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A protocol for Metagenomic RNA extraction from bacterial consortium in the presence of crude oil

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Abstract

Most extraction protocols for purifying RNA from consortia for high throughput analysis are cumbersome. Commercial kits are widely used, but normally produce low quantities of RNA, requiring multiple isolations for large-scale analyses. Based on two previous protocols, a novel procedure was developed aimed at isolating large quantities of good quality RNA of a bacterial consortium in liquid culture with crude oil. Gel electrophoresis demonstrated isolation of undegraded RNA suitable for downstream molecular applications such as RT-PCR. RNA functionality was verified in a crude oil degradation cycle through the identification of early changes in expression levels of catechol 2, 3-dioxygenase (XyEl).

Keywords: biodegradation, catechol 2, 3-dioxygenase, gene expression, microbial consortium, RNA extraction.

Introduction

Extraction of good quality RNA is the first step in gene expression research. Consequently, extraction of high quality RNA involves preservation of transcriptomes essential to exploring expression of particular genes in a system (ROSIC AND HOEGH-GULDBERG [5]). However, RNA molecules are extremely sensitive and can be easily degraded by ribonucleases (RNases) or contaminated by metagenomic DNA during extraction. Isolation of sufficient RNA is essential for construction of complementary DNA (cDNA) libraries, reverse transcription coupled to polymerase chain reaction (RT-PCR), quantitative real time PCR (qRT-PCR) and differential display reverse transcription polymerase chain reaction (DDRT-PCR). Commercial RNA extraction kits for bacteria are widely available, but reports exist of isolation bias from consortia. These biases have been caused by difficulties in lysing cell walls, the inability of detergents to protect nucleic acid from extensive RNase cleavage, and the presence of complex pollutants such as hydrocarbons (SHARKEY & al. [6]). Protocols have been reported successfully for RNA extraction from bacterial pure cultures. Several cetryltrimethylamoonium bromide (CTAB)-based methods have been employed to RNA extraction from samples containing high levels of polysaccharides and phenol. Other methodologies have been used to RNA extraction from soil contaminated with pollutants like as pesticides and hydrocarbons (GASIC & al. [7]; CRUZ-LEYVA & al. [8]). However isolation methodologies from microbial consortia cultures are still scarce and further protocols are required to better understand the metabolic mechanisms and population succession during complex compounds degradation such as hydrocarbons (benzene, phenol, crude oil, etc). Hydrocarbons enter the water and soil due to

crude oil leaks during refining, storage and distribution as well as accidental spills, because of this monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (BTEX) are pollutants commonly found in contaminated water and soil (MARIANO & al. [2]; ZHANG & al. [1]). Several studies on biodegradation of BTEX contaminants by native microorganisms have showed that aromatic ring cleavage is a key step for complete mineralization during aromatic hydrocarbon biodegradation (MESARCH & al.[3]; NYYSSÖNEN & al. [4]). Cleavage is catalyzed by dioxygenases such as catechol 2, 3-dioxygenase (*XyEl*), which are often detected and named as a catabolic gene in native microorganisms involved in crude oil degradation. Analysis of catabolic genes may play an important role in understanding the pathways of recalcitrant pollutant biodegradation by mixed microorganisms and native consortium (ZHANG & al. [1]). Obtaining large quantities of high-quality RNA from a consortium during crude oil degradation in liquid cultures has proved challenging. In an effort to study the temporal and spatial expression of genes involved in genetic regulation and molecular physiology during hydrocarbon biodegradation in liquid cultures, a protocol was developed to isolate high quality RNA from a bacterial consortium during crude oil degradation in an aqueous medium. This protocol was developed by modifying two previously reported methodologies (GASIC & al. [7]; CRUZ-LEYVA & al. [8]). The resulting novel protocol allowed isolation of high quality mgRNA whose functionality was tested in detecting *XyEl* gene through RT-PCR during crude oil biodegradation in liquid cultures.

Material and Methods

Liquid cultures

All experiments were conducted in Erlenmeyer flasks with 100 mL Bushnell Hass mineral medium (BUSHNELL AND HAAS, [9]) at 37 ºC and 150 rpm. Each liquid culture was inoculated with 5% v/v (0.043 µg protein/mL) of native microbial consortium isolated from oil contaminated soil of Mérida, Yucatán. Crude oil was added to the microbial suspension at 0.5% v/v. The functionality studies of RNA were realized with microbial consortium samples obtained during crude oil degradation at 4, 8 and 12 days. All studies were carried out at least in duplicate.

Metagenomic RNA extraction

To evaluate the RNA extraction protocol on microbial consortium in presence of crude oil, the biomass present in 30 mL (0.2 mg) of liquid culture was harvested by centrifugation (10,000 x g for 15 min at 4 °C). Cell pellet was washed twice using 1 mL TEN buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl) to eliminate residual hydrocarbons, and supernatant was discarded. The saline phosphate buffer (PBS) used by CRUZ-LEYVA & al. [8] was replaced with TEN buffer. Washed cells were resuspended in 20 µL TEN buffer and then lysed using glass beads with a mortar and pestle and liquid nitrogen. After lysing, 500 µL TRIzol Reagent (Invitrogen™, Eugene, OR, USA) were added and incubated at room temperature for 10 min. TRIzol was used instead of the thermic shock and CTAB-based buffer reported by GASIC & al. [7]. The vials were centrifuged at 10,000 x g for 15 min at 4 °C, 500 µL chloroform-isoamylic alcohol (24:1 v/v) added and the solution mixed for 2 minutes to eliminate polysaccharides and cellular residues. They were then centrifuged again at 10,000 x g for 15 min at 4 °C, the supernatant recovered in a fresh, cold microcentrifuge tube and 300 µL isopropanol and 500 µL 7.5 M LiCl added. The tubes were mixed gently and incubated for 12 h at 4 °C to precipitate the RNA. After incubation, the tubes were centrifuged at 10,000 x g for 30 min at 4 °C. The supernatant was discarded and the mgRNA pellet air-dried at room temperature and resuspended in 20 µL DEPC-treated water. A final

precipitation was done with 1 vol SSC (0.8 M sodium citrate, 1 M sodium chloride) and 5 vol cold ethanol. This double precipitation was done according to the methodology of GASIC & al. [7]. Tubes were incubated for 3 hours at -20 °C and then centrifuged at 10,000 x g for 30 min at 4 °C. The mgRNA pellet was washed twice with 500 µL ethanol (75% V/V), air-dried, resuspended in 25 µL DEPC-treated double distilled water and stored at -80 °C until use.

Assessment of RNA quantity and quality

RNA was quantified with a spectrophotometer (NanoDrop, Thermo Scientific) at 260/ 280 nm. Its integrity was verified by electrophoresis in 1.2% agarose gel in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). The gel was viewed and recorded with a Gel Doc™ System (BioRad).

Functional RNA analysis by RT-PCR

Functional analysis was carried by expression of catechol 2, 3 dioxygenase gene at different time exposition of crude oil. One hundred nanograms (ng) of each mgRNA obtained from different exposition time (4, 8 and 12 days) to crude oil were transcribed into a single strand of cDNA using SuperScript® III Transcriptase Invitrogen™, with random primers and a mix of ten 11-mer primers targeting the Shine-Dalgarno sequence of bacterial mRNA (Fislage & al. [11]). After reverse transcription, a PCR was run with the primers XyElF (5'-CTGAAAGGTATGGCGGCTGTG-3') and XyElR (5'-CATCAGGTCAGCACGGTCAT-3') and targeting a 724 bp fragment of gene coding for catechol 2, 3-dioxygenase (NYYSSÖNEN & al. [4]). The PCR was set to a final volume of 25 µL containing 1X reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% stabilizer); 3.0 mM $MgCl_2$; 0.4mM dNTPs mix; 0.4 mM of each primer; 1 U Taq Polymerase (Invitrogen™); 14.75 µL H_2O and 50 ng of cDNAs as template. The PCR conditions were initial denaturation at 90 °C for 5 min; 40 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 5 min. A positive control was included of mgDNA obtained from the same culture used for mgRNA preparation, as well as a negative control with ddH_2O instead of cDNA. PCR products were viewed in 1.2% agarose gel in 1x TAE buffer, as described above.

Results and Discussion

An initial attempt was made to isolate RNA from a microbial consortium in liquid culture during hydrocarbon biodegradation using the protocols reported by GASIC & al. [7] and CRUZ-LEYVA & al. [8], but no mgRNA was isolated (Fig 1A). This may have occurred because the protocol published by GASIC & al. [7] was originally designed for RNA isolation from apple tissues rich in polyphenols and polysaccharides, and requires large amounts of sample (1 g). Likewise, the method reported by CRUZ-LEYVA & al. [8] was originally used for RNA isolation from bacterial flora associated with octopus.

The presence of high concentrations of hydrocarbons, phenolic substances and RNases can hamper the RNA isolation process. It is well known that extraction conditions (e.g. RNA source [water or soil pollutants], sampling and isolation technique) can affect reproducibility, and therefore the relevance, of gene expression profile results. Therefore, a series of modifications were made to the GASIC & al. [7] and CRUZ-LEYVA & al. [8] protocols. One key step was application of initial washes with TEN buffer in compassion to PBS buffer reported by CRUZ-LEYVA & al. [8], TEN buffer helped to eliminate residual hydrocarbons. Use of liquid nitrogen and glass beads helped to release cellular components, thus avoiding RNA degradation. The TRIzol reagent concentration was lowered to 0.5 mL, below manufacturer recommendations. Use of TRIzol improved results compared to the extraction buffer reported by GASIC & al. [7] as could be visualized in Fig.1A. These modifications resulted in a protocol that allowed isolation of high quality mgRNA from liquid cultures

containing crude oil. When viewed on 1.2% agarose gels, the ribosomal RNA bands were sharp; indeed, characteristic undegraded 23S and 16S bands were visible for all tested mgRNA samples (Fig 1B).

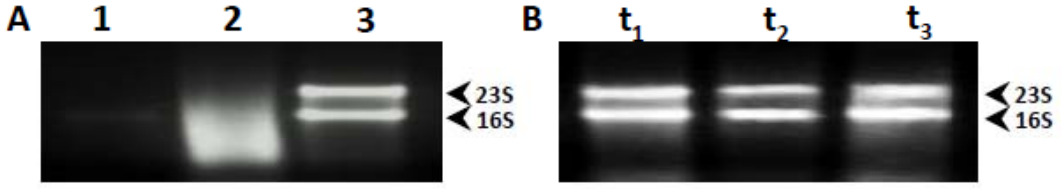

Figure 1- Integrity of mgRNA shown by electrophoresis in 1.2% agarose gel. (A) Lane 1, RNA extraction following CRUZ-LEYVA & al. [8] protocol; Lane 2, GASIC & al. [7] protocol; Lane 3, present protocol. (B) t_1, mgRNA sample isolated after 4 days exposure to crude oil; t_2, mgRNA sample after 8 days; t_3, mgRNA sample after 12 days.

Table 1- Comparison of RNA isolation methods.

Method	RNA [ng/µL] ratio	$A_{260/280}$ ratio	Sample source
Present protocol	85.6 to 120.9	2.07 to 2.09	Microbial consortia with hydrocarbons
ROSIC AND HOEGH-GULDBERG [5]	47.95 to 159.05	1.77 to 2.12	*Symbiodinium* sp. in f/2 medium
SHARKEY & al. [6]	100.2 to 116.6	Not reported	*Geobacillus thermoleovorans* in nutrient broth
GASIC & al. [7]	4.8 to 89.8	1.91 to 2.02	Apple tissues

Concentrations ranged from 85.6 to 120.9 ng/µL, indicating low protein contamination, and $A_{260/280}$ ratios ranged from 2.07 to 2.09 (Table 1). RNA purity and concentrations were similar to those reported by ROSIC AND HOEGH-GULDBERG [5]; SHARKEY & al. [6] and GASIC & al. [7], those methods are based on commercial kits, TRIzol reagent, a lysing matrix containing a mixture of ceramic and glass beads, and CTAB buffer extraction. The present protocol is an easier and cheaper alternative for isolating high quality RNA of a microbial consortium in presence of recalcitrant compounds such as crude oil. A final addition in the present protocol was the treating of all glassware with 0.1% DEPC, and baking of the glass beads overnight at 180 °C. Also, use of 0.1% DEPC-treated water in solution preparation and the final resuspension buffer kept the isolated RNA free of RNase activity (CRUZ-LEYVA & al. [8]; Sessitsch & al. [12]).

Funtional analysis was done through isolated mgRNA conversion to cDNA using the SuperScript kit (Invitrogen™), and changes in catechol 2, 3-dioxygenase gene expression analyzed by PCR. Changes in expression levels of *XyEl* allowed observation of microbial growth at different times when consortium was exposed to crude oil (Fig 2). Change in the catechol 2, 3-dioxygenase gene is related to degradation pathways of monoaromatic hydrocarbons presents in crude oil (toluene, benzene, xylenes, phenol, biphenyl and naphthalene), catalyzing meta-cleavage of the aromatic ring (MESARCH & al.[3]). Expression was higher at 8 days exposure than at 4 and 12 days, possibly due to initial presence of aromatic hydrocarbons in the crude oil and presence of the catechol-like intermediate. A single *XyEl*-related band was observed between 506 and 1018 pb (Figure 2). A second band was also present, and may be due to different copy *XyEl* genes present in other non-*Pseudomonas* bacteria since the primers used here were designed from the *Pseudomonas putida XyEl* gene (NYYSSÖNEN & al. [4]).

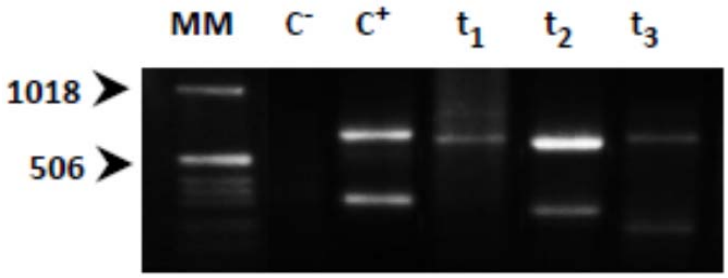

Figure 2- RT-PCR results using specific primers from *XyEl* gene. PCR products were separated by electrophoresis on a 1.0% (w/v) agarose gel. MM, 1 kb molecular marker; C^-, negative control; C^+, DNA amplification as positive control; t_1, cDNA sample isolated after 4 days exposure to crude oil; t_2, cDNA sample isolated after 8 days; and t_3, cDNA sample isolated after 12 days.

Other authors have reported the presence of the *XyEl* gene in other genera and *Pseudomonas* species (MESARCH & al.[3]; NYYSSÖNEN & al. [4]; Táncsics & al. [13]; ZHANG & al. [1]), and FERRERO & al. [10] reported the coexistence or two distinct genes related to hydrocarbons degradation in *Pseudomonas*.

Conclusions

This is the first report of a protocol for mgRNA extraction from microbial consortia in liquid cultures containing crude oil. This protocol allows isolation of good quality mgRNA that was effectively used in analysis of *XyEl* gene expression, and provided evidence of its activity during crude oil degradation in liquid cultures.

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