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Role of DNase in Recovery of Plasmid DNA from *Clostridium perfringens*

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Recovery of plasmid DNA from *Clostridium perfringens* 10543A and 3626B cleared lysates was significantly improved by the addition of 0.2% (vol/vol) diethylpyrocarbonate (DEP) before protoplast disruption in the cleared lysate protocol. Three previously undetected, large-molecular-mass plasmids (45.2, 51.9, and 68.2 megadaltons) were isolated from modified DEP-treated cleared lysates of *C. perfringens* 3626B. Two plasmids (9.4 and 30 megadaltons) were recovered from *C. perfringens* 10543A modified DEP-treated cleared lysates which previously required dye-buoyant density gradient centrifugation for visualization on agarose gels. Unsuccessful attempts to isolate plasmid DNA from Brij 58 cleared lysates of extracellular DNase-negative mutants of *C. perfringens* suggested the deleterious DNase activity was not extracellular. Cellular localization studies indicated that the cell wall-compartmentalized cell fraction contained 72.2% of the total DNase activity, whereas the extracellular and intracellular fractions demonstated much less (26.8 and 1.0%, respectively). Cleared lysates prepared with DEP demonstrated much less DNase activity than cleared lysates prepared without DEP. The variable and irreproducible recovery of plasmid DNA from *C. perfringens* cleared lysates was attributed to cell wall-compartmentalized DNase.

The isolation of large (>10 megadalton) covalently-closed circular (CCC) plasmid DNA from cleared lysates of the gram-positive bacillus, *Clostridium perfringens*, continues to be variable and irreproducible. Investigators from different laboratories have reported on the recovery of multiple configurational forms of plasmid DNA (CCC, open circular, and linear) from *C. perfringens* strains (2, 14, 17, 19). To optimize the recovery of plasmid DNA, several workers have resorted to large-culture-input volumes combined with enrichment by dye-buoyant density gradient centrifugation (2, 3, 6, 16, 18, 19).

A number of bacterial genera (e.g., *Staphylococcus*, *Clostridium*, and *Serratia*) are known to produce extracellular nucleases (7, 20, 21). Extracellular DNase is produced by strains of all five *C. perfringens* types (20) and may be responsible for variable agarose electrophoresis plasmid band patterns. The variable and irreproducible isolation of plasmid DNA from the gram-negative bacteria *Serratia marcescens* and *Pseudomonas cepacia* 4G9 was attributed to nuclease activity (21–24). The objective of this study was to examine the role played by endogenous DNase in the recovery of plasmid DNA from *C. perfringens* cleared lysates.

MATERIALS AND METHODS

Strains, media, and cultural conditions. *C. perfringens* 10543A and 3626B were obtained from the American Type Culture Collection, Rockville, Md., and M. Solberg, Department of Food Science, Rutgers University, New Brunswick, N.J., respectively. Stock cultures were maintained as described previously (19). TGY medium (11) was used as the growth medium for all experiments. A 1.0% cooked-meat medium (Difco Laboratories, Detroit, Mich.) inoculum was aseptically transferred to fresh TGY medium and incubated overnight (12 to 16 h) at 37°C. Another volume of TGY medium was inoculated at 5.0% with the overnight culture. Incubation proceeded until the absorbance at 600 nm was 1.0 as measured by a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.). This resulted in a *C. perfringens* culture in the late logarithmic phase of growth (ca. 10^9 cells per ml). Harvesting the culture by centrifugation (10,000 × *g*, 4°C, 5 to 10 min) yielded 4 mg (wet weight) of cells per ml.

Plasmid isolation. *C. perfringens* plasmid DNA was isolated by using either the cleared lysate procedure described by Blaschek and Solberg (2) minus the RNA digestion step (15) or a modification of the above procedure in which 0.2% (vol/vol) diethylpyrocarbonate (DEP; Sigma Chemical Co., St. Louis, Mo.) nuclease inhibitor was added immediately after the EDTA addition step to inactivate cell wall-compartmentalized DNases released during protoplast formation. Cleared lysates were purified for CCC plasmid DNA by using dye-buoyant density gradient centrifugation (18). Low-temperature (4°C) centrifugations were carried out for 48 h at 145,000 × *g* with a Beckman type 50 fixed-angle Ti rotor. Agarose gel electrophoresis was carried out as described previously (2, 19) in Tris-acetate buffer (12). The molecular weight of plasmid DNA was calculated from relative mobilities in 0.7% agarose gels (2).

Isolation of extracellular DNase-negative mutants of *C. perfringens*. The procedure of Adelberg et al. (1) as described by Carlton and Brown (4) was modified for the mutagenesis of *C. perfringens* 10543A with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). After growth to late exponential phase in 10 ml of TGY medium, the culture was harvested and washed twice in 1 volume of sterile 0.1% peptone–0.5% sodium thioglycolate (pH 6.0) (PT buffer). The washed cells were standardized to an absorbance at 600 nm of 1.0 in sterile PT buffer. NTG in sterile water (1.0 mg/ml) was added to a final concentration of 125 μg of NTG per ml (50% killing dose). After incubation at 37°C for 30 min without shaking, the mutagenized culture was washed in 1 volume of sterile PT buffer to remove residual NTG. After overnight expression in TGY medium, direct selection for extracellular DNase-negative mutants was done on DNase test agar (Difco) supplemented with 1.5% glucose, 1.0%

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yeast extract, and 0.0025% purified methyl green. Methyl green was purified of methyl violet contaminant by repeated extractions with chloroform until the organic phase was clear (13).

Preparation of cell fractions. The detailed procedure for the preparation of extracellular, cell wall-compartmentalized, and intracellular cell fractions was as follows. One volume of late-log-phase liquid TGY culture of *C. perfringens* 10543A was harvested (10,000 × *g*, 4°C, 5 min). The culture supernatant containing extracellular enzyme was saved. The cell pellet was suspended in 1/10 (0.1) volume of TES A buffer (3). The cells were centrifuged as before, and the cell wash was combined with the extracellular fraction. The washed cell pellet was resuspended in 1/10 (0.1) volume of Tris-sucrose buffer (3). Crystalline lysozyme (grade I; Sigma) was added at 1.0 mg/ml, and incubation followed for 10 min at 37°C. The resulting suspension was centrifuged gently (4,250 × *g*, 10 min) to yield a clear supernatant (designated the cell wall-compartmentalized fraction) and a gelatinous white protoplast pellet. The intracellular lysate was obtained by adding 1/10 (0.1) volume of modified Brij 58 buffer (1% Brij 58, 0.4% sodium deoxycholate, 0.05 M Tris [pH 8.0]) (5) to the well-drained protoplast pellet and incubating at 37°C for 10 min to release intracellular contents. Large cell debris was removed by centrifugation (10,000 × *g*, 5 min).

Quantitative assay of DNase activity. The procedure of Houck (10) as modified by Erickson and Deibel (7) was further modified to quantitate the DNase activity of various cell fractions of *C. perfringens* 10543A. To determine the DNase activity of a given cell fraction, 0.75 ml of a 1-mg/ml DNA (type III; Sigma) stock solution and 7.25 ml of DNase buffer (20 mM Tris-hydrochloride, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol [pH 8.0]) (19) were added to a test tube. The solution was prewarmed at 37°C for 15 min. A 1.0-ml volume of the cell fraction to be assayed was added and mixed. The polymerized DNA present at time zero and remaining after 60 min at 37°C was precipitated with 1.0 ml of 5 N HCl. Turbidity was developed at 37°C for 10 min and was estimated by reading the absorbance at 600 nm. The DNase activity present in each cell fraction was based on the amount of substrate DNA depolymerized in 60 min with a standard curve.

RESULTS

Effect of DEP on recovery of plasmid DNA. The addition of DEP to the cleared lysate protocol (see above) resulted in the recovery of two distinct CCC plasmid bands from *C. perfringens* 10543A (Fig. 1, lane B) not present when DEP was omitted (Fig. 1, lane A). These plasmids correspond to the 9.4- and 30-megadalton plasmids previously recovered only from dye-buoyant density-purified preparations of *C. perfringens* 10543A (19). In addition to plasmids pHB101 and pHB102, the modified DEP-treated cleared-lysate technique allowed for the recovery of three large-molecular-mass plasmid bands (45.2, 51.9, and 68.2 megadaltons; Fig. 1, lane D) from *C. perfringens* 3626B that were not previously detected in this strain (2). The CCC nature of these plasmids was suggested by denaturation studies during which the lysate was boiled for 60 to 90 s, followed by quick chilling on ice (data not shown). No plasmids could be visualized in *C. perfringens* 3626B cleared lysates when DEP was omitted (Fig. 1, lane C). The usefulness of the modified DEP-treated cleared-lysate technique is further evident when one compares the modified DEP-treated cleared-lysate plasmid profile of *C. perfringens* 3626B (Fig. 1, lane D) with the corresponding dye-bouyant density-purified preparation (Fig. 1, lane E). An additional striking feature apparent in Fig. 1 is the elimination of RNA smearing in the modified DEP-treated cleared lysates of both *C. perfringens* 10543A and 3626B (Fig. 1, lanes B and D, respectively).

Cellular localization of DNase activity. NTG mutagenic treatment of *C. perfringens* 10543A resulted in the recovery on modified DNase test agar of two extracellular DNase-negative mutants designated NTG-3 and NTG-4. Repeated attempts at recovering plasmid DNA from either the *C. perfringens* 10543A extracellular DNase-positive strain or NTG-3 and NTG-4 extracellular DNase-negative mutants by using Brij 58 lysis (5) at 0 or 37°C were unsuccessful. Brij 58 cleared lysates of strains 10543A, NTG-3, and NTG-4 demonstrated equivalent positive DNase activities when assayed by using a modified DNase test agar well assay (data not shown).

To target the deleterious DNase activity present in *C. perfringens* cleared lysates, cellular localization was carried out. The DNase activity of each of three cell fractions of *C. perfringens* 10543A can be seen in Table 1. The cell wall-compartmentalized cell fraction possessed the most DNase activity (72.2% of total activity), followed in descending order by the extracellular and intracellular cell fractions (26.8 and 1.0% of total activity, respectively).

Incorporating DEP into the plasmid isolation protocol has an effect on the DNase activity of *C. perfringens* 10543A cleared lysates. The cleared lysate to which DEP had been added immediately after protoplast formation demonstrated DNase activity of 0.13 U/ml, which was nearly threefold less than that of the cleared lysate prepared without DEP (0.33 U/ml). (Activity [1 U] was defined as the amount of enzyme

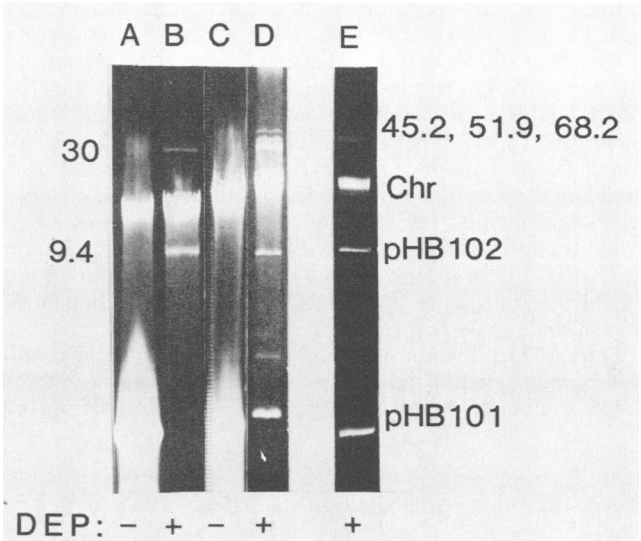

FIG. 1. Effect of DEP on the recovery of plasmid DNA from ethanol precipitates of *C. perfringens* 10543A and 3626B cleared lysates. Lane A, *C. perfringens* 10543A; lane B, *C. perfringens* 10543A plus 0.2% DEP; lane C, *C. perfringens* 3626B; lane D, *C. perfringens* 3626B plus 0.2% DEP; lane E, dye-buoyant density-purified plasmid DNA of a DEP-treated cleared lysate of *C. perfringens* 3626B. Two 1-liter cultures of *C. perfringens* 10543A and 3626B were split in half and processed identically up to the DEP addition step. One protoplast suspension of each strain received 0.2% DEP, whereas the other acted as a control. The remaining steps of the cleared lysate protocol were performed identically on all four lysates.

depolymerizing 1 μg of DNA per min at 37°C. Values are averages of separate duplicate determinations.)

DISCUSSION

The similarity in the plasmid profiles of the modified DEP-treated cleared lysate of *C. perfringens* 3626B (Fig. 1, lane D) and the corresponding dye-buoyant density-purified preparation (Fig. 1, lane E) suggests that the modified DEP-treated cleared-lysate technique may have general utility for isolating both large and small plasmids from *C. perfringens* strains without additional processing in the form of dye-buoyant density gradient centrifugation. The mechanism responsible for the elimination of RNA smearing from modified DEP-treated cleared lysates is not clear. This phenomenon has important consequences from the point of view of unmasking low-molecular-weight plasmids (e.g., pHB101) without the use of an RNase treatment.

Since Brij 58 lysates of *C. perfringens* NTG-3 and NTG-4 extracellular DNase-negative mutants demonstrated DNase activities equivalent to that of the 10543A wild-type strain, NTG-3 and NTG-4 appear to be DNase transport mutants. Consequently, the isolation of extracellular DNase-negative mutants for the specific purpose of improving plasmid recovery from *C. perfringens* cleared lysates may have little value. These results differ from those of Timmis and Winkler (21), who reported that nuclease could not be detected in toluene-lysed extracellular nuclease-negative mutants of *S. marcescens* HY. Furthermore, these same workers demonstrated that improved, reproducible yields of plasmid DNA could be obtained from extracellular nuclease-negative mutants of *S. marcescens* but not from the corresponding wild-type strain by using a modification of the Clewell and Helinski cleared lysate technique (5).

An important factor for efficient chromosomal DNA precipitation is the retention of its higher molecular weight relative to plasmid DNA (9). DEP appears to inactivate DNase (see above) that would normally degrade plasmid and chromosomal DNA, thereby improving the efficiency of the cleared lysate salt precipitation step (8) in preferentially separating CCC DNA from cellular membrane and chromosomal DNA. This observation is further substantiated by a procedural alteration made necessary after the use of 0.2% DEP. Specifically, the clearing spin of the salt-precipitated crude lysate had to be increased from 17,000 × *g* for 30 min to 50,000 × *g* for 1 h to the extremely viscous nature of the DEP-treated crude lysate, indicative of a higher concentration of undegraded chromosomal DNA.

Cellular localization and inhibition studies identified cell wall-compartmentalized DNase as primarily responsible for the loss of plasmid DNA during processing of *C. perfringens* cleared lysates. DEP is currently being utilized in the development of a rapid, small-input-volume, cleared-lysate plasmid isolation protocol for *C. perfringens*.

TABLE 1. Cellular localization of DNase activity in *C. perfringens* 10543A

Cell fraction	DNase activity (U/ml)[a]	% Total activity
Extracellular[b]	0.52	26.8
Cell wall compartmentalized	1.4	72.2
Intracellular	0.02	1.0

[a] Activity (1 U) was defined as the amount of enzyme depolymerizing 1 μg of DNA per min at 37°C. Values are averages of separate duplicate determinations.

[b] The culture supernatant was concentrated to a volume equal to that of the other cell fractions by dialysis against polyethylene glycol 6000. The extracellular cell fraction includes DNase activity present in the first cell wash.

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