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Localization and inactivation of DNase activity in *Clostridium pasteurianum* NRRL-B598

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SUMMARY

Each of four cell fractions of *Clostridium pasteurianum* NRRL-B598 contained DNase. The culture supernatant contained 31.3% of the total cellular DNase activity, while the cell wash, cell-wall compartmentalized and intracellular fractions contained 27.8, 27.8 and 13.1%, respectively. This enzyme was thermoresistant and heat treatment at various stages of the plasmid isolation protocol reduced chromosomal smearing, but did not improve plasmid recovery. The use of a new density gradient material (cesium trifluoroacetate; CsTFA) known to strongly inactivate DNase was compared to standard isopycnic centrifugation with cesium chloride. The consistent recovery of a 2.3 MDa plasmid only in the presence of CsTFA suggests that DNase continues to be a problem during ultracentrifugation as well as throughout the cleared lysate protocol.

INTRODUCTION

There are a number of reports which suggest that DNase activity is present in different species of the genus *Clostridium* [11,18,21,24, Swiatek et al., Abstracts of the 1986 Meeting of the American Society for Microbiology]. Recently, there has been an increased awareness on the role of DNase in the recovery of plasmid DNA from this genus [3,22,25; Kahn and Pierson, unpublished results]. Work in our laboratory [3] suggested that diethylpyrocarbonate (DEP) inactivates DNase activity and thereby improves plasmid recovery from *C. perfringens* strains 10543A and 3626B. However, because of the inherent instability of DEP [9], alternative means for inactivating DNase need to be examined. Heat treatment at 65°C for 3 min or 55°C for 15 or 20 min inactivated the protoplast-associated DNase of *C. acetobutylicum* SA-1 [13], although heating *C. botulinum* cell preparations to 50°C before, after, or both before and after lysis did not control DNA degradation [22].

The purpose of this investigation was to examine the presence of DNase in *C. pasteurianum* NRRL-B598, a strain reported to contain one 5.2 MDa plasmid [23]. The effect of heat treatments on the DNase associated with various cellular fractions and on plasmid recovery was also examined. Finally, the effect of a new density gradient material, cesium trifluoroacetate (CsTFA), known to specifically inhibit nuclease activity, was compared with

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cesium chloride on plasmid recovery by density gradient centrifugation.

MATERIALS AND METHODS

Microorganism, media and cultural conditions

C. pasteurianum NRRL-B598 was obtained from the Northern Regional Research Laboratory of the United States Department of Agriculture (Peoria, IL). Stock cultures were stored in cooked meat medium (CMM, Difco, Detroit, MI) at 4°C following growth in trypticase glucose yeast extract (TGY; [10]). Clostridium Basal Medium (CBM; [16]) containing 0.4% glycine [13] was used as the primary growth medium in these studies. Unless stated otherwise, the reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

All manipulations were carried out in an anaerobic atmosphere (85% N_2, 10% CO_2 and 5% H_2) in a Coy Anaerobic Chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) at 37°C. A late logarithmic phase culture (A_{600} = 0.7) was harvested by centrifugation at 10000 × *g* for 10 min at 4°C and used for DNase localization and plasmid isolation studies.

Preparation of cell fractions and DNase assay

The procedures for the preparation of the cell fractions and assay of DNase were essentially as described previously [3], except that the cell wash was not combined with the culture supernatant fraction. Also, cellular lysis was carried out using 1% sodium dodecyl sulfate (SDS) instead of sodium deoxycholate. One ml fractions were heated at 100°C/1 min, 70°C/10 min, 55°C/15 min or 44°C/20 min. Calculations of the standard error (S.E.) of the mean of replicate determinations were carried out as described by Snedecor and Cochran [19].

Plasmid isolation

(a) Cleared lysate procedure. The cleared lysate procedure described by Brefort et al. [5] and modified by Blaschek and Solberg [4] was used to isolate plasmid DNA from 1 liter of culture. When used, DEP was added immediately prior to the SDS-induced lysis step. Enrichment of cleared lysate preparations was carried out using dye-buoyant density gradient centrifugation with cesium chloride [17] or CsTFA (Pharmacia Fine Chemicals, Piscataway, NJ) adjusted to a density of 1.62 g/ml. The density of CsTFA was determined by measuring the refractive index on a refractometer (Abbe-3L, Bausch and Lomb Optical Co., Rochester, NY) and applying the formula as described by Andersson and Hjorth [2]. Since SDS forms insoluble salts in CsTFA-EtBr gradients, sodium *N*-lauroyl sarcosinate was used to prepare cleared lysates for density gradient centrifugation in CsTFA containing 0.4 mg EtBr. The centrifugations were carried out using 13.5 ml polyallomer tubes in a Beckman Ti 50 fixed angle head rotor at 40000 rpm for 48 h at 4°C. DNA bands in the gradients were observed under long-wave ultraviolet light using a hand-held UV lamp (Ultraviolet Products, Inc.). In the case of centrifugation in cesium chloride, ethidium bromide was removed by three extractions with cold (4°C) CsCl-saturated isopropanol. Ethidium bromide was removed from CsTFA gradients by three extractions with cold (4°C) CsTFA-saturated 1-pentanol [2]. The clear aqueous phase was recovered and dialyzed extensively against TES buffer to remove CsCl or CsTFA. Agarose gel electrophoresis was carried out as described previously [4,20] in Tris-acetate buffer [12]. The molecular weight of plasmid DNA was calculated using *Escherichia coli* V517 standard plasmids [14].

(b) Alkaline procedures. An additional procedure used to isolate plasmid DNA from *C. pasteurianum* was a modification of the alkaline cleared lysate protocol described previously [1]. Cell lysis was carried out using a combination of achromopeptidase (Wako Chemicals Inc., Dallas, TX; 300 U/ml) and mutanolysin (100 U/ml) in Tris-sucrose buffer [5] containing 25 mM $MgCl_2$ and $CaCl_2$. The effect of heat on plasmid recovery was examined by boiling the TES washed cell and the lytic enzyme treated suspensions for 1 min.

A modification of the alkaline cell lysis procedure described by Crosa and Falkow [8] was also used to recover plasmid DNA from *C. pasteurian-*

um. Modifications included increasing the culture volume to 4 ml, increasing the incubation time of the cell suspension containing lysis buffer (pH = 12.45) to 30 min, and extraction of the salt-precipitated lysate with phenol-chloroform (1:1) followed by chloroform, prior to ethanol precipitation.

RESULTS AND DISCUSSION

Cellular localization of DNase activity

Each of four cell fractions of *C. pasteurianum* NRRL-B598 was found to contain DNase. The culture supernatant contained 31.3%, while the cell wash, cell-wall compartmentalized and intracellular fractions contained 27.8, 27.8, and 13.1% of the total cellular DNase activity, respectively (Table I). From the point of view of plasmid recovery, DNase activity in the culture supernatant and cell wash fraction is not a great concern, since these fractions are routinely discarded prior to cellular lysis. The presence of substantial amounts of DNase activity in the cell-wall compartmentalized and intracellular fractions may, however, affect the recovery of plasmid DNA from *C. pasteurianum*. The variable and irreproducible recovery of plasmid DNA from *C. perfringens* 10543A cleared lysates was previously attributed to cell-wall compartmentalized DNase activity [3]. A summation of the DNase activities in the individual cell fractions indicated that there was 1.94 U/ml total activity in *C. perfringens* 10543A [3] and 3.2 U/ml total activity in *C. pasteurianum* NRRL-B598. Furthermore, it was observed that the DNase present in the cell fractions from *C. pasteurianum* degraded highly polymerized substrate DNA rapidly. This may be a critical factor affecting the isolation and detection of plasmid DNA in this microorganism (see below).

Inactivation of DNase activity

The effect of various heat treatments on the DNase activity of the four cellular fractions of *C. pasteurianum* NRRL-B598 was examined. Although heat partially inactivated the DNase present in each cell fraction, the low percent inhibition values (Table 2) suggest that *C. pasteurianum* DNase was resistant to thermal inactivation. The DNase present in the cell wash fraction appears even more resistant to high temperatures than that present in the other fractions. It is not clear why this is so, although cell wall associated proteins may be exerting a 'protective effect' on the thermal inactivation of this enzyme. For each cell fraction, the percent inhibition was highest for the 70°C/10 min treatment. Although activity could be reduced up to approx. 60% by heating the cell-wall compartmentalized fraction at 70°C for 10 min, heat treatment at 44°C for 20 min stimulated DNase activity. This is in agreement with earlier results [13] which demonstrated that this particular time/temperature combination stimulated the DNase associated with *C. acetobutylicum* SA-1 protoplasts.

Table 1

DNase activity of various cell fractions of *C. pasteurianum* NRRL-B598

Cell fraction[a]	DNase activity ± S.E. (U)[b]	Percent cellular activity[c]
Culture supernatant	1.0 ± 0.26	31.3
Cell wash	0.89 ± 0.08	27.8
Cell-wall compartmentalized	0.89 ± 0.01	27.8
Intracellular	0.42 ± 0.13	13.1

[a] Isolated as described in Materials and Methods.

[b] Activity (1 U) was defined as the amount of enzyme that depolymerizes 1 μg of DNA per min per ml at 37°C. Standard error is based on the mean of triplicate determinations.

[c] Percent cellular activity = $\frac{\text{Cell fraction DNase activity}}{\text{Total cellular DNase activity}} \times 100$

Table 2

Effect of various heat treatments on the DNase activity of cellular fractions of *C. pasteurianum* NRRL-B598

Treatment	Percent inhibition of DNase activity[a]			
	culture supernatant	cell wash	cell wall compartmentalized	intracellular
100°C/1 min	16.4	6.6	56.1	30.0
70°C/10 min	22.1	7.5	60.8	36.0
55°C/15 min	8.3	−2.8[b]	11.2	26.1
44°C/20 min	1.7	−6.5	−4.3	−29.5

[a] Values based on non-heat-treated control samples (see Table 1).
[b] Negative values reflect a stimulatory effect on DNase activity.

Recovery of plasmid DNA from C. pasteurianum

Plasmid DNA was not recovered from *C. pasteurianum* with the standard *C. perfringens* cleared lysate procedure. Agarose gels showed extensive chromosomal DNA smearing in these lysates (Fig. 1, lane B). The application of heat at various stages of the alkaline cleared lysate plasmid isolation protocol resulted in a reduction of chromosomal DNA smearing, but did not improve the recovery of plasmid DNA (data not shown). Strom et al. [22] reported that heating *C. botulinum* preparations to 50°C before or after lysis did not control degradation due to DNase. The addition of the nuclease inhibitor and denaturant, DEP, previously successful with *C. perfringens* [3] and *C. acetobutylicum* SA-1 [13], resulted in variable recovery of plasmid DNA from this strain. A comparison of the inhibition of the cell-wall compartmentalized DNases in this study (39% inhibition) with that seen earlier [3] for inhibition of *C. perfringens* 10543 cleared lysate DNase (60% inhibition) suggested that *C. pasteurianum* NRRL-B598 DNase is more resistant to inactivation by DEP.

Because of the variability in plasmid recovery from this DNase positive strain, enrichment of ethanol-precipitated DNA was attempted using cesium chloride–ethidium bromide dye-buoyant density gradient centrifugation. Agarose gel electrophoresis failed to detect plasmid DNA in these preparations (Fig. 1, lane C). Failure to recover plasmid DNA may result from the presence of DNase activity during the cleared lysate protocol as well as ultracentrifugation. In order to test this hypothesis, an alternative form of plasmid enrichment, CsTFA containing ethidium bromide, was utilized. CsTFA removes protein contamination by

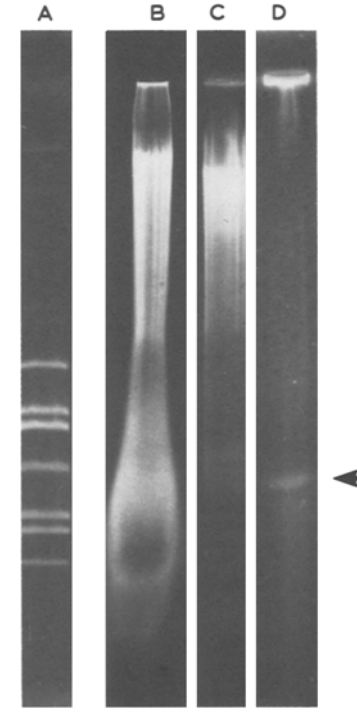

Fig. 1. Plasmid recovery from *C. pasteurianum* NRRL-B598. Lane A, *E. coli* V517 reference plasmids; lane B, cleared lysate preparation; lane C, cesium chloride density gradient purified sample; lane D, cesium trifluoroacetate density gradient purified sample showing the presence of a 2.3 MDa plasmid.

solubilization and denaturation, thereby strongly inhibiting DNase activity [7]. This procedure resulted in the recovery of a 2.3 MDa plasmid from *C. pasteurianum* NRRL-B598 (Fig. 1, lane D). Truffaut and Sebald [23] previously isolated a 5.2 MDa cryptic plasmid from *C. pasteurianum* NRRL-598 with an alkaline lysis procedure developed for *Rhizobium* [6]. In our laboratory, the application of a modification of the alkaline-lysis procedure described by Crosa and Falkow [8] also resulted in the recovery of a 5.2 MDa plasmid from this strain (data not shown). It is possible that this plasmid is present as a DNA–protein complex as suggested earlier for *C. perfringens* plasmids pHB101 and pHB102 [4]. The difference in plasmid molecular weight may therefore be related to the protein-solubilizing and dissociating effects of CsTFA. The consistent recovery of a 2.3 MDa plasmid with CsTFA, plus the fact that CsTFA specifically inhibits DNase activity, suggests that this enzyme continues to be a significant problem during ultracentrifugation as well as the cleared lysate protocol.

Because of the difficulties inherent in using DEP [9], the thermoresistance of *C. pasteurianum* DNase, and the expense in using isopycnic centrifugation, alternative means need to be developed to effectively eliminate DNase activity prior to cell lysis. The successful applications of a small-input-volume alkaline-lysis protocol based on a modification of a method described by Crosa and Falkow [8] for the isolation of plasmid DNA from *C. pasteurianum* (see above), *C. botulinum* [25] and *C. perfringens* [15] suggest that clostridial DNase may be inactivated at high pH. Whether this is also the case for *C. acetobutylicum* and other *C. perfringens* strains is currently under investigation.

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