A rapid and simple method for *Bacillus subtilis* transformation on solid media

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Cells of *Bacillus subtilis* strains 168 and W23 deprived of an amino acid or a base on a given solid medium were found to develop competence. We describe a rapid and simple method of genetic transformation of this organism consisting in spreading a sample containing 1 µg DNA and 10⁷ exponentially growing cells of an auxotrophic mutant onto plates devoid of the required amino acid or base. After overnight incubation, about 100–200 prototrophic transformants per plate were obtained, i.e. a frequency of about 10⁻⁵, as compared to 10⁻⁴ routinely obtained by the method of transformation in liquid medium with frozen competent cells. Plasmids and other chromosomal or plasmid-borne markers, which cannot be directly selected for, were transferred by congression. The dependence of the transformation efficiency on cell density, medium richness, incubation time and the nature of transforming DNA was investigated. We conclude that the development of competence accompanies amino acid or base starvation of cells under appropriate physiological conditions.

Keywords: *Bacillus subtilis*, transformation on plates, competence, plasmid

INTRODUCTION

Bacillus subtilis is a Gram-positive bacterium capable of developing competence and undergoing DNA-mediated transformation (Dubnau, 1991). Spizizen (1958) and Anagnostopoulos & Spizizen (1961) described the conditions under which cells growing in liquid medium attain the state of competence allowing uptake of donor DNA and transformation. This method, which results in relatively high transformation frequencies with appropriate strains, has undergone a few minor modifications but is still in use. The process of obtaining competent cells is rather delicate, and takes 5–6 h. Therefore, more recently, competent cells were frozen and then thawed before use. This was accompanied by a 5–10-fold lower transformation efficiency. To simplify the preparation of competent cells, several methods have been proposed. Ephrati-Elizur (1965) described a method involving 60 min growth followed by 30 min exposure to transforming DNA. Transformants were obtained at a frequency of 10⁻³ to 10⁻⁴. However, to obtain reproducible results, the overnight culture must be inoculated with a

defined number of spores. Thus, the method requires preliminary preparation, purification and counting of spores. Tanooka (1973) proposed spreading a mixture of germinating spores of an auxotrophic strain and wild-type DNA onto plates which were only partly supplemented for the recipient's requirement. Development of competence occurred on the plates and transformants arose at a frequency about 100-fold lower than that obtained in parallel crosses performed by the method of Anagnostopoulos & Spizizen (1961). However, the absolute frequency of transformation was not calculated. In addition, the method requires preliminary determination of the concentration of the limiting amino acid in the plates. Recently, Kunst *et al.* (1994) described a protocol in which competence was attained 60 min after the end of the exponential phase in a minimal medium containing glutamate and casein hydrolysate. Nevertheless, although this method is much simpler than that of Anagnostopoulos & Spizizen (1961), it still takes several hours. Galizzi *et al.* (1976), and subsequently Hahn *et al.* (1987), achieved transformation by simply replicating fresh colonies onto selective plates seeded with donor DNA. However, this method, used for screening purposes, was not quantitative.

In this paper we describe a simple and rapid transformation procedure on solid media which yields about

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100 transformants per plate, i.e. a transformation frequency of about 10^{-5} .

METHODS

Strains and media. *B. subtilis* strains are listed in Table 1. SR is Schaeffer's rich sporulation medium (Schaeffer *et al.*, 1965). LA is solid L medium (Karamata & Gross, 1970), containing, per litre: tryptone (Difco) 10 g, yeast extract (Difco) 5 g, NaCl 10 g, agar (Gibco) 12 g, thymidine 20 mg; pH 7.0. TS medium (Karamata & Gross, 1970) is Spizizen minimal medium, containing, per litre: $(\text{NH}_4)_2\text{SO}_4$ 2 g, K_2HPO_4 14 g, KH_2PO_4 6 g, trisodium citrate dihydrate 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, glucose 5 g and supplemented with MnSO_4 0.85 mg, sodium glutamate 5 g and yeast extract (Difco) 0.01 g. When required, $20 \mu\text{g ml}^{-1}$ of amino acids and $50 \mu\text{g ml}^{-1}$ of bases were added to this medium.

DNA preparation. Chromosomal DNA was prepared according to Marmur (1961), and purified by centrifugation on CsCl density gradients (Maniatis *et al.*, 1982). Plasmid and bacteriophage DNA were prepared as described by Del Sal *et al.* (1988) and Grossberger (1987), respectively.

Transformation on plates. Cells grown on LA plates or spores, generated at 30°C on SR medium slants or plates, were spread onto LA plates and grown overnight at 30°C . The cells thus obtained were resuspended in sterile double-distilled water and their concentration determined either by counting or by nephelometric density (ND) measurements on an appropriate dilution. ND was measured on a Unigalvo (Corning-EEL) nephelometer. For cells originating from fresh colonies on LA plates, an ND reading of 100 corresponds to about 1×10^8 c.f.u. and 1.5×10^8 cells per ml. A 0.1–0.5 ml sample containing 10^7 cells was mixed with 3–40 μl of a DNA preparation (a total of 0.6–3 μg DNA); the mixture was spread immediately on appropriately supplemented TS plates (8.5 cm diameter) and incubated at 37°C .

Table 1. *B. subtilis* strains

Strain	Genotype or description	Reference or remarks
L1440	Prototroph	Mauël & Karamata (1984)
168	<i>trpC2</i>	Laboratory stock
GSY1127	<i>bisH2 ilvC1/ilvC+</i>	Anagnostopoulos & Trowsdale (1976); merodiploid strain
M22	<i>purA16 leuA8 metB5 ilvA1</i>	Karamata & Gross (1970)
L5256	<i>flaA15 purA16 ilvA1</i>	Pooley & Karamata (1984)
Ni15	<i>flaA15 trpC2 thyA1 thyB1 xcin-15</i>	Pooley (1976)
2A1	<i>thr</i>	W23 strain; BGSC* original code SB623
2A2	prototroph	W23 strain; BGSC* original code WB672

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Transformation in liquid medium. Competent cells were obtained according to the protocol of Pooley & Karamata (1984). They were centrifuged at 4°C , resuspended at 0°C in one-tenth of the initial volume of the supernatant, supplemented with 0.5% glucose and 15% (v/v) glycerol, frozen in liquid nitrogen and stored at -80°C . For transformation, 0.1 ml cells were thawed at 37°C , added to a tube containing 0.1 ml supplemented transformation medium and 0.1 ml DNA (final concentration of 3–5 $\mu\text{g ml}^{-1}$), and incubated for 60 min at 37°C with shaking. They were then diluted in non-supplemented transformation medium, plated onto appropriate TS plates and incubated overnight at 37°C .

RESULTS AND DISCUSSION

To achieve transformation from auxotrophy to prototrophy cells of *B. subtilis* strains 168 and W23, a mixture of donor DNA and recipient cells was spread on solid medium (TS) using the following protocol: (i) Cells from an overnight culture on rich medium (LA) were resuspended in double-distilled water and supplemented by donor DNA of different origin and degree of purity (plasmid, purified recombinant phage DNA, lysate of *Escherichia coli* infected by recombinant phage or chromosomal DNA, either purified or not purified on a CsCl density gradient). (ii) The 0.1–0.5 ml samples of the mixture spread on the plates contained a total of 10^7 cells and 1–3 μg of DNA. (iii) Limited growth was allowed before the beginning of starvation for one of the recipient's requirements. Following 24 h incubation, about 100–200 colonies of prototrophic transformants per plate are obtained for all recipient strains and auxotrophic markers tested, i.e. about 10^{-5} transformants per cells initially present on the plate (Table 2). For strain 168, this frequency is one order of magnitude lower than that which we routinely obtain with thawed competent cells. However, strain W23 is not transformable in liquid medium with the standard protocol. That the prototrophic colonies were transformants was indicated by their absence (i) when DNA was treated with DNase prior to mixing with cells, and (ii) when both the donor and the recipient strain carried the same relevant auxotrophic mutation. The size of recombinant colonies, relatively small when compared to those obtained in parallel crosses performed by standard methods, suggests that competence development and DNA uptake took place on the plate after several hours of incubation.

Under the experimental conditions used, i.e. spreading 1 μg of DNA per plate (5675 mm^2) which, for purified DNA, corresponds on the average to about 4×10^{10} molecules of 1.5×10^7 Da, we can calculate that each cell (about $1.2 \mu\text{m}^2$) is in contact with about 50 molecules of DNA. That this corresponds to a saturating DNA concentration is demonstrated by a congression of about 10% between unlinked markers *purA16* and *metB5* or *ilvA1* even with 0.6 μg DNA per plate, i.e. a co-transfer comparable to that obtained in liquid medium at saturating DNA concentrations (data not presented).

Occurrence of high co-transfer during transformation on plates allowed the introduction, by congression, of plasmids carrying markers unsuitable for direct selection

Table 2. Transformation of *B. subtilis* on solid medium

Cells grown overnight on LA were resuspended in sterile water, mixed with transforming DNA, plated onto TS selective medium and incubated at 37 °C. Except W23 strains 2A1 and 2A2, all strains are 168 derivatives.

Donor*	Recipient†	Selected marker‡	No. of cells per plate	Transformants per cell plated	
				Incubation time§: 24 h	48 h
L1440	M22 <i>leuA8</i>	Leu ⁺	10 ⁸	5 × 10 ⁻⁶	5 × 10 ⁻⁶
			10 ⁷	1 × 10 ⁻⁵	3 × 10 ⁻⁵
			10 ⁶	1 × 10 ⁻⁵	4 × 10 ⁻⁵
			10 ⁵	0	2 × 10 ⁻⁴
			10 ⁴	0	3 × 10 ⁻³
GSY1127	L5256 <i>ilvA1</i>	Ilv ⁺	10 ⁸	5 × 10 ⁻⁶	5 × 10 ⁻⁶
			10 ⁷	2 × 10 ⁻⁵	2 × 10 ⁻⁵
			10 ⁶	1 × 10 ⁻⁵	3 × 10 ⁻⁵
			3 × 10 ⁵	3 × 10 ⁻⁵	1 × 10 ⁻⁴
			10 ⁵	3 × 10 ⁻⁵	7 × 10 ⁻⁴
L1440	168 <i>trpC2</i>	Trp ⁺	3 × 10 ⁴	2 × 10 ⁻⁵	5 × 10 ⁻³
			10 ⁸	3 × 10 ⁻⁶	3 × 10 ⁻⁶
			10 ⁷	5 × 10 ⁻⁶	5 × 10 ⁻⁶
2A2	2A1 <i>thr</i>	Thr ⁺	10 ⁶	3 × 10 ⁻⁶	3 × 10 ⁻⁶
			10 ⁷	2 × 10 ⁻⁵	2 × 10 ⁻⁵

* 3 µg of CsCl purified DNA per plate was used except in the L1440 × 168 cross, where 2 µg was added.

† Relevant markers only are shown.

‡ The reversion frequency of the *leuA8* marker was about 6 × 10⁻⁸, while those of *ilvA1* and *trpC2* were below 10⁻⁸.

§ The same plates were examined at 24 and 48 h.

|| The marker *ilvA1* is not duplicated in the merodiploid strain GSY1127.

by this method. A sample containing 2.4 µg of strain 168 IlvA⁺ chromosomal DNA and 0.15 µg of concatemers of plasmid pMTL500C was added to M22 *ilvA1* recipient cells and spread on a TS plate. Plasmid pMTL500C, a replicative plasmid conferring chloramphenicol resistance, was constructed by N.P. Minton, Centre for Applied Microbiology, Porton Down, UK, and provided by M. Young, Aberystwyth, UK. About 20% of selected IlvA⁺ transformants were resistant to 5 µg chloramphenicol ml⁻¹. Ligase treatment of the plasmid, leading to concatamer formation, greatly increased the frequency of transformation (data not presented), in agreement with previous observations showing that transfer of plasmid DNA requires a multimeric form (Canosi *et al.*, 1978; Mottes *et al.*, 1979). Integration into the chromosome of non-replicative plasmids, introduced by congression, occurred at a frequency of about 1% among the selected transformants (data not presented). This relatively low efficiency may be related to the size of the chromosomal DNA insert and thus the probability of plasmid insertion into the chromosome.

To characterize some of the factors relevant for plate transformation efficiency, the incubation time and the medium composition were varied. At cell densities of less

than 10⁸ per plate, an additional 24 h incubation of recipients M22 and L5256 yielded many more transformant colonies (Table 2). The frequency of these late transformants increased with a decreasing number of cells initially spread on the plate, reaching nearly 10⁻² transformants for an initial density of 10⁴ cells per plate. This observation could be interpreted as indicating the presence of a limiting factor, which is consumed at a rate hand in hand with the number of cells initially spread on the plate. Exhaustion of this factor would be a prerequisite for the development of competence. To test this possibility, we prepared plates with media of different 'richness' (Table 3). Relatively few transformants were obtained on TS plates devoid of yeast extract, which strongly suggests that the latter component corresponds to the limiting factor. However, despite the fact that transformation efficiency and yeast extract concentration went hand in hand, we do not recommend concentrations of yeast extract as high as 0.05 g l⁻¹ since they result in too heavy a lawn due to residual growth. The appearance of transformants in the absence of yeast extract but in the presence of glutamate suggests that the latter compound does play a role in competence development. Incidentally, Kunst *et al.* (1994) recently showed that glutamate stimulates the expression of competence genes. In con-

Table 3. Effect of medium richness on transformation efficiency

The medium richness of the TS plates was determined by yeast extract and glutamate concentrations.

Yeast extract (g l ⁻¹)	Glutamate (%)	Relative transformation frequency
0.01*	0.5*	1
0.05	0.5	5–10
0	0.5	0.3
0	0	0

* The conventional TS medium (see Methods).

clusion, preliminary observation of competence development as a function of time and residual growth suggest that the key steps are the establishment of proper physiological conditions for colony growth, followed by amino acid or base starvation, while glutamate seems to act as an effector. Growth and starvation conditions on plates might well, to some extent, be related to those encountered in natural environments which were shown to be appropriate for efficient natural transformation (Graham & Istock, 1978; Lorenz *et al.*, 1988).

The method described was simplified so as to allow extensive screening and strain constructions. To that end, donor DNA was spotted onto plates previously seeded with cells, either resuspended in distilled water (see Methods) or directly spread onto plates. The occurrence of transformation as well as congression was illustrated by spotting onto the non-motile strain L5256 *ihvA1 flaA15* 10 µl of a mixture of diluted chromosomal DNA (1 µg ml⁻¹) obtained from strain Ni15 *flaA15* *IlvA*⁺ and a saturating concentration of recombinant phage λUF7 DNA, which contains part of the *flaA* operon (Albertini *et al.*, 1991; Hauser *et al.*, 1991). About 50% of selected *IlvA*⁺ transformants were found to be motile, due to correction of the unlinked *flaA15* mutation.

In conclusion, plate transformation offers a rapid and simple method for the following applications: (i) screening of gene libraries by spotting DNA preparations onto plates and identifying the relevant marker by direct or indirect selection, (ii) simultaneous construction of a series of strains through back-crossing and congression, (iii) measurement of co-transfer and recombination indexes, and (iv) introduction of replicative plasmids and, at a rather low frequency, integration into the chromosome of plasmids with relevant inserts, by congression with reference DNA able to correct the auxotrophic mutation responsible for the development of competence on plates.

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REFERENCES

- Albertini, A. M., Caramori, T., Crabb, W. D., Scoffone, F. & Galizzi, A. (1991). The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. *J Bacteriol* **173**, 3573–3579.
- Anagnostopoulos, C. & Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* **81**, 741–746.
- Anagnostopoulos, C. & Trowsdale, J. (1976). Production of merodiploid clones in *Bacillus subtilis*. In *Microbiology – 1976*, pp. 44–57. Edited by D. Schlessinger. Washington, DC: American Society for Microbiology.
- Canosi, U., Morelli, G. & Trautner, T. A. (1978). The relationship between molecular structure and transformation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. *Mol & Gen Genet* **166**, 259–267.
- Del Sal, G., Manfioletti, G. & Schneider, C. (1988). A one-tube plasmid DNA miniprep suitable for sequencing. *Nucleic Acids Res* **16**, 9878.
- Dubnau, D. (1991). Genetic competence in *Bacillus subtilis*. *Microbiol Rev* **55**, 395.
- Ephrati-Elizur, E. (1965). Development of competence for transformation in an overnight culture of germinating spores of *Bacillus subtilis*. *J Bacteriol* **90**, 550–551.
- Galizzi, A., Siccardi, A. G., Mazza, G., Canosi, U. & Polsinelli, M. (1976). A recombination test to classify mutants of *Bacillus subtilis* of identical phenotype. *Genet Res* **27**, 47–58.
- Graham, J. B. & Istock, C. A. (1978). Genetic exchange in *Bacillus subtilis* in soil. *Mol & Gen Genet* **166**, 287–290.
- Grossberger, D. (1987). Minipreps of DNA from bacteriophage lambda. *Nucleic Acids Res* **15**, 6737.
- Hahn, J., Albano, M. & Dubnau, D. (1987). Isolation and characterization of Tn917lac-generated competence mutants of *Bacillus subtilis*. *J Bacteriol* **169**, 3104–3109.
- Hauser, P. M., Crabb, W. D., Fiora, M. G., Scoffone, F. & Galizzi, A. (1991). Genetic analysis of the *flaA* locus of *Bacillus subtilis*. *J Bacteriol* **173**, 3580–3583.
- Karamata, D. & Gross, J. D. (1970). Isolation and genetic analysis of temperature-sensitive mutants of *B. subtilis* defective in DNA synthesis. *Mol & Gen Genet* **108**, 277–287.
- Kunst, F., Msadek, T. & Rapoport, G. (1994). Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*. In *Regulation of Bacterial Differentiation*, pp. 1–19. Edited by P. J. Piggot, P. Youngman & C. P. Moran (in press).
- Lorenz, M. G., Aardema, B. W. & Wackernagel, W. (1988). Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains. *J Gen Microbiol* **134**, 107–112.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Mauël, C. & Karamata, D. (1984). Characterisation of proteins induced by mitomycin C treatment of *Bacillus subtilis*. *J Virol* **49**, 806–812.
- Mottes, M., Grandi, G. & Sgarbetta, V. (1979). Different specific activities of the monomeric and oligomeric forms of plasmid DNA

in transformation of *B. subtilis* and *E. coli*. *Mol & Gen Genet* **174**, 281–286.

Pooley, H. M. (1976). Turnover and spreading of old wall during surface growth of *Bacillus subtilis*. *J Bacteriol* **125**, 1127–1138.

Pooley, H. M. & Karamata, D. (1984). Genetic analysis of autolysin-deficient and flagellaless mutants of *Bacillus subtilis*. *J Bacteriol* **160**, 1123–1129.

Schaeffer, P., Millet, J. & Aubert, J. P. (1965). Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci USA* **54**, 704–711.

Spizizen, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc Natl Acad Sci USA* **44**, 1072–1078.

Tanooka, H. (1973). Transformation of germinated spores of *Bacillus subtilis* on agar plates. *J Bacteriol* **114**, 445–447.

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