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Transformation of Heat-Treated *Clostridium acetobutylicum* Protoplasts with pUB110 Plasmid DNA

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Heat treatment of *Clostridium acetobutylicum* SA-1 protoplasts at 55°C for 15 min before transformation resulted in expression in this microorganism of the kanamycin resistance determinant associated with plasmid pUB110. No heat treatment, or heat treatment at 65 or 44°C for various time intervals, resulted in no kanamycin resistance transformants being recovered on selective kanamycin-containing regeneration medium. DNase plate assay indicated that treatment at 55°C for 15 min completely inactivated the DNase activity associated with SA-1 protoplasts. Treatment of protoplasts at 65 or 55°C for various periods under simulated transformation conditions had an inhibitory effect, although prolonged treatment at 55 or 44°C appeared to stimulate DNase activity. Inactivation of protoplast-associated DNase activity by heat treatment at 55°C for 15 min correlated with successful expression of kanamycin resistance and suggests that an extremely active, heat-sensitive, protoplast-associated DNase may be a factor in the polyethylene glycol-induced transformation of *C. acetobutylicum* SA-1 protoplasts. Plasmid pUB110 DNA was isolated from *C. acetobutylicum* SA-1 kanamycin-resistant (Km^r) transformant cultures by a modification of the procedure used for *C. perfringens* plasmids. Detection of pUB110 DNA was possible only when diethyl pyrocarbonate was incorporated into isolation protocols to inactivate DNase activity. Restriction studies further verified the presence of pUB110 DNA in *C. acetobutylicum* SA-1 Km^r transformants.

The acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum* has a long history of using molasses or cornstarch as carbohydrate source (18, 21, 22, 32). Recent work in this laboratory with a butanol-tolerant mutant of *C. acetobutylicum* grown in extruded corn broth suggested a need for amplification of α-amylase activity in this microorganism (12). Genetic manipulation to enhance α-amylase activity in the *C. acetobutylicum* SA-1 butanol-tolerant strain may increase the final butanol concentration as well as the corn-to-butanol conversion efficiency at high concentrations of extruded corn broth.

The development of a plasmid gene transfer system for *C. acetobutylicum* SA-1 is a prerequisite for plasmid genetic manipulation of this microorganism. Normally, *Clostridium* spp. show such strong DNase activity within the cell that transformation or isolation of plasmid DNA is very difficult (23, 30). A recent localization study of *C. perfringens* DNase indicated that cell wall compartmentalized DNase was strikingly active and predominant, making up 72.2% of the total cellular DNase activity (2a). The DNases of *C. acetobutylicum* have never been investigated. Also, attempts to transfer plasmids into *C. acetobutylicum* by conjugation, conventional transformation, or protoplast transformation have been unsuccessful (1, 20). Although there is one report on the transformation of untreated *C. acetobutylicum* protoplasts by bacteriophage CA-1 DNA, the phage DNA was inactivated by the protoplasts at a high rate (20).

In this paper, we show that plasmid pUB110 DNA can be taken up and expressed by suitably heat-treated *C. acetobutylicum* SA-1 protoplasts that are able to regenerate. The effect of various heat treatments on protoplast-associated DNase activity and the relationship with polyethylene glycol (PEG)-induced transformation of *C. acetobutylicum* SA-1 protoplasts was also examined. Finally, a protocol was developed for isolation of plasmid pUB110 DNA from *C. acetobutylicum* SA-1 kanamycin-resistant (Km^r) transformants.

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MATERIALS AND METHODS

Strains, media, and culture conditions. Culture maintenance and experimental conditions for the butanol-tolerant *C. acetobutylicum* SA-1 mutant were described previously (12). *Bacillus subtilis* BD366 harboring plasmid pUB110 (11) was a gift from P. S. Lovett, Department of Biological Sciences, University of Maryland, Catonsville.

C. acetobutylicum SA-1 cells were preconditioned by growth to exponential phase (optical density of 1.0 at 680 nm; 10^8 CFU/ml) in *Clostridium* basal medium (CBM [17]) containing 0.4% (wt/vol) glycine. *C. acetobutylicum* SA-1 protoplasts were regenerated on the regeneration medium (RM) described by Allcock et al. (1) which contained 2.5% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich.).

Transformation of *C. acetobutylicum* SA-1 protoplasts. The formation and regeneration of *C. acetobutylicum* SA-1 protoplasts were carried out essentially as described by Allcock et al. (1). Transformation of *C. acetobutylicum* SA-1 protoplasts was carried out by using a modification (see below) of the procedure developed by Chang and Cohen (4) for *B. subtilis*.

Preconditioned *C. acetobutylicum* SA-1 cells were harvested at room temperature for 10 min at 12,000 × *g* and suspended in CBM containing 0.3 M sucrose, 25 mM $MgCl_2 \cdot 6H_2O$, and 25 mM $CaCl_2 \cdot 2H_2O$. Lysozyme (1 mg/ml) was added, and the suspension was incubated at 37°C for 30 min. Protoplasts were recovered by centrifugation at 2,600 × *g* for 10 min and gently washed twice with CBM containing 0.3 M sucrose. The washed protoplast suspension was brought to one-tenth of the volume of the initial culture by suspension in CBM containing 0.3 M sucrose. Samples (1 ml) of protoplast suspension were treated as shown in Table 1. All treatments were conducted in 30-ml Sorvall poly-

TABLE 1. Effect of various treatments on protoplast-associated DNase activity and transformation of *C. acetobutylicum* SA-1 protoplasts with plasmid pUB110[a]

Treatment	DNase activity[b]	CFU/ml		Transformation frequency[c]
		Viable regenerants	Transformants	
No heat treatment (control)	+++	2.7×10^8	ND[d]	0
65°C, 1 min	++	1.5×10^7	ND	0
65°C, 2 min	+	3.0×10^6	ND	0
65°C, 3 min	−	3.8×10^2	ND	0
55°C, 10 min	++	2.4×10^6	ND	0
55°C, 15 min	−	3.2×10^5	2.1×10^2[e]	6.6×10^{-4}
55°C, 20 min	−	1.1×10^2	ND	0
44°C, 20 min	+++	2.5×10^8	ND	0
44°C, 40 min	+++	1.5×10^8	ND	0
44°C, 60 min	+++	7.8×10^7	ND	0
EDTA, 25 mM		8.5×10^6	ND	0
EDTA, 2.5 mM		1.2×10^8	ND	0

[a] Final DNA concentration in the transformation mixtures was 7.5 μg/ml.
[b] Detected with modified DNase agar with methyl green. +++, Extremely active; ++, active; +, slightly active; and −, no activity.
[c] Number of transformants per viable regenerant.
[d] ND, Not detectable.
[e] Additional experiments yielded similar results for the 55°C, 15-min heat treatment, i.e., 1.1×10^2, 3.1×10^2, and 1.8×10^2 transformants per ml.

carbonate centrifuge bottles (catalog no. 03543; Du Pont Co., Biomedical Products Div., Newton, Conn.) with airtight sealing caps. The transformation mixture contained 0.5 ml of protoplast suspension, 1.5 ml of 40% (wt/vol) PEG 6000 (Du Pont Co., Wilmington, Del.) in CBM containing 0.3 M sucrose, and pUB110 DNA (final concentration, 7.5 μg/ml). The mixture was incubated at 37°C for 2 min and diluted with 5 ml of CBM containing 0.3 M sucrose, 25 mM $MgCl_2 \cdot 2H_2O$, and 25 mM $CaCl_2 \cdot 2H_2O$. The protoplasts were recovered by centrifugation at 2,600 × *g* for 10 min and suspended in 5 ml of CBM containing 0.3 M sucrose, 25 mM $MgCl_2 \cdot 6H_2O$, and 25 mM $CaCl_2 \cdot 2H_2O$. Further incubation was carried out at 37°C for 2 h. The protoplasts were diluted with CBM containing 0.3 M sucrose and plated on RM with or without added Km (125 μg/ml). RM plates were incubated at 37°C for 36 h to allow regeneration of the protoplasts.

Determination of DNase activity. The DNase activity of *C. acetobutylicum* SA-1 protoplast suspensions was evaluated after various treatments (Table 1). Two different assay techniques were used.

(i) DNase plate assay. DNase test agar with methyl green (Difco) was supplemented with cysteine hydrochloride, 0.5 g/liter, $MgCl_2 \cdot 6H_2O$, 5.08 g/liter, and $CaCl_2 \cdot 2H_2O$, 3.67 g/liter; the final pH was adjusted to 7.0. Protoplast suspensions (40 μl) in CBM containing 0.3 M sucrose were applied to wells (produced by a no. 3 cork borer) in modified DNase test agar and incubated anaerobically for 12 h at 37°C. DNase activity was indicated by a yellow zone surrounding the wells in a blue-green background.

(ii) Electrophoretic assay. The in vitro electrophoretic assay described by Williams et al. (34) was modified to evaluate the DNase activity associated with *C. acetobutylicum* protoplasts during transformation. The reaction mixture consisted of 125 μl of a twice-washed protoplast suspension, 50 μl of dye-buoyant density-purified plasmid pUB110 in CBM containing 0.3 M sucrose (final concentration, 7.5 μg/ml), and 325 μl of CBM containing 0.3 M sucrose. After incubation at 37°C for 2 min, the reaction was terminated by adding 70 μl of a solution containing 5% (wt/vol) sodium dodecyl sulfate, 25% (vol/vol) glycerol, and 0.025% (wt/vol) bromophenol blue. Samples were applied directly to wells and subjected to agarose gel electrophoresis in Tris-borate buffer as described previously (3, 29). To obtain a reference banding pattern for the covalently closed circular (CCC), open circular (OC), and linear forms of pUB110 DNA in 0.7% agarose, a limited DNase I treatment of dye-buoyant density-purified pUB110 DNA was carried out as described by Solberg et al. (29).

Effect of glycine on growth and lysis of *C. acetobutylicum* SA-1. Five percent (vol/vol) of a 24-h CBM culture of *C. acetobutylicum* SA-1 (optical density of 1.0 at 600 nm) was inoculated into CBM containing 0, 0.2, 0.4, 0.6, and 0.8% (wt/vol) glycine. For cultures containing 0, 0.2, and 0.4% glycine, growth at 37°C was monitored until an optical density of 1.0 at 600 nm was reached. A 10-ml sample of each culture was pelleted by centrifugation at 12,000 × *g* for 10 min at room temperature. The cells were immediately suspended in 9 ml of 50 mM Tris-hydrochloride buffer (pH 7.4) in Coleman cuvettes (14 by 105 mm; Coleman Instruments, Maywood, Ill.). A 1-ml portion of lysozyme (10 mg/ml in Tris-hydrochloride buffer) was added to a final concentration of 1 mg/ml. Growth and cell lysis were monitored at 37°C by measuring the optical density at 600 nm as a function of time with a Coleman model 6A spectrophotometer.

Plasmid isolation. Plasmid pUB110 was isolated from *B. subtilis* BD366 and purified of chromosomal DNA by dye-buoyant density gradient centrifugation as described by Blaschek and Solberg (3). Extensive dialysis against TE buffer was performed to ensure the removal of sodium dodecyl sulfate detergent. Essentially the same procedure was used for isolation of pUB110 DNA from *C. acetobutylicum* SA-1 Km^r transformants, with some modifications. *C. acetobutylicum* culture input was increased to 3 liters. For more efficient production of protoplasts, glycine (0.4% [wt/vol]) was incorporated into the CBM growth medium containing 10 μg of Km per ml, and lysozyme (3 mg/ml) treatment was carried out for 15 min. Diethyl pyrocarbonate (DEP; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.2% (vol/vol) immediately before disruption of protoplasts by sodium dodecyl sulfate. Viscous cleared lysates obtained after the salt precipitation step were centrifuged at 120,000 × *g* for 30 min to recover the supernatant. To avoid plasmid separation difficulties due to diffuse chromosomal banding in density gradient tubes, we used acid-phenol extraction (36) to eliminate the chromosomal DNA background before agarose gel electrophoresis was carried out. The agarose gel electrophoresis procedure with Tris-borate buffer was described previously (3).

Restriction endonuclease analysis. Restriction endonucleases were obtained from either Bethesda Research Laboratories (Gaithersburg, Md.) or Sigma. All restriction endonuclease digestions were carried out for 30 min at 37°C in a 50-μl reaction volume containing the reaction buffers recommended by the manufacturers. *Eco*RI and *Hae*III enzyme reactions were terminated by heat treatment at 65°C for 10 min, whereas phenol extraction was used to terminate the *Bam*HI reaction.

RESULTS

Effect of various treatments on transformation and DNase activity of *C. acetobutylicum* SA-1 protoplasts. The effects of various treatments on protoplast-associated DNase activity

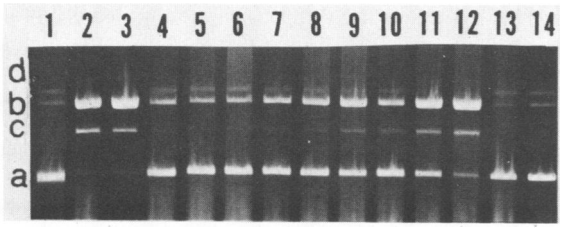

FIG. 1. Effect of various treatments on DNase activity of *C. acetobutylicum* SA-1 protoplasts after incubation for 2 min at 37°C in a simulated transformation mixture. Lanes show pUB110 DNA incubated with twice-washed protoplasts after various treatments. 1, control (no protoplasts); 2, untreated protoplasts (after two washes); 3, untreated supernatant of protoplasts (before wash); 4, 65°C, 1 min; 5, 65°C, 2 min; 6, 65°C, 3 min; 7, 55°C, 10 min; 8, 55°C, 15 min; 9, 55°C, 20 min; 10, 44°C, 20 min; 11, 44°C, 40 min; 12, 44°C, 60 min; 13, EDTA, 25 mM; 14, EDTA, 2.5 mM. Plasmid forms are marked as follows: a, CCC monomer; b, OC monomer; c, linear monomer; d, CCC oligomer.

and the PEG-induced transformation of *C. acetobutylicum* SA-1 protoplasts with plasmid pUB110 are shown in Table 1. Transformation was successful only when the protoplasts were heated at 55°C for 15 min. The experiment was repeated four times with similar results (Table 1). The transformation frequency was 6.6×10^{-4} transformants per viable regenerated protoplast or 28 transformants per μg of pUB110 DNA. Prolonged incubation of the transformation mixture (up to 10 min at 37°C) did not improve the transformation frequency, and attempts to transform treated *C. acetobutylicum* SA-1 protoplasts at 0°C were unsuccessful (data not shown).

The DNase plate assay showed the presence of an extremely active DNase associated with untreated protoplasts of *C. acetobutylicum* SA-1 (Table 1). Heat treatment at 65°C for 3 min, 55°C for 15 min, or 55°C for 20 min completely inactivated the protoplast-associated DNase. The heat treatment at 55°C for 15 min impaired the cell wall regenerative capability of *C. acetobutylicum* SA-1 protoplasts, since the number of viable regenerants was ca. 3 orders of magnitude lower than that of the untreated control. Heat treatments at 65°C for 3 min and 55°C for 20 min drastically reduced (ca. 6 orders of magnitude) the number of viable regenerants on RM plates, making the selection of Km^r transformants impossible after these treatments. Furthermore, heat treatments which had no effect or only partially inactivated the protoplast-associated DNase activity of *C. acetobutylicum* SA-1 did not result in the recovery of detectable amounts of transformants. Untreated protoplast suspensions produced a yellow zone of DNase test agar after only 30 min of incubation, whereas heat-treated samples required 4 to 5 h for zone development (data not shown).

The effect of various treatments on protoplast-associated DNase activity of *C. acetobutylicum* SA-1 under simulated transformation conditions was evaluated by the electrophoretic assay for DNase (Fig. 1). The conversion of the CCC monomeric and oligomeric forms of plasmid pUB110 into the OC and linear forms after 2 min of incubation at 37°C indicated that the twice-washed *C. acetobutylicum* SA-1 protoplast suspension (Fig. 1, lane 2) and the protoplast supernatant before washing (lane 3) showed significant DNase activity under simulated conditions of transformation. Heat treatment of *C. acetobutylicum* SA-1 protoplasts at 65°C (lanes 4 to 6) or 55°C (lanes 7 to 9) for designated periods resulted in a reduced conversion of pUB110 CCC monomeric and oligomeric DNA to OC and linear forms relative to the untreated control (lane 2). After heat treatment at 65 or 55°C, the majority of pUB110 DNA was still present as CCC monomer, although the OC bands were slightly more intense than those of the control with no added protoplasts (lane 1). Although heat treatment at 44°C for 20 min (lane 10) appeared to be as effective as heat treatment at 55°C for 20 min (lane 9), treatment at 44°C for 40 or 60 min (lanes 11 and 12) reduced the extent of pUB110 conversion by protoplast-associated DNase but was less effective than treatments at either 65 or 55°C. Interestingly, as the time of heating at 44°C increased, the intensity of the OC and linear species also increased, with a concurrent decrease in the CCC monomeric and oligomeric forms. Furthermore, incubation at 55°C for 20 min (lane 9) resulted in a slightly increased accumulation of linear plasmid DNA. The addition of 25 or 2.5 mM EDTA to the protoplast suspension did not result in the recovery of any transformants (Table 1), despite the fact that this treatment was very effective in inactivating *C. acetobutylicum* SA-1 protoplast-associated DNase activity (lanes 13 and 14). Only 25 mM EDTA was effective in preventing significant breakdown of pUB110 DNA when assayed after treatment for 30 min at 37°C (data not shown).

Effect of glycine on growth and lysis of *C. acetobutylicum* SA-1. Despite a 17-h lag phase, *C. acetobutylicum* cells grown in CBM containing 0.4% (wt/vol) glycine showed a growth rate similar to that of the CBM control. Concentrations of glycine higher than 0.4% completely inhibited growth of the *C. acetobutylicum* SA-1 culture (data not shown).

The effect of added glycine in CBM growth medium on *C. acetobutylicum* SA-1 cell lysis is shown in Fig. 2. The extent of cell lysis of CBM-grown cells treated with lysozyme (70% lysis in 30 min) was less dramatic in light of autolytic effects in the absence of lysozyme (27% lysis in 30 min). On the other hand, *C. acetobutylicum* SA-1 cells cultivated in CBM containing 0.2 or 0.4% glycine were rapidly lysed by

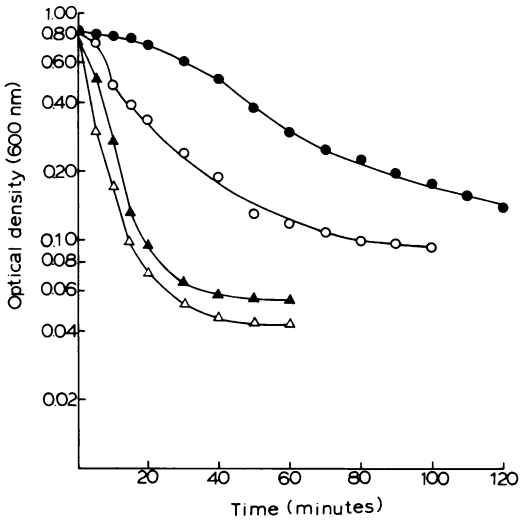

FIG. 2. Effect of glycine in CBM on *C. acetobutylicum* SA-1 cell lysis in the presence of lysozyme. Symbols: ●, CBM-grown cells without lysozyme; ○, CBM-grown cells treated with lysozyme (1 mg/ml); ▲, cells grown in CBM + 0.2% glycine treated with lysozyme; △, cells grown in CBM + 0.4% glycine treated with lysozyme. Tris-hydrochloride (50 mM, pH 7.4) was used as lysis buffer.

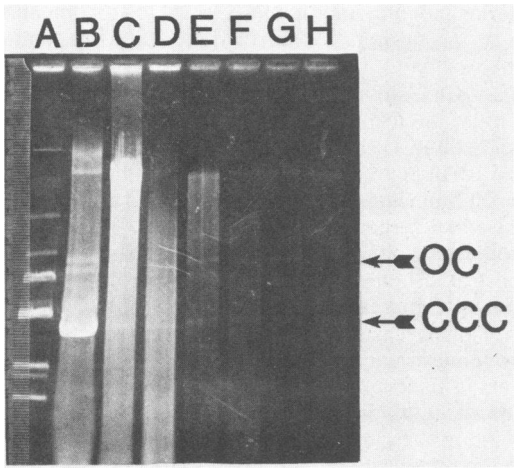

FIG. 3. Agarose gel electrophoresis of cleared lysates of *B. subtilis* BD366 and *C. acetobutylicum* SA-1 Km^r transformants before and after phenol extraction. Lanes A, Molecular weight marker *Escherichia coli* V517 plasmids; B, lysate of *B. subtilis* BD366; C, lysate of the SA-1 transformant; D, lysate from lane C boiled for 40 s; E, lysate of the SA-1 transformant after the first phenol extraction; F, boiled lysate from lane E; G, lysate of the SA-1 transformant after the second phenol extraction; H, boiled lysate from lane G.

lysozyme, and >95% lysis was obtained after a 30-min incubation period. The incorporation of 0.4% glycine into CBM resulted in the most efficient lysis of cells. This concentration of glycine was routinely added to CBM during the preparation of *C. acetobutylicum* protoplasts for either transformation or plasmid isolation.

Isolation of plasmid pUB110 from *C. acetobutylicum* SA-1 Km^r transformants. Plasmid pUB110 was isolated from Km^r transformants of *C. acetobutylicum* SA-1 by the modified DEP-treated cleared-lysate procedure (see above) together with enrichment by repeated acid-phenol extraction (Fig. 3). Although the ethanol-precipitated, modified DEP-treated cleared lysate of the *C. acetobutylicum* SA-1 Km^r transformant (Fig. 3, lane C) was masked by chromosomal DNA fragmentation, a plasmid band with the same mobility as the CCC form of pUB110 from *B. subtilis* BD366 (lane B) was barely visible after boiling (lane D). Acid-phenol extraction was very effective in unmasking CCC, OC, and oligomeric forms of pUB110 (lanes E and G). By denaturing and thereby removing linear and nicked OC DNA, boiling served as a means of distinguishing between supercoiled (CCC) monomer and oligomers and nicked (OC) forms of pUB110 (lanes F and H). Plasmid DNA was not recovered from *C. acetobutylicum* SA-1 isolates which had not undergone transformation (data not shown).

Restriction endonuclease analysis of pUB110 isolated from *B. subtilis* BD366 and *C. acetobutylicum* SA-1 Km^r transformants is shown in Fig. 4. Dye-buoyant density-purified plasmid DNAs isolated from *B. subtilis* BD366 (Fig. 4, lane A) or the *C. acetobutylicum* SA-1 Km^r transformant (lane D) each possessed single restriction sites for *Eco*RI (lanes B and E) and *Bam*HI (lanes C and F) and three sites for *Hae*III (data not shown). These sites have been reported previously (7, 11) for pUB110 isolated from *B. subtilis*.

DISCUSSION

Heat treatment of protoplasts at 55°C for 15 min allowed successful transformation of *C. acetobutylicum* with pUB110 DNA. This is a major breakthrough in the molecular biology of the clostridia. Results of the DNase plate assay (Table 1) suggested that a heat-labile DNase may be interfering with the PEG-induced transformation of *C. acetobutylicum* SA-1 protoplasts. The successful heat treatment appears to inactivate protoplast-associated DNase, without severely compromising the cell wall regenerative capability of *C. acetobutylicum* protoplasts. Heat treatment at 55°C for 15 min appears to achieve a balance in this regard, whereas more severe treatments (65°C for 3 min or 55°C for 20 min) reduce the cell wall regenerative capability of protoplasts to a point at which the transformants cannot be recovered. Preliminary evidence suggesting that protoplast-associated DNase may be a factor in the uptake of foreign DNA came from a recent report by Reid et al. (20), who showed that *C. acetobutylicum* protoplasts inactivate phage CA1 DNA at a high rate. Furthermore, these workers were unable to transfer *Staphylococcus aureus*, *B. subtilis*, or *C. perfringens* plasmids to untreated *C. acetobutylicum* protoplasts.

The use of physical treatments to inactivate interfering DNases has also been reported for $CaCl_2$-mediated transformation of *Serratia marcescens* cells. Reid et al. (19) showed that for two strains of *Serratia marcescens* no transformants were obtained with the $CaCl_2$-mediated transformation procedure unless the cells first received a heat treatment to inactivate extracellular endonuclease. The highest frequency of transformation was obtained after heat treatment at 65°C for 1 min.

Neither PEG-induced protoplast transformation nor $CaCl_2$-mediated transformation involves an uptake system that is selective for multimeric plasmid DNA, nor is the incoming DNA converted to a single-stranded intermediate during uptake, as for *B. subtilis* competent cell transformation (9, 14, 28). It was recently reported that protoplasts of competence-negative *B. subtilis* mutants could be transformed by

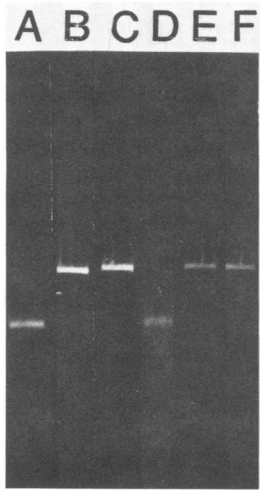

FIG. 4. Analysis of dye-buoyant density-purified plasmid pUB110 from *B. subtilis* BD366 and the Km^r transformant of *C. acetobutylicum* SA-1 after restriction endonuclease digestion. Plasmid pUB110 was digested with *Eco*RI and *Bam*HI at 37°C for 30 min and analyzed by 0.7% agarose gel electrophoresis. Lanes: A, Undigested pUB110 DNA from *B. subtilis*; B, *Eco*RI-digested pUB110 from *B. subtilis*; C, *Bam*HI-digested pUB110 from *B. subtilis*; D, undigested pUB110 DNA from *C. acetobutylicum*; E, *Eco*RI-digested pUB110 from *C. acetobutylicum*; F, *Bam*HI-digested pUB110 from *C. acetobutylicum*.

plasmid pUB110 DNA (10). Consequently, inactivation of DNases which degrade incoming exogenous DNA should not diminish the success of either protoplast- or $CaCl_2$-mediated transformation.

The DNase associated with either twice-washed or unwashed *C. acetobutylicum* SA-1 protoplast suspensions was strikingly active under simulated transformation conditions (Figure 1, lanes 2 and 3). These results suggest that a fraction of the DNase can be tightly bound or loosely associated with the protoplasts. On the basis of the electrophoretic assay, one can calculate that the amount of DNase present in 0.5 ml of protoplast suspension is sufficient to degrade 15 μg of DNA in 30 min at 37°C. Such DNase activity undoubtedly is a significant barrier to the transformation of *C. acetobutylicum* SA-1 protoplasts. Also, prolonged heat treatment of *C. acetobutylicum* SA-1 protoplasts at either 55 or 44°C appeared to increase the extent of DNA degradation (Fig. 1, lanes 9 and 11 and 12, respectively). The protoplast-associated DNase of *C. acetobutylicum* SA-1 appears to have greater heat resistance than the extracellular nuclease from *Serratia marcescens*, which was completely inactivated by heat treatment at 44°C for 40 min (15). The lack of absolute correlation between results obtained with the DNase plate assay (Table 1) and the electrophoretic assay (Fig. 1) suggests that heat-labile DNase may not be the sole factor interfering with the PEG-induced transformation of *C. acetobutylicum* SA-1 protoplasts. It is possible that heat treatment may alter the cell membrane or inhibit a heat-sensitive restriction-modification system (24) in *C. acetobutylicum* and thereby allow for plasmid uptake.

Although EDTA caused the greatest inhibition of protoplast-associated DNase activity (Fig. 1, lanes 13 and 14), addition of this compound to the protoplast suspension did not result in successful transformation (Table 1). EDTA is known to exert a destablizing effect on the cell wall and cell membrane (25). It is frequently used in combination with lysozyme to convert cells of many gram-negative bacteria to true protoplasts (13, 33). In some cases, EDTA alone is sufficient to cause osmotic shock in hypotonic solution by eliminating the cell wall and outer membrane (16). Recently, Klacik (M.S. thesis, University of Illinois, Urbana, 1983) found that autolysis and cytoplasmic leakage of *C. perfringens* cells in 0.3 M sucrose was increased dramatically by the addition of 10 mM EDTA. In *B. subtilis* cells, the DNA-binding receptors associated with the cytoplasmic membrane were inactivated by the presence of EDTA (28). These factors may contribute to the unsuccessful transformation of EDTA-treated *C. acetobutylicum* SA-1 protoplasts.

This is the first report of successful transformation of *C. acetobutylicum* with plasmid DNA. Although the transformation frequency was below that obtained during transformation of *B. subtilis* protoplasts with pUB110 DNA (4), the reaction conditions for PEG-induced transformation of *C. acetobutylicum* SA-1 protoplasts appeared to be optimized at 37°C for 2 min, since neither cold shock (°C) nor prolonged incubation at 37°C gave better results. However, increasing the protoplast input or plasmid DNA concentration in the reaction mixture may increase the number of transformants recovered from RM plates. Although the mechanism of PEG-induced transformation is not well understood, it is known that *B. subtilis* protoplasts can be transformed by CCC monomeric plasmid DNA at about the same efficiency as by dimer and higher oligomers of DNA (6, 14). Linear plasmid DNA transforms *B. subtilis* protoplasts as well as circular molecules, and nicked monomers transform as well as the CCC form (6). In view of results of the in vitro electrophoretic assay (Fig. 1) and the transformation experiments (Table 1), the CCC form of plasmid pUB110 DNA may be required for the transformation of *C. acetobutylicum* SA-1 protoplasts, unlike the case in *B. subtilis*.

C. acetobutylicum SA-1, a butanol-tolerant strain capable of growing in the presence of at least 20 g of butanol per liter (12), showed extensive autolysis when suspended in Tris-hydrochloride buffer (Fig. 2). This finding disagrees with the report by Van Der Westhuizen et al. (31), who suggested that there is an inverse relationship between butanol tolerance and autolysis in *C. acetobutylicum*. They found that an autolysis-deficient mutant (*lyt-1*) was able to grow in higher concentrations of butanol than could the P262 parent strain. The *C. acetobutylicum* SA-1 strain used in this study is even more butanol-tolerant than the *lyt-1* autolysis-deficient mutant described by Van Der Westhuizen et al. (31); however, the SA-1 strain still undergoes significant autolysis.

Growth of bacteria in the presence of high concentrations of glycine has been reported to result in destabilization of the cell wall owing to replacement of D-alanine residues by glycine in the peptidoglycan chain, thereby interfering with cross-linking (8). As a result of a weakened cell wall structure, degradation by lysozyme is more efficient. For *C. acetobutylicum* SA-1, optimal cell lysis was achieved by growth in CBM containing 0.4% (wt/vol) glycine and subsequent lysozyme treatment (1 mg/ml) at 37°C for 30 min. These results are in agreement with those of other workers (1, 20) for the optimal production of protoplasts from *C. acetobutylicum* P262 and P262J, respectively.

Isolation of plasmid DNA from *C. acetobutylicum* and other related species is difficult because of the resistance of the cell wall to the lytic action of lysozyme (30), significant DNase activity (2a, 30), and autolysis induced by hypertonic sucrose or NaCl buffer (1, 35). The modified DEP-treated cleared-lysate procedure was developed to minimize these problems. However, the yield of pUB110 DNA from *C. acetobutylicum* SA-1 Km^r transformants was low in contrast to that obtained from *B. subtilis* (Fig. 3). The coexistence of fragmented DNA and pUB110 DNA in the agarose gel suggests that the modified DEP-treated cleared-lysate procedure is not as effective at inhibiting the DNase of *C. acetobutylicum* SA-1 when compared with *C. perfringens* 10543A (2a). Chloramphenicol (200 μg/ml) amplification of plasmid DNA did not improve the yield of pUB110 DNA from the *C. acetobutylicum* SA-1 Km^r transformant, as was the case for *B. subtilis* (27).

The problems inherent in attempting to retransform the starting SA-1 strain with DNA derived from *C. acetobutylicum* are twofold. First, the DNase activity of *C. acetobutylicum* cleared lysates appears to be even greater than that of *C. perfringens* 10543A (2a). Purified CCC pUB110 DNA added to the cell pellet from 500 ml of *C. acetobutylicum* culture could not be recovered from cleared lysates (data not shown). Consequently, it is very difficult to recover intact plasmid DNA for retransformation experiments. Second, plasmid pUB110 DNA was isolated from *C. acetobutylicum* SA-1 only when DEP was incorporated into the isolation protocol (Fig. 3). DEP, however, appears to adversely affect the biological activity of nucleic acids (5). The competent cell transformation frequency of *B. subtilis* 1A297 with DEP-isolated pUB110 was 10-fold lower than the frequency of transformation with pUB110 isolated without DEP (data not shown). This decrease in biological activity of DEP-isolated DNA is important in light of the already low number of *C. acetobutylicum* transformants recovered in the primary transformation (Table 1).

Attempts to transform *C. acetobutylicum* SA-1 heat-treated (55°C for 15 min) protoplasts with plasmids other than pUB110, such as pIP401 (26) and pBC16 (2), were not successful. The pUB110 plasmid-vector system has the potential for introducing recombinant molecules into *C. acetobutylicum* SA-1 and other related strains. The usefulness of this system for molecular cloning of native and foreign DNA is currently under investigation.

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LITERATURE CITED

1. **Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods.** 1982. *Clostridium acetobutylicum* protoplast formation and regeneration. Appl. Environ. Microbiol. **43**:719–721.
2. **Bernhard, K., H. Schrempf, and W. Goebel.** 1978. Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J. Bacteriol. **133**:897–903.
2a. **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.
3. **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.
4. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
5. **Ehrenberg, L., I. Fedorcsak, and F. Solymosy.** 1976. Diethyl pyrocarbonate in nucleic acid research. Prog. Nucleic Acid Res. Mol. Biol. **16**:189–262.
6. **Gryczan, T., S. Contente, and D. Dubnau.** 1980. Molecular cloning of heterologous chromosomal DNA by recombination between a plasmid vector and a homologous resident plasmid in *Bacillus subtilis*. Mol. Gen. Genet. **177**:459–467.
7. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
8. **Hammes, W., K. H. Schleifer, and O. Kandler.** 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. J. Bacteriol. **116**:1029–1053.
9. **Hopwood, D. A.** 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. **35**:237–272.
10. **Jandova, Z., and P. Tichy.** 1982. Transformation of protoplasts of nontransformable *Bacillus subtilis* mutants by plasmid pUB110 DNA. Folia Microbiol. **27**:465–467.
11. **Keggins, K. M., P. S. Lovett, and E. J. Duvall.** 1978. Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75**:1423–1427.
12. **Lin, Y.-L., and H. P. Blaschek.** 1983. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl. Environ. Microbiol. **45**:966–973.
13. **Matsushita, K., E. Shinagawa, O. Adachi, and M. Armeyama.** 1981. Spheroplast of acetic acid bacteria. Agric. Biol. Chem. **45**:1515–1518.
14. **Mottes, M., G. Grandi, V. Sgaramella, V. Canosi, G. Morelli, and T. A. Trautner.** 1979. Different specific activities of the monomeric and oligomeric forms of plasmid DNA in transformation of *B. subtilis* and *E. coli*. Mol. Gen. Genet. **174**:281–286.
15. **Nestle, M., and W. K. Roberts.** 1969. An extracellular nuclease from *Serratia marcescens*. I. Purification and some properties of the enzyme. J. Biol. Chem. **244**:5213–5218.
16. **Neu, H. C., and L. A. Hepple.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3692.
17. **O'Brien, R. W., and J. A. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
18. **Prescott, S. C., and C. G. Dunn.** 1959. Industrial microbiology, p. 250–284. McGraw-Hill Book Co., New York.
19. **Reid, J. D., S. S. Stoufer, and D. M. Ogrydziak.** 1982. Efficient transformation of *Serratia marcescens* with pBR322 plasmid DNA. Gene **17**:107–112.
20. **Reid, S. J., E. R. Allcock, D. T. Jones, and D. R. Woods.** 1983. Transformation of *Clostridium acetobutylicum* protoplasts with bacteriophage DNA. Appl. Environ. Microbiol. **45**:305–307.
21. **Rose, A. H.** 1961. Acetone-butanol fermentation, p. 161–166. *In* Industrial microbiology. Butterworth Publishers, Inc., Washington, D.C.
22. **Ryden, R.** 1958. Development of anaerobic fermentation processes: acetone-butanol, p. 125–145. *In* R. Steel (ed.), Biochemical engineering. Heywood Books, London.
23. **Sakaguchi, K.** 1980. Species barrier to the maintenance and expression of foreign DNA, p. 1–7. *In* K. Sakaguchi and M. Okanishi (ed.), Molecular breeding and genetics of applied microorganisms. Kodansha International Ltd., Tokyo.
24. **Schell, J., and W. Glover.** 1966. The effect of heat on host-controlled restriction of phage λ in *Escherichia coli* K(P1). J. Gen. Microbiol. **45**:61–72.
25. **Schnaitman, C. A.** 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. **108**:553–563.
26. **Sebald, M., D. Bouchaud, and G. Bieth.** 1975. Nature plasmidique de la resistance à plusieurs antibiotiques chez *Clostridium perfringens* type A, souche 659. C. R. Acad. Sci. Ser. D **280**:2401–2404.
27. **Shivakumar, A. G., and D. Dubnau.** 1978. Plasmid replication in *dna* Ts mutants of *Bacillus subtilis*. Plasmid **1**:405–406.
28. **Smith, H. O., D. B. Danner, and R. A. Deich.** 1981. Genetic transformation. Annu. Rev. Biochem. **50**:41–68.
29. **Solberg, M., H. P. Blaschek, and P. Kahn.** 1981. Plasmids in *Clostridium perfringens*. J. Food Saf. **3**:267–289.
30. **Urano, N., I. Karube, S. Suzoki, and T. Yamada.** 1983. Isolation and partial characterization of large plasmids in hydrogen-evolving bacterium *Clostridium butyricum*. Eur. J. Appl. Microbiol. **17**:349–354.
31. **Van Der Westhuizen, A., D. T. Jones, and D. R. Woods.** 1982. Autolytic activity and butanol tolerance of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **44**:1277–1281.
32. **Walton, M. T., and J. L. Martin.** 1979. Production of butanol-acetone by fermentation, p. 197–209. *In* H. J. Peppler and D. Perlman (ed.), Microbial technology, vol. 1: Microbial processes. Academic Press, Inc., New York.
33. **Weiss, R. L.** 1976. Protoplast formation in *Escherichia coli*. J. Bacteriol. **128**:668–670.
34. **Williams, J. A., J. P. Yeggy, and A. J. Markovetz.** 1980. Role of nucleases in the isolation of plasmid deoxyribonucleic acid from *Pseudomonas cepacia* 4G9. J. Bacteriol. **143**:1057–1059.
35. **Yoshino, S., S. Ogata, and S. Hayashida.** 1982. Some properties of autolysis of *Clostridium saccharoperbutylacetonicum*. Agric. Biol. Chem. **46**:1243–1248.
36. **Zasloff, M., G. D. Ginder, and G. Felsenfeld.** 1978. A new method for the purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1152.