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Short Communication

High Frequency Transformation of *Bacillus subtilis* Protoplasts by Plasmid DNA

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Summary. A highly efficient method for transformation of *Bacillus subtilis* by plasmid DNA is reported. The procedure, which involves polyethylene glycol-induced DNA uptake by protoplasts and subsequent regeneration of the bacterial cell wall, yields up to 80% transformants with an efficiency of 4×10^7 transformants per μg of supercoiled DNA. Plasmids constructed by in vitro ligation or endonuclease-generated fragments of linear plasmid DNA can also transform PEG-treated protoplasts, but at a lower frequency.

The ability to introduce individual molecules of plasmid DNA into bacterial cells by transformation has been of central importance to the recent rapid advancement of plasmid biology and to the development of DNA-cloning methods. Since establishment of the initial gene-cloning system in *Escherichia coli* (Cohen et al., 1973), the gram positive organism *Bacillus subtilis* has received increasing attention as a possible host for recombinant DNA molecules. While as many as 10% of recipient cells have been reported to undergo genetic alteration when determinants located on chromsomal DNA are introduced into competent *B. subtilis* cells (Bettinger and Young, 1975), the frequency of transformation of *plasmid* DNA has been reported to be several orders of magnitude lower (Ehrlich, 1977; Gryczan et al., 1978). Chimeric plasmids constructed in vitro have yielded even less effective transformation, thus limiting the use of DNA-cloning methods in this microorganism.

We report here a procedure for plasmid DNA transformation in *B. subtilis* that involves polyethylene glycol (PEG) induction of DNA uptake in protoplasts and subsequent regeneration of the bacterial cell wall. The procedure is highly efficient (4×10^7 transformants per μg of plasmid DNA) and yields up to 80% transformants, making the method suitable even for the introduction of phenotypically cryptic plasmids.

Our early attempts to develop a generally applicable high frequency transformation procedure involved the fusion of bacterial protoplasts with liposomes (Ostro et al., 1977) that contain entrapped plasmid DNA. *B. subtilis* was chosen as a model system for these experiments since a variety of plasmids capable of replication in this species have been identified, and protoplast fusion and regeneration can be achieved with high efficiency (Fodor and Alföld, 1976; Schaeffer et al., 1976). From a series of early experiments we learned that PEG-treatment can accomplish efficient plasmid DNA uptake by *B. subtilis* protoplasts directly and in the absence of lipsomes. Our subsequent experiments have elucidated the optimal conditions necessary for plasmid-mediated transformation.

All recipient strains used for this study were derivatives of *B. subtilis* Marburg 168. Media and solutions are described in the legend to Fig. 1. Midlog phase cell cultures freshly grown in Penassay broth (PAB) at 37° C to $1–2 \times 10^8$ cfu/ml were harvested and resuspended in 1/10 volume of SMMP solution. Lysozyme was added to 2 mg/ml final concentration and the suspension was incubated at 37° with gentle shaking. Although protoplasts formed within 30 min, incubation usually was carried out for 2 h to ensure complete protoplasting. Cells were pelleted in a horizontal rotor (Sorvall HB-4) at 2600 g for 15 min, washed once by resuspending them gently in SMMP, and pelleted a second time. The washed protoplast suspension was brought to 1/10–1/15 volume of the starting culture by addition of SMMP. The resulting preparation is stable at room temperature for at least 5 h; when plated onto regeneration media, the viable cell count was normally 10–25% of the colony form-

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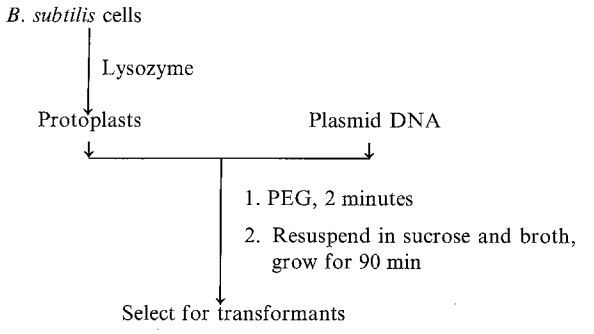

Fig. 1. Plasmid transformation procedure. Protoplasts of *B. subtilis* were prepared and treated as described in the text. Media and solutions have the following compositions; SMM buffer (Wyrick and Rogers, 1973) consists of 0.5 M sucrose, 0.02 M Maleate and 0.02 M $MgCl_2$, pH 6.5 adjusted with NaOH. SMMP medium is prepared by mixing equal volumes of 4X strength Penassay broth (Difco) and 2X strength SMM. PEG solution (40%, w/v) contains 40 g PEG (Sigma, approx. M.W. 6000) and 50 ml 2X SMM buffer in 100 ml. The DM3 regeneration medium consists of following sterile solutions (per liter; 200 ml 4% agar, 500 ml 1 M sodium succinate (pH 7.3), 100 ml 5% Difco Casamino acids, 50 ml 10% Difco yeast extract, 100 ml 3.5% K_2HPO_4 and 1.5% KH_2PO_4, 25 ml 20% glucose, 20 ml 1 M $MaCl_2$, and 5 ml filter-sterilized 2% bovine serum albumin (added to the mixture when the temperature is ~55° C). This preparation is a modification of the DPA medium described by Wyrick and Rogers and later used by Schaeffer et al., for protoplast fusion (Schaeffer et al., 1976). We adopted the modification because it reduces the time required for protoplast regeneration and also eliminates the "crowding effect" observed on DPA plates (Schaeffer et al., 1976)

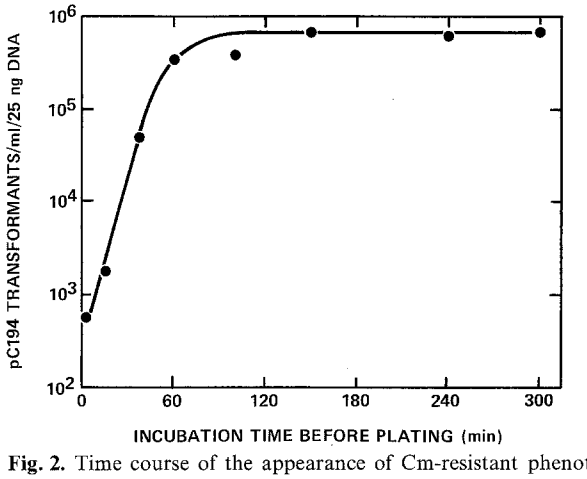

Fig. 2. Time course of the appearance of Cm-resistant phenotype in pC194 transformants. Protoplasts of BD170 (*trp*C2, *thr*-5) strain were transformed with 25 ng of pC194 plasmid DNA as described. PEG-treated protoplasts were pelleted and then resuspended in SMMP at time zero. 0.1 ml aliquots were plated at the indicated times on DM3 regeneration plates containing 20 μg/ml Cm

ing units (cfu) observed for the starting culture. However, cfu were decreased by at least five orders of magnitude when the protoplast preparation was plated on media (i.e. Penassay agar plates) which did not contain the osmotic stabilizer required for cell wall regeneration.

The protoplast transformation procedure is summarized in Fig. 1. 1 pg to 5 μg plasmid DNA in 50 μl or less TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was mixed with equal volume of 2X SMM solution in a sterile culture tube. 0.5 ml protoplast suspension was added, followed immediately by addition of 1.5 ml of 40% PEG (w/v) solution and gentle mixing of the contents of the tube. After two minutes exposure to PEG, 5 ml of SMMP medium was added to the mixture to dilute the PEG, and protoplasts were recovered by centrifugation for 10 min at 2600 × g. The treated protoplasts were resuspended in 1 ml SMMP and were routinely incubated for 1.5 h at 30° C in a gently shaking water bath to enable phenotypic expression of genetic determinants carried by the plasmid (see Fig. 2) before plating them onto antibiotic media for direct selection of transformants. Alternatively, samples of appropriate dilutions of PEG-treated protoplasts made in SMMP were plated onto DM3 medium (Fig. 1) without selection, and after regeneration of the cell wall, transformants were identified by transfer or replica-plating of cells onto selective media. In both instances, scoring of transformation frequency usually was done after incubation of protoplasts on regeneration plates at 37° C for 2 days.

We have used the plasmids pC194 (M.W., 2.0×10^6; Ehrlich, 1977) and pUB110 (M.W., 3.0×10^6; Gryczan et al., 1978), which specify resistance to chloramphenicol (Cm) and kanamycin (Km) respectively, for the transformation experiments described here. Covalently-closed circular (CCC) transforming plasmid DNA was prepared from *B. subtilis* strain BD170 (pC194), and BD170 (pUB110) essentially as described (Gryczan et al., 1978). Since protoplasts are extremely sensitive to trace amounts of detergent which might be present in the DNA preparation, we dialyze the plasmid DNA extensively (4 days with at least four changes of buffer) against TE buffer, precipitate it with ethanol, and re-dissolve it in TE buffer before use. Selection of transformants was carried out using the Cm and Km resistance determinants expressed by the plasmids. Although the *B. subtilis* strains employed usually are sensitive to 5 μg/ml of Km on Bacto-Penassay agar plates, the MIC (minimal inhibitory cencentration) of Km is much higher on DM3 plates, possibly because of high concentration of succinate on these plates. For the selection of Km^R transformants, we commonly replica-plated regenerated colonies from DM3 plates onto Penassay agar plates containing Km (5 μg/ml); however, direct selection of Km^R tranformants on freshly prepared DM3 plates containing 100 μg/ml Km also gives good results (T.J. Gryczan and D. Dubnau, personal communication). Transformants

Table 1. Some properties of the protoplast transformation system

Exp. No.	DNA	DNA amount (μg)	Treatment and source	Transformants/ μg DNA
1	pC194	1	None	1.8×10^7
2	pC194	1	DNase before PEG treatment	<5
3	pC194	1	DNase after PEG treatment	2.0×10^7
4	pCS540	1	None	2[a]
5	pCS540	1	*Hpa*II	2.1×10^4
6	pCS540	1	*Hpa*II + ligase	6.2×10^5
7	pCS540	0.5		
	pUB110	0.25	*Eco*RI + ligase	2.4×10^5
8	pCS489	0.01	from *E. coli*	2.7×10^7
9	pCS489	0.01	From *B. subtilis*	1.8×10^7
10	Chromosomal DNA Nal^R determinant	2	Nal^R strain VUB210	$<2.5 \times 10^2$ (background)

Transformants were selected on DM3 regeneration plates containing chloramphenicol (Cm) (20 μg/ml, experiments 1–9) or nalidixic acid (10 μg/ml, experiment 10). Restriction endonuclease digestions and ligation with T4 DNA ligase were performed as previously described (Sharp et al., 1973; Bahl et al., 1976). Plasmid pCS540, a recombinant molecule of 5.85 Kb, was constructed by linking linear pC194 DNA to the pSC101 replicon using *Hap*II endonuclease and T4 DNA ligase and was introduced by transformation (Cohen et al., 1972) into *E. coli*. It contains a unique *Eco*RI site which originated on pSC101 as well as the replication region of pSC101. Insertion of the pSC101 sequence at the single *Hap*II site on pC194 abolished its replication function in *B. subtilis*. However, an intact pC194 plasmid can be regenerated from *Hap*II endonuclease digests of pCS540 DNA, either by in vitro ligation or by in vivo re-circularization (unpublished). Plasmid pCS489 is a bifunctional replicon consisting of pC194 and *E. coli* plasmid pBR322 sequences which are joined at their respective *Hind*III sites. In experiments 2 and 3, pancreatic DNA's (20 μg in 5 μl of SMM) was added to the transforming DNA 5 min before, or to the resuspended protoplasts after, the PEG treatment

[a] Two Cm^R clones were selected, but no CCC plasmid DNA was detected in these clones. They may represent chromosomal mutations to Cm-resistance or alternatively could have resulted from integration of pCS540 into a short homologous sequence that potentially could be present on the chromosome (Duncan et al., 1978)

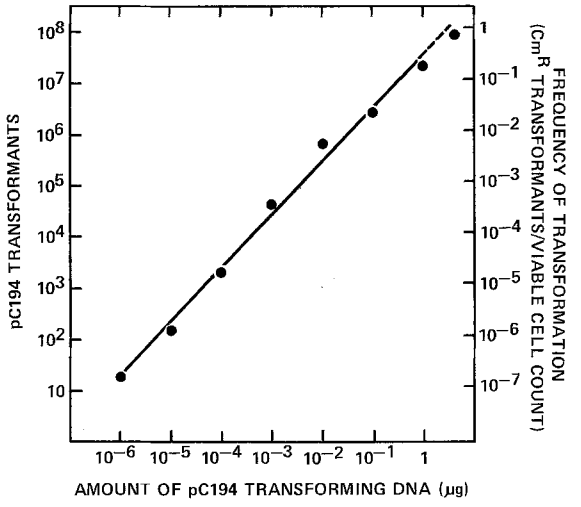

Fig. 3. Efficiency and frequency of plasmid transformation as a function of pC194 DNA concentration. The total viable cell count (i.e. regenerated protoplast) in each transformation mixture is 1.1×10^8

harboring pC194 plasmid can be directly selected on DM3 plates containing 20 μg/ml chloramphenicol.

The kinetics of the appearance of pC194 transformants on DM3 regeneration plates containing Cm is shown in Figure 2. As in the case of *E. coli* K12 (Cohen et al., 1972) the fraction of cells expressing the antibiotic resistance phenotype encoded by the plasmid increases 1000-fold during incubation in antibiotic-free medium and reaches a maximum after 60–75 min. Addition of pancreatic DNA'se immediately after the two minute PEG-treatment step does not affect the transformation frequency, indicating that uptake of DNA by protoplasts is completed during this step in the procedure (Table 1). However, addition of DNA'se prior to PEG treatment results in a sharp decrease in transformation frequency. As shown in Fig. 2, the number of antibiotic resistant cells present in the SMMP medium remains constant for at least a period of 3 h after reaching a plateau, which suggests that the transformed protoplasts are incapable of undergoing cell division in SMMP under the conditions used. This finding is consistent with an earlier report that *Bacillus* protoplasts do not divide in liquid media (Landman et al., 1968). Therefore, all transformants isolated from DM3 regeneration plates must represent individual clones. This property of the system is useful for a variety of genetic experiments.

Study of transforming ability of different concentrations of CCC plasmid DNA (Fig. 3) shows a linear relationship of transformation frequency to DNA

concentration over a 10^6 fold range and a sensitivity potentially capable of detecting less than 0.1 pg of DNA; in a typical experiment, 1 pg of pC194 CCC DNA (equivalent to 3×10^5 molecules) yielded 19 transformants. Using pC194 or pUB110 plasmid DNA, at amounts lower than 2 μg per transformation mixture, we have observed up to 40% transformants and have consistently obtained transformation frequences ranging from 5×10^6 to 4×10^7 transformants per μg of DNA. Although frequencies are less reproducible when DNA amounts greater than 2 μg per transformation mixture are used, transformation of 80% of input cells has been observed using 5 μg DNA. Since a similarly high efficiency of transformation was obtained using the replica-plating method in which no direct selection is involved, the protoplast transformation procedure appears to be a uniquely powerful tool for cloning and studying many phenotypically cryptic *Bacillus* plasmids (Le Hégarat and Anagnostopoulos, 1977; Tanaka et al., 1977; Lovett and Bramucci, 1975) that otherwise cannot easily be transferred into strains appropriate for their genetic and biochemical analysis.

In addition to its much higher yield of plasmid-containing transformants, the protoplast transformation system differs in a number of other respects from the traditional *B. subtilis* transformation system that uses physiologically competent cells (Table 1): 1) Linear plasmid DNA (experiment 5) and non-supercoiled circular plasmid DNA molecules constructed by in vitro ligation (experiments 6 and 7) can be introduced at high efficiency into *B. subtilis* by the protoplast transformation system, although at a frequency one to three orders of magnitude lower than the frequency observed for CCC plasmid DNA; in contrast, attempts to detect transformation by linear plasmid DNA using competent *B. subtilis* cells have been unsuccessful (Ehrlich, 1978; Gryczan and Dubnau, 1978); 2) No evidence of phenotypic restriction of DNA isolated from another bacterial species was seen with the protoplast method; the efficiency of transformation by plasmid pCS489 which consists of pC194 linked to an *E. coli* replicon (pBR322), is the same whether the DNA has been isolated from *B. subtilis* (experiment 9) or *E. coli* (experiment 8). 3) While competent cells can be transformed easily for genetic determinants located on the *B. subtilis* chromosome, no detectable transformation with chromosomal DNA was seen using the protoplast assay (experiment 10), suggesting that physiological and/or enzymatic functions associated with "competence" (Notani and Setlow, 1974; Dubnau, 1976) which are required for integration of chromosomal DNA fragments may be absent in protoplasts.

The transformation procedure described here seems to have a number of significant advantages over previously employed transformation methods for investigating the biology of plasmids capable of replication in *B. subtilis* and for introducing constructed chimeric plasmids into this bacterial species. Moreover, since the special physiological state of competence, which is often a species-related event (Notani and Setlow, 1974), is not required for the introduction of plasmid DNA into protoplasts, this procedure may have general applicability to other microorganisms. Recent reports of successful transformation of *Streptomyces* species (Bibb et al., 1978) and *Saccharomyses cerrevisiea* (Hinnen et al., 1978) using PEG-trated protoplasts are consistent with this view.

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