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Rapid Extraction of Plasmids from *Clostridium perfringens*

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Two rapid methods were evaluated for their extraction of plasmids from *Clostridium perfringens*. The first method involved lysis of 1 to 2 ml of *C. perfringens* culture by treatment with hyaluronidase, lysozyme, and sarcosyl. DNA, extracted with phenol-chloroform, was treated with RNase, boiled, and electrophoresed in a 1.2% agarose gel. The second method involved lysis of 2 ml of culture by lysozyme treatment and extraction with alkaline sodium dodecyl sulfate (SDS). Extracted DNA was treated with RNase, boiled, and electrophoresed in a 0.7% agarose gel. Of 57 strains of *C. perfringens* analyzed by both extraction procedures, 11 were shown to have plasmids by the alkaline SDS method which were missed by the phenol-chloroform extraction method. These new plasmids were of higher molecular mass and ranged up to 68 megadaltons. Use of the DNase inhibitor diethyl pyrocarbonate did not further improve the yield of plasmid DNA. An additional 159 isolates of *C. perfringens* screened by the alkaline SDS method revealed plasmids up to 80 megadaltons in mass and an overall plasmid carriage rate of 69%.

Plasmid DNA was first observed in *Clostridium perfringens* by Ionesco and Bouanchaud (5), although it was Ionesco et al. (4) who first isolated different-molecular-weight plasmids in this species. Bréfort et al. (2) described an extraction procedure for plasmids of *C. perfringens*. This procedure and that described by several investigators since then utilized density gradient centrifugation, a process requiring heavy equipment and considerable time. Strom et al. (8) described four methods for the isolation of plasmids from *C. botulinum*, some of which worked better for certain strains than did others. Nucleases which degraded plasmid DNA were proposed to present difficulty in obtaining good plasmid preparations from some strains of *C. botulinum* (8), and the presence of nucleases has been described by Blaschek and Klacik (1) to be an obstacle to the isolation of plasmids in some strains of *C. perfringens*.

To date there has been no report describing rapid methodologies for the isolation of plasmids from *C. perfringens*. This paper describes two approaches for the relatively rapid extraction of plasmids from *C. perfringens* without using density gradient centrifugation. The first approach is a micro modification of that described by Bréfort et al. (2), and the second is a modification of a method described by Crosa and Falkow (3) for the isolation of large plasmids from bacteria.

MATERIALS AND METHODS

Bacterial cultures. Cultures of *C. perfringens* representing many countries of the world were provided by the Central Public Health Laboratory, London, United Kingdom. Twenty-seven isolates were also obtained from N. D. Verma of India. The bacteria were grown at 37°C in cooked meat medium (Difco Laboratories, Detroit, Mich.) and stored in this medium at room temperature. Boiled brain heart infusion broth (Difco) was used for routine culturing of *C. perfringens*.

Plasmid extraction. (i) Method 1. A micro adaptation of the plasmid extraction procedure described by Bréfort et al. (2) was developed by G. A. Clark of our laboratory. One milliliter of an overnight cooked meat culture was subcultured into 9 ml of brain heart infusion broth and incubated at 37°C for 3 h. One milliliter of this culture was centrifuged for 1.5 min in an Eppendorf microcentrifuge (model 5412; Brinkmann Instruments, Inc., Westbury, N.Y.), and the cell pellet was suspended in 90 μl of TES buffer (0.05 M Tris hydrochloride, 0.005 M EDTA, 0.15 M NaCl [pH 7.4]). Ten microliters of hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) dissolved in TES buffer (1 mg/ml) was added to the cell suspension and incubated at 37°C for 1 h. This was centrifuged for 1.5 min, and the pellet was suspended in 39.6 μl of Tris-sucrose buffer (0.05 M Tris hydrochloride, 25% sucrose, 0.001 M EDTA [pH 8.0]) and 4.4 μl of lysozyme (1 mg/ml; grade I; Sigma). The suspension was incubated at 37°C for 10 min followed by 0°C for 20 min, and 6.3 μl of Tris-EDTA buffer (0.05 M Tris hydrochloride, 0.04 M EDTA [pH 8.0]) was added for 10 min. Then 50 μl of sarcosyl buffer (0.05 M Tris hydrochloride, 0.005 M EDTA, 5% sodium lauryl sarcosinate sarcosyl [pH 8.0]) was added and incubated for 20 min at 0°C. DNA was extracted from the final 100 μl of lysed cells by the method of Kado and Liu (6). Phenol-chloroform (100 μl; 1:1) was added to the cell lysate, which was shaken until a confluent white mixture formed. This was centrifuged, the aqueous phase was carefully removed, and the DNA extract was placed in a new Eppendorf tube. Ten microliters of RNase A (type II-A from bovine pancreas; Sigma; 1 mg/ml in 0.01 M Tris hydrochloride [pH 8.0]) was added, and the mixture was incubated at 45°C for 1 h.

To eliminate open circular and linear forms of plasmid DNA as well as contaminating chromosomal DNA, we drew 50 μl of the RNase-treated DNA up into a 50-μl capillary pipette, which was then flame sealed at both ends and placed in a boiling water bath for 1 min (7). The pipette was rapidly cooled at 0°C and broken open, and the total 50 μl was added to 5 μl of dye solution (0.05 M Tris-acetate, 50% glycerol, 0.25% bromocresol purple). The sample was applied to a 1.2% agarose gel (agarose type II; Sigma) and electrophoresed for 18 h at 30 V. The running buffer (TEAS) consisted of 0.04 M Tris, 0.002 M EDTA, 0.02 M sodium acetate, and 0.018 M NaCl adjusted to pH 8.0 with acetic acid. Gels were stained with ethidium bromide (1 μg/ml) for 0.5 h, destained

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in running water for 0.5 h, and photographed under UV light with a Polaroid MP-4 Land camera fitted with a red filter and Polaroid Land film T-667. Alternatively, 0.7% agarose gels were used, and a shorter electrophoresis time of 5 h was achieved at 100 V. Plasmids used as molecular weight markers for gel electrophoresis were extracted from *C. perfringens* 28 (7), *Escherichia coli* V517, and *E. coli* RP4.

(ii) Method 2. This method of plasmid extraction is basically as described by Crosa and Falkow (3) with some minor modifications. Two milliliters of a young broth culture of *C. perfringens* provided sufficient DNA for detection. The pellet obtained from centrifuging 2 ml of culture was suspended in 45 μl of Tris-sucrose buffer containing 1.25 mg of lysozyme per ml and incubated for 20 min at 37°C. One modification of the method was the addition of penicillin G sodium (Crystapen; Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom; final concentration, 2 U/ml) to a 2-h culture prior to lysozyme at 3 h. This step was not essential for a good plasmid yield but occasionally provided better results. The lysis buffer containing 4% sodium dodecyl sulfate was freshly prepared each day and carefully adjusted to pH 12.4 as described by Crosa and Falkow (3). Our procedure included RNase treatment and boiling and cooling of the final DNA preparation as described for method 1. The electrophoresis buffer (TEAS) was also that described for method 1. DNA was electrophoresed in 0.7% agarose at 100 V for 5 h.

RESULTS AND DISCUSSION

Figure 1 shows an ethidium bromide-stained agarose gel in which three plasmids extracted by method 1 from our reference strain 28 are visible. Based on a standard curve plotted for the mobility of *E. coli* V517 plasmids, the three clostridial plasmids were 7.1, 5.4, and 1.1 megadaltons (MDa) in mass. These plasmids possessed the same molecular mass as those plasmids we obtained from this strain by the dye-buoyant density gradient technique, in which molecular masses were determined by mobility against known covalently closed circular DNA markers and by the Kleinschmidt method (7). Li et al. (7) showed that denaturation of the DNA by boiling was required to remove noncovalently closed circular DNA bands. The rapid method, therefore, eliminated such bands, since only plasmids corresponding to previously confirmed covalently closed circular DNA bands appeared. Hyaluronidase treatment was important to this lysing procedure, and failure to incorporate the enzyme produced negative results. The addition of RNase decreased RNA staining, which was often apparent at the distal end of the gel. Method 2 also revealed these same plasmids in *C. perfringens* 28, although RNA digestion seemed poorer under those experimental conditions.

Fifty-seven strains of *C. perfringens* were analyzed by both extraction procedures. Of these, 29 had demonstrable plasmids. All plasmids detected by the phenol-chloroform extraction method (method 1) were also detected by the alkaline extraction method (method 2); however, 11 of the 57 strains (19%) were shown to have plasmids by method 2 which were missed by method 1 (Fig. 2). Plasmids ranging from 8.9 to 68 MDa were detected in five strains in which plasmids had not been detected before. Six other strains were found to carry plasmids additional to those found by method 1. These ranged from 15.5 to 64 MDa in mass. The largest plasmid detected in these strains by method 1 was 9.7 MDa. Of the 14 new plasmids detected by method 2, 12 (86%) were larger than 9.7 MDa. The two plasmids that were smaller than 9.7 MDa were both 8.9 MDa. Figure 3 demonstrates the difference observed in the plasmid profiles of two selected strains (A21 and E119) of *C. perfringens* by both methods of extraction. The larger plasmids were not observed when method 1 was used. Since DNA extracted by both methods was run on 0.7% agarose gels, failure to find large plasmids with method 1 on 1.2% agarose gels was not a reflection of gel concentration.

To determine whether a DNase inhibitor would further enhance extraction of larger plasmids, we added diethyl pyrocarbonate (Sigma) to the extraction procedure as described by Blaschek and Klacik (1), with no observable change in results. This indicates that either nucleases do not represent a problem or else the nuclease inhibitor is without effect in our system.

Method 2 was used to extract plasmids from an additional 159 strains of *C. perfringens*. Some of these strains had plasmids estimated to be about 80 MDa. The incidence of plasmid carriage in the total 216 strains of *C. perfringens* determined by method 2 (Table 1) was 69%. Since some of these strains were from studies of food poisoning or other clostridial enteric syndromes and may have represented more than one isolate of the same organism, the real incidence of plasmid carriage may actually have differed somewhat from 69%. However, this incidence of plasmid-containing strains collected from various parts of the world does indicate universal carriage of plasmids by the species. The incidence and range of molecular masses were similar to

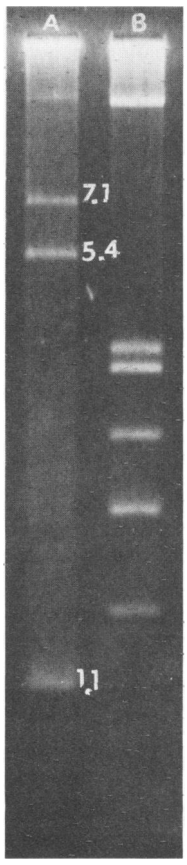

FIG. 1. Plasmids of *C. perfringens* 28 extracted by the phenol-chloroform method (method 1) and electrophoresed on a 1.2% agarose gel (lane A). Plasmid sizes are expressed in megadaltons. *E. coli* reference plasmids appear in lane B.

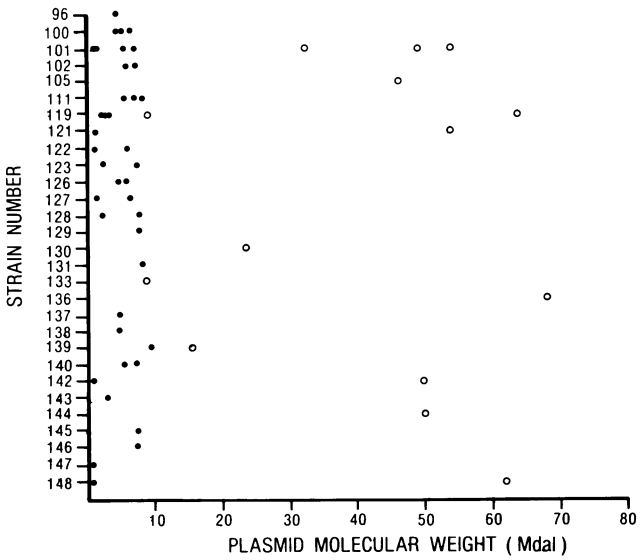

FIG. 2. Plasmids extracted from 29 strains of *C. perfringens* by method 1 (hyaluronidase, lysozyme, sarcosyl, and phenol-chloroform) and method 2 (lysozyme and alkaline sodium dodecyl sulfate). Extracted plasmid DNA detected in 29 of 57 strains was boiled and cooled to eliminate open circular and linear DNAs and electrophoresed on 1.2% agarose gel for 18 h at 30 V (method 1) and on 0.7% agarose gel for 5 h at 100 V (method 2). Plasmids detected by the phenol-chloroform method (●) were also detected by the alkaline sodium dodecyl sulfate method (○).

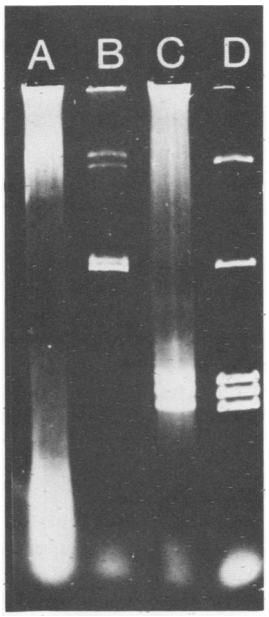

FIG. 3. Electrophoresis of plasmids from two strains of *C. perfringens*. Plasmids in lanes A and C were extracted by the phenol-chloroform method; plasmids in lanes B and D were extracted by the alkaline sodium dodecyl sulfate method. Lanes A and B represent strain A21, a fecal isolate from Scotland, and lanes C and D represent strain E119, a bacteriocin-producing strain from The Netherlands. Electrophoresis was performed in a 0.7% agarose gel.

TABLE 1. Plasmid carriage in *C. perfringens*

Country of origin	No. of strains surveyed	No. (%) of plasmid-carrying strains
Israel	10	4 (40)
The Netherlands	31	16 (52)
India	34	21 (62)
Australia	1	1 (100)
Britain	117	97 (83)
Nigeria	21	8 (38)
Thailand	2	2 (100)

those observed in *C. botulinum* (8), in which the incidence of plasmid carriage was reported to be 56% for plasmids ranging up to 81 MDa. Certain of our strains possessed what appeared to be common plasmids, some of which are associated with bacteriocin synthesis. Restriction endonuclease digestion experiments should further reveal the degree of similarity among such plasmids.

In conclusion, we have described two methods of plasmid isolation for *C. perfringens* . Both methods were useful for the isolation of low-molecular-mass plasmids, but the alkaline sodium dodecyl sulfate extraction method was required for detection of plasmids over 10 MDa. High-molecular-mass plasmids were detectable, and a nuclease inhibitor was not required for detection of such plasmids. Both methods were reasonably easy to perform, and both required small volumes of culture and relatively inexpensive equipment. The time saving over density gradient procedures is considerable and, where analytical studies on plasmid carriage or transfer might be important, the method offers many advantages.

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