METHOD PAPER

Lysis efficiency of standard DNA extraction methods for Halococcus spp. in an organic rich environment

S. Leuko F. Goh R. Ibáñez-Peral B. P. Burns
M. R. Walter B. A. Neilan

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Abstract The extraction of nucleic acids from a given environment marks a crucial and essential starting point in any molecular investigation. Members of Halococcus spp. are known for their rigid cell walls, and are thus difficult to lyse and could potentially be overlooked in an environment. Furthermore, the lack of a suitable lysis method hinders subsequent molecular analysis. The effects of six different DNA extraction methods were tested on Halococcus hamelinensis, Halococcus saccharolyticus and Halobacterium salinarum NRC-1 as well as on an organic rich, highly carbonated sediment from stromatolites spiked with Halococcus hamelinensis. The methods tested were based on physical disruption (boiling and freeze/thawing), chemical lysis (Triton X-100, potassium ethyl xanthogenate (XS) buffer and CTAB) and on enzymatic lysis (lysozyme). Results showed that boiling and freeze/thawing had little effect on the lysis of both Halococcus strains. Methods based on chemical lysis (Triton X-100, XS-buffer, and CTAB) showed the best results, however, Triton X-100 treatmemolecular analysis.

Keywords Halococcus · Cell lysis · XS-buffer · Organic rich environment · Stromatolites

Introduction

In the past decade, applications of molecular approaches have provided unique insights into the uncultured microbial communities of environments as they avoid certain biases inherent in traditional culture-based microbiological methods (Miller et al. 1999). However, investigating diversity with non-culturing approaches, avoiding culture-dependent methods, can also result in new obstacles that can significantly bias any study (Miller et al. 1999; v. Wintzingerode et al. 1997).

During a molecular investigation of an environment, the lysis of all microorganisms within the given habitat is important and has to comply with two requirements: the effective lysis of cells, and the removal of any possible inhibitors for further analysis (e.g. humic acids). Further-

the biodiversity present, and thus the choice of the optimal extraction method is critical.

Presently, many different DNA extraction methods exist (Leff et al. 1995; Ochsenreiter et al. 2002; Radax et al. 2001; Tillett and Neilan 2000), and are specifically designed for a particular environment and/or a particular family of microorganisms. However, each protocol usually includes one, two or all three of the following basic elements: physical disruption, chemical lysis and enzymatic lysis (Miller et al. 1999). Methods employing physical disruption techniques can be divided into four subgroups, that comprises freeze/thawing (Hugenholtz et al. 1998; Tsai and Olson 1991), grinding in liquid nitrogen (Zhou et al. 1996), ultrasonication (Gabig-Ciminska et al. 2005), and bead mill homogenization (Radax et al. 2001). A combination of freeze/thawing and bead mill homogenization is one of the most common techniques in use (Miller et al. 1999). Protocols belonging to the chemical lysis group can be further categorized into procedures that contain detergents, such as sodium dodecyl sulphate (Kuske et al. 1998; Ogram et al. 1987), potassium ethyl xanthogenate (XS) (Tillett and Neilan 2000) and cetyl trimethyl ammonium bromide (CTAB) (Wilson 1990), and procedures that contain various buffers (Miller et al. 1999). The enzymatic lysis employs specific enzymes for defined types of microorganisms, including lysozyme for Gram-negative bacteria, achromopeptidase for Gram-positive bacteria, and lyticase for fungal cells (Purdy 2005). A

121 C for 60 min (Zhou et al. 1996). Cells of Hcc. hamelinensis were washed three times with 4 M TN buffer (contained 4 M NaCl and 100 mM Tris, pH 7.4) and were mixed with the sterilised ground stromatolite to a final concentration of 10^6 and 10^4 cells/g ground stromatolite, respectively. A 0.5 g aliquot of ground stromatolite was used for each experiment. Unspiked ground stromatolite was used as a negative control for every extraction and subsequent PCR amplification.

DNA extraction methods

Extraction method A: boiling samples for 10 min

Pure cultures were resuspended in 200 l dH_2O, vortexed and boiled for 10 min. Following this step, samples were centrifuged for 5 min at 5,000g and supernatant was transferred into a fresh tube. Ground stromatolitic samples were resuspended in 500 l dH_2O and treated as stated above. DNA was purified as stated below.

Extraction method B: freeze/thawing cycles

Pure cultures were resuspended in 200 l dH_2O and vortexed for 1 min. Samples were placed at 40 C for 15 min and then transferred to 70 C for 15 min. This procedure was repeated five times. Following these steps, samples were centrifuged for 5 min at 5,000g and the supernatant was transferred into a fresh tube. Ground stromatolitic samples were resuspended in 500 l dH_2O and treated as stated above. DNA was purified as stated below.

Extraction method C: triton X-100

Pure cultures were resuspended in 40 l 0.1% Triton X-100 and 10 l 0.4 M NaCl. Samples were incubated for 5 min at 95 C and an additional 10 l of 1 M Tris–HCl pH 7.4 was added. Ground stromatolitic samples were resuspended in 400 l 0.1% Triton X-100 and 100 l of 0.4 M NaCl and additional 100 l of 1 M Tris–HCl pH 7.4 was added. Samples were centrifuged for 15 min at 12,000g and the supernatant was transferred into fresh tubes and DNA was purified as described below.

Extraction method D: potassium ethyl xanthogenate (XS) buffer

This method is based on the use of potassium ethyl xanthogenate followed by an incubation of the samples for 2 h at 65 C. Pure cultures were resuspended in 500 l XS buffer containing 1% potassium ethyl xanthogenate, 20 mM EDTA, 1% SDS, 800 mM ammonium acetate and 100 mM Tris–HCl pH 7.4. Ground stromatolitic samples were resuspended in 1 ml of XS buffer. Samples were vortexed for 2 min and incubated for 2 h at 65 C and briefly mixed every half hour. Following incubation, cells were vortexed for 10 s and transferred on ice for 10 min. Samples were then centrifuged for 10 min at 12,000g and the supernatant was collected in a fresh tube and DNA was purified as described below.

Extraction method E: cetyl trimethyl ammonium bromide (CTAB)

Pure cultures, as well as ground stromatolitic samples, were resuspended in 576 l TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA), 30 l 10% SDS, 3 l proteinase K (10 mg/ml) and 6 l RNase (10 mg/ml). The solution was incubated for 3 h at 37 C with intermittent shaking (150 rpm). Following this step, 100 l of 5 M NaCl and 80 l of CTAB (4.1 g NaCl and 10 g CTAB dissolved in 100 ml dH_2O) were added to the solution and incubated for 20 min at 65 C. To remove the remaining CTAB and proteins from the solution, 1 V of Chloroform:Isoamylalcohol (24:1) was added, the solution gently inverted and centrifuged for 5 min at 12,000g. The aqueous layer was transferred into a fresh tube and DNA was further purified as described below.

Extraction method F: Combination of enzymatic (lysozyme), chemical (SDS), and physical lysis methods (bead beating and thermal shocks)

Pure cultures and ground stromatolitic samples were resuspended in 500 l of TE buffer (100 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% SDS) and 50 mg glass beads. This mixture was vortexed for 2 min after which 25 l lysozyme (dissolved in 1% TE buffer) was added and the samples incubated for 1 h at 37 C. Following this step the samples were boiled for 10 min. Subsequently, the solution was vortexed for 2 min and 500 l lysis buffer (4% SDS, 50 mM Tris–HCl, 100 mM EDTA) and 20 l proteinase K (10 mg/ml) were added. Samples were vortexed and incubated for 1 h at 56 C. Finally, samples were freeze/thawed five times at 40 C and 70 C, respectively. Samples were centrifuged for 5 min at 5,000g and the supernatant was transferred into a fresh tube. DNA was purified as described below.

DNA purification

DNA extracted from pure cultures was purified using phenol–chloroform–isoamylalcohol (PCI) and isopropanol

precipitation as previously described by Sambrook et al. (1989). Purified DNA was resuspended in 100 l dH_2O and stored at 4

Table 1 Comparison of DNA yield from pure cultures, subjected to different extraction methods

Treatment	Hcc. saccarolyticus	Hcc. hamelinensis	Hbt. salinarum NRC-1
(A) Boiling	96 (31%)	86 (13%)	239 (20%)
(B) Freeze/thawing	71.6 (10.2%)	46 (8%)	347 (36%)
(C) Triton X-100	90 (13.2%)	119 (14%)	353 (30%)
(D) XS–buffer	188 (29.1%)	209 (37%)	400 (36%)
(E) Lysozyme	242.7 (3.3%)	296.3 (12.4%)	449.1 (15.4%)
(F) CTAB	203.5 (30%)	193.7 (21.1%)	378 (16.9%)

DNA yields were measured using the Nanodrop ND-1000 spectrophotometer and the average yield of at least triplicate DNA extractions is given in ng/ l. Standard deviation is indicated in brackets

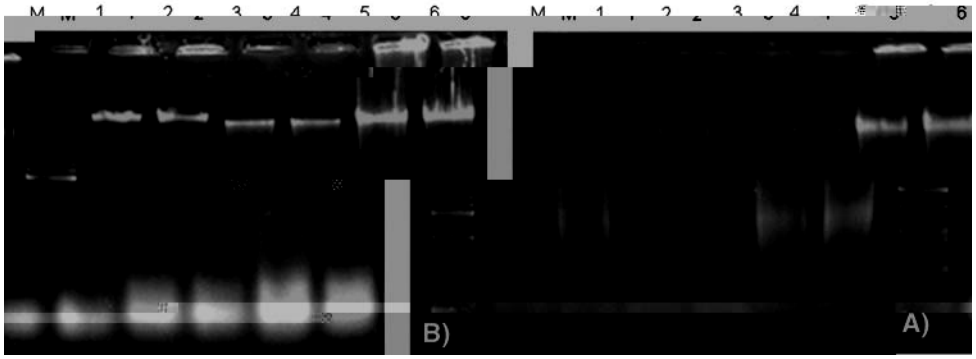

Fig. 1 Agarose gel electrophoresis of total DNA extraction from Hcc. saccharolyticus, Hcc. hamelinensis and Hbt. salinarum NRC-1 using (a) XS-buffer and (b) lysozyme. Lane M: Molecular marker; lanes 1 and 2 DNA isolated from Hcc. saccharolyticus; lanes 3 and 4 DNA isolated from Hcc hamelinensis; lanes 5 and 6 DNA isolated from Hbt. salinarum NRC-1

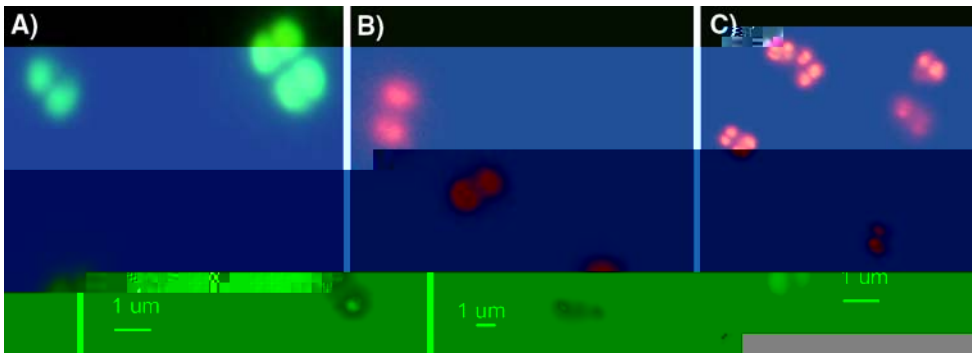

Fig. 2 Picture (a) shows a representative sample of Hcc. hamelinensis stained with the LIVE/DEAD [1] BacLight Kit without treatment. In comparison, cells of Hcc. saccharolyticus (b) and Hcc. hamelinensis (c) stained with the LIVE/DEAD [1] BacLight Kit following extraction using the XS-buffer method. Intact cell wall can be seen, however cells are biologically inactive as indicated by the red fluorescence

colony forming units were observed following each extraction method.

Efficiency of lysis methods on seeded sediment

Stromatolitic samples were sterilised and spiked with a known concentration of Hcc. hamelinensis and the six previously tested methods were used to extract DNA from these samples. Autoclaved, unspiked stromatolite samples were used as a negative control. None of these methods could recover visible quantities of DNA using samples spiked with 10^4 cell/g, nor was it possible to obtain any PCR amplification following several steps of extraction and purification. For stromatolitic material spiked with 10^6 cells/g the XS-buffer method proved again to be the most efficient method, producing a clear genomic DNA band following extraction (Fig. 3, lane 6). Extraction

using CTAB was the only other method resulting in a visible band after gel electrophoresis (Fig. 3, lane 7). Every other method failed to produce a visible band of genomic DNA, and was not successful in PCR amplifications.

DNA purification and PCR efficiency

Following every extraction a PCR was performed to test the purity of the recovered DNA. Although three consecutive PCI and isopropanol extractions were performed [Method (I)] for all the extraction procedures, it did not result in sufficiently pure DNA suitable for further investigations. None of the resulting DNA samples allowed amplification of a PCR product. Spiking the samples with a known amount of DNA or dilutions of the obtained DNA used as a template also did not result in a PCR product. Purification and precipitation employing method (II) was more efficient than method (I) with a faint PCR product visible following two PCI extractions and isopropanol precipitation using DNA extracted with the XS-buffer method (Fig. 4a). Every other method failed to produce suitable DNA for PCR amplification following three puri-

though every method follows a clear protocol, the results often vary, due to the fact that there are many steps involved which can not be standardized, e.g. removing the aqueous phase following PCI extraction. During the course of investigations we encountered this problem in particular when trying to determine the quantity of DNA recovered (Table 1). While the extractions were repeated numerous times, the amount recovered varied strongly within a method, resulting in a high standard deviation. Despite the high standard deviations, the results allowed us to determine the efficiency of the cell lysis protocol.

The focus of this study was to test six lysis methods on two representatives of Halococcus spp. This genus is known for its tough and rigid cell wall (Kandler and König 1998). Methods solely based on physical disruption of cells (boiling and freeze/thawing) have been previously used to isolate DNA from microorganism such as yeast (Harju et al. 2004) and Mycobacterium avium (O'Mahony and Hill 2004), as well as from hypersaline environments (Antón et al. 2000). These methods, however, proved to be ineffective in lysing pure cultures of Hcc. hamelinensis and Hcc. saccharolyticus in the present study. These methods also failed to both extract visible amounts of DNA and provide sufficient DNA for PCR amplification from stromatolite samples.

Chemical lysis showed the best results with respect to cell lysis and DNA purity. Although all four chemical methods tested had some beneficial effects, the methods differed with respect to time consumption, DNA quality and quantity recovered. The method employing a combination of enzymatic lysis (lysozyme), chemical lysis (SDS) and physical disruption (bead beating and thermal shocks) resulted in the greatest DNA recovery, but sheared the DNA badly during the course of extraction and was also the most labour intensive. The CTAB and XS-buffer methods, both recovered less DNA compared to the combination of enzymatic, chemical lysis and physical disruption procedure. However, the CTAB and XS-buffer methods lyse the cells more gently, resulting in very little sheared DNA. Both methods recovered similar amounts of DNA with similar quality. Comparing both CTAB and XS-buffer methods, the XS-buffer has the advantage of only using one buffer and one incubation step compared to two incubation steps and more reagents required for the CTAB. The Triton X-100 method is less labour intensive and uses few reagents, however, only very low quantities of DNA could be recovered from pure cultures with no bands visible on an agarose gel (data not shown).

An important issue in current estimates of bacterial/archaeal diversity is related with the sensitivity of post DNA extraction PCR-based procedures (Luna et al. 2006). Several previous studies emphasise that the presence of Ca^{2+} ions interferes with Mg^{2+}, thus decreasing the PCR efficiency (Bickley et al. 1996; Wilson 1997). Stromatolites, largely composed of calcium carbonate in combination with organic material, present a difficult environmental sample for a successful DNA extraction and subsequent PCR reaction. None of the tested extraction methods completely removed all of the contaminants from these samples using one standard purification step. Additional purification steps after extraction using PCI were required to reduce polysaccharide and humic acid contaminations, respectively (Malik et al. 1994; Miller et al. 1999). During the course of this study we found that, using the standard purification method (I), three consecutive purification steps employing PCI and isopropanol did not result in sufficiently clean DNA for subsequent PCR amplification. However, by conducting an isopropanol step before the first PCI step [method (II)], only two purifications were necessary.

By applying this purification step (method II) to all six extraction methods we found the best results for this environment were obtained by using the XS-buffer method. All other procedures failed to provide DNA suitable for PCR under experimental conditions. Although PCR amplification following XS-buffer extraction could be observed, the amplification efficiency was weak suggesting that inhibitor may still have been present. To overcome this problem, diluting the samples provided a rapid and straightforward way of permitting amplification (Wilson 1997). Using a 1:100 dilution of DNA, we were able to amplify the 16S rRNA gene (Fig. 4), however 1:20 and 1:50 dilutions did not result in PCR amplification. Although diluting the recovered DNA resulted in an improved amplification, care must be taken with dilutions

Bickley J, Short JK, McDowell DG, Parkes HC (1996) Polymerase chain reaction (PCR) detection of