Use of a ‘Miniprep’ for rapid extraction of plasmids from vancomycin- and gentamicin-resistant Enterococcus faecium

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Summary

Conventional methods of plasmid extraction are largely unsuited to diagnostic laboratories. The ‘Miniprep’ is a rapid method that utilises a centrifugable column to separate plasmid DNA from chromosomal DNA. We have modified this technique to extract plasmid DNA from seven strains of vancomycin- and gentamicin-resistant Enterococcus faecium (VGREF): 1% mannitol was added to the growth medium and cell lysis was achieved by incubation in 10 mg of lysozyme/ml in 10 mM Tris, 1 mM EDTA and 25% sucrose at pH 8.0. RNase A was added to plasmid eluate rather than at the lytic step. In comparison to a standard phenol/chloroform method, Miniprep completely eliminated chromosomal interference in gel electrophoresis but otherwise produced identical plasmid profiles. Plasmids obtained from the VGREF ranged from 42 to 1.3 Md. Band densities on a single elution from the Miniprep varied from 8.3 to 106.3 relative units. Double elution increased band densities from the same preparation from 30.4 to 196 relative units; mean percentage increases per track between 7.0 and 34.6%. This method is suitable to achieve plasmid DNA extraction from VGREF within 1 h, making the process more suitable for diagnostic laboratories.

Introduction

Plasmid analysis is useful in the study of the epidemiology of resistance of nosocomial gram-positive cocci (Schaberg and Zervos 1986). Rapid techniques for the isolation of plasmid DNA were described in the late 1970s (Birnboim & Doly 1979; Broda 1979) and have since been the subject of various modifications (Sambrook et al. 1989). However, conventional methods use agents that are toxic or carcinogenic and do not eliminate chromosomal DNA from plasmid preparations. These methods are also subject to considerable variability and take several hours to complete, making them unsuitable for diagnostic laboratories.

A recent introduction in molecular biology is the ‘Miniprep’ method, which allows extraction of plasmid DNA from genetically engineered micro-organisms such as Escherichia coli to be completed in about 1 h. This methodology uses a miniature silica membrane column which eliminates chromosomal contamination and yields plasmid DNA of sufficient purity for use in further molecular manipulations without additional processing.

We describe here our experience and modification of a Miniprep method to extract plasmids from isolates of vancomycin- and gentamicin-resistant Enterococcus faecium (VGREF).

Materials and methods

Bacterial strains

(a) Seven epidemiologically distinct clinical isolates of VGREF namely E316, S635, S644, S649, S624, S326 and E418 were cultured from liver patients at King’s College Hospital. In addition, two vancomycin- and gentamicin-resistant transconjugants namely GE-2 and GE-3 were used; the recipient was a plasmid-free strain of E. faecium, GE-1, that was kindly provided by N. Woodford at the Division of Hospital Infection, Central Public Health Laboratory, but was originally isolated by Eliopoulos et al. (1988).
(b) E. coli strains 39R861 (Threlfall et al. 1986) and V517 (Macrina et al. 1978) that contained plasmids
of known molecular weight were used for molecular weight markers.

**Media and reagents**

Luria Bertani broth (Sambrook et al. 1989) was used as growth medium but this was supplemented with 1% mannitol, vancomycin (8 mg/l) and gentamicin (40 mg/l). ‘QIA prep-spin’ columns and associated buffers ‘P2, PB, PE, and N3’ were supplied as one kit by Qiagen Ltd. (UK). Freshly prepared Tris-EDTA buffer containing 25% sucrose and 10 mg of lysozyme/ml, and Tris-Borate-EDTA buffer were prepared according to Sambrook et al. (1989).

**Extraction of plasmid DNA using the Miniprep**

After overnight culture in Luria Bertani broth at 37 °C, VGREF cells were harvested by centrifugation at 3000 rev/min. Centrifugation was for 30 min, but at 4 °C (not at room temperature as in the commercial method). The cells were resuspended in 100 μl of freshly prepared TE buffer but the lysis conditions were different to the commercial method in that Tris-EDTA containing 25% sucrose and 10 mg lysozyme/ml was used. The lysis mixture was incubated at 37 °C for 35 min to form a lysate but not at room temperature as in the commercial method.

All further centrifugation was carried out in an Eppendorf centrifuge operating at 13,000 rev/min. A sample of 250 μl of buffer P2 containing NaOH/SDS (Birnboim 1983) was added to the lysate and the sample left for 5 min at room temperature. Unlike the commercial method, 350 μl of N3 buffer which contained guanidine hydrochloride, was chilled on ice before being added and mixed by gentle inversion, and then kept on ice for 5 min. The samples were then centrifuged for 10 min and the supernatant decanted onto the QIAprep-spin column, which was then placed in a clean 2 ml microcentrifuge tube and centrifuged for 30–60 s. The QIAprep-spin column was washed by adding 0.5 ml of PB buffer that contained guanidine hydrochloride, and then centrifuged for 30–60 s. The column was finally washed with 0.75 ml of PE buffer that contained 96–100% ethanol, and centrifuged at 13,000 rev/min for 30–60 s. Plasmid DNA was eluted from the column by adding 100 μl of 10 mM Tris/HCl pH 8.5 or TE pH 8.5 to the column, followed by centrifugation for 60 s. A major change that was introduced was a second elution of DNA for all plasmid preparations. Before electrophoresis, RNase A was added to each sample to achieve a final concentration of 1 mg/ml.

None of the steps in the commercial method, or in the modifications to them, required the use of phenol/chloroform extraction.

The *E. coli* strains 39R861 and V517 with plasmids of known molecular weight, were processed in a similar fashion, according to the protocol described in the Qiagen handbook (Qiagen 1999).

**Comparator method**

The comparator method for rapid extraction of plasmid DNA (Woodford et al. 1992) was a standard modification of the rapid alkaline extraction method of Birnboim & Doly (1979). The standard modification involves lysing the bacterial cells in a lysozyme solution that contained 25% sucrose instead of glucose and allowing time for lysis by incubation for 35 min at 37 °C. In addition, the standard modifications require phenol/chloroform extraction which complexes with proteins in the preparation (Woodford et al. 1992). The *E. coli* controls were processed in the same manner.

**Electrophoresis of DNA and analysis of gels**

Plasmid DNA was analysed by gel electrophoresis in 0.7% agarose gel as previously described, using TBE buffer (Sambrook et al. 1989) and a ‘Sub-Cell Submarine’ unit (Bio-Rad, UK) run at 80 V for 2 h. Ethidium bromide at a final concentration of 1 mg/l was added to the agarose which was cooled to 50 °C before pouring. Gels were illuminated with ultraviolet and photographed with an MP4 Polaroid camera fitted with a Wratten filter type 22A.

Molecular weights were determined from the known standards contained in the *E. coli* control strains, by using an inverse logarithmic formula (Sealey & Southern, 1982). The relative grey-scale density of bands was determined from the photographs by use of a CCD videocamera (Cobu Inc., USA) linked to a 486 PC running the BioScan ‘Snapshot Analyse’ programme (BioScan Inc., USA). The relative grey-scale density of paired tracks was analysed by the ‘paired t-test’ using ‘Statview’ (Abacus Concepts, Inc., USA).

**Results**

Addition of 1% mannitol to the Luria Bertani culture broth increased the yield of *E. faecium* for plasmid extraction after overnight incubation from 10⁷ to 10⁸ c.f.u/ml. The plasmid profiles of clinical isolates and transconjugants from VGREF are illustrated in Figures 1–3. Figure 1 shows the electrophoresis of DNA obtained from the seven patient isolates of VGREF plus two transconjugants, GE-2 and GE-3, by the comparator extraction method. Molecular weights of plasmid bands resolved in Figure 1 are shown in Table 1. The molecular weight markers in tracks 1 and 12 of Figure 1 clearly show interference characteristic of chromosomal DNA which appears at the top of each track and at the 27 Md position.

Figure 2 shows the plasmid profiles of the seven clinical isolates of VGREF and two transconjugants using the Miniprep. In comparison with Figure 1, it can be seen that the Miniprep eliminated the chromosomal DNA band interference but otherwise produced identical plasmid patterns. However, a band that is reported