY

In order to ensure accurate gene expression analyses, it is important that the analyzed RNA truly represents *in vivo* transcript ratio. Generally, isolation of RNA from Gram-positive bacteria is difficult because these bacteria have rigid and thick cell walls. Moreover, to ensure maintenance of RNA integrity during purification is difficult because ribonucleases (RNases) break down RNA molecules. Hence, the extraction procedures should be simple, rapid and effective. In this study we report a rapid, reproducible, high yield optimization method for the preparation of RNA from Gram-positive bacteria. The enzymatic lysis was compared with the mechanical disruption with glass beads. As cell wall disruption methods we applied lysosthaphin (37°C, 5 min, 56U) and then RNA was isolated using the TRIzol Reagent. We found that a short time of incubation is essential for the high yield recovery of bacterial RNA from Gram-positive bacteria.

STRESZCZENIE

Celem pracy było zoptymalizowanie metody izolacji całkowitego RNA z bakterii Gram-dodatnich do badań ekspresyjnych. Bakterie Gram-dodatnie ze względu na budowę ściany komórkowej wymagają lizy enzymatycznej. Czas lizy zalecany przez producentów zestawów do izolacji często przekracza 30 min. Uzasadnieniem podjęcia badań było efektywne zniszczenie ściany komórkowej i szybka izolacja niezdegradowanego RNA o niezmienionym stosunku ilościowym transkryptów. Postawiony cel zrealizowano poprzez zastosowanie metody lizy enzymatycznej w czasie 5 minut z większą ilością lizostafiny. Zoptymalizowana metoda izolacji pozwala na obiektywne badania ekspresyjne. Szybkość i efektywność niszczenia ściany komórkowej oraz stabilizacja wyizolowanego RNA o niezaburzonych proporcjach to główne zadanie postawione efektywnej izolacji materiału służącego do badań z wykorzystaniem techniki *real - time PCR*.