Purification of an *Enterobacter aerogenes* plasmid DNA using MnCl_2 as compaction agent

Sandhya R. Shenoy, Mohammad Khalid, Anshu Gupta, Rajni Singh, Sunil K. Khare,^{*} and Munishwar N. Gupta

Chemistry Department, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

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Development of an efficient process for purification of DNA plasmids continues to be a technological challenge [1-3]. While earlier applications of DNA plasmids were mostly in cloning, the last few years have seen its increasing applications in gene therapy [1] and preparation of DNA vaccines [4]. A crucial step in the production process is the downstream processing stage which encompasses separation of plasmid DNA from cellular components of the host strain (including chromosomal DNA and its fragments). In the case where the intended applications are in therapeutics, it is also necessary to separate the plasmid from materials such as toxic solvents, mutagenic reagents, and animal-derived enzymes (e.g., bovine pancreatic RNase A, used for removal of abundant RNA present in cell lysate) which may have been used in the production process. The existing protocols for plasmid production are, in general, time consuming, expensive, and difficult to scale-up [5]. Almost all of these require at least one chromatographic step although recently a process using a multicompartiment electrolyser separated by ultrafiltration has been described [6].

In this paper, we describe a mini-prep protocol that is a simple, inexpensive, and quick method for plasmid purification which in principle can be easily scaled-up. As the protocol described here does not require any aggressive chemicals and/or use any animal-origin enzymes it is potentially useful for therapeutic applications. The method is based upon two observations reported earlier by others: the possibility of plasmid DNA purification by selective precipitation [5,7] and condensation of DNA induced by MnCl_2 [8]. The protocol described herein has been developed for purification

of plasmid DNA from *Enterobacter aerogenes* and *Escherichia coli* but should be of general utility. *E. aerogenes* used here is a bacterial strain reported from our laboratory recently [9].

Materials and methods

Plasmid DNA isolation. Overnight cultures of *E. aerogenes* and *E. coli* were subjected to alkaline lysis following the protocol of Birnboim and Doly [10]. Cells were collected by centrifugation at 13,500g at 4°C for 30 min and resuspended in 100 μl of GTE buffer (50 mM glucose, 25 mM Tris/HCl, 10mM EDTA, pH 8.0). Then 200 μl freshly prepared lysis solution (1% SDS, 0.2 M NaOH) was added into the cell suspension, gently mixed, and held on ice for 5 min; 150 μl neutralization solution (3 M sodium acetate solution) was then added into the lysate, gently mixed, and held on ice for another 5 min. After the removal of cellular debris by centrifugation at 13,500g, at 4°C for 15 min, the supernatant was extracted with equal volume of isopropanol and allowed to stand at room temperature for 30 min. The plasmid DNA was recovered in the supernatant by centrifugation at 13,500g at 4°C for 15 min. Isolated plasmid DNA was extracted with equal volumes of phenol/ CHCl_3 , precipitated with two volumes of cold ethanol and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The yield of purified plasmid was calculated by using *To talLab V2.01* software, Model GelDoc Mega-V 4.00 gel documentation system (Biosystematica, UK).

Compaction agent precipitation. The plasmid DNA(s) was precipitated by the compaction agent MnCl_2 . The solution of plasmid DNA (400 μl) was precipitated with an equal volume of 10 mM MnCl_2 at room temperature.

After vortexing, the plasmid was pelleted in a microcentrifuge at 13,500g for 5min and washed with 800 μ l 50% isopropanol solution of 10 mM $MgCl_2$, 300 mM NaCl, and 25 mM EDTA. The whole process took about 12-15 min. The preparation was washed repeatedly (three times) and finally suspended in TE buffer. $MnCl_2$ concentration in final product was found to be 0.02 ppm by atomic absorption spectrophotometer (Perkin-Elmer, USA). The purified plasmids were analyzed using agarose gel electrophoresis.

Protein analysis. Protein concentration was measured by the bicinchonic acid (BCA)¹ assay [11]. Samples (20 μ l) were added to 2 ml of BCA reagent (Sigma Chemical Co., USA; Cat. D 8284) in test tubes and incubated for 1 h at 37 °C. Absorbance was measured at 562 nm. A calibration curve was made with bovine serum albumin standards. The purified plasmid pools were analyzed by sodium dodecyl-polyacrylamide gel (12%) electrophoresis (SDS-PAGE) using a vertical slab gel electrophoresis unit from Bangalore Genei, Bangalore, India. Samples were run at 100 V and visualized using silver staining.

Results and discussion

The selectivity of precipitation can be seen in Fig. 1, which illustrates a typical compaction agent-based plasmid purification of *E. aerogenes*. Lane 1 is a plasmid ladder, *k* *Hind*III size marker; lane 2 is a pellet after ethanol precipitation (plasmid and RNA); lane 3 is the resuspended pellet of the compaction precipitation with $MnCl_2$ (showing pure plasmid only). Fig. 2 illustrates compaction agent-based plasmid purification of *E. coli*, demonstrating the generality of the method. The plasmids DNA yields were found to be 94 and 97% in the cases of *E. aerogenes* and *E. coli*, respectively. Figs. 1 and 2 clearly show the removal of RNA and residual g-DNA and the absence of any plasmid isoform, as only a single band of purified plasmid is seen in lane 3. Protein was undetectable by the BCA assay. SDS-PAGE of the purified plasmid pools of *E. aerogenes* and *E. coli* showed no protein contamination.

In this process, the primary contribution of the compaction agent is to remove abundant RNA by selectively precipitating plasmid DNA only. Compaction of DNA involves charge neutralization in combination with stabilization of inter-helix interactions. The compaction agent binds in either the major or the minor groove, in direct contact with negatively charged phosphate groups. Precipitation occurs when adjacent DNA helices are affected simultaneously, with the compaction

Fig. 1. A 1% agarose gel showing purification of DNA from *Enterobacter aerogenes*. Lane 1 is a plasmid ladder, *k* *Hind*III size marker; lane 2 is a pellet after ethanol precipitation (plasmid and RNA); lane 3 is the resuspended pellet of the compaction precipitation with $MnCl_2$ (pure plasmid free of RNA).

agent not only reducing the helix-helix repulsion but also bridging the helices [5].

Nearly all the DNA in living organisms is negatively supercoiled. Ma and Bloomfield [8] have reported that supercoiling appears to cooperate with Mn^{2+} in stabilizing helix distortions, which provides a pressure which enhances lateral association and condenses DNA. The mechanism of condensation of plasmid DNA by Mn^{2+} appears to be quite different from that believed to underlie the more extensively studied condensation induced by higher-valence cations. Divalent cation Mn^{2+} condenses DNA in water-alcohol mixtures as opposed to multivalent cations that condense DNA in aqueous solution [12]. Supercoiling brings long stretches of the DNA into close, nearly parallel juxtaposition; Mn^{2+} like any cation, facilitates this close approach by reducing the columbic repulsion between DNA segments, which somehow produce, or augment, an attractive force between helices condensing DNA [8].

We have used this *E. aerogenes* strain as a model system for the purification of plasmid DNA [13], using

¹ Abbreviation used: BCA, bicinchonic acid.

Fig. 2. A 1% agarose gel showing purification of DNA from *Escherichia coli*, demonstrating the generality of the method. Lane 1 is a plasmid ladder, *k* *HindIII* size marker; lane 2 is a pellet after ethanol precipitation (plasmid and RNA); lane 3 is the resuspended pellet of the compaction precipitation with $MnCl_2$ (pure plasmid free of RNA).

selective precipitation by a compaction agent, $MnCl_2$. This strain of *E. aerogenes* is promising as it is reported to exhibit a unique solvent-tolerant property [9]. The generality of the method has been demonstrated using *E. coli* strain as another parallel system.

To assess the suitability of purified plasmids for molecular biological applications, the transformation of competent *E. coli* $\delta DH 5\alpha$ cells by purified *E. aerogenes* plasmid was carried out as a preliminary experiment. The *Enterobacter* plasmid is known to carry a mercury-resistant marker. The transformed *E. coli* cells were able to grow in the presence of mercury after transformation. These results with regard to simplicity of method, purity, yield, and biological activity of purified plasmid

make the described method a promising technique for plasmid purification.

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