APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1986, p. 197–199
0099-2240/86/070197-03$02.00/0

Vol. 52, No. 1

NOTES

Modified Plasmid Isolation Method for *Clostridium perfringens* and *Clostridium absonum*

IRVING ROBERTS,* W. MICHAEL HOLMES, AND PHILLIP B. HYLEMON

Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Received 12 November 1985/Accepted 3 April 1986

A rapid plasmid isolation procedure for *Clostridium perfringens* and *C. absonum* is described. The ratio of culture volume to lysis buffer volume was found to be crucial for efficient plasmid isolation. The method can be scaled up, without difficulty, for large-scale plasmid preparation.

Published methods for plasmid isolation from most bacteria (1–6, 8) generally make use of most or all of the following steps: (i) treatment of the cells with Tris-sucrose buffer and lysozyme; (ii) disruption of the resulting osmotically sensitive cells with EDTA and sodium dodecyl sulfate; (iii) for some methods, treatment of the mixture with NaOH to denature the chromosomal DNA, followed by neutralization with Tris hydrochloride buffer; (iv) addition of NaCl to precipitate chromosomal DNA; and (v) centrifugation to obtain a clear lysate suitable for plasmid extraction. Among the various methods, the conditions for individual steps may differ, but all of the published methods seem to work well with *Escherichia coli*.

With *Clostridium* spp., however, we obtained variable results. We attempted large-scale production of plasmids from *Clostridium perfringens* VPI 12502, reported by Squires et al. (9) to have four plasmids ranging in size from 3.3 to 38.8 kilobases (kb), and from *C. perfringens* VPI 11268, reported by Squires et al. (9) to have one plasmid of 42.8 kb. We used the method of Rood et al. (8), developed by them for *C. perfringens* strains, and the preparative method of Anderson and McKay (1), developed by them for strains of lactic streptococci, and we failed to obtain useful yields of plasmid DNA by either method. Cesium chloride centrifugation of the lysates appeared to yield only chromosomal DNA. Electrophoresis of the lysate DNA on an agarose gel showed smears of chromosomal DNA plus faint plasmid bands. One such gel is shown in Fig. 1, in which the Rood et al. method is compared for the same culture with the method described in this paper. Electrophoresis of the low-yield lysate DNA on polyacrylamide gels showed stronger smears of chromosomal DNA, indicating that most of the DNA in the cell lysate was present as fragments of less than 1 kb in size, even though we had been careful to use gentle agitation of the DNA-containing solutions to avoid physical breakdown of the DNA. These results suggested the presence of active endogenous DNases, which are known to be present in members of the *Clostridium* genus. The presence of such DNases was reported by Blaschek and Klacik (3), who found that improved plasmid yields were obtained from two *C. perfringens* strains after the addition of diethyl pyrocarbonate, a nuclease inhibitor.

When we tried the screening (small-scale) method of Anderson and McKay (1), we began to obtain agarose gels that showed significant yields of plasmids. Further experimentation revealed that most of our problems were avoided by careful control of the ratio of the volume of cells being treated to the volume of lysis solution. This led to the development of the modified plasmid method reported here, in which cells from 1.5 ml of culture were treated with 0.4 ml of buffer plus 0.1 ml of lysozyme solution.

We look upon these components as being in a treatment ratio of 3, i.e., a ratio of 1.5 ml of culture to 0.5 ml of lysis solution. At this ratio, good plasmid yields were obtained with all *Clostridium* strains tested, but when we attempted to double or triple the volume of cells treated, i.e., when we

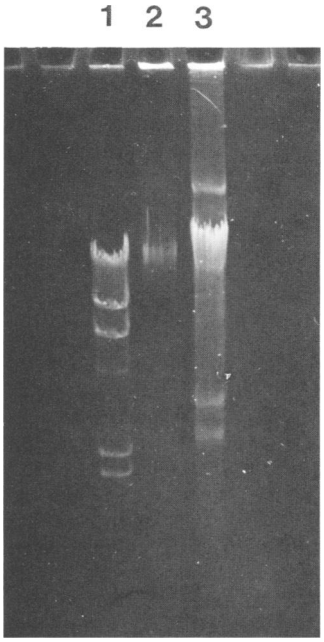

FIG. 1. Plasmid isolation from 1.5-ml samples of a late log-phase culture of *C. perfringens* VPI 12502. Electrophoresis performed on a 0.7% agarose gel at 100 V. Lanes: 1, lambda DNA cut with *Hin*dIII; 2, method of Rood et al. (8); 3, method described in this paper.

* Corresponding author.

TABLE 1. Comparison of treatment ratios and lysozyme contents in published methods

Method	Vol of culture (ml)	Vol of buffer (ml)	Vol of lysozyme (ml)	Buffer plus lysozyme (ml)	Ratio
Rood et al. (7)	100	2.0	0.4 (10 mg/ml)	2.4	42
Guerry et al. (6)	30	1.0	0.2 (5 mg/ml)	1.2	25
Birnboim and Doly (2)	0.5			0.1[a]	5
Anderson and McKay (1)					
Preparative	600	30	7.5 (10 mg/ml)	37.5	16
Screening	1.5–10	0.379	0.096 (10 mg/ml)	0.475	3–21

[a] The Birnboim and Doly (2) buffer includes 2 mg of lysozyme per ml.

increased the treatment ratio to 6 or 9, we found that the plasmid yield decreased and that the chromosomal DNA increased.

A review of the literature indicated a wide range of treatment ratios (Table 1). The high ratios used in the method of Rood et al. (8) and the preparative method of Anderson and McKay (1) may explain our inability to obtain good yields of plasmid by these methods, whereas the low ratio used in the screening method of Anderson and McKay (1) gave us our first successes. The method of Birnboim and Doly (2), developed specifically for *E. coli* and widely used as such, is similar in principle to the method developed here.

We offer the following as a possible explanation of these results. In the first step (treatment of the cells with Tris-sucrose buffer and lysozyme), at low ratios peptidoglycan digestion is complete and endogenous DNase is released completely into the medium, where it is inactivated. At high ratios, cell lysis may be incomplete and continue after addition of EDTA and sodium dodecyl sulfate, releasing additional DNase, which attacks the plasmid and chromosomal DNA that are released at the same time. Supporting this hypothesis is our observation that at low ratios, substantially all the cell material appears to go into solution within minutes after addition of EDTA and sodium dodecyl sulfate, whereas at high ratios, curds of solid material remain undissolved in this second step, indicating possibly incomplete peptidoglycan digestion in the first step.

We also determined that careful control of pH for the NaOH step is crucial for successful plasmid isolation. With the quantities shown, NaOH brings the solution to pH 12.0, and the Tris hydrochloride, pH 7.0, brings the solution back to pH 8.5. We found that bringing the solution to pH 9.0 instead of pH 8.5 resulted in complete loss of plasmids.

Our small-scale rapid plasmid method is as follows. A 5- or 10-ml volume of BHI medium (brain heart infusion broth supplemented with hemin and cysteine) (7) was inoculated from an overnight starter culture or glycerol stock (25% glycerol, stored at −20°C) and grown to late log phase (optical density at 650 nm, about 1.0). A 1.5-ml volume of this culture was transferred to an Eppendorf tube, and the cells were harvested by centrifugation. The pelleted cells could be used immediately or frozen overnight at −70°C. Cells more than a few days old should not be used, as we found that the plasmid yield deteriorated as cells aged.

The pelleted cells were resuspended by vortexing in 400 μl of Tris–EDTA–sucrose buffer (50 mM Tris, 1 mM EDTA, 6.7% sucrose [pH 8]). This suspension was warmed to 37°C, and 100 μl of lysozyme solution (20 mg/ml in 25 mM Tris [pH 8.0]) was added. This was mixed by inversion and incubated at 37°C for 5 to 10 min. This mixture should not be vortexed. One hundred microliters of 0.25 M EDTA–50 mM Tris (pH 8) and 60 μl of 20% sodium dodecyl sulfate (in 50 mM Tris, 20 mM EDTA [pH 8]) were added and mixed by inversion, and the mixture was incubated at 37°C for 10 to 15 min. After this, 28 μl of fresh 3.0 N NaOH was added and again mixed by inversion, and the mixture was allowed to stand at room

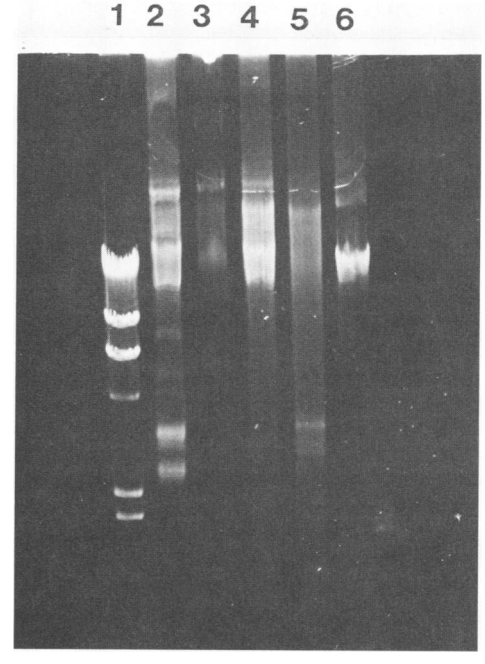

FIG. 2. Rapid plasmid isolation results. Electrophoresis performed on 0.7% agarose gel at 100 V. Lane 1, lambda DNA cut with *Hin*dIII. Reading upwards, bands are 2.0, 2.3, 4.3, 6.7, 9.4, and 23.1 kb. Lane 2, *C. perfringens* VPI 12502. Squires et al. (9) state that the plasmids are 3.3, 3.9, 17, and 38.8 kb. Clearly, relaxed as well as covalently closed circular forms are present in this gel. Lane 3, *C. perfringens* VPI 11268. Squires et al. (9) state that there is one plasmid, 42.8 kb. Some chromosomal DNA, at about 25 kb, is also present. Lane 4, *C. perfringens* ATCC 10543. Blaschek and Klacik (3) obtained two plasmids, 9.4 and 30 megadaltons (14 and 45 kb), from this strain by using diethyl pyrocarbonate. This gel, also obtained without the addition of diethyl pyrocarbonate, shows both plasmids; the smaller is partly masked by chromosomal DNA. Lane 5, *C. absonum* ATCC 27637. Two plasmids, about 3.5 and 4.0 kb, are visible. These are newly discovered and are reported here for the first time. Lane 6, *E. coli*, containing a 9-kb plasmid, probably a relaxed form located close to the chromosomal DNA.

temperature for 10 min. Then 100 μl of 2.0 M Tris hydrochloride, pH 7, was added and mixed by inversion, and the mixture was allowed to stand at room temperature for 3 min. A 75-μl volume of 5.0 M NaCl was added and mixed by inversion, and the mixture was allowed to stand at 4°C for 90 min or more. This mixture was centrifuged for 5 min, and the pellet, which contained the bulk of the chromosomal DNA, was discarded. The supernatant was treated with 2 μl of pancreatic RNase (10 μg/ml, boiled for 2 min before use) and allowed to stand at room temperature for 15 min or more.

To the RNase-treated solution was added 600 μl of phenol, saturated with 3% NaCl, and 100 μl of chloroform–isoamyl alcohol (24:1). This was mixed thoroughly by vortexing and centrifuged for 2 min. The upper (aqueous) phase was removed and precipitated with an equal volume of isopropanol, incubated at −20 or −70°C for 10 min, and centrifuged at 4°C for 5 min. The supernatant was discarded, and the tube was inverted to allow the DNA pellet to drain. The pellet was dried under vacuum for 15 min to remove isopropanol and then taken up in 20 μl of TE buffer for agarose gel electrophoresis (0.7% agarose, 100 V).

Increased purity for restriction enzyme cutting was obtained by two precipitations with ethanol, as follows: (i) from the isopropanol precipitation, the undried pellet was taken up in 180 μl of water and treated with 20 μl of 5 M potassium acetate and 500 μl of cold 95% ethanol; (ii) this was mixed, incubated at −20 or −70°C for 10 min, and centrifuged at 4°C for 5 min; and (iii) the supernatant was poured off, the tube was inverted, and the DNA pellet was allowed to drain. Steps (i), (ii), and (iii) were then repeated, and the pellet was washed by addition of 500 μl of cold 80% ethanol, without mixing, and then centrifuged for 30 s. The supernatant was pipetted off, and the pellet was dried under vacuum for 15 min and taken up in 20 μl of TE buffer for restriction endonuclease digestion.

In considering the results shown in Fig. 1 and 2, one should bear in mind that some chromosomal DNA appears in all samples as a smear at about 25 kb. Furthermore, two or three forms of each plasmid may be present, and although the covalently closed circular form usually migrates fastest, it may appear at a position above or below that of the true linear size. We identified and sized individual plasmids by electroelution of the bands from the gel segments and by restriction enzyme analysis of each. We found that the degree of plasmid purity obtained by this rapid plasmid isolation method was entirely adequate for this purpose.

This method can be scaled up without difficulty for large-scale plasmid preparation, so long as the treatment ratio of culture volume to lysis volume, as defined herein, is kept at approximately 3. This was demonstrated by experiments in which 30-ml cultures of *C. perfringens* VPI 12502 and *C. absonum* ATCC 27637 were processed by the method described in this paper for 1.5-ml cultures, at 20 times the reagent quantities. Each of these experiments resulted, after the NaCl precipitation step, in about 14 ml of cleared lysate. Aliquots (700 ml) (1/20) of the lysates were carried through the remaining steps and yielded agarose gels substantially identical to those given in lanes 2 and 5 of Fig. 2.

This work was supported, in part, by a grant from the Center for Innovative Technology of the Commonwealth of Virginia and Public Health Service grant CA17747 from the National Cancer Institute.

LITERATURE CITED

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Blaschek, H. P., and M. A. Klacik.** 1984. Role of DNase in recovery of plasmid DNA from *Clostridium perfringens*. Appl. Environ. Microbiol. **48**:178–181.
4. **Blaschek, H. P., and M. Solberg.** 1981. Isolation of a plasmid responsible for caseinase activity in *Clostridium perfringens* ATCC 3626B. J. Bacteriol. **147**:262–266.
5. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
6. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
7. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
8. **Rood, J. I., E. A. Maher, E. B. Somers, E. Campos, and C. L. Duncan.** 1978. Isolation and characterization of multiply antibiotic-resistant *Clostridium perfringens* strains from porcine feces. Antimicrob. Agents Chemother. **13**:871–880.
9. **Squires, C. H., D. L. Heefner, R. J. Evans, B. J. Kopp, and M. J. Yarus.** 1984. Shuttle plasmids for *Escherichia coli* and *Clostridium perfringens*. J. Bacteriol. **159**:465–471.