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Regeneration of Protoplasts of *Bacillus subtilis* 168 and Closely Related Strains

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Regeneration of protoplasts to bacilli was attempted in several strains of *Bacillus* closely related to *Bacillus subtilis* 168. On DM3 and similar media using succinate as osmotic support, only *B. subtilis* 168 and *Bacillus natto* ATCC 15245 were able to regenerate. Media containing mannitol as osmotic support, and agar as gelling agent gave rise to L-form colonies with *Bacillus licheniformis* NCTC 6346. Many of the L-form colonies were able to regenerate to the bacillary form when plated on the mannitol medium solidified with gelatin. All of the *Bacillus* species tested were able to regenerate on the latter medium at rates sufficient to allow protoplast transformation and fusion experiments.

INTRODUCTION

There is increasing interest in the use of protoplast-mediated techniques such as fusion (Fodor & Alföldi, 1976; Schaeffer *et al.*, 1976) and transformation (Chang & Cohen, 1979) for the genetic study and manipulation of *Bacillus* species. To use these techniques effectively it is necessary to have methods that allow quantitative conversion of cells to protoplasts and subsequent reversion to the bacillary form. Such systems are established for a few species including *Bacillus subtilis* 168 (Landman *et al.*, 1968) and *Bacillus megaterium* KM (Fodor *et al.*, 1975) but not for many others.

Strains of *Bacillus* produce a wide range of products of potential and frequently real industrial importance. Food-grade and other exoenzymes (Priest, 1977), antibiotics (Katz & Demain, 1977), entomotoxins (de Barjac, 1981) and other products are produced by strains for which gene transfer techniques to permit genetic analysis are seldom available. It is likely that the actual processes of protoplast fusion and transformation would vary little from one *Bacillus* species to another so that these techniques could have wide-ranging applications. However, it seems that few *Bacillus* species regenerate on the media that are effective for *B. subtilis* 168 and *B. megaterium* KM. By adopting an *ad hoc* approach to formulation a number of groups have described regeneration media for use with individual species, e.g. *Bacillus sphaericus* (McDonald & Burke, 1984); *Bacillus popilliae* and *Bacillus larvae* (Bakhiet & Stahly, 1985); *Bacillus stearothermophilus* (Imanaka *et al.*, 1982). However, with some exceptions, rates of regeneration from protoplasts to bacilli remain poor and variable. We have tried to establish more general conditions and have developed media that allow regeneration of a number of strains closely related to *B. subtilis* 168.

METHODS

Strains and growth conditions. The bacterial strains were chosen to represent a range of bacilli all relatively closely related to *B. subtilis* 168 (Seki *et al.*, 1979). They include representatives that produce extracellular amylases, proteases, β-lactamase and polyglutamic acid (Table 1). Stocks of bacteria were maintained in 5% (v/v) glycerol at −70 °C.

The composition of the liquid growth medium was found in preliminary experiments to have an important effect on subsequent quantitative conversion of cells to protoplasts. Cells grown in the absence of sugars were

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Table 1. *Bacterial strains*

Strain	Product	Source or reference*
B. subtilis 168 (*trpC2*)	—	J. Mandelstam, Oxford University, UK
B. licheniformis NCTC 6346 (His⁻)	—	Elliott *et al.* (1975)
B. licheniformis NCIB 8874	Bacitracin	NCIB
B. licheniformis ATCC 10716	Bacitracin	ATCC
B. licheniformis 749/C	β-Lactamase	J. O. Lampen, Rutgers University, NJ, USA
B. globigii NCIB 8649	—	R. Sharp, CAMR, Porton, UK
B. amyloliquefaciens NCIB 10785	α-Amylase	NCIB
B. subtilis NCIB 8565	β-Glucanase	Hinchliffe (1984)
B. natto ATCC 15245	Polyglutamate	ATCC
B. pumilis 8A1	—	BGSC
Bacillus sp. N7	α-Amylase	R. Piggot, Biocon Ltd, Carrigaline, Eire
Bacillus sp. 16A	α-Amylase	R. Piggot, Biocon Ltd, Carrigaline, Eire

* NCIB, National Collection of Industrial Bacteria, Aberdeen; ATCC, American Type Culture Collection, Rockville, Md, USA; BGSC, *Bacillus* Genetic Stock Center, University of Ohio, USA.

converted to protoplasts more readily and synchronously than cells grown in sugar-supplemented media or rich, undefined media, such as tryptone soya broth, so that only a low background of lysozyme-resistant cells remained. The preferred growth medium was minimal salts (Spizizen, 1958) supplemented with 1% (w/v) Bacto Casamino acids (Difco) and 20 μg tryptophan ml^{-1}. However, for *Bacillus natto* this medium was insufficient and so tryptone soya broth, despite its disadvantages, was used.

Cultures were inoculated from fresh nutrient agar (NA) stock plates and shaken overnight at 30 °C. The following morning cultures with an OD_{600} of less than 0·6, and which had not sporulated were used to inoculate fresh medium to an OD_{600} of approx. 0·01. The subcultures rapidly attained exponential phase on shaking at 37 °C and when the OD_{600} reached approx. 0·5 the cells were harvested by centrifugation.

Preparation of protoplasts. Pellets of harvested cells were resuspended in a 0·1 volume of osmotically-supported protoplast buffer. A viable count was taken by diluting cells in growth medium and plating on NA. Hen egg white lysozyme (Sigma) was added to a final concentration of 100 μg ml^{-1} and the culture incubated with shaking for 20 min at 37 °C. Protoplast formation was assessed semiquantitatively by phase-control microscopy and, if a significant proportion of bacillary cells remained (>1%), incubation was continued for up to 60 min. Once satisfactory protoplast formation had occurred a second viable count was taken, as before, to determine the number of lysozyme-resistant survivors.

The protoplast buffer used was tryptone/yeast extract/sucrose (TYS; Elliott *et al.*, 1975) supplemented with 1% (w/v) bovine serum albumin (Sigma fraction V) and sterilized by filtration.

Cell wall regeneration. Protoplasts were diluted in TYS buffer and spread on osmotically-supportive regeneration agar plates. The plates were incubated at 30 °C until no further colonies appeared (up to 10 d).

Three regeneration media were tested. DP (Elliott *et al.*, 1975) and DM3 (Chang & Cohen, 1979) use succinate for osmotic support. MRA is a mannitol-based medium described by Nimi *et al.* (1983) for regeneration of *Bacillus brevis* protoplasts. Modifications of these media are described, where appropriate, in the results.

Mean chain length. Bacillus strains often form chains of cells, the mean length of which varies. Conversion of cells to protoplasts incidentally causes dechaining so that the apparent initial viable count (i.e. colony forming units) is an underestimate with regard to potential regenerants from a culture of protoplasts. To correct for this, mean chain lengths of cells in cultures were determined immediately before lysozyme addition. A drop of culture was mixed with a drop of saturated CsCl solution and the plasmolysed cells were viewed by phase-contrast microscopy. The cell content of 300 chains was counted for each culture and the mean chain length determined.

The initial viable count was calculated as the product of colony forming units and mean chain length. Regeneration is then given as the ratio (bacillary colony count on regeneration medium − survival count on NA)/(initial viable count). This assumes that lysozyme-resistant survivors are either dechained or that only one cell per chain is resistant.

RESULTS AND DISCUSSION

In initial experiments cells were converted to protoplasts in TYS buffer and regeneration to bacilli was tested on DM3 medium. Typical results for lysozyme survival and protoplast regeneration are shown in Table 2. For all strains tested at least 99·5% of cells were converted to protoplasts. *B. subtilis* 168, *B. natto* and to a lesser extent *B. subtilis* 8565 regenerated readily at

Table 2. *Typical values for frequencies of lysozyme resistance and regeneration*

Values were determined as described in Methods and mean chain lengths are the results of observations of 300 chains. Initial viable counts were all in the range 6×10^8 to 6×10^9.

Strain	Mean chain length	Lysozyme resistance frequency	Regeneration frequency		Improved regeneration with MRG (fold)
			DM3 medium	MRG medium	
B. subtilis 168	2·60	$1·05 \times 10^{-3}$	$1·30 \times 10^{-1}$	$1·27 \times 10^{-1}$	1
B. licheniformis 6346	1·26	$1·20 \times 10^{-4}$	$1·42 \times 10^{-4}$	$2·32 \times 10^{-2}$	163
B. licheniformis 8874	2·33	$1·44 \times 10^{-5}$	$3·42 \times 10^{-5}$	$1·51 \times 10^{-2}$	442
B. licheniformis 10716	1·80	$1·86 \times 10^{-4}$	$5·02 \times 10^{-5}$	$5·04 \times 10^{-3}$	100
B. licheniformis 749/C	1·47	$5·66 \times 10^{-5}$	$4·91 \times 10^{-5}$	$4·16 \times 10^{-2}$	847
B. globigii 8649	1·82	$2·00 \times 10^{-3}$	$5·09 \times 10^{-3}$	$7·06 \times 10^{-2}$	14
B. amyloliquefaciens 10785	9·48	$5·20 \times 10^{-6}$	$5·84 \times 10^{-5}$	$3·91 \times 10^{-3}$	67
B. subtilis 8565*	1·63	$1·45 \times 10^{-3}$	$1·33 \times 10^{-2}$	$1·12 \times 10^{-1}$	8
B. natto 15245	17·80	$2·66 \times 10^{-7}$	$5·05 \times 10^{-2}$	$1·14 \times 10^{-2}$	0·2
B. pumilis 8A1	1·29	$1·27 \times 10^{-6}$	$1·49 \times 10^{-3}$	$2·04 \times 10^{-2}$	14
Bacillus sp. N7	3·08	$6·27 \times 10^{-5}$	$7·17 \times 10^{-3}$	$1·95 \times 10^{-2}$	3
Bacillus sp. 16A	13·47	$1·54 \times 10^{-6}$	ND	$1·78 \times 10^{-2}$	–

ND, Not determined.

* *B. subtilis* 8565 grew in clumps which were dispersed during protoplast formation. This makes frequencies of lysozyme resistance and regeneration probable overestimates.

rates significantly above the lysozyme survival counts. The rates of regeneration are lowered by the correction for mean chain length. Without this correction, apparent regeneration frequencies would have been $3·38 \times 10^{-1}$ for *B. subtilis* 168 and $8·99 \times 10^{-1}$ for *B. natto*. The uncorrected value for *B. subtilis* 168 is comparable with that obtained by other workers (e.g. Schaeffer *et al.*, 1976) and the rate for *B. natto* in particular illustrates the error that can occur due to dechaining. Apart from these two strains, the regeneration frequencies obtained with DM3 medium were generally very low. The failure of *Bacillus licheniformis* 6346 to regenerate was particularly unexpected since Elliott *et al.* (1975) using similar conditions observed regeneration sufficiently well to allow biochemical and physiological investigations of the process. We therefore concentrated on establishing regeneration conditions for *B. licheniformis* 6346 to the temporary exclusion of the other strains.

DP medium (Elliott *et al.*, 1975) and DM3 medium (Chang & Cohen, 1979) are both modifications of the DPA medium of Wyrick & Rogers (1973). DM3 was used by Chang & Cohen (1979) because with *B. subtilis* 168 it reduced the time required for cell wall regeneration and eliminated the crowding effect reported by Schaeffer *et al.* (1976). It was possible that modification had made the medium unsuitable for regeneration of *B. licheniformis* 6346 protoplasts. However, in a comparison experiment it was shown that while DM3 medium is clearly superior to DP for cell wall regeneration of *B. subtilis* 168 (regeneration frequencies of $2·73 \times 10^{-2}$ and $1·24 \times 10^{-2}$ respectively), *B. licheniformis* 6346 protoplasts failed to regenerate significantly for practical purposes on either medium (regeneration frequencies of $1·45 \times 10^{-4}$ on DM3 and $9·26 \times 10^{-5}$ on DP), despite using identical strains and conditions as close as possible to those of Elliott *et al.* (1975). This highlights a problem with regeneration media, in that they are complex in composition and there are reports of batch-to-batch variation of media greatly affecting rates of regeneration (King & Gooder, 1970). Thus many components may be changed or modified without significant improvement in regeneration. Moreover, it is possible that the growth medium and protoplast buffer could have effects on subsequent regeneration. We have tried many modifications to the media and conditions without significantly increasing the rate of regeneration.

An alternative to testing further modifications of DM3 was to use a new medium altogether. A mannitol-based regeneration medium (MRA) described by Gray & Chang (1981) and modified by Nimi *et al.* (1983) for regeneration of *B. brevis* protoplasts was chosen. With *B. licheniformis* 6346 the count of bacillary colonies was as low as that on DM3 medium. However, L-form

colonies, representing about 30% of the initial viable count, developed on MRA but not on DM3 medium. The problem remained then to find conditions that would allow L-form reversion.

Spontaneous reversion of L-forms to bacilli after prolonged incubation has been reported for *B. subtilis* 168 (Landman & Halle, 1963). However, incubation prolonged for 10 d led to no significant reversion of the L-forms of *B. licheniformis* 6346 on MRA medium. Landman *et al.* (1968) reported that gelatin (25%, w/v) as sole gelling agent was effective in allowing reversion of L-forms and regeneration of protoplasts of *B. subtilis* 168 to the bacillary state. When we used 25% (w/v) gelatin as sole gelling agent (MRG medium) a significant increase in regeneration of protoplasts of *B. licheniformis* 6346 was obtained (Table 2). The number of bacillary colonies formed was not as great as the number of L-form colonies formed on MRA but, nevertheless, the extent of regeneration would be sufficient to allow protoplast-mediated gene-transfer techniques to be used in this organism.

The other *Bacillus* species used in this investigation were also able to regenerate on MRG (Table 2). *B. subtilis* 168 regenerated to about the same extent on DM3 and MRG, and *B. natto* regenerated better on DM3. Nevertheless, in the latter case, rates of regeneration on MRG are sufficient to allow its use, if a series of experiments require that only a single regeneration medium be used. In all other cases, MRG medium allowed more extensive regeneration than DM3. The most marked improvements were seen with the *B. licheniformis* strains and *B. amyloliquefaciens*, and even though the rates of regeneration are still quite low in some cases, in practice they are suffcient to allow effective uptake of plasmids in protoplast transformations (unpublished observation). MRG is inconvenient to make up and requires incubation at 30 °C or below. Liquefaction of the gelatin by some strains causes difficulties on extended incubation but this can be avoided by enumerating and subculturing early. Despite these problems the use of MRG significantly extends the range of *Bacillus* strains available for protoplast-mediated genetic experiments to include many strains of interest as exoenzyme producers (e.g. *B. amyloliquefaciens*, *Bacillus* sp. N7, *Bacillus* sp. 16A) and antibiotic producers (e.g. *B. licheniformis* 10716, *B. licheniformis* 8874). Thus, genetic analysis and manipulation of these strains of practical importance becomes more feasible.

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