Transformation of *Bacillus licheniformis* protoplasts by plasmid DNA

Zoltán Prágai, András Holczinger and Tibor Sík

Author for correspondence: Zoltán Prágai. Fax: +36 28 310804. e-mail: pz@rkt.gau.hu

Department of
Biotechnology, Gödöllő
University of Agricultural
Sciences, Gödöllő, Páter
K. u. 1., H-2103, Hungary

A simple and efficient protoplast regeneration and PEG-induced transformation procedure was developed for *Bacillus licheniformis*. The protoplast stabilizing osmoticum was 0.5 M sucrose in the regenerating media, and the restoration of cell wall was carried out at pH 8.4. The mean frequency of regeneration was 83 %. The transformation efficiency was studied with four different strains and five plasmids, and ranged from 3.5×10^5 to 7.2×10^6 transformants per μg plasmid DNA depending on the plasmid, and on the donor and recipient strains used. Endonuclease digested and ligated plasmid DNA could also be used to transform the protoplasts, but at a lower frequency.

Keywords: *Bacillus licheniformis*, protoplast transformation, plasmid

INTRODUCTION

Bacillus licheniformis is used for the industrial production of the peptide antibiotic bacitracin, α -amylase, β -lactamase, alkaline protease and alkaline phosphatase (Kleinkauf & von Döhren, 1987; Harwood, 1992). In a strain improvement and genetic manipulation programme plasmid DNA transfer is indispensable. There are several methods to introduce plasmid DNA into *Bacillus* species, such as transformation of competent cells, PEG-mediated transformation of protoplasts, electroporation, transduction and conjugation (Bron, 1990), but in the case of some *Bacillus licheniformis* strains only protoplast transformation was successful (Jensen & Hulett, 1989; A. Holczinger, unpublished results). The method described for *Bacillus subtilis* by Chang & Cohen (1979) cannot be applied to *B. licheniformis* because of deficient cell wall regeneration of the protoplasts. Regenerants could only be obtained by changing the composition of the growth and regeneration media, or the incubation temperature. Even so, the efficiency of regeneration and transformation was low and variable (Grosch & Wollweber, 1982; Bourne & Dancer, 1986; Jensen & Hulett, 1989; Bron, 1990; Wati *et al.*, 1990).

A transformation procedure is reported here for protoplasts of *B. licheniformis* strains with different plasmids. The method is a reproducible variant of the procedure of Chang & Cohen (1979), in which the media are modified to increase regeneration and transformation frequencies.

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; EtBr, ethidium bromide; MIC, minimal inhibitory concentration; PEG, polyethylene glycol; RT, room temperature.

METHODS

Bacterial strains and plasmids. Strains used in the experiments are described in Table 1 and plasmids in Table 2.

Media. P-solution: 1.25 g NH_4Cl , 15 g Tris, 0.075 g NaCl, 0.045 g KCl, 0.375 g $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 5.33 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 171.15 g sucrose per litre, pH 8.4 [modified hypertonic medium of Fodor *et al.* (1975)]. P-lys medium: P-solution containing 2 mg lysozyme (Serva) ml^{-1} and then filter-sterilized. PEG solution: 60 % (w/v) PEG 6000 (Serva) in P-solution. Lysozyme solution, neutral phenol/chloroform and TE buffer were according to Kieser (1984). YTA media were described by Sík *et al.* (1980). ART liquid medium: 5 g NaCl, 5 g yeast extract (Oxoid L21), 5 g tryptone (Oxoid L42), 6.057 g Tris, 5 g glucose, 171.15 g sucrose, 1 ml 0.5 M CaCl_2 , 1 ml 1.0 M MgSO_4 , 1 ml 0.5 M MnSO_4 per litre, pH 8.4. ART soft-agar and ART agar media consisted of ART liquid medium containing 0.8 % (w/v) or 1.8 % (w/v) agar (Oxoid L11), respectively.

DNA isolation. Plasmid DNA was isolated from *B. subtilis* 27 and *B. licheniformis* 19 by a large-scale plasmid purification method using alkaline lysis and CsCl-EtBr centrifugation (Bron, 1990). Plasmid DNA content of *B. licheniformis* transformants was examined by a quick-test method. From selective YTA agar medium one loopful of bacteria was suspended in 50 μl lysozyme solution and incubated for 20 min at 37 °C. Then 25 μl 2 % SDS solution was added and the mixture was extracted with 25 μl neutral phenol/chloroform. After centrifugation (RT, 5 min, 6000 g), DNA and RNA structures in the supernatant were separated by 0.7 % agarose gel electrophoresis.

Preparation of protoplasts. Bacteria were grown in YTA broth overnight (14–16 h) at 37 °C. The cultures were subsequently diluted 100-fold into 5 ml fresh YTA medium and grown for 1.5–2 h at 37 °C (the number of c.f.u. was around $3\text{--}6 \times 10^7 \text{ ml}^{-1}$). The cells were centrifuged at RT for 5 min at 6000 g and resuspended in the same volume of P-lys medium. After 90 min at 37 °C (without shaking), almost 100 % of the cells

Table 1. Strains

Strain	Product	Source
<i>B. licheniformis</i>		
ATCC 9800	—	OKI, Budapest, Hungary
ATCC 10716 UM12	Bacitracin	T. Imanaka, Osaka University, Osaka, Japan
19	Bacitracin	Our laboratory
16*	Bacitracin	Our laboratory
<i>B. subtilis</i> 27	pUB110 donor	T. Imanaka, Osaka University, Osaka, Japan

* A derivative of *B. licheniformis* 19 cured of phage BLF.

Table 2. Plasmids

Plasmid	Antibiotic resistance conferred in <i>Bacillus</i>	Size (kb)	Copy number (per chromosome)	Reference
pUB110	Km	4.548	50	Sueoka <i>et al.</i> (1986)
pUBUC18*	Km	7.234	50	Our laboratory
pAT187	Km	10.5	15	Trieu-Cuot <i>et al.</i> (1987)
pHV33	Cm	7.278	10	Primrose & Ehrlich (1981)
pLTV1	Cm, Tc	20.6	6	Youngman (1990)

* A shuttle plasmid between *Bacillus* and *E. coli*, carrying Km and Ap resistances, prepared by ligating pUB110 and pUC18 plasmids at their single *EcoRI* sites.

were protoplasted. The protoplasts were harvested by centrifugation (RT, 5 min, 6000 *g*) and resuspended in the same volume of P-solution.

PEG-induced transformation. Protoplasts were centrifuged (RT, 5 min, 6000 *g*) and resuspended in the same volume of P-solution containing CsCl-purified plasmid DNA (final concentration: 1 µg ml⁻¹). This was mixed with equal volume of PEG solution. After 3 min exposure to PEG the mixture was diluted 12.5-fold into ART liquid medium and incubated for 90 min at 30 °C with agitation. Then non-inhibitory amounts of appropriate antibiotic were added during the expression period of resistance genes (0.1 µg Km or Cm ml⁻¹), which was 30 min at 30 °C.

Regeneration of protoplasts. Protoplasts were diluted in ART liquid medium and plated in ART soft-agar overlays on ART agar medium. For direct selection of transformants, selective antibiotic (Km 5 µg ml⁻¹ or Cm 20 µg ml⁻¹) was added to both ART agar media. For colony formation the incubation time at 30 °C depended on the strain and plasmid used (3–6 d).

Restriction endonuclease digestion and ligation. All of the enzymes were purchased from Amersham and were used according to the manufacturer's specifications. Reaction products were analysed by agarose gel electrophoresis.

RESULTS

Cell wall regeneration

In order to develop a simple and efficient protoplast regeneration method for *B. licheniformis* 0.5 M sucrose was used in all media as osmotic stabilizer. Optimal conditions for cell wall regeneration were worked out for *B.*

licheniformis 19. The effect of pH was tested at pH 7–10 in ART media. The protoplasts were prepared (from 4.6×10^7 c.f.u. ml⁻¹) and the cell walls were regenerated as described in Methods. The results are shown in Fig. 1. For cell wall regeneration, the optimum was pH 8.4. The efficiency of protoplast preparation and regeneration was determined at this pH optimum.

Since *B. licheniformis* cells form long chains during early exponential growth, the number of c.f.u. was only an

Fig. 1. Influence of pH in ART media on regeneration of cell wall. The data shown are means of three independent experiments. The optimum was pH 8.4, as determined by second-order polynomial function.

Fig. 2. Effect of PEG concentration on transformation of protoplasts. The data are means of three independent experiments. The optimal PEG final concentration was 30% (w/v), as determined by second-order polynomial, two-sample analysis (performed in pairs) and one-way analysis of variance.

Fig. 3. Effect of plasmid DNA concentration on transformation using pUB110 DNA purified from *B. licheniformis* 19 (□) or *B. subtilis* 27 (○). The number of regenerants was 2.0×10^7 ml⁻¹. The recipient was *B. licheniformis* 19.

approximate value. Three independent experiments were carried out with *B. licheniformis* 19 cultures (4.2×10^7 c.f.u. ml⁻¹). Protoplasts were prepared as described in Methods. To evaluate the effect of lysozyme treatment, the number of surviving c.f.u. was determined after bursting the protoplasts in sterile distilled water by plating on YTA agar medium: 6.1×10^3 c.f.u. ml⁻¹ were detected and the frequency of osmotic shock resistant cells was around 1.5×10^{-4} . To determine the efficiency of regeneration the cell wall of protoplasts was restored as described in Methods. The number of regenerants was

3.5×10^7 ml⁻¹. Regeneration frequencies were on the average 83%, although in some cases we observed 140–180% regeneration. In these cases more than one protoplast was formed from one bacterial chain.

Conditions for optimum protoplast transformation

Using the conditions for efficient regeneration described above, the effects of PEG and plasmid DNA concentration on transformation frequencies were studied.

The optimal final concentration of PEG 6000 was determined for *B. licheniformis* 19 protoplasts using $1 \mu\text{g ml}^{-1}$ pUB110 DNA (purified from the same strain) in the transformation mixture. Increasing volumes of PEG solution were added to equal volumes of protoplast and plasmid DNA suspensions, so that the final percentages were from 10 to 50 (w/v) PEG. Transformants were isolated by direct selection ($5 \mu\text{g Km ml}^{-1}$). The results are shown in Fig. 2. At the optimal concentration of PEG (30%, w/v) 4.1×10^7 regenerated protoplasts ml⁻¹ were detected; 16.6% of these were transformed.

To study the effect of plasmid DNA concentration on transformation, *B. licheniformis* 19 protoplasts were transformed with increasing amounts of pUB110 DNA (10^{-4} – $10^2 \mu\text{g ml}^{-1}$), purified from *B. licheniformis* 19 or *B. subtilis* 27. Transformants were isolated by direct selection on ART agar medium containing Km (Fig. 3). The Km^r transformants were transferred to YTA agar medium containing $5 \mu\text{g Km ml}^{-1}$, and their plasmid DNA content was detected by the quick-test method. The same plasmid was found in all samples (results not shown). Transformation efficiency depended on the bacterium from which the plasmid DNA was prepared. A significant difference in efficiency was found between DNAs extracted from *B. licheniformis* 19 and *B. subtilis* 27 (4.8×10^6 and 1.5×10^6 Km^r transformants per μg plasmid DNA, respectively; Fig. 3). Transformation frequencies were determined as the ratios (in percentage) of transformants and regenerated protoplasts. The transformation frequencies were 24.0% and 7.5% using $1 \mu\text{g ml}^{-1}$ pUB110 purified from *B. licheniformis* 19 or *B. subtilis* 27, respectively. Both curves follow a saturation function (Fig. 3). At high DNA concentrations ($100 \mu\text{g ml}^{-1}$) all protoplasts became transformed with both donor DNAs.

Transformation of different strains of *B. licheniformis*

The transformation efficiencies were compared with four *B. licheniformis* strains in three independent experiments using $1 \mu\text{g ml}^{-1}$ of pUB110 (Table 3). The numbers of Km^r transformants were determined and their plasmid content was tested as described before. The transformation efficiency of strain ATCC 9800 was significantly lower than that of strains ATCC 10716 UM12, 19, and 16, as determined by two-sample analysis (95% significance level). There were also differences among the strains with respect to the time required for cell wall regeneration. This time was shorter for *B. licheniformis* 16 or 19, and longer for ATCC 9800.

Table 3. Transformation frequencies of *B. licheniformis* strains by pUB110

pUB110 DNA was purified from *B. subtilis* 27. Values are means of three independent experiments.

Strain	No. of transformants ($\mu\text{g DNA}$) ⁻¹	Transformation frequency (%)	Incubation time (d)
ATCC 9800	3.5×10^5	1.8	5–6
ATCC 10716 UM12	1.1×10^6	6.1	5
19	1.3×10^6	5.9	3–4
16	1.5×10^6	5.8	3–4

Table 4. Transformation frequency of *B. licheniformis* 19

Plasmid	Selected marker	No. of transformants ($\mu\text{g DNA}$) ⁻¹	Transformation frequency* (%)
pUBUC18	Km ^r	7.2×10^6	25.7
pUB110	Km ^r	5.8×10^6	20.7
pAT187	Km ^r	3.3×10^6	11.8
pHV33	Cm ^r	1.8×10^6	6.4
pLTV1	Cm ^r	1.3×10^6	4.6

* The number of regenerated protoplasts was $2.8 \times 10^7 \text{ ml}^{-1}$.

Table 5. Transformation with cut and ligated pUBUC18 DNA

pUBUC18 (Table 2) contains a unique *Hind*III site which originated on pUC18, and two *Eco*RI sites where pUB110 (4.548 kb) and pUC18 (2.686 kb) are joined. Restriction endonuclease digestions and ligation with T4 DNA ligase were performed as described in Methods. The recipient was *B. licheniformis* 19.

Plasmid DNA	No. of transformants ($\mu\text{g DNA}$) ⁻¹
Untreated	6.4×10^6
<i>Eco</i> RI treated	4.5×10^3
<i>Eco</i> RI + T4 ligase	1.9×10^6
<i>Hind</i> III treated	2.7×10^4
<i>Hind</i> III + T4 ligase	8.1×10^5

Protoplast transformation by different plasmids

Five plasmids of different size and copy number, carrying Km or Cm resistances, were introduced into the protoplasts of *B. licheniformis* 19. We used $1 \mu\text{g ml}^{-1}$ plasmid DNAs (from *B. licheniformis* 19) and transformants were isolated by direct selection (Table 4). Transformants and their plasmid contents were tested as described in foregoing sections. The size and the *Eco*RI or *Bam*HI restriction endonuclease digestion patterns of the plas-

mids were the same in the transformants as in the original strain, suggesting that the plasmids were maintained in *B. licheniformis* without any deletions or rearrangements. The transformation frequencies were compared by two-sample analysis, and the results were the following. There was no significant difference between pUB110 and pUBUC18, which are based on the same replicon, though their sizes are different. Likewise, no significant difference in transformation efficiency was found between various low-copy-number plasmids of different size (pLTV1 is nearly three times bigger than pHV33). Significant differences were found, however, between the high-copy-number plasmids (pUBUC18, pUB110) and the low-copy-number plasmids (pAT187, pHV33, pLTV1). Cm^r transformants formed colonies 2–3 d later than Km^r transformants.

Protoplast transformation by cut and ligated plasmid DNA

The transformation efficiencies of restriction endonuclease digested and ligated plasmid DNA were determined for *B. licheniformis* 19 protoplasts using $1 \mu\text{g ml}^{-1}$ uncut, *Eco*RI treated, *Hind*III treated and ligated pUBUC18 DNAs in the transformation mixtures (Table 5). Transformants were selected on ART agar medium containing Km ($5 \mu\text{g ml}^{-1}$). The numbers of transformants were determined and their plasmid content was tested as described above. In the case of *Eco*RI digested and T4 DNA ligase treated plasmid DNA, 16% of the tested Km^r colonies (231) contained pUBUC18 ('recombinant DNA'), and 84% contained pUB110.

DISCUSSION

The most limiting step in the transformation of *B. licheniformis* protoplasts seems to be the regeneration of the cell wall, because the L-form colonies arising from protoplasts appear unable to revert to normal cells (Bourne & Dancer, 1986; Jensen & Hulett, 1989). Regenerants have been obtained by several investigators, for instance when succinate in the regeneration medium was replaced by mannitol (Wati *et al.*, 1990), or gelatin was added to this medium (Bourne & Dancer, 1986), or when the incubation temperature was increased to 46 °C (Jensen & Hulett, 1989), or when the growth and regeneration media were different (Bron, 1990). The frequencies of

regeneration described so far in the literature are low, in the range of 6×10^{-2} – 1×10^{-3} per protoplasted cell (Bourne & Dancer, 1986; Jensen & Hulett, 1989). The procedures described up to now were only suitable for a few *B. licheniformis* strains and were poorly reproducible (Jensen & Hulett, 1989; Wati *et al.*, 1990). The method described in the present work is simpler and the frequency of regeneration (83%) was almost as high as that reported by Gabor & Hotchkiss (1979) for *B. subtilis* protoplasts. Efficient regeneration was achieved without using special protoplast stabilizing agents such as gelatin or bovine serum albumin. Osmotic protection was obtained with 0.5 M sucrose and salts (i.e. NH_4^+ , K^+ , Na^+ , Mg^{2+}), as in earlier studies with *B. licheniformis* protoplasts (Snoke & Cornell, 1965).

The maximum number of transformants was obtained using 30% (w/v) PEG 6000 for DNA uptake, although there were no big differences in the range from 20% to 40% PEG (Fig. 2).

There was a linear relationship between transformation frequency and DNA concentration in the range 10^{-4} to $1 \mu\text{g DNA ml}^{-1}$ (Fig. 3), where 1 Km^r transformant was detected per $1\text{--}3 \times 10^4$ molecules of pUB110 (from *B. licheniformis* 19). These efficiencies are three to five orders of magnitude higher than those obtained by Jensen & Hulett (1989), Wati *et al.* (1990), and Bron (1990) for *B. licheniformis*, and were nearly the same as those observed by Chang & Cohen (1979) for *B. subtilis*.

The MIC of Km was increased in the succinate-based regeneration medium from 5 to $100 \mu\text{g ml}^{-1}$ for *B. subtilis* (Chang & Cohen, 1979) and from 20 to $300 \mu\text{g ml}^{-1}$ for *B. licheniformis* (Grosch & Wollweber, 1982), and Jensen & Hulett (1989) found that concentrations of Km up to 2 mg ml^{-1} were ineffective in the direct selection of Km^r transformants. Since ART media do not contain any succinate, direct selection could be performed with $5 \mu\text{g Km ml}^{-1}$ in our experiments.

Significant differences were found between the transformation efficiencies in *B. licheniformis* 19 with the same plasmid isolated from *B. subtilis* or from *B. licheniformis* (Fig. 3). This may indicate that different restriction–modification systems exist in the two species. If this idea is correct, it appears that the plasmid DNA extracted from *B. subtilis* is inappropriately modified for *B. licheniformis*.

Efficient transformation of four *B. licheniformis* strains was achieved with pUB110 DNA (from *B. subtilis* 27) (Table 3). Significant differences were found among the transformation frequencies of the strains, and yields were 3.5×10^5 – 1.5×10^6 transformants per μg plasmid DNA, depending on the strain.

Comparing five different plasmids (Table 4), a negative correlation was not noticed between transformation frequency and plasmid size, but significant differences were found between high- (pUBUC18, pUB110) and low-copy-number plasmids (pAT187, pHV33, pLTV1), as Zhang *et al.* (1988) reported for *B. stearothermophilus*.

Endonuclease digested and ligated plasmid DNA could

be introduced at high efficiency into *B. licheniformis* protoplasts by this transformation system (Table 5), although the frequencies were half to three orders of magnitude lower than that for uncut plasmid DNA, as observed by Chang & Cohen (1979) for *B. subtilis*. Without any selection for insert DNA, a high proportion (16%) of the transformants carried recombinant DNA. These results demonstrate that the transformation procedure described here seems to be a useful tool for cloning in *B. licheniformis*.

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REFERENCES

- Bourne, N. & Dancer, B. N. (1986). Regeneration of protoplasts of *Bacillus subtilis* 168 and closely related strains. *J Gen Microbiol* **132**, 251–255.
- Bron, S. (1990). Plasmids. In *Molecular Biological Methods for Bacillus*, pp. 75–174. Edited by C. R. Harwood & S. M. Cutting. Chichester: John Wiley.
- Chang, S. & Cohen, S. N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol & Gen Genet* **168**, 111–115.
- Fodor, K., Hadlaczký, G. & Alföldi, L. (1975). Reversion of *Bacillus megaterium* protoplasts to the bacillary form. *J Bacteriol* **121**, 390–391.
- Gabor, M. H. & Hotchkiss, R. D. (1979). Parameters governing bacterial regeneration and genetic recombination after fusion of *Bacillus subtilis* protoplasts. *J Bacteriol* **137**, 1346–1353.
- Grosch, J. C. & Wollweber, K. L. (1982). Transformation of *Bacillus licheniformis* and *Bacillus amyloliquefaciens* protoplasts by plasmid DNA. In *Genetic Exchange*, pp. 97–105. Edited by U. N. Streips, S. H. Goodgal, W. R. Guild & G. A. Wilson. New York: Marcel Dekker.
- Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *TIBTECH* **10**, 247–256.
- Jensen, K. K. & Hulett, F. M. (1989). Protoplast transformation of *Bacillus licheniformis* MC14. *J Gen Microbiol* **135**, 2283–2287.
- Kieser, T. (1984). Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- Kleinkauf, H. & von Döhren, H. (1987). Biosynthesis of peptide antibiotics. *Annu Rev Microbiol* **41**, 259–289.
- Primrose, S. B. & Ehrlich, S. D. (1981). Isolation of plasmid deletion mutants and study of their instability. *Plasmid* **6**, 193–201.
- Sik, T., Horváth, J. & Chatterjee, S. (1980). Generalized transduction in *Rhizobium meliloti*. *Mol & Gen Genet* **178**, 511–516.
- Snoke, J. E. & Cornell, N. (1965). Protoplast lysis and inhibition of growth of *Bacillus licheniformis* by bacitracin. *J Bacteriol* **89**, 415–420.
- Sueoka, N., Hoshino, T., McKenzie, T. & Sonis, D. (1986). Membrane binding and regulation of replication initiation of pUB110 in *Bacillus subtilis*. In *Bacillus Molecular Genetics and Biotechnology Applications*, pp. 47–54. Edited by A. T. Ganesan & J. A. Hoch. Orlando: Academic Press.
- Trieu-Cuot, P., Carlier, C., Martin, P. & Courvalin, P. (1987). Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. *FEMS Microbiol Lett* **48**, 289–294.

Wati, M. R., Priest, F. G. & Mitchell, W. J. (1990). Mutagenesis using Tn917 in *Bacillus licheniformis*. *FEMS Microbiol Lett* **71**, 211–214.

Youngman, P. (1990). Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species. In *Molecular Biological Methods for Bacillus*, pp. 221–266. Edited by C. R. Harwood & S. M. Cutting. Chichester: John Wiley.

Zhang, M., Nakai, H. & Imanaka, T. (1988). Useful host–vector systems in *Bacillus stearothermophilus*. *Appl Environ Microbiol* **54**, 3162–3164.

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