FEMS Microbiology Letters 40 (1987) 1-5
Published by Elsevier

FEM 02625

A simple medium for rapid regeneration of *Bacillus subtilis* protoplasts transformed with plasmid DNA

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Received 1 September 1986
Accepted 4 September 1986

Key words: *Bacillus subtilis*; Protoplast; Transformation; Plasmid; (Regeneration medium)

1. SUMMARY

A sucrose-based simple medium (c-R5) is described on which protoplasts prepared from *Bacillus subtilis* are able to revert rapidly to bacillary forms. Transformations of *B. subtilis* protoplasts were performed using polyethylene glycol and various plasmid DNAs, followed by regeneration of the protoplasts on c-R5 medium. Regenerated bacteria were easily detectable on selective c-R5 medium after only 16 h of incubation at 37°C. This medium can be used for direct selection of various antibiotic-resistance markers. In addition, the c-R5 medium can be simplified to make it chemically defined, allowing the selection of prototrophs.

2. INTRODUCTION

Introduction of plasmid DNA into *Bacillus subtilis* by the method of the polyethylene glycol (PEG)-induced protoplast transformation [1], constituted a landmark in the development of these bacteria as a cloning system. One of the main advantages of this method is that all plasmid forms are biologically active. In addition, a very high transformation efficiency can be achieved with covalently closed circular DNA of small plasmids [1]. It has been demonstrated [2] that plasmid DNA is incorporated into *B. subtilis* protoplasts in the double-stranded form with little, if any, damage. These findings would explain the high biological activity of plasmid DNA in this system of transformation [1,2]. In addition to *B. subtilis* [1], PEG-induced plasmid transfer has been widely used in other organisms, such as *Streptomyces* spp. [3] and several members of the corynebacteria [4–7]. In some of these cases [6], a high efficiency of plasmid DNA transformation has been reported.

Several drawbacks of this system have been indicated [8,9]. In the first place, regeneration of protoplasts to complete cells may take between 2 days for *B. subtilis* [1] and 10–14 days for some glutamate-producing corynebacteria [7]. Difficulties with the selection of certain antibiotic resistance markers may mean that the colonies have to be regenerated into non-selective medium, followed by replica plating on selective medium [1,2].

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Moreover, the regeneration medium DM3 described by Chang and Cohen [1] is not suitable for the selection of nutritional genetic markers due to its complexity [8,9]. In addition, significant variations in the number of regenerants are sometimes observed, probably due to the source of the osmotic stabilizer (sodium succinate) employed for cell wall regeneration of *B. subtilis* protoplasts (unpublished observations).Other osmotic stabilizers have also been used, such as mannitol [10] or sorbitol [7], although rather less frequently.

We report here a simple medium for the regeneration of *B. subtilis* protoplasts after transformation with plasmid DNA. Detection of transformed regenerants can be achieved after overnight incubation on selective plates. The complete medium, called c-R5, is a modification of the medium previously employed for *Streptomyces* [3] and corynebacteria [5,11], and contains sucrose as osmotic stabilizer. Direct selection for antibiotic resistance markers is possible and, in addition, we observed a high reproducibility in the number of transformants obtained, independently of the source of plasmid DNA and the recipient strain employed. Furthermore, the c-R5 medium can be modified to make it totally defined, so that selection of prototrophs should be possible.

3. MATERIALS AND METHODS

3.1. Strains and plasmids

The *B. subtilis* strains and the plasmids used are listed in Table 1. As recipients of plasmid DNAs, we chose the highly transformable *B. subtilis* strain MB11 [12] and the, in our hands, poorly transformable minicell-producing strain CU403. When we tried to chemically define the regeneration medium, the wild-type strain MB51 was employed. In addition, the strain BD170, used by Chang and Cohen [1], was also selected as a control. *Streptococcus pneumoniae* R61 (wild type) was used to prepare heterologous plasmid DNA [12].

3.2. Preparation of plasmid DNA

Pure plasmid DNA was prepared from either *B. subtilis* or *S. pneumoniae* by published procedures [12], taking into consideration the precautions indicated by Chang and Cohen [1] aimed at the removal of any trace of detergent or ethidium bromide in the DNA preparations. The amount of plasmid DNA employed was usually 0.05–0.08 μg per 0.2 ml of protoplast suspension. In some cases, cleared lysates of plasmid DNA [12] were employed after extensive dialysis without any detectable loss in their transformation efficiency (not shown).

3.3. Preparation of protoplasts

For the preparation of protoplasts, 1 ml of an overnight culture in penassay broth (antibiotic medium No. 3, Difco) was diluted into 50 ml of the same medium and incubated at 37°C with vigorous shaking. When the culture reached an absorbance at 550 nm of 1.0, cells were harvested

Table 1

Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype [a]	Origin and comments
B. subtilis		
MB11	*lys3 metB10 hisH2*	Madrid collection
MB51	wild type	Madrid collection
BD170	*trpC2 thr5*	D. Dubnau [1]
CU403	*thyA thyB metB divIVB1*	Minicell-producing strain, Madrid collection
Plasmid		
pC194 (2.9 kb)	Cm^R	J.C. Alonso [9]
pUB110 (4.5 kb)	Km^R	D. Dubnau [9]
pLS1 (4.4 kb)	Tc^R	S.A. Lacks [12]

[a] Cm, chloramphenicol; Km, Kanamycin; Tc, tetracycline.

by centrifugation, and suspended in 5 ml of SMMP (a mixture, v/v, of 2 × penassay broth and 1 M sucrose, 0.04 M maleate, 0.04 M $MgCl_2$, pH 6.5) [1]. Cells were placed in a 100-ml flask, and lysozyme (final concentration, 2 mg/ml) was added. Cell suspension was incubated at 37°C with gentle shaking for 45–60 min, and protoplast formation was monitored by phase-contrast microscopy. At the end of the incubation period, protoplasts were collected by centrifugation at 2000 × *g*, 10 min, washed with SMMP and suspended in 5 ml of the same medium.

3.4. Transformation and regeneration of protoplasts

Samples (0.2 ml) of protoplast suspension were transformed essentially as described [1], in the presence of 2 vols. of 40% PEG 6000. After incubation with plasmid DNA for 3 min at 0°C 3 volumes of SMMP were added, protoplasts were pelleted as above and suspended in 2 vols. of SMMP. After incubation at 37°C for 90 min to allow phenotypic expression, protoplasts were plated onto the appropriate regeneration medium. Selection for antibiotic resistance markers was at 5 μg/ml for chloramphenicol, 10 μg/ml for kanamycin and 15 μg/ml for tetracycline. The percentage of regenerants was calculated as described [6].

The regeneration media usually employed were DM3 [1], and a modified complete R2YE [3], named c-R5 and containing (per l): 103 g sucrose, 0.25 g K_2SO_4, 10 g $MgCl_2 \cdot 6\ H_2O$, 10 g glucose, 6.5 g 4-morpholinepropanesulfonic acid (MOPS) 0.66 g NaOH, 6 g proline, 0.2 g vitamin-free casamino acids, 10 g yeast extract, 0.05 g KH_2PO_4 and 2.2 g $CaCl_2$. In addition, 2 ml · l $^{-1}$ of trace element solution [3] and 1.1% agar (w/v) were added to the medium. Preparation of this c-R5 medium was performed following the steps described for R2YE medium [3].

3.5. Chemicals

All reagents were purchased from Merck, with the exception of yeast extract (Difco) and the antibiotics (Sigma). Sucrose from various sources (Fluka, Serva) was also employed in a set of experiments.

4. RESULTS AND DISCUSSION

A comparison between the results obtained in DM3 and in c-R5 media is presented in Table 2.

Table 2

Transformation efficiency of *B. subtilis* protoplasts by plasmid DNA

Regeneration medium	Recipient strain	Donor DNA	Source of DNA	Transformants/ml [a] (×10^3)
DM3	BD170	pC194	*S. pneumoniae*	18
		pLS1	*S. pneumoniae*	7.6
	MB11	pC194	*S. pneumoniae*	8.1
		pC194	*B. subtilis*	8.7
		chromosomal	*B. subtilis*	N.D. [b]
c-R5	BD170	pC194	*S. pneumoniae*	8.7
		pLS1	*S. pneumoniae*	8.3
	MB11	pC194	*B. subtilis*	15
		pC194	*S. pneumoniae*	12
		pUB110	*B. subtilis*	6.9
		chromosomal	*B. subtilis*	N.D.
	CU403	pC194	*B. subtilis*	8.8
		pC194	*S. pneumoniae*	9.5

[a] No transformants were detected for any strain in DNA-free control samples.

[b] N.D., none detected.

The percentage of regenerants obtained in both media was approximately the same, 5–10%. However, when a different batch of sodium succinate was used for the preparation of DM3 plates, the proportion of regenerants consistently dropped to 1% (not shown). We did not observe any significant variation when different batches or sources of sucrose were employed (not shown). As illustrated in Table 2, the number of transformants obtained in DM3 or in c-R5 was very similar. These results were independent of the bacterial strain employed and of the antibiotic resistance marker selected. In addition, no differences were found when plasmid DNA was purified from *S. pneumoniae* (plasmid pLS1 and some preparations of pC194) or from *B. subtilis* (Table 2). This finding contrasts with the differences previously reported for competent *B. subtilis* cells [12], and agrees with the reported uptake of plasmid DNA by *B. subtilis* protoplasts in the double stranded configuration, since in the latter case all plasmid forms would be biologically active [2]. As expected [1] no colonies were detected either in samples treated with chromosomal DNA, or in the DNA-free controls.

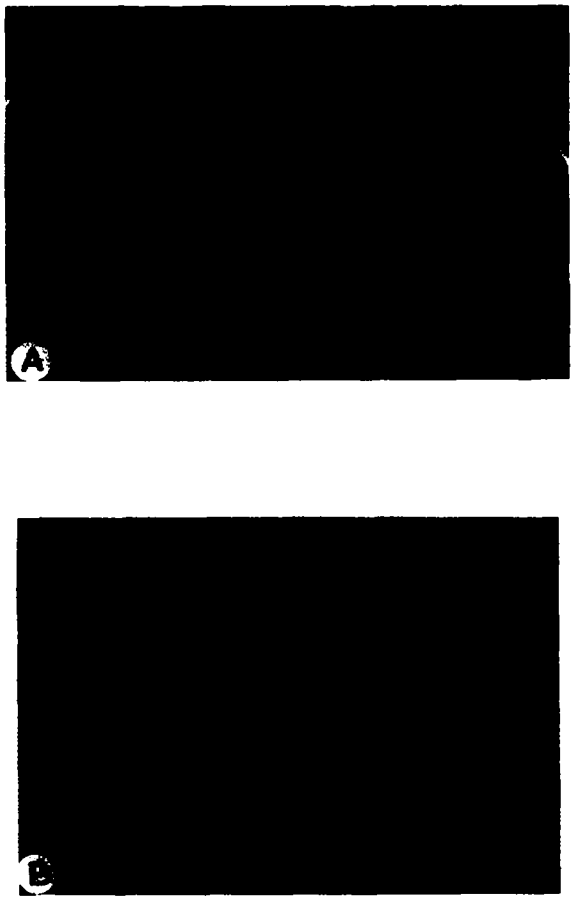

Fig. 1. Morphology of *B. subtilis* colonies grown on c-R5 medium (A) or in hypotonic LB medium (B). Regenerants obtained in c-R5 medium after incubation at 37°C for 24 h (A) were grown in penassay broth, diluted, plated on LB medium and incubated at 37°C for 24 h (B).

Regenerants on DM3 plates were detected after about 40 h of incubation at 37°C. On c-R5 plates, regeneration was obtained after overnight incubation (about 14–16 h); after about 24 h at 37°C, the regenerated bacilli grew in colonies as depicted in Fig. 1A. The viscous appearance of these colonies was more pronounced as the incubation time was prolonged. Such morphology of the colonies on c-R5 plates is most likely due to the secretion of saccharolytic enzymes involved in the *B. subtilis* sucrose metabolic system [13]. Nevertheless, this appearance of the regenerants did not interfere with the selection for antibiotic-resistant transformants, although the mucoid nature of the colonies could cause cross-contamination of colonies if plates with a high density of regenerants are incubated for periods longer than 24 h. To avoid this problem, a low number of regenerants per plate, or picking the colonies after no more than 20 h of incubation, is recommended. Transfer to plates containing a hypotonic medium, such as LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) resulted in the formation of colonies with the normal *B. subtilis* morphology (Fig. 1B).

Modifications of the c-R5 medium were later performed. They were aimed at the simplification and definition of the c-R5 medium and included the omission of the trace elements solution (vy-R5), of the yeast extract (v-R5) and of the yeast extracts and casamino acids, which were then replaced by a mixture of the 20 amino acids to give a final concentration of 20 μg/ml each. Thus, a chemically defined minimal regeneration medium (m-R5) was prepared. To evaluate these modifications, protoplasts of *B. subtilis* MB51 (0.2 ml) were transformed with 0.07 μg of plasmid pC194 DNA and, after allowing the 90-min expression

Table 3

Regeneration of *B. subtilis* MB51 protoplasts transformed with plasmid pC194 DNA on different media

Media	Viable cell count/ml in medium containing		Percentage of transformants [a]	Regeneration time (h)
	No Cm	5 μg Cm/ml		
C-R5	3×10^5	2.9×10^4	9.7	16
vy-R5	2.8×10^5	2.6×10^4	9.3	16
v-R5	10^5	4.5×10^3	4.5	25–30
m-R5	5×10^4	2.4×10^3	4.8	38–42

[a] No Cm-resistant colonies appeared in the DNA-free control samples.

period, they were spread on plates containing the aforementioned modifications of the c-R5 medium. Results are presented in Table 3. Omission of trace elements (vy-R5 plates) did not affect either the number of the regenerants, or the percentage of transformations. Elimination of yeast extract (v-R5) or substitution of casamino acids for a mixture of amino acids (m-R5) caused a decrease in the percentage of transformants as well as in the number of regenerants. In addition, the time needed for the completion of regeneration was also lengthened. When one or two amino acids were omitted from the m-R5 medium, the number of transformants and the percentage of regenerants did not change. It is likely that some improvements can be made to increase the number of transformants and to diminish the regeneration time in m-R5 medium. However, as is described here, this medium may solve some of the problems discussed concerning the protoplast transformation system of *B. subtilis*. Moreover, for the first time it has been reported that a chemically defined medium allows regeneration of *B. subtilis* protoplasts in a very reproducible form. The minimal medium previously described [14], which permits selection of prototrophs resulting from PEG-fusion of *B. subtilis* protoplasts, is a modification of DM3 medium [1], which has the disadvantage of not being totally defined, since it contains horse serum, which seems to be essential for good regeneration [14]. In summary, a convenient, inexpensive and reproducible regeneration medium for *B. subtilis* protoplasts is described which gives a high yield of transformants and allows a regeneration time totally comparable with any classical plating procedure, i.e., overnight.

ACKNOWLEDGEMENTS

Research supported by Grant 618/501 of the C.A.I.C.Y.T. and C.S.I.C.

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