Journal of Industrial Microbiology, 4 (1989) 325–332
Elsevier

SIM 00190

Protoplast formation, L-colony growth, and regeneration of *Clostridium beijerinckii* NRRL B-592 and B-593 and *Clostridium acetobutylicum* ATCC 10132

G.A. Birrer, W.R. Chesbro and R.M. Zsigray

Department of Microbiology, University of New Hampshire, Durham, NH, U.S.A.

Received 12 August 1988
Accepted 16 December 1988

Key words: Clostridium genetics; *Clostridium beijerinckii; Clostridium acetobutylicum;* Protoplast regeneration; L-colony

SUMMARY

Protocols for protoplast formation, L-colony cultivation, and regeneration of *Clostridium beijerinckii* NRRL B-592, B-593 and *C. acetobutylicum* ATCC 10132 were developed. Two osmotically reinforced media were formulated. Protoplasts of B-592, B-593, and ATCC 10132 grew as cell wall-deficient forms (L-colonies) when plated on the first medium (BLM) and continued to do so through at least 3 passages on this medium. The second (BRM) permitted the L-colonies to regenerate cell walls after transfer to this medium. Transferred *C. beijerinckii* B-592 L-colonies reverted to bacillary colonies at a frequency of 25%. Likewise, L-colonies of B-593 and *C. acetobutylicum* ATCC 10132 could be regenerated at frequencies of 7.0 and 8.6%, respectively. Thus, these procedures are suitable for genetic engineering of these industrial microorganisms using protoplast manipulation techniques.

INTRODUCTION

Clostridium beijerinckii (butylicum) and *Clostridium acetobutylicum* have industrial potential because of their ability to produce solvents from various carbohydrates via a two-stage fermentation. In the acidogenic stage acetate and butyrate are the primary products produced, while small amounts or no ethanol may also be produced. In the solventogenic stage industrially significant amounts of acetone and butanol are produced by *C. acetobutylicum* [8]. Similar amounts of butanol and ethanol are produced by solventogenic *C. beijerinckii* along with either acetone or isopropanol, or both [1,3,4]. Propanol and 1,2-propanediol are also produced in this stage by *C. beijerinckii* when grown on certain pentoses [1,3].

Correspondence: Robert M. Zsigray, Department of Microbiology, University of New Hampshire, Durham, NH 03824, U.S.A.

A system for the genetic manipulation of *C. acetobutylicum* and the closely related *C. beijerinckii* would permit strain improvement, which could improve the economic feasibility of this industrial process, while also increasing our knowledge of the regulation of the fermentation [8]. The primary methods for producing genetic recombination in Gram-positive bacteria of industrial importance have been protoplast fusion [7,9] and transformation [11]. We have studied the formation and regeneration of protoplasts of *C. beijerinckii* NRRL B-592 as a step in developing a genetic system for this species.

Although there are no reports of protoplast manipulation techniques for *C. beijerinckii*, there have been reports of protoplast formation and regeneration of certain strains of *C. acetobutylicum*, [2,13,14]. Two of these were reported by Allcock et al. [2], and Reilly and Rogers [13], for strains P262 and B643 of *C. acetobutylicum*, respectively. (Neither of these procedures were successful in regenerating bacillary forms from protoplasts of *C. beijerinckii*.) The procedures described by Allcock et al. [2] and Reilly and Rogers [13] seemed directly adaptable for protoplast formation of *C. beijerinckii*. However, when we tested these procedures, neither allowed protoplasts of *C. beijerinckii* to regenerate cell walls. The media described by these authors differed primarily with respect to ionic strength, osmotic strength, and agar concentration. Examination of the effects on *C. beijerinckii* B-592 protoplast regeneration of these critical medium ingredients yielded two distinct osmotically reinforced media: one that supports the growth of large L-colonies, and one that allows transferred B-592 L-colonies to regenerate and grow as the bacillary form. These media also supported L-colony growth and regeneration of *C. beijerinckii* B-593 and *C. acetobutylicum* ATCC 10132. (The terms L-form(s) and L-colony(s) have been defined [10,16] either as cell-wall deficient bacteria which regenerate to the parental form when the inducing agent is no longer present or as wall-deficient bacteria other than mycoplasma which continue to grow as such without reverting to the parental form. The cell wall-deficient *C. beijerinckii* described here are L-forms by the first definition on the latter medium and by the second definition on the former.)

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Clostridium beijerinckii NRRL B-592 and B-593 were obtained from L.K. Nakamura of the Northern Regional Research Center, Peoria IL. *Clostridium acetobutylicum* ATCC 10132 and ATCC 4259 were obtained from the American Type Culture Collection. Cultures were prepared from heat-shocked spore suspensions grown overnight in CBM broth [12] under stringent anaerobic conditions. All manipulations involving cells and protoplasts were carried out in a Coy anaerobic hood (Coy Laboratory Products Inc., Ann Arbor, MI) at a mean temperature of 32 ± 3°C.

BLM and BRM media formulation

BLM and BRM were prepared by the addition of stock solutions to a basal mixture. Stock solution A contained D-biotin, 0.1 g; PABA, 0.1 g; thiamine-HCl, 0.01 g; $FeSO_4 \cdot 7\,H_2O$, 0.1 g; $MnSO_4 \cdot 4\,H_2O$, 0.1 g; and $MgSO_4 \cdot 7\,H_2O$, 2.0 g, all in 100 ml H_2O. The solution was filter sterilized, and kept in a N_2/CO_2 atmosphere. Stock solution B consisted of 25 g glucose in 100 ml of H_2O while stock solutions C and D consisted of 2.5 M solutions of $MgCl_2$ and $CaCl_2$, respectively. Stock solution E contained K_2HPO_4, 7.0 g, and KH_2PO_4, 3.0 g, dissolved in 100 ml H_2O. Stock solutions B through E were autoclaved separately.

The basal mixture contained gelatin (Oxoid), 50.0 g; agar (Difco), 15.0 g; yeast extract (Difco), 8.0 g; casamino acids (Difco), 2.5 g and L-asparagine, 1.0 g. These ingredients were mixed with either 930 ml H_2O for BRM or 910 ml for BLM and the mixture stirred and brought to boiling before autoclaving at 121°C for 15 min. Upon cooling, 10 ml of stock solution A and 40 ml of solution B were added to the basal ingredients. In the preparation of BRM, 5 ml of each of solutions C and D were added while 15 ml of these solutions were used to prepare BLM. Stock solution E (10 ml) was added as the final component in preparing both BRM and BLM.

Protoplast Formation

Overnight cultures were diluted 1:4 with fresh, sterile CBM broth containing 0.4% glycine [2]. When the cultures became nearly 100% motile as determined by phase contrast microscopy, (45–60 min), the osmotic strength of each culture was increased by adding sucrose to 0.5 M along with lysozyme (2 mg/ml final concentration). After 5 min $MgCl_2$ and $CaCl_2$ were added to final concentrations of 12.5 mM each. After 60 min, protoplast formation was complete.

Growth of C. beijerinckii *as L-colonies*

Prior to plating, protoplast suspensions were diluted in CPM broth [2]. CPM broth consisted of CBM broth supplemented with sucrose to 0.5 M as an osmotic stabilizer and with $MgCl_2$ and $CaCl_2$ added to final concentrations of 12.5 mM. In this medium the protoplasts remained intact for prolonged periods of time. Samples were plated onto *C. beijerinckii* L-colony Medium (BLM). Large L-colonies routinely grew after 4 days of incubation at 32°C. A small number of bacillary colonies also appeared on some of the BLM plates. The numbers of these colonies usually correlated with the numbers of osmo-resistant forms counted by plating protoplast samples that had been diluted in anaerobic H_2O on non-osmotically reinforced media.

Transfer and regeneration of L-colonies on BRM

Large, well isolated L-colonies were transferred to *C. beijerinckii* regeneration medium (BRM) for regeneration. L-colonies were transferred as agar plugs taken with sterile Pasteur pipettes, and the agar plug extruded, inverted and then pressed onto marked sectors of BRM plates. These plates were incubated for 2–4 days until regeneration occurred.

RESULTS

Formation of protoplasts

Maximum conversion of bacillary forms to protoplasts occurred when rapidly growing, exponential phase cultures were treated with 2 mg/ml lysozyme in the presence of 0.5 M sucrose. The degree of lysozyme sensitivity varied directly with the motility of the cultures. Protoplast formation was either absent or greatly reduced in overnight cultures or in any cultures containing clostridial forms. Cultures whose growth rates were upshifted by a fourfold dilution in fresh CBM broth (containing 0.4% glycine [2]) became increasingly susceptible to lysozyme treatment until 1 h after upshift, when >99% wall-less forms could be obtained. Post-upshift times longer than 1 h resulted in lower protoplast yields (data not shown). Protoplasts were observed extruding from the sides or poles of the cells.

Formulation of L-colony and regeneration media

A comparison of the regeneration media reported by Allcock et al. [2] and Reilly and Rogers [13] for *C. acetobutylicum* showed that the concentrations of agar, gelatin and $CaCl_2$ + $MgCl_2$ used varied considerably and were most likely influencing regeneration. Both media were based on CBM (a basal growth medium for *Clostridium* [12]), however, the concentrations of agar and gelatin were 2% and 5%, for the former, and 0.8% and 0%, for the latter. Therefore, a Box-Behnken 3-parameter optimization strategy [5] was employed to test the effect of various concentrations of these ingredients on regeneration and L-colony growth of *C. beijerinckii* NRRL B-592. The results showed that two distinct media could be formulated. The first supported growth of L-colonies (BLM), and the second permitted regeneration (BRM). Fig. 1 shows a typical L-colony (arrow) growing next to a bacillary colony of *C. beijerinckii* B-592 on a BLM plate. Some bacillary colonies appeared on BLM plates, but because the number of these correlated with counts of osmotically resistant cells, they were assumed to have arisen from lysozyme resistant bacteria. B-592 protoplasts plated on BLM routinely grew as large L-colonies (1 mm in diameter) and at frequencies ranging between 2.4 and 5.5% of the total number of protoplasts (Table 1). When protoplasts were plated directly onto BRM regeneration plates the frequencies of L-colony formation and regeneration were lower than when protoplasts were first plated on BLM, grown as L-colonies, and then transferred to BRM (data not shown). The data in Table 2

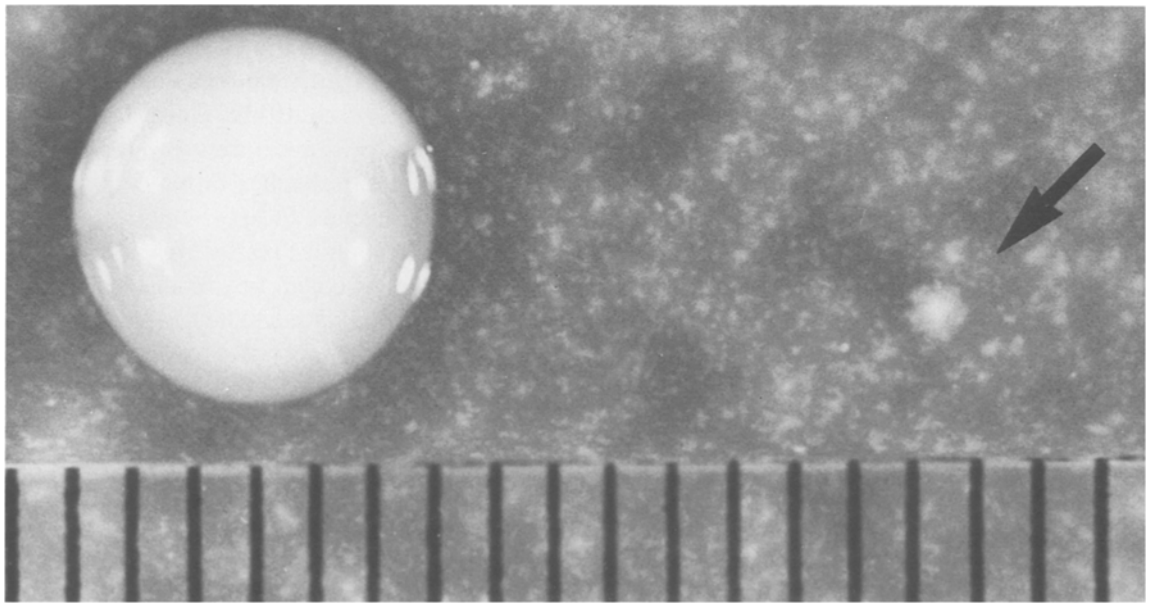

Fig. 1. *Clostridium beijerinckii* B-592 growing on BLM medium. An L-colony (arrow) growing next to a bacillary colony can be seen which has the characteristic 'fried egg' morphology. The ruled lines define a distance of approximately 1 mm.

show that the frequency of regeneration of transferred B-592 L-colonies ranged between 9 and 49% and that the average final regeneration frequency was 25%. Fig. 2 is a photograph of a BRM plate onto which L-colonies had been transferred. It illustrates the drastic change in colonial morphology that accompanies regeneration of an L-colony. The large spreading colonies are bacillary colonies of B-592.

L-colony growth and regeneration of other solventogenic clostridia on BLM and BRM

Three other solvent-producing clostridial strains were examined for their ability to grow as L-colonies on BLM and to regenerate on BRM. Protoplasts of *C. beijerinckii* NRRL B-593, and *C. acetobutylicum* strains ATCC 10132 and ATCC 4259 could be produced by the method developed for B-592. Table 3 shows that the B-593 strain of *C.*

Table 1

L-colony growth of *C. beijerinckii* on BLM medium

Experiment	CFU/ML[a]	OSMO-Resistant[b] CFU/ML	L-colony[c] CFU/ML	% L-colony[d]
1	8.6×10^6	1.0×10^3	4.1×10^5	4.8
2	1.1×10^7	5.3×10^3	6.0×10^5	5.5
3	5.1×10^6	<10	1.2×10^5	2.4
Mean:	8.2×10^6	2.1×10^3	3.8×10^5	4.2

[a] Viable cells prior to addition of 2 mg/ml lysozyme.
[b] Lysozyme-treated samples diluted 1:10 in anaerobic H_2O and plated on CBM agar plates.
[c] Number of L-colonies appearing after 6 days of incubation on BLM plates.
[d] c/a × 100.

Table 2

Regeneration of L-colonies to bacillary colonies

Experiment	L-colonies transferred	L-colonies regenerated	% regeneration
1	114	56	49
2	90	8	9
3	77	12	16

Average % regeneration from L-colonies = 25.

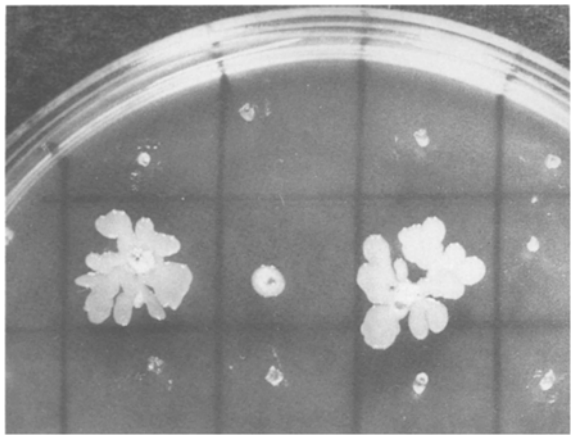

Fig. 2. L-colonies of *C. beijerinckii* that have reverted to the bacillary state after three days growth on BRM plates.

Table 3

L-colony growth and regeneration of L-colonies of other solventogenic *Clostridium* species

Organism	% L-colonies	% Regeneration
C. beijerinckii NRRL B-593	0.23	6.9
C. acetobutylicum ATCC 10132	4.1	8.6
C. acetobutylicum ATCC 4259	–	–

% L-colonies calculated as follows:
Number of L-colonies divided by the number of viable cells before lysozyme addition, minus the osmo-resistant forms times 100%

beijerinckii and the 10132 strain of *C. acetobutylicum* grew as L-colonies and regenerated to bacillary colonies when L-colonies were transferred to BRM medium. Strain 10132 formed L-colonies at a frequency of 4.1% and its transferred L-colonies regenerated at a frequency of 8.6%. Strain B-593 formed L-colonies on BLM at a frequency of 0.23% of the number of cells protoplasted. The L-colonies regenerated 7% of the time when transferred to BRM. *C. acetobutylicum* strain ATCC 4259 did not grow as either form on these media.

DISCUSSION

The original purpose of this work was to develop a protocol and medium that would yield stable protoplasts of *C. beijerinckii* B-592 and allow the protoplasts to regenerate their cell walls. In addition to meeting this purpose, a second medium was formulated that permitted protoplasts to replicate into colonies of wall-deficient, osmotically-sensitive forms that could be transferred to the regeneration medium on which they gave rise to the bacillary form.

Our method of forming protoplasts from *C. beijerinckii* is similar to the method reported for *C. acetobutylicum* by Reilly and Rogers [13]. They used 50 mM $CaCl_2$ + $MgCl_2$ in the protoplast formation broth because this concentration allowed the highest frequency of regeneration. We found that 12.5 mM or greater concentrations of these salts resulted in stable protoplast formation, but higher concentrations did not increase the frequency of L-colony growth or regeneration of *C. beijerinckii*. Optimum protoplast formation occurred when homogeneous rapidly growing cultures were treated with lysozyme in CBM medium (with 0.4% glycine) containing 0.5 M sucrose and 12.5 mM $MgCl_2$ + $CaCl_2$. Cultures of all clostridial strains used in this study were converted to over 99% protoplasts with this procedure.

It was possible to cultivate lysozyme-generated protoplasts of *C. beijerinckii* on BLM as wall-deficient, or L-, colonies. These colonies, when removed as agar plugs and spread onto fresh BLM

plates, gave rise to numerous other L-colonies which continued to grow as L-forms through at least 3 passages without reverting to the bacillary state. However, L-colonies transferred from BLM to BRM plates either regenerated cell walls 25% of the time or continued to slowly grow as L-colonies. This represents the first report of a medium capable of supporting solventogenic clostridia as wall-deficient colonies. The only report to our knowledge of clostridial L-forms and colonies was by Heefner et al. [6]. They were able to transform autoplasts and L-phase variants of *C. perfringens* with plasmid DNA, but only the autoplasts would regenerate to rod-shaped cells.

The only difference between BLM and BRM is the concentration of the $CaCl_2$ and $MgCl_2$ (see above). It seems that high concentrations of these salts (37.5 mM in BLM) support the growth of these strains as wall-less colonies. The need for high concentrations of these salts in clostridial protoplast regeneration media has been shown before [2,13–15]. Reilly and Rogers [13] observed the highest frequencies of regeneration of *C. acetobutylicum* only when protoplasts were formed in the presence of 50 mM $CaCl_2$ + $MgCl_2$ and then plated on a soft agar medium containing 25 mM concentrations of these salts. We observed a similar sequence with respect to L-colony regeneration. L-colonies were induced to regenerate when plated on the threefold lower concentrations of $CaCl_2$ + $MgCl_2$ in BRM.

Protoplasts of *C. beijerinckii* NRRL B-593 and *C. acetobutylicum* ATCC 10132 showed a growth and regeneration pattern on BLM and BRM similar to that of B-592 for which these media were formulated. These strains grew as wall-deficient colonies on BLM and reverted to the bacillary form when transferred to BRM. *Clostridium beijerinckii* B-593 formed fewer L-colonies per input protoplast but regenerated at a frequency more comparable to that of B-592. Interestingly, *C. acetobutylicum* strain 10132 formed L-colonies at frequencies equal to B-592 and regenerated at only a slightly lower frequency (Table 3). Although *C. acetobutylicum* strain 4259 formed >99% protoplasts by this method, it failed to grow on either BLM or BRM, even when untreated cultures were plated directly.

Protoplast regeneration among clostridia has been cited [12] as being species-specific. Our protocol is the first reported that allows regeneration of protoplasts from more than one species of *Clostridium*. However, strains of the same species varied in their ability to regenerate to the bacillary form when our protocol was used.

ACKNOWLEDGEMENT

We thank Dr. L.K. Nakamura for kindly providing the strains of *C. beijerinckii* used in this study. We also thank R.E. Mooney for his technical assistance. R.M.Z., W.R.C. and G.A.B. were supported in part by Grant DAAL03-86-K-0018 from the U.S. Army Research Office.

REFERENCES

1 Ahmed, I., R.A. Ross, V.K. Mathur and W.R. Chesbro. 1988. Growth rate dependence of solventogenesis and solvents produced by *Clostridium beijerinckii*. Appl. Microbiol. Biotechnol. 28: 182–187.

2 Allcock, E.R., S.J. Reid, D.T. Jones and D.R. Woods, 1982. *Clostridium acetobutylicum* protoplast formation and regeneration Appl. Envir. Microbiol. 43: 719–721.

3 Forsberg, C.W., L. Donaldson and L.N. Gibbins. 1987. Metabolism of rhamnose and other sugars by strains of *Clostridium acetobutylicum* and other *Clostridium* species. Can. J. Microbiol. 33: 21–26.

4 George, H.A., J.L. Johnson, W.E. Moore, L.V. Holdeman and J.S. Chen, 1983. Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. Appl. Environ. Microbiol. 45: 1160–1163.

5 Greasham, R. and E. Inamine. 1986. Nutritional improvement of processes. In: Manual of Industrial Microbiology (A.L. Demain and N.A. Solomon, eds.), pp. 41–47, American Society for Microbiology. Washington DC.

6 Heefner, D.L., C.H. Squires, R.J Evans, B.J. Kopp and M.J. Yarus. 1984. Transformation of *Clostridium perfringens*. J. Bacteriol. 159: 460–464.

7 Hotchkiss, R.D. and M.H. Gabor. 1980. Biparental products of bacterial protoplast fusion showing unequal parental chromosome expression. Proc. Nat. Acad. Sci. U.S.A. 77: 3553–3557.

8 Jones, D.T. and D.R. Woods. 1986. Acetone-Butanol Fermentation Revisited. Microbiol. Rev. 50: 484–524.

9 Jones, D.T., W.A. Jones and D.R. Woods. 1985. Production

of recombinants after protoplast fusion in *Clostridium acetobutylicum* P262. J. Gen. Microbiol. 131: 1213–1216.

10 Landman, O.E. and M.R. De Castro-Costa. 1976. Reversion of protoplasts and L-forms of Bacilli. In: Microbial and Plant Protoplasts. (Peberdy, J.F., A.H. Rose, H.J. Rogers and E.C. Cocking, eds.), pp. 201–217, Academic Press, New York, NY.

11 Lin, Y. and H.P. Blaschek. 1984. Transformation of heat-trated *Clostridium acetobutylicum* protoplasts with pUB110 plasmid DNA. Appl. Envir. Microbiol. 48: 737–742.

12 O'Brien, R.W. and J.G. Morris. 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. 68: 241–273.

13 Reilly, P.M. and P. Rogers. 1987. Regeneration of cells from protoplasts of *Clostridium acetobutylicum* B643. J. Ind. Microbiol. 1: 329–334.

14 Reysset, G., J. Hubert, L. Podvin and M. Sebald. 1987. Protoplast formation and regeneration of *Clostridium acetobutylicum* strain N1-4080. J. Gen. Microbiol. 133: 2595–2600.

15 Stahl, M.H. and H.P. Blaschek. 1985. Protoplast formation and cell wall regeneration in *Clostridium perfringens*. Appl. Environ. Microbiol. 50: 1097–1099.

16 Weibull, C. 1968. The morphology of protoplasts, spheroplasts, and L-forms. In: Microbial Protoplasts, spheroplasts and L-Forms. (Guze, L.B., ed.), pp. 62–73, The Williams and Wilkins Co., Baltimore.