Rapid Screening for Plasmid DNA

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Summary. A procedure is described for demonstrating plasmid DNA and its molecular weight, based on rate zonal centrifugation of unlabelled DNA in neutral sucrose gradients containing a low concentration of ethidium bromide. Each DNA species is then visualized as a discrete fluorescent band when the centrifuge tube is illuminated with ultra-violet light. Plasmids exist as closed circular and as relaxed circular molecules, which sediment separately, but during preparation of lysates, closed circular molecules are nicked so that each plasmid forms only a single band of relaxed circles within the gradient.

1. Introduction

As a growing number of bacterial functions are tentatively being attributed to plasmids, there is a place for a simple and economical method for screening bacterial strains for the presence of plasmid DNA. The generally used methods depend on isotopic labelling of the bacterial DNA followed by either dye-buoyant isopycnic density gradient centrifugation or rate zonal centrifugation and measurement of the distribution of isotope within the gradient. Performed on any scale, these methods become extremely time-consuming whereas the procedure described here using unlabelled bacteria and visual identification of plasmid DNA achieves the same result far more rapidly.

In principle, unlabelled bacterial DNA is separated into predominantly plasmid and chromosomal fractions by differential centrifugation, and the plasmid fraction, after treatment with RNase, is analysed by rate zonal centrifugation through neutral sucrose gradients containing ethidium bromide. The subsequent position of plasmid DNA within the gradient, and hence its molecular weight, is determined by its fluorescence when the centrifuge tube is illuminated with ultra-violet light.

2. Materials and Methods

Details of the procedure are first described, followed by notes in the succeeding section.

Cultures. These were incubated at 37° on a reciprocating shaker (50 mm stroke; 100 strokes/min) in 250 ml capped conical flasks containing 50 ml YE2 broth (NaH₂PO₄·2H₂O, 3 g; K₂HPO₄, 14 g; NH₄Cl, 5 g; NH₄NO₃, 1 g; Na₂SO₄, 2 g; Oxoid acid-hydrolysed casein, 5 g; Oxoid yeast extract, 5 g; L-cysteine HCl, 50 mg; DL-tryptophan, 50 mg; and glucose, 2 g per 1 distilled water). After 18-22 h incubation, the optical density at 650 nm was 2.5-2.9. The organisms were deposited by low speed centrifugation and resuspended as evenly as possible by a pasteur pipette in 1.1 ml 0.85% (w/v) NaCl to give ca. 1.3 ml suspension (Note 1).

Lysis. One ml 5% (w/v) sodium dodecyl sulphate (SDS) and 3 ml TES4 (0.1 M-Tris, 0.07 M-di-Na EDTA, 0.05 M-NaCl, pH 8.0) were placed in a 5 ml polycarbonate centrifuge tube (MSE 3441-111). One ml bacterial suspension was squirted into this lysing mixture from a calibrated pasteur pipette and the tube immediately inverted several times to mix its contents. Lysis often began immediately and was completed by transferring the tube to a 60° water-bath for 8 min, by which time the dense milky bacterial suspension had become uniformly semi-translucent (Note 2).

Enrichment for Plasmid DNA. The lysate was centrifuged in the same polycarbonate tube for 15 min at 30,000 rpm at 20° in the SW50.1 rotor of a Spinco L3-40 centrifuge. This deposited most of the chromosomal DNA (Note 3), leaving as the supernatant a 'cleared lysate' containing most of the plasmid DNA (Clewell and Helinski, 1969). The upper 3.0-3.5 ml supernatant was retained, the remainder being discarded with the deposit (Note 4).

RNase. The cleared lysate was placed either at 4° overnight or at 0° for 2-4 h until most of the SDS precipitated. The supernatant was removed and mixed with 0.1 vol RNase solution, 250 µg/ml (E.C.2.7.7.16. Bovine pancreatic ribonuclease, British Drug Houses, Poole, Dorset, England, ref. 39039. This was dissolved in distilled water and heated in a bath of boiling water for 10 min to inactivate contaminating DNase). The mixture was incubated at 37° for 30 min (Note 5). Finally, 0.1 vol ethidium bromide (40 µg/ml) was added (Note 6).
Sucrose Gradients. All sucrose solutions were prepared in TES4 and were autoclaved at 121°C for 15 min to inactivate nucleases. Ethidium bromide was added to 4 μg/ml (Note 6). Linear sucrose gradients were prepared in 5 ml cellulose nitrate centrifuge tubes (Spinco 305050) by diffusion overnight at room temperature with the tubes capped to prevent evaporation. Each tube received 0.6 ml 21% (w/v) sucrose followed by 1.1 ml each of 17%, 13%, 9%, and 5% (w/v) sucrose.

Centrifugation. A sample of 0.2-0.3 ml cleared lysate containing ethidium bromide was placed on the gradient (Note 7) which was immediately centrifuged at 35,000 r.p.m. in the SW50.1 rotor at 20°C. The period of centrifugation is specified in Figure 1 and does not include either the run-up or the unbraked run-down times (ca. 1.8 and 12.7 min, respectively).

Visualization of Bands. The tubes were illuminated in a dark room with a hand-held 80 W mercury discharge lamp fitted with a Woods filter transmitting predominantly at 365 nm (Hanovia Ltd., 480 Bath Road, Slough, Bucks SL1 6BJ, England, ref. 16744/1). Plasmid DNA appeared as a thin fluorescent red band within the gradient (Note 6). The distance of migration was measured in mm from the interface between the sample layer and the top of the gradient.

3. Notes

1. Tests with calf thymus DNA in different concentrations of ethidium bromide suggested that as little as 0.5 μg double-stranded DNA could be seen in these gradients (Radloff, Bauer and Vinograd, 1967). Since the minimum volume of sample applied to the gradient was 0.2 ml, the minimum concentration of plasmid DNA required was 12.5 μg/ml lysate. Assuming plasmids comprise 1.5% of total bacterial DNA and that 10^3 Escherichia coli contain 8 μg DNA, then 12.5 μg plasmid should be found in ca. 10^11 E. coli (e.g. 50 ml culture of O.D. 2.5). Certain plasmids comprise far more than 1.5% of total DNA. One is R6K, which contributes ca. 20% in overnight culture (Kontomichalou, Mitani and Clowes, 1970) and, in that case, sufficient plasmid DNA can be isolated from as little as 7.5 ml overnight culture.

2. Successful lysis depends on prompt mixing of the organisms with the lysing mixture before lysis begins. Otherwise, partial lysis makes the dense suspensions so viscous that further mixing is impossible and large skeins of unlysed organisms persist in the tube. It was for this reason that lysates were prepared by SDS alone at 60°C (Marmur, 1963), rather than with lysozyme and EDTA followed by detergent which never gave adequate mixing with these suspensions. Heating at 60°C has the further advantages, first, of producing a homogenous collection of relaxed plasmid molecules (see Results) and second, taken with SDS, of being likely to render most Gram-negative pathogens harmless. In addition to Escherichia coli, this lysis procedure was found satisfactory with Alcaligenes faecalis (NCIB 8156), Citrobacter freundii (NCTC 9750), Enterobacter aerogenes (NCTC 10006), Proteus vulgaris (NCTC 5751), Pseudomonas aeruginosa (NCTC 10332), Ps. fluorescens (KH 597) and Serratia marcescens (NCTC 1377); but not with Bacillus subtilis (KH 468) or Staphylococcus pyogenes (UB 4008).

3. The conditions for the clearing spin were calculated initially on the assumption that, in these lysates, the chromosome of Escherichia coli would be unfolded but intact with a sedimentation coefficient (s) of ca. 250 (Stonington and Pettijohn, 1971). However, its observed s value appeared appreciably greater and centrifugation for 15 min at 30,000 r.p.m. was finally used. Excessive forces are undesirable at this stage for, although the chromosome is some 20 times bigger than a large plasmid like ColV-K94 (mol. wt. 94 x 10^6), its predicted s value is only about twice as great.

4. Results

After centrifugation, the tubes showed one of three appearances. The most usual was that expected: a sharply defined fluorescent band of plasmid DNA perhaps 0.5 mm deep in the main part of the gradient, with a more brilliant band just below the interface between the sample and the gradient, presumed to consist of small DNA and RNA fragments. Occasionally, the main part of the gradient showed either a diffuse band perhaps 10 mm deep (Note 7) or no band whatsoever which, with known plasmid-carrying strains, was taken to follow loss of plasmid during the clearing spin or too small a sample. When either of the latter occurred, the expected result was usually obtained by running another sample or by preparing another lysate. No sharp band was ever seen with known plasmid-free strains.

The behaviour of plasmids whose molecular weights ranged from 4.8 x 10^6 - 61 x 10^6 is shown in Figure 1. These fell into three groups, depicted by curves A, B and C, each of whose members sedimented at the same rate. In each group, the distance sedimented was proportional to the period of centrifugation. That is, in these linear gradients the increased centrifugal force on passing down the gradient cancelled out the increasing viscosity and density of sucrose to produce isokinetic conditions in which the velocity of sedimentation (v) was constant. The