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High-yielding plasmid extraction method from acidophilic heterotrophic bacteria of the genus *Acidiphilium*

Samarendra K. Singh, Pataki C. Banerjee *

Indian Institute of Chemical Biology, Kolkata 700032, India

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Abstract

Plasmid yield from *Acidiphilium* strains always had been poor following various standard methods. We adopted some simple modifications in the alkaline lysis procedure to get a better yield of plasmid from these bacteria. An approximately 10- to 20-fold increase in the plasmid yield was achieved when harvested *Acidiphilium* cells were preincubated 16–20 h at pH 6 in nitrogen-free medium. Another independent approach showed that freezing (−18 to −20 °C) of the harvested cells initially and at two subsequent steps in the alkaline lysis procedure of plasmid DNA extraction improved the yield further by 1.5- to 3-fold. The combination of these changes yielded at least 15- to 30-fold more plasmid from various *Acidiphilium* strains as compared with standard methods.

Keywords: *Acidiphilium cryptum*; *Acidiphilium multivorum*; *Acidiphilium symbioticum*; Plasmid preparation

The natural habitat of many acidophilic bacteria includes sulfide mining regions and acidic mine waters. These microorganisms cause natural leaching of ores and are being exploited in industrial operations for the recovery of metals from low-grade and recalcitrant ores [1,2]. Plasmid vectors are the tools through which genetic engineering of bacterial cells is widely implemented. Although many natural and chimeric plasmid vectors have been developed and used for a wide variety of bacteria [3], there exists a dearth in the case of these acidophilic bacteria [4]. Although the majority of the chemolithoautotrophic (*Acidithiobacillus ferrooxidans*) and heterotrophic (*Acidiphilium* species) biomining bacteria contain one or more plasmids [4,5], studies on these genetic elements are limited. With a view to explore the plasmids of *Acidiphilium* species as vectors for biomining bacteria, we prepared plasmid DNA from *Acidiphilium* strains following various published and commercial methods [6–10], but the yields always were very poor. In this article, we describe simple modifications to the plasmid isolation protocol that yielded at least 15- to 30-fold more plasmid DNA from the acidophilic heterotrophic bacterial strains of the genus *Acidiphilium*.

Materials and methods

Bacterial strains

The *Acidiphilium* [11] strains used in this work were *Acidiphilium cryptum* BS18, RB4, RB8 [12], and P2 [13]; *Acidiphilium multivorum* JCM8867 [14]; and *Acidiphilium symbioticum* H8 [15]. *Escherichia coli* DH5α [16] was used as the transforming strain for cloning of plasmid DNA fragments.

Media and solutions

MGY (mineral salt–glucose–yeast extract)[1] medium [15] was composed of 2 g/L $(NH_4)_2SO_4$, 0.25 g/L K_2HPO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KCl, 1.0 g/L glucose, and

* Corresponding author. Fax: +91 33 24735197.
E-mail address: pcbanerjee@iicb.res.in (P.C. Banerjee).

[1] *Abbreviations used:* MGY, mineral salt–glucose–yeast extract; LB, Luria–Bertani; TAE, Tris–acetate–EDTA; TE, Tris–EDTA; SAP, shrimp alkaline phosphatase.

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0.1 g/L yeast extract, with pH adjusted to 3 with 10 N H_2SO_4 before sterilization. MGY1 medium was composed of 0.3 g/L K_2HPO_4, 0.8 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L KCl, 10 g/L glucose, and 1 g/L yeast extract, with pH adjusted to 6 with 1 N H_2SO_4 before sterilization. Glucose solutions (10×) were sterilized separately and mixed with proportionate volumes of the respective sterile solution of other components to attain the final medium composition. Luria–Bertani (LB) broth and nutrient agar-containing ampicillin (100 μg/ml) were prepared as described by Sambrook and coworkers [16].

The following reagents used in plasmid preparation were prepared according to Sambrook and coworkers [16]: cocktail lysozyme solution (50 mM glucose, 25 mM Tris buffer [pH 8], 10 mM EDTA [pH 8], 5 mg/ml lysozyme); solution 1 (50 mM glucose, 25 mM Tris buffer [pH 8], 10 mM EDTA [pH 8]); solution 2 (0.2 M NaOH, 1% SDS); solution 3 (5 M potassium acetate, 2 M glacial acetic acid); TAE (Tris–acetate–EDTA: 40 mM Tris base, 19.74 mM glacial acetic acid, 1 mM EDTA [pH 8]); and TE (Tris–EDTA: 10 mM Tris buffer [pH 8], 1 mM EDTA [pH 8]).

Culture conditions and harvesting of cells

Acidophilic strains were routinely maintained in MGY medium. Bacteria were grown at 30 °C on a rotary shaker (250 rpm) to late log or early stationary phase (30–48 h, 0.8–1.0 OD_{660}). Half of the culture volume was centrifuged, and the cell pellet was suspended in an equal volume of nongrowth MGY1 medium. The suspension was incubated for 16–18 h at 30 °C with shaking (150 rpm). The other half of the culture (in MGY medium) was also incubated similarly. Harvested cells from the above two incubations were either treated immediately for plasmid extraction or preserved at approximately −20 °C before processing.

E. coli DH5α and its derivatives were grown in LB broth with shaking (300 rpm) and on antibiotic-enriched nutrient agar medium, respectively, at 37 °C overnight.

Agarose gel electrophoresis, densitometric scanning, and other methods

A modified alkaline lysis method of Birnboim and Doly [6] was applied for plasmid preparation from the *Acidiphilium* strains [16] and the transformed *E. coli* DH5α [17]. Quantification and purity of the DNA preparations were monitored from the ratios of absorbance values at 234, 260, and 280 nm [17].

Agarose (medium EEO, 0.6%, w/v) gel was cast in TAE buffer and run in the same buffer. The samples loaded contained either the same amount of DNA or plasmid DNA prepared from culture volumes that contained equal amounts of whole cell protein. Documentation and densitometric scanning of the gels were done by the Bio-Rad gel documentation system. Intensity of the corresponding bands was analyzed through Quantity-1 software.

Cloning and sequencing of plasmid DNA

Restriction endonucleases and T4 DNA ligase were purchased from Gibco-BRL. Shrimp alkaline phosphatase (SAP) and pUC19 plasmid vector were obtained from Promega. Restriction digestion of purified plasmids, purification of plasmid DNA bands from the gel, ligation of DNA fragments in pUC19, preparation of *E. coli* competent cells, transformation in *E. coli*, and selection of transformants were done following standard procedures [16].

One of the *Hin*dIII-digested plasmid DNA fragments of approximately 0.7 kb size from the *A. symbioticum* H8 strain was sequenced after cloning in pUC19. Universal primers M13 forward and M13 reverse were used for sequencing the clones. Sequencing reactions were performed with ABI dye terminator sequencing reagents using an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Results

The plasmid preparation methodology that was finalized at different steps after several trial experiments is outlined in Table 1. Ratios of absorbance values of plasmid preparations at 234, 260, and 280 nm confirmed that the samples consisted primarily of DNA.

The amounts of plasmid extracted from *A. symbioticum* H8 by introducing various modifications are shown in Table 2. In comparison with the small amount (0.40 μg) of plasmid DNA obtained per milliliter culture following the standard procedure, 31-fold more plasmid DNA could be recovered following the modified method. A significant increase (~22-fold) in plasmid yield resulted when the harvested cells were preincubated in an ammonium-free medium (first modification, Table 1, step 2). The yield of plasmid increased further by freezing the cells before and after the addition of solution 1 (second and third modifications, Table 1, steps 3 and 4). An approximately 1.5-fold increase in DNA resulted when the cells were frozen before lysis by the addition of solution 1 (Table 2). More (~3-fold) plasmid DNA was extracted when the cells plus the solution 1 mixture were frozen for a brief period (Table 2). It was also noted that, in the gel electrophoretogram, plasmid prepared from preincubated harvested cells exhibited not only strong DNA bands but also additional high- and low-molecular size plasmid bands (Fig. 1A, "Exp") in comparison with those observed with plasmids prepared from non-incubated control cells (Fig. 1A, "Std"). Densitometric scanning of the DNA bands in the gel revealed at least a 10-fold increase in concentration of each band in the experimental set.

For the *A. multivorum* strain, quantification of plasmid DNA prepared by the modified method revealed at least a 25-fold increase in the yields in various preparations in comparison with those obtained following the standard protocol. The plasmid bands from the strain prepared by the adopted method (i.e., preincubation of cells at pH 6 and