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Regeneration of cells from protoplasts of *Clostridium acetobutylicum* B643

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SUMMARY

Conditions that allow regeneration of cells from *Clostridium acetobutylicum* strain B643 protoplasts were studied. Protoplast formation and stabilization in minimal media with 50 mM $CaCl_2$, 50 mM $MgCl_2$ and 0.3 M sucrose were crucial to subsequent regeneration on soft yeast extract agar containing 25 mM $CaCl_2$ and 25 mM $MgCl_2$. A regeneration frequency of 8–25% was consistently obtained.

INTRODUCTION

C. acetobutylicum is a gram-positive soil anaerobe that ferments glucose to butyric and acetic acids until the later phase of vegetative growth when it switches to form the solvents butanol, acetone and ethanol [3]. In order to understand the regulation of this switching mechanism, development of a genetic system in this organism is essential [11].

Cell fusion and transformation are the most promising approaches to genetic studies in gram-positive organisms [7]. Fusion and transformation in gram-positive bacteria necessitate the formation of protoplasts that can be regenerated back into walled cells. Since the original reports of fusion of *Bacillus subtilis* protoplasts [12] and the high frequency transformation of *Bacillus* protoplasts [2], regeneration for use in genetic studies has been accomplished in a variety of bacteria. The regeneration of *Clostridium* species has been achieved using different types of regeneration media. Allcock et al. [1] were able to regenerate the cells from protoplasts of *C. acetobutylicum* p262 plated on the defined medium of O'Brien and Morris [10] with 2% agar supplemented with gelatin, divalent cations, and casein hydrolysate. Regeneration of cells from *C. pasteurianum* protoplasts [9] depended upon 15% lactose as a stabilizer and *N*-acetylglucosamine as a cell wall precursor. The protoplasts of *C. saccharoperbutylacetonicum* were regenerated to bacillary form in a yeast extract-based agar medium with 10% PEG added as a stabilizing agent [14]. *C. perfringens* autoplasts can be regenerated into walled cells when grown on nitrocellulose filters and then inoculated into brain heart infusion (BHI)

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medium supplemented with sucrose and 25% gelatin [6]. *C. perfringens* protoplasts can be regenerated on thioglycolate medium supplemented with $CaCl_2$, $MgCl_2$ and agar [13].

None of these available methods gave sufficient regeneration of *C. acetobutylicum* strain B643 for use in genetic studies. Therefore, conditions that allow regeneration of *C. acetobutylicum* B643 were determined and are reported here. Portions of this work have been presented previously (P. Reilly and P. Rogers, First Annual ASM Conference on Biotechnology, Washington DC, 1986; Program and Abstracts, p. 26, American Society for Microbiology, Washington DC).

MATERIALS AND METHODS

Spore cultures of *Clostridium acetobutylicum* strain B643 were obtained from the Northern Regional Research Center, Peoria, IL 61604. *C. acetobutylicum* strain SA-1 [8], a butanol-resistant mutant of strain ATCC 824, was obtained from Hans Blaschek. *Clostridium* basal medium (CBM) as described by O'Brien and Morris [10] contains per liter: glucose, 10 g; $MgSO_4$ anhydrous, 0.08 g; $MnSO_4 \cdot H_2O$, 0.0063 g; $FeSO_4 \cdot 7H_2O$, 0.0083 g; K_2HPO_4, 0.784 g; KH_2PO_4, 0.224 g; casein hydrolysate, 0.8 g; PABA, 0.001 g; biotin, 2 μg. Yeast extract media (YEM) is CBM without PABA and biotin, and supplemented (per liter) with: casein hydrolysate, 2.5 g; asparagine, 1 g; cysteine, 0.5 g; yeast extract, 8 g. The pH was adjusted to 7.0.

Standard Regeneration agar (SRA) contains CBM, without biotin or PABA, supplemented (per liter) with: yeast extract, 8.0 g; casein hydrolysate, 2.2 g; asparagine, 1.0 g; cysteine, 0.5 g; $CaCl_2 \cdot 2H_2O$, 4.0 g; $MgCl_2 \cdot 6H_2O$, 5.0 g; and agar, 8.0 g. All organic components were autoclaved separately from salt components and then added together. The pH was adjusted, prior to autoclaving, to 7.0 with 3.0 M NaOH.

Protoplasting media (PPM) contained, for standard regeneration experiments: CBM; 0.3 M sucrose; 50 mM $CaCl_2 \cdot 2H_2O$; and 50 mM $MgCl_2 \cdot 6H_2O$. The pH was adjusted to 7.5.

All manipulations, except centrifugations, were carried out in a Forma Anaerobic Chamber, model 1024 (Forma Scientific, Marietta, OH).

C. acetobutylicum B643 spores were inoculated into 25 ml of CBM containing 1.0% glucose and then incubated overnight at 35°C. Strain SA-1 spores were heat-shocked for 90 s at 80°C prior to inoculation. Mid-exponentially growing cells were harvested by centrifugation at 10 600 × *g* for 10 min and then suspended in 5 ml of PPM. Lysozyme (2.5 mg/ml, chicken egg white grade 1, Sigma) was added to the cell suspension and protoplasts formed after incubation at 35°C for 30–60 min. Protoplasts were centrifuged at 3300 × *g* for 5 min and suspended in fresh PPM without lysozyme. Percent protoplast formation was determined by phase contrast microscopy.

Polyethylene glycol (PEG) (Sigma Chemical Co.), M.W. 1000, was dissolved in PPM by heating gently. The warm PEG solutions were sterilized by passage through a 0.22 μm Millipore filter. Treatment of protoplasts with PEG was accomplished by dilution of 0.25 ml of washed protoplasts with 0.75 ml of PEG solution (final concn. 25%), incubation at room temperature for 2 min followed by a 1:100 dilution in PPM.

To regenerate the cell wall, protoplasts were diluted in PPM, plated at various concentrations on SRA and incubated 24–48 h at 35°C. Percent protoplast regeneration was determined by the following equation:

$$\% \text{ Regeneration} = \frac{\text{CFU/ml regenerated protoplasts} - \text{CFU/ml non-protoplasted units}}{\text{CFU/ml cells before protoplasting}} \times 100$$

where CFU are colony forming units present after incubation. See Table I for example data.

RESULTS

The optimum growth phase of *C. acetobutylicum* for protoplast formation was determined by re-

moval of 5 ml samples at several points during growth and comparison of their ability to form protoplasts as described in Materials and Methods. Percent protoplast formation was determined microscopically at 40 min after the addition of lysozyme. Protoplast formation was optimum from cultures in early exponential phase. Fig. 1 shows data from one representative experiment. The type of stabilizer used in PPM to prevent osmolysis had a large effect on protoplast formation. Succinate (0.3 M) instead of sucrose in PPM allowed lysis of forming protoplasts. Mannitol (0.3 M) instead of sucrose in PPM yielded only 20% conversion of the cells to protoplasts. Glucose, lactose and sucrose (0.3 M) permitted greater than 90% protoplast formation. Other investigators [1,9] have suggested that the addition of *N*-acetylglucosamine (GlcNAc) and/or bovine serum albumin (BSA) to PPM or a recovery period in high nutrient media prior to regeneration would enhance protoplast regeneration. These modifications did not increase regeneration in *C. acetobutylicum* B643.

Two types of growth media were tested for their effect on regeneration; YEM and CBM. We observed an increase in percent regeneration when *C.*

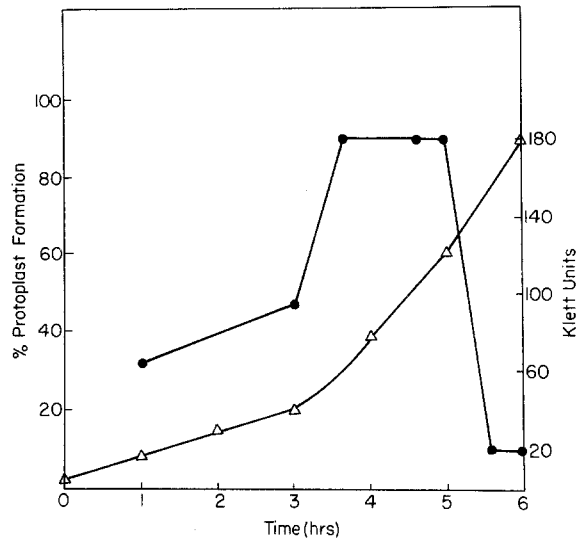

Fig. 1. Correlation of growth phase with induction of protoplast formation by lysozyme. *C. acetobutylicum* spores were inoculated into 25 ml of CBM containing 1% glucose and allowed to germinate at 35°C. Growth of the 25 ml culture was determined by measuring turbidity (100 Klett units = 0.2 O.D. units), (△—△). When the Klett reached 5 (≈18 h post-inoculation) and once per hour thereafter, samples of cells were washed and resuspended in CBM containing 0.3 M sucrose and lysozyme (2 mg/ml). The percent protoplasts formed at 40 min was determined microscopically, (●—●).

Table I

Regeneration of *C. acetobutylicum* B643

Expt. No.	Cells[a] (CFU/ml)	Regenerated protoplasts[b] (CFU/ml)	Non-protoplasted units[c] (CFU/ml)	Percent regeneration[d]
1	$7 \cdot 10^4$	$1.0 \cdot 10^4$	$1.0 \cdot 10^2$	14.2
2	$5 \cdot 10^6$	$1.2 \cdot 10^6$	$1.0 \cdot 10^4$	24.0
3	$22 \cdot 10^7$	$2.7 \cdot 10^7$	$0.7 \cdot 10^7$	9.5
4	$6.8 \cdot 10^7$	$0.75 \cdot 10^7$	$1.0 \cdot 10^5$	11.0
5	$8.5 \cdot 10^7$	$0.69 \cdot 10^7$	$2.0 \cdot 10^5$	8.1
6	$1.3 \cdot 10^7$	$0.64 \cdot 10^7$	$3.0 \cdot 10^6$	26.5
7[e]	$4.4 \cdot 10^6$	$1.0 \cdot 10^4$	$1.0 \cdot 10^3$	0.2

[a] CFU/ml of cells grown in CBM prior to protoplast formation was determined from a viable count after 48 h growth at 35°C on SRA.

[b] Protoplasts were formed in PPM by treatment with lysozyme (2.5 mg/ml) for 60 min, centrifuged, suspended in PPM and plated on SRA. CFU/ml of regenerated protoplasts was determined after 48 h growth at 35°C.

[c] Non-protoplasted units were determined from a viable count of the protoplast suspension after dilution in CBM to bring about osmotic lysis of protoplasts.

[d] Percent of regeneration was calculated as described in Materials and Methods.

[e] The cell culture was grown in yeast extract medium. This experiment was done in parallel with Expt. 6.

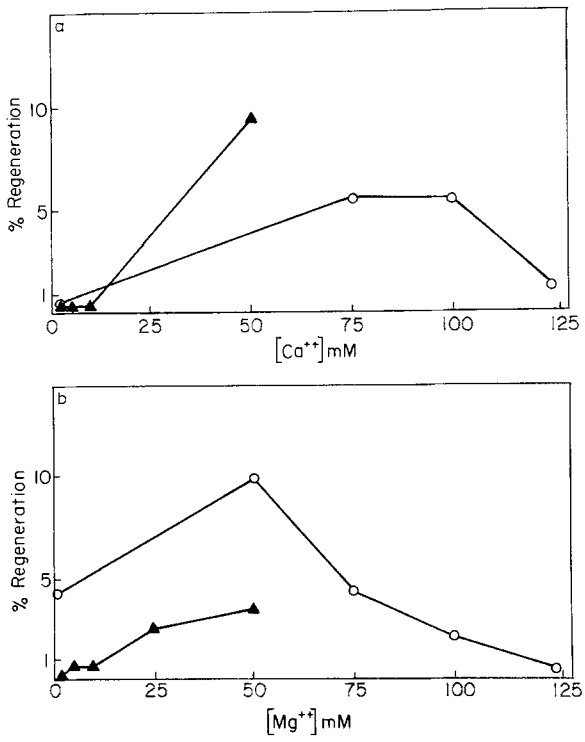

Fig. 2. Effect of Ca^{2+} and Mg^{2+} on regeneration. Harvested exponentially grown cells of *C. acetobutylicum* were suspended in protoplasting media (PPM) without Ca^{2+} or Mg^{2+}. Prior to addition of lysozyme to form protoplasts, the desired concentrations of Ca^{2+} or Mg^{2+} were added. Protoplasts were diluted in standard PPM and spread on Standard Regeneration agar (SRA). Colonies were counted and percent regeneration calculated as described in Materials and Methods. Panel a: varying concentrations of $CaCl_2 \cdot 2H_2O$ with constant concentration of $MgCl_2 \cdot 6H_2O$ at 25 mM (▲) and 50 mM (○). Panel b: varying concentrations of $MgCl_2 \cdot 6H_2O$ with constant concentration of $CaCl_2 \cdot 2H_2O$ at 25 mM (▲) and 50 mM (○).

acetobutylicum was grown in CBM rather than the richer YEM (Table I, Expts. 6 and 7). There was an 8.1–26.5% regeneration of the protoplasts from cells grown in CBM, while only about 0.2% regeneration of protoplasts from cells grown in YEM was found.

Divalent cations, usually Mg^{2+} and Ca^{2+}, are used to stabilize the protoplast membrane, whereas the addition of a chelator such as EDTA destabilizes the membrane [5]. Fig. 2 demonstrates that Ca^{2+} and Mg^{2+} in the PPM during protoplasting effect subsequent protoplast regeneration. Both Ca^{2+} and Mg^{2+} seem to be required for maximum regeneration at concentratrions over 25 mM. Little or no regeneration occurred when the Ca^{2+} concentration in the PPM was 10 mM or below.

Four types of plating medium for regeneration were used to attempt regeneration of *C. acetobutylicum* B643 protoplasts: *C. pasteurianum* yeast extract agar with added lactose [9]; *Bacillus* regeneration agar [2]; *C. perfringens* BHI with added sucrose and gelatin [6]; *C. acetobutylicum* strains p262 and SA-1 CBM with 5% gelatin [1,8]. When compared to the SRA described in this paper, the four published regeneration methods did not result in adequate regeneration of *C. acetobutylicum* strain B643. However, initial studies indicate that *C. acetobutylicum* strain SA-1 [8] can be regenerated using the SRA reported here.

Sucrose (0.3 M), lactose (0.5 M), gelatin 5% and BSA 1% added to SRA did not increase regeneration, and, in fact, both sucrose and lactose reduced regeneration several-fold. Variation in percent agar in the SRA from 0.8% to 1.5% did not have a great effect on regeneration; however, 2.5 and 3.0% agar decreased the number of regenerating protoplasts to 1–4%.

Polyethylene glycol (PEG) is used to promote protoplast fusion and uptake of DNA by protoplasts [2,4]. Since protoplasts are often treated with PEG prior to regeneration, the effect of PEG on regeneration of protoplasts was investigated. Treatment for 2 min at concentrations of PEG from 0 to 25% had no effect on percent regeneration or caused only a slight decrease in regeneration. PEG at concentrations greater than 30% decreased the percent regeneration by a factor of a thousand.

DISCUSSION

The number of procedures reported for protoplast formation and regeneration is testimony to the species-specific nature of regeneration. Therefore, the main focus of this work was to develop a system to regenerate walled cells from protoplasts of *C. acetobutylicum* strain B643. Protoplasts of *C. acetobutylicum* strain SA-1 [8], the only other strain tested with this procedure, were also regenerated.

This procedure may be adapted for other *Clostridium* species and strains.

Cell growth and protoplast formation have a critical effect on the percent regeneration. Data presented here show that very little regeneration occurs when cell growth and protoplast formation are carried out in a rich yeast extract medium. A hypothesis offered by Gabor and Hotchkiss [5] suggests that protoplast formation in rich media may cause early and efficient restoration of cell metabolism and lead to growth and expansion before adequate cell wall formation. Since protoplasts cannot divide they literally burst. A prompt vigorous metabolic rate also encourages segregation in recombinants and may also reduce regeneration. These data and the hypothesis offered by Gabor and Hotchkiss are also supported by *Streptomyces* data that show increased regeneration in the presence of low phosphate even though cell growth is optimal at high phosphate concentrations [7].

The best regeneration of strain B643 occurred when protoplast formation was carried out with mid-exponential phase cells grown in CBM, harvested and resuspended in CBM with added Mg^{2+} and Ca^{2+} (50 mM), 0.3 M sucrose and treated with 2.5 mg/ml of lysozyme for 1 h at 35°C. A similar protoplasting medium (PPM) was reported by Allcock et al. [1] for protoplast formation in *C. acetobutylicum* strain p262. The optimum Mg^{2+} and Ca^{2+} concentration was 25 mM for strain p262 as compared with 50 mM described here for strain B643.

Some investigators report a large increase in regeneration when GlcNAc is added to regenerating protoplasts, acting as a cell wall precursor or binding excess lysozyme [9]. In our studies, GlcNAc added to PPM had no effect on regeneration but it did inhibit protoplast formation. GlcNAc added to regeneration media also had no effect on regeneration. This may indicate that added GlcNAc binds to lysozyme, inhibiting its enzymatic action on the newly forming cell wall. Sufficient washing of protoplasts prior to regeneration may substitute for the use of GlcNAc.

The physical and chemical stabilization of the protoplast membrane is the most critical consideration for regeneration. It is therefore not surprising that those parameters tested that contributed to a stable undamaged membrane also increased regeneration by the greatest factor. An important consideration is gentle treatment of the protoplasts. It was observed that repeated centrifugation at higher speeds decreased regeneration. The use of soft agar (0.8%) increased regeneration to approximately 2–3-fold. Certain species of *Bacillus* and *Streptomyces* also show an increase in regeneration when plated on soft agar or in a soft agar overlay [7].

The optimal regeneration agar (SRA) for strain B643 was CBM containing yeast extract, casein hydrolysate, Mg^{2+} (25 mM) and Ca^{2+} (25 mM), and agar (0.8%). SRA does not contain any component typically used by other workers as an osmotic stabilizer. Gelatin, PEG, sucrose or lactose did not enhance regeneration as reported for *C. acetobutylicum* strains p262 and SA1, *C. saccharoperbutylacetonicum, C. perfringens* and *C. pasteurianum* [1,8,14,6,9]. In fact, gelatin was reported to reduce regeneration of *C. pasteurianium* and *C. perfringens* [9,13], and we observed inhibition of growth of *C. acetobutylicum* with high concentrations of added sucrose or lactose. The reason for this reported difference is unknown.

Usually protoplasts will be treated with the fusogen PEG, prior to regeneration. PEG has been shown to induce fusion of protoplasts [5] and transformation of protoplasts by plasmid DNA [2]. Other investigators have used PEG concentrations of 22.5–50% for cell fusion with little reduction in percent regeneration [7]. Treatment of *C. acetobutylicum* B643 protoplasts with PEG (25%) induced cell aggregation but only reduced regeneration slightly. Thus, the regeneration procedure reported here may facilitate studies using PEG for fusion and transformation of *C. acetobutylicum* B643 protoplasts.

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