SHORT COMMUNICATION

Rapid Methods for the Study of both Stable and Unstable Plasmids in *Pseudomonas*

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Rapid methods for the analysis of degradative plasmids in *Pseudomonas* are described. Bacterial lysates prepared with alkaline sodium dodecyl sulphate were vigorously stirred in the presence of antifoam agent. Samples were subjected to agarose gel electrophoresis to detect plasmid DNA. After a short period of centrifugation on a sucrose gradient, the lysates yielded plasmid DNA of sufficient purity for restriction endonuclease digestion without further treatment. The methods have proved most useful in the extraction of previously undetected plasmid DNA, some of which appears to be unstable.

INTRODUCTION

A variety of extraction methods (Humphreys *et al.*, 1975; Currier & Nester, 1976; Palchaudhuri & Chakrabarty, 1976; Duggleby *et al.*, 1977; Hansen & Olsen, 1978) have been used for the physical analysis of the large plasmids involved in the degradation of aromatic compounds by *Pseudomonas* (Duggleby *et al.*, 1977; Benson & Shapiro, 1978; Downing & Broda, 1979; Franklin & Williams, 1980; Nakazawa *et al.*, 1980). The methods presented here, initially developed from the work reported by Freifelder (1971), were designed for the analysis of those plasmids which had proved refractory to other extraction procedures. The methods have since acquired a more general application on account of their speed and simplicity.

METHODS

Bacterial strains and plasmids. The archetype TOL plasmid, pWWO, enables its hosts to utilize toluene, *m*-xylene and *p*-xylene as sole carbon sources (Worsey & Williams, 1975); the DNA has a molecular weight of 7.8×10^7 , corresponding to 117×10^3 base pairs, and restriction endonuclease cleavage sites have been mapped (Downing & Broda, 1979). Its existence was first suggested in *Pseudomonas putida* (*arvilla*) mt-2 by Nakazawa & Yokota (1973). This strain, now called PaW1 (ATCC 33015), was used as a standard since the plasmid DNA is relatively easy to extract. Strains PaW8 and PaW85 are derivatives of PaW1 in which the degradative capability has been lost (Bayley *et al.*, 1977). Strain MT20 is a pseudomonad carrying the plasmid pWW20; the DNA comprises 275×10^3 base pairs (Pickup *et al.*, 1980) and codes for the same degradative capacity as pWWO (Williams & Worsey, 1976). Strain P3,5X (NCIB 9869) is a strain of *Pseudomonas putida* capable of degrading 3,5-xylene; this strain, and its derivatives in which the degradative capability has been lost, were described by Hopper & Kemp (1980).

Growth conditions. Cell cultures inoculated from single colonies were grown overnight at 30 °C in nutrient broth or selective media.

Buffers. TE buffer contained 50 mM-Tris and 50 mM- Na_2EDTA . Gel running buffer contained 89 mM-Tris, 2.5 mM- Na_2EDTA and 89 mM-boric acid (Greene *et al.*, 1974).

Reagents. Reagent A was TE buffer containing 5% Dow Corning Antifoam RD emulsion (Hopkin & Williams, Chadwell Heath, Essex) and 0.1 mg xylene cyanol FF ml⁻¹ (BDH). This mixture was made up in quantity and could be stored at room temperature for many months. It needed to be vigorously shaken before use. Reagent B was 1M-NaOH saturated at 20 °C with sodium dodecyl sulphate (BDH).

Gel electrophoresis. Plasmid DNA preparations were examined on horizontal gels containing 0.7% (w/v) electrophoresis grade agarose (BDH). For screening, a pair of slot formers in parallel provided up to 40 sample slots in a 'double-decker' gel (Barnes, 1977). Whole plasmid DNA could be detected after electrophoresis for 30 min at 200 V but a longer time, routinely 3 h, was needed to compare the sizes of large plasmids. Restriction endonuclease digests were run overnight on gels containing 1 µg ethidium bromide ml⁻¹ (BDH) and flooded with running buffer. The fluorescence of pre-stained gels was photographed directly on a C-62 UV transilluminator (Ultraviolet Products, San Gabriel, Calif., U.S.A.) using a Polaroid MP-4 Land camera, 665 film and appropriate filters (Hansen & Olsen, 1978). Other gels were stained for 15 min in 2 µg ethidium bromide ml⁻¹ before viewing.

Sucrose gradients. A solution of 20% (w/v) sucrose (BDH) in sterile water was slowly frozen solid and then thawed in centrifuge tubes to form sucrose gradients (Baxter-Gabbard, 1972). These were again frozen and stored at -20 °C, and finally thawed slowly just before use.

Detection of plasmid DNA: rapid screening procedure. Cells freshly harvested from 1 ml of culture in 1.5 ml Eppendorf vials, or the equivalent taken from an agar plate, were resuspended in 100 µl Reagent A. Then 25 µl Reagent B was added and the vials were inverted 20 times during 1 min; the suspension became viscous, with a characteristic colour change from blue towards green, as the cells lysed. The suspension was mixed vigorously on a vortex mixer; several vials were agitated together in a 35 mm internal diameter centrifuge tube (Richards & Takai, 1979). Samples of 20–40 µl were loaded directly into the slots of a gel and allowed to stand for 5 min before commencing the electrophoresis. The xylene cyanol reverted to blue in the gel and travelled slightly faster in the direction of the anode than the broad band of chromosomal debris; plasmid DNA was recognizable as discrete bands (see Fig. 1a).

Plasmid DNA extraction for endonuclease digestion. Freshly harvested cells from 50 ml volumes of bacterial cultures were usually resuspended in 2 ml Reagent A, but this volume could be adjusted to equalize the cell concentration between samples in a batch. A 1.6 ml sample of the suspension was transferred to a 15 mm internal diameter centrifuge tube (10 ml) and 0.4 ml Reagent B was added as the tube was agitated gently. Pairs of samples were then stirred vigorously by holding the tubes in a 35 mm internal diameter centrifuge tube clamped on a vortex mixer operating at full speed for 5 min; a rubber bung faced with Parafilm and fitted in the outer tube ensured that the sample tubes did not ride up, or spill. The suspensions thus prepared were layered on to 12.5 ml sucrose gradients in 16.5 ml centrifuge tubes. These were centrifuged in a 6 × 16.5 ml swing-out rotor at 100 000 g for 1 h at 20 °C. After centrifugation, a glass siphon tube (2.5 mm internal diameter) was lowered into the centrifuge tube and clamped so that its bevelled tip was just above the pelleted matter at the bottom. Fractions (1 ml) were collected into sterile Eppendorf vials.

Electrophoresis of 20 µl samples of these fractions was carried out for 30 min at 200 V in a 'double-decker' gel to reveal the presence of plasmid DNA. To ensure that material from the upper deck did not obscure that of the lower, thymol violet (BDH) was used as a marker of the front. Samples (40 µl) of the fractions apparently containing the most pure, or the most concentrated, plasmid DNA were used immediately for restriction endonuclease digestions, carried out according to the recommendations of the enzyme suppliers (Bethesda Research Laboratories, Rockville, Md., U.S.A.).

When necessary the DNA was concentrated by centrifugation. The vial containing the relevant sucrose fraction was filled with sterile water and tightly sealed. It was then floated on water in a 15 mm internal diameter centrifuge tube (10 ml) and centrifuged in a 10 × 10 ml angle rotor at 200 000 g for 1 h at 20 °C. As the vial continued to float, the material within it sedimented. The upper portion of the supernatant was removed leaving a 200 µl sample of concentrated DNA suitable for digestion without further treatment.

Sucrose gradient fractions containing stable plasmid DNA gave reproducible digestion patterns after storage at -20 °C. However, in the study of unstable plasmids, fresh preparations were made each time; miniature gradients (1.25 ml) have been successfully used for these, with proportionately less materials (see Fig. 1b).

RESULTS AND DISCUSSION

The methods described here rely first upon the resistance of plasmid DNA to extraction conditions which disrupt the bacterial chromosome, and secondly upon the ease with which the plasmid DNA may then be separated from the chromosomal debris – by agarose gel electrophoresis for detection (Fig. 1a), or by sucrose gradient centrifugation for restriction enzyme digestion (Fig. 1b, c).

By these rapid means, the DNA of certain previously undetected plasmids has been revealed. The DNA of the TOL plasmid of strain MT20 (Fig. 1a, track 4) was previously

Fig. 1. (a) Agarose gel electrophoresis (3 h) of crude cell lysates prepared by treatment with alkaline sodium dodecyl sulphate of the following *Pseudomonas* strains and plasmids (estimated molecular weights $\times 10^{-6}$ in parentheses): (1) PaW1 carrying TOL plasmid pWWO (78); (2) PaW8 carrying pWWO-8, a derivative of pWWO, (52); (3) PaW85 carrying no detectable plasmids (control); (4) MT20 carrying TOL plasmid pWW20 (180); (5) P3,5X carrying 3,5-xyleneol degradative plasmid (>200); (6) derivative of P3,5X without degradative capability carrying plasmid of reduced size (70).

(b) Agarose gel electrophoresis (15 h) of restriction endonuclease digestions of plasmid pWWO DNA extracted from *Pseudomonas putida* PaW1: (1) from 1.25 ml sucrose gradient, sample digested with *Hind*III; (2–4) from 12.5 ml sucrose gradients, samples digested with *Hind*III (2), *Xho*I (3) and *Eco*RI (4).

(c) Agarose gel electrophoresis (24 h) of *Hind*III digestions of plasmid DNA extracted from the following strains of *Pseudomonas*. (1) PaW1 – Hansen & Olsen (1978) extraction control. (2–5) Extractions from 12.5 ml sucrose gradients: (2) MT20; (3) P3,5X; (4) derivative of P3,5X without degradative capability; (5) PaW1 – pWWO DNA fragments of known size (Downing & Broda, 1979).

refractory to extraction (Duggleby *et al.*, 1977), as was the plasmid DNA of strain P3,5X (Fig. 1*a*, track 5). Indeed, extracts of P3,5X prepared by the method described here appeared to be unstable and had to be used at once for the plasmid DNA to be detected. Such instability in vitro may account for some plasmid DNA being undetected in more protracted procedures.

The plasmid of strain P3,5X is unstable in vivo unless selective medium is used to maintain it (Hopper & Kemp, 1980). Of the derivatives of P3,5X which have lost the capacity to degrade 3,5-xyleneol, all those investigated were shown to retain some, but not all, of the plasmid DNA of the parental type (Fig. 1*a*, tracks 5 and 6; Fig. 1*c*, tracks 3 and 4), and this plasmid DNA was more stable in vitro than the original plasmid DNA.

In contrast to strain P3,5X, the plasmid DNA of strains PaW1 and MT20 (Fig. 1*a*, tracks 1 and 4; Fig. 1*c*, tracks 2 and 5) appeared to be stable in our extracts. The plasmids of these strains are also relatively stable in vivo in conditions not specifically designed to maintain them (Williams & Murray, 1974; Worsey & Williams, 1977).

Figure 1(*b*, *c*) shows that the sucrose gradient centrifugation of the crude cell extracts yields plasmid DNA of sufficient purity for digestion by a variety of restriction endonucleases without further treatment. It is therefore possible to obtain plasmid DNA restriction patterns within a day of harvesting the cells. This is essential in the case of unstable plasmids, like that of P3,5X (Fig. 1*c*, track 3).

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