Rapid Extraction of Plasmids from \textit{Clostridium perfringens}

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Two rapid methods were evaluated for their extraction of plasmids from \textit{Clostridium perfringens}. The first method involved lysis of 1 to 2 ml of \textit{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 \textit{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 \textit{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 \textit{Clostridium perfringens} by Ionesco and Bouanuch (5), although it was Ionesco et al. (4) who first isolated different-molecular-weight plasmids in this species. Brefort et al. (2) described an extraction procedure for plasmids of \textit{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 \textit{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 \textit{C. botulinum} (8), and the presence of nucleases has been described by Blaschek and Klacić (1) to be an obstacle to the isolation of plasmids in some strains of \textit{C. perfringens}.

To date there has been no report describing rapid methodologies for the isolation of plasmids from \textit{C. perfringens}. This paper describes two approaches for the relatively rapid extraction of plasmids from \textit{C. perfringens} without using density gradient centrifugation. The first approach is a micro modification of that described by Brefort 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 \textit{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 \textit{C. perfringens}.

**Plasmid extraction.** (i) **Method I.** A micro adaptation of the plasmid extraction procedure described by Brefort 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 \( \mu \)l 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 \( \mu \)l of Tris-sucrose buffer (0.05 M Tris hydrochloride, 25\% sucrose, 0.001 M EDTA [pH 8.0]) and 4.4 \( \mu \)l 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 \( \mu \)l of Tris-EDTA buffer (0.05 M Tris hydrochloride, 0.04 M EDTA [pH 8.0]) was added for 10 min. Then 50 \( \mu \)l of sarcosyl buffer (0.05 M Tris hydrochloride, 0.005 M EDTA, 5\% sodium laurel sarcosinate sarcosyl [pH 8.0]) was added and incubated for 20 min at 0°C. DNA was extracted from the final 100 \( \mu \)l of lysed cells by the method of Kado and Liu (6). Phenol-chloroform (100 \( \mu \)l; 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 \( \mu \)l of the RNase-treated DNA up into a 50-\( \mu \)l 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 \( \mu \)l was added to 5 \( \mu \)l 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 \( \mu \)g/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.
pefiringens 28 (7), Escherichia coli V517, and E. coli RP4.

(b) Method 2. This method of plasmid extraction is basi-
cally as described by Crosa and Falkow (3) with some minor
modifications. Two milliliters of a young broth culture of C.
pefiringens provided sufficient DNA for detection. The
pellet obtained from centrifuging 2 ml of culture was sus-
pended 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 dode-
cyl 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 etidium 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 molecu-
lar 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 denatura-
tion of the DNA by boiling was required to remove non-
covalently closed circular DNA bands. The rapid method,
therefore, eliminated such bands, since only plasmids corre-
sponding to previously confirmed covalently closed circular
DNA bands appeared. Hyaluronidase treatment was impor-
tant 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. pefiringens 28, although RNA digestion
seemed poorer under those experimental conditions.

Fifty-seven strains of C. pefiringens 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 demon-
strates the difference observed in the plasmid profiles of two
selected strains (A21 and E119) of C. pefiringens 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 de-
scribed 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. pefiringens. Some of these strains had
plasmids estimated to be about 80 MDa. The incidence of
plasmid carriage in the total 216 strains of C. pefiringens
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 inci-
dence of plasmid carriage may actually have differed some-
what 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. pefiringens 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.
the 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. 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 from 0.7% agarose gel. The time required was not reasonable and, therefore, was not considered important, where analytical studies on plasmid carriage or transfer might be important, the method offers many advantages.

ACKNOWLEDGMENTS

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LITERATURE CITED


TABLE 1. Plasmid carriage in C. perfringens

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

Those observed in C. botulinum, 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.