JOURNAL OF BACTERIOLOGY, July 1983, p. 432–434
0021-9193/83/070432-03$02.00/0
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Vol. 155, No. 1

Regeneration of Protoplasts of *Clostridium pasteurianum* ATCC 6013

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Received 4 March 1983/Accepted 26 April 1983

A procedure has been developed for the regeneration of *Clostridium pasteurianum* protoplasts with frequencies of up to 10% reversion being obtained.

The obligately anaerobic gram-positive clostridia are noted for their catholic distribution and for their ability to ferment a wide variety of substrates (2, 13, 18). The diverse fermentative ability has been exploited in the industrial production of organic solvents (e.g., acetone-butanol fermentation by *clostridium acetobutylicum* (17), a process receiving renewed attention in the light of growing recognition of the limited petroleum resources (19). It is frequently overlooked that the ability to utilize a diverse range of substrates without costly aeration affords clostridia great potential in the rapidly expanding field of biotechnology. This would be greatly facilitated by the development of a gene transfer system to allow exploitation of recombinant DNA techniques. As a first step in the development of a protoplast transformation/fusion technique similar to that used with other gram-positive bacteria (3, 4, 7, 8, 16, 20), we have examined the optimal conditions necessary for the regeneration of *Clostridium pasteurianum* protoplasts. We believe *C. pasteurianum* to be a good candidate as a host for genetic studies because, unlike the vast majority of clostridia, it can be grown on chemically defined medium (10), it can tolerate short-term exposure to air (i.e, it is a moderate anaerobe) (12), mutants are easily obtainable (5, 10; N. P. Minton, Ph.D. thesis, University College of Wales, Aberystwyth, 1981), and the organism has no known association with disease.

The strain employed was *C. pasteurianum* ATCC 6013. Cells were cultured at 37°C in CY medium, which consisted of the glucose basal medium of O'Brien and Morris (15) supplemented with 0.1% Casamino Acids, 0.3% yeast extract, and 0.05% cysteine hydrochloride. Growth on agar medium was under an atmosphere of hydrogen (50%), nitrogen (47.5%), and carbon dioxide (2.5%).

A routine procedure for the formation of stable *C. pasteurianum* protoplasts has been developed previously (5) which used an isotonic buffer containing lactose (15%, wt/vol), 20 mM $MgCl_2$, and 100 mM potassium phosphate, pH 6.5. When exponential-phase cells were suspended in this buffer supplemented with lysozyme (200 μg/ml) and held at 37°C, more than 99% of the cells were converted to protoplasts in 20 to 30 min. Out of a variety of regeneration media tested, "clostridial" glucose, basal medium supplemented with 2% agar, 0.5% Casamino acids, 0.3% yeast extract, 0.05% cysteine hydrochloride, and 15% lactose (HCYL agar), was found to support the best protoplast reversion frequency. The surface of the agar medium was treated immediately before use with 0.1 ml of a freshly prepared solution of catalase (20,000 U/ml).

In a typical regeneration experiment, cells from an exponential-phase culture of *C. pasteurianum* were harvested and suspended in 0.1 volumes of protoplast buffer. The number of viable cells present was estimated from the CFU obtained after serial dilutions on HCYL agar. Lysozyme was added to a final concentration of 200 μg/ml, and the cell suspension was incubated at 37°C for 30 min. A crude estimate of the percentage converting to protoplasts was obtained by microscopic counts of 10 random fields. The protoplasts were diluted in both the isotonic buffer and sterile distilled water and plated out on HCYL agar. The percentage regeneration was calculated (see Table 1) essentially by comparing the CFU (on HCYL) obtained from the protoplasts diluted in isotonic buffer to the CFU of the cell suspension before lysozyme treatment. A comparison of the CFU obtained from protoplasts diluted in distilled water with the CFU of the cell suspension gave an indication of the number of osmotically resistant cells present in each protoplast suspen-

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TABLE 1. CFU on HCYL agar

Expt	Cells (10^9) (a)	CFU per ml for protoplasts diluted in: Isotonic buffer (10^7) (b)	Distilled water (10^4) (c)	% Reversion
I	1.15	1.0	<0.1	0.87
II	0.69	0.21	0.85	0.34
III	0.97	2.37	1.31	2.44
IV	1.15	1.2	2.2	1.04

[a] Cells were protoplasted in the modified isotonic buffer discussed in the text. Incubation with lysozyme was at 37°C for 30 min, at which point N-acetylglucosamine was added to 0.1 M. The percent reversion frequency was calculated according to the following formula: $100 \times (b - c)/a$ (a, b, and c indicated in the table). In practice, c was insignificant compared to b, and so could be discounted. Control experiments demonstrated that the CFU of cell suspensions was unaffected by the osmotic shock procedure and by incubation in isotonic buffer lacking lysozyme at 37°C for 60 min.

sion. Typical regeneration frequencies obtained varied from 0.01 to 0.1% of the total protoplasts present, whereas the number of osmotically resistant cells represented between 0.0005 to 0.005% of the protoplast population. The underlying population of the osmotically resistant cells remained constant with extended lysozyme treatment (up to 2 h of incubation), whereas the percentage of regeneration exhibited a gradual decline. To increase the number of regenerative protoplasts, a number of modifications were made to the isotonic protoplast buffer, suggested by the results obtained in other systems (7, 8, 16). These included the provision of a more favorable metabolic environment by the addition of 0.3% Casamino Acids, 0.3% yeast extract, all the other constituents of the otherwise nitrogen-free basal medium, and 2% (wt/vol) bovine serum albumin. The toxic effects of any inadvertent introduction of oxygen were minimized by supplementation of the protoplast buffer with 0.05% cysteine hydrochloride and catalase at a final concentration of 400 U/ml. Finally, *N*-acetylglucosamine (0.1 M) was added at the end of the lysozyme treatment and was also included in the isotonic dilution buffer. These modifications produced a significant increase in the reversion frequencies (Table 1), the percentage regeneration varying from 0.3 to 2.4%. The number of osmotically resistant cells did not increase significantly. The presence of *N*-acetylglucosamine was shown to account for 50% of this increase in reversion, presumably by both inhibiting further action of lysozyme (14) and acting as a precursor in cell wall biosynthesis.

In view of the findings of Gabor and Hotchkiss (9), two further modifications were examined in conjunction with those described above. The organism was cultured in CY medium containing 15% (wt/vol) lactose and protoplast formation was carried out at the higher temperature of 42°C (Table 2). The presence of lactose in the growth medium was found to cause a consistent increase in regeneration frequency of the resultant protoplasts, with up to 10% reversion occurring. Protoplast formation at 42°C also appeared to improve the regeneration frequency (Table 2).

Further changes to the regeneration agar had no significant effect on reversion. These included substitution of lactose with maltose or sorbitol, inclusion of autoclaved clostridial cells, or the presence of the four cell wall sugars (glucose, mannose, rhamnose, and galactose) of *C. pasteurianum* (6) at a final concentration of 0.5% (wt/vol). In contrast to other systems (21), the inclusion of 0.5% gelatin in the medium caused a 60% reduction in the reversion frequency.

A protoplast regeneration protocol has been developed for *C. pasteurianum* which compares favorably with a number of procedures for other gram-positive bacteria (7, 8, 16), yielding frequencies of between 1 and 10% protoplast regeneration. *C. acetobutylicum* protoplasts were reported to give 80% regeneration (1). However, little comparison with our, and other, work can be made, as the reversion frequencies of the *C. acetobutylicum* protoplasts appear to have been calculated as the percentage difference between CFU obtained on regeneration medium after

TABLE 2. CFU on HCYL agar

Expt	Lysozyme incubation temperature (°C)	Lactose (15%) in initial growth medium	Cells (10^8)	CFU per ml of protoplasts diluted in: Isotonic buffer (10^6)	Distilled water (10^3)	% Reversion[a]
I	37	+	4.5	26	10	5.8
	37	−	3.5	2.6	7	0.74
II	37	+	1.76	12	0.125	6.82
	42	+	1.76	17.8	0.227	10.1

[a] Percent reversion was calculated as described in Table 1, footnote *a*.

diluting protoplasts in isotonic buffer and the CFU of osmotically shocked protoplasts. This may account for their higher apparent regeneration frequency, i.e., reversion frequencies of *C. pasteurianum* protoplasts exeed 99.999% when calculated by the same means. The protoplast regeneration procedure described in this report should enable genetic studies to be carried out in *C. pasteurianum*, using fusion and transformation techniques. Accordingly, double auxotrophic mutants of *C. pasteurianum* have been isolated (N. P. Minton, Ph.D. thesis, University College of Wales, Aberystwyth, 1981), and a number of naturally occurring clostridial plasmids (11) are currently being investigated as possible vectors.

N.P.M. acknowledges a research studentship from the Science & Engineering Research Council, United Kingdom.

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. **Barker, H. A.** 1961. Fermentation of nitrogenous organic compounds, p. 151–207. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 2: metabolism. Academic Press, Inc., New York.
3. **Bibb, M. J., J. M. Ward, and D. A. Hopwood.** 1978. Transformation of plasmid DNA into *Streptomyces* at high frequency. Nature (London) 274:398–400.
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. **Clarke, D. J., D. B. Kell, C. D. Morley, and J. G. Morris.** 1982. Butyricin 7423 and the membrane H^+-ATPase of *Clostridium pasteurianum*. Arch. Microbiol. 131:81–86.
6. **Cummins, C. S., and J. L. Johnson.** 1971. Taxonomy of the clostridia. Wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostrida. J. Gen. Microbiol. 67:33–46.
7. **Ferenczy, L.** 1981. Microbial protoplast fusion, p. 1–34. *In* S. W. Glover and D. A. Hopwood (ed.), Genetics as a tool in microbiology. Society for General Microbiology, symposium 31. Cambridge University Press, Cambridge.
8. **Fodor, K., and L. Alfoldi.** 1976. Fusion of protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. U.S.A. 73:2147–2150.
9. **Gabor, M. H., and R. D. Hotchkiss.** 1979. Parameters governing bacterial regeneration and genetic recombination after fusion of *Bacillus subtilis* protoplasts. J. Bacteriol. 137:1346–1353.
10. **Mackey, B. M., and J. G. Morris.** 1974. Isolation of a mutant strain of *Clostridium pasteurianum* defective in granulose degradation. FEBS Lett. 48:64–67.
11. **Minton, N. P., and J. G. Morris.** 1982. Isolation and partial characterisation of three cryptic plasmids from strains of *Clostridium pasteurianum*. J. Gen. Microbiol. 127:325–331.
12. **Morris, J. G.** 1975. The physiology of obligate anaerobiosis. Adv. Microb. Physiol. 12:169–246.
13. **Nakhmanovich, B. M., and N. A. Shcheblykina.** 1959. Fermentation of pentoses of corn cob hydrolysates by *Clostridium acetobutylicum*. Microbiologiya 28:99–104.
14. **Neuberger, A., and B. M. Wilson.** 1967. Inhibition of lysozyme by derivatives of D-glucosamine. Biochim. Biophys. Acta 147:473–486.
15. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. 68:307–318.
16. **Schaeffer, P., and R. D. Hotchkiss.** 1978. Fusion of bacterial protoplasts. Methods Cell Biol. 20:149–158.
17. **Spivey, M. J.** 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 13:2–4.
18. **Stadtman, T. C.** 1973. Lysine metabolism by Clostridia. Adv. Enzymol. Relat. Areas Mol. Biol. 38:413–448.
19. **Tong, G. E.** 1979. Industrial chemicals from fermentation. Enzyme Microb. Technol. 1:173–179.
20. **Vorobjeva, I. P., I. A. Khmel, and L. Alfoldi.** 1980. Transformation of *Bacillus megaterium* protoplasts by plasmid DNA. FEMS Microbiol. Lett. 1:261–263.
21. **Ward, J. B.** 1978. The reversion of bacterial protoplasts and L-forms, p. 249–271. *In* R. Y. Stanier, H. J. Rogers, and B. J. Ward (ed.), Relations between structure and function in the procaryotic cell. Society of General Microbiology Symposium 28. Cambridge University Press, Cambridge.