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High-Frequency Transformation of *Brevibacterium lactofermentum* Protoplasts by Plasmid DNA

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An efficient polyethylene glycol-assisted method for transformation of *Brevibacterium lactofermentum* protoplasts that uses plasmid vectors has been developed. Two small plasmids, pUL330 (5.2 kilobases) and pUL340 (5.8 kilobases), both containing the kanamycin resistance gene from transposon Tn*5* and the replication origin of the natural plasmid pBL1 of *B. lactofermentum*, were selected as vectors. Supercoiled forms of the plasmids yielded a 100-fold higher transformation frequency than did linear forms. The optimal transformation frequency was achieved with 10 ng of DNA in 1 ml of transformation buffer. Higher concentrations of plasmid DNA resulted in a decrease in transformation frequency per microgram of DNA. Optimal transformation was obtained with 25 to 35% polyethylene glycol 6000. Under optimal conditions, 10^6 transformants per μg of DNA were obtained.

Nonpathogenic soil corynebacteria (e.g., *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*) are widely used for the industrial production of amino acids (13). Isolation of endogenous plasmids and construction of hybrid derivatives carrying antibiotic resistance markers in *B. lactofermentum* and *Corynebacterium callunae* has been reported previously (9, 10). Preparation of *B. lactofermentum* protoplasts has been reported by Kaneko and Sakaguchi (7). In a previous publication, we described a new regeneration medium (RS) for *Brevibacterium* protoplasts that allowed rapid protoplast regeneration and a preliminary polyethylene glycol (PEG)-assisted transformation system (10).

Because of the industrial relevance of amino acid-producing corynebacteria, it was important to develop an efficient transformation system. In this paper, we describe the characteristics of vectors and host strains and the optimal parameters for transformation of corynebacteria protoplasts with plasmid DNA.

Bacterial strains and plasmid vectors. The phenotypes of the *B. lactofermentum* strains and plasmid vectors are listed in Table 1.

Conditions for formation of protoplasts and reversion of protoplasts. *B. lactofermentum* protoplasts were obtained by the method of Santamaría et al. (10). Protoplasts were regenerated in RS medium (10). Several dilutions of the protoplast suspension were spread on plates of hypertonic (RS) and hypotonic (TSA) regeneration medium and incubated at 30°C. The number of protoplasts was calculated from the difference between the numbers of colonies grown in hypertonic and hypotonic media.

Protoplast transformation. Protoplasts obtained from 25 ml of culture in MMYC medium (minimal medium supplemented with 1 g of yeast extract and 0.5 g of Casamino Acids per liter) (10) were collected by centrifugation for 5 min at 1,200 × *g* and gently suspended in 2 ml of TSMC buffer (10 mM $MgCl_2 \cdot 6H_2O$, 30 mM $CaCl_2 \cdot 2H_2O$, 500 mM sodium succinate, 50 mM Tris-HCl [pH 7.5]). After a second centrifugation, protoplasts were suspended in 0.1 ml of TSMC buffer. Plasmid DNA (30 ng) was dissolved in 10 μl of TE buffer, 0.7 ml of PEG 6000 (Sigma Chemical Co.) (25% [wt/vol] sodium in TSMC) was added, and the protoplast suspension was spread directly on regeneration medium RS. For direct selection of antibiotic-resistant transformants, the regeneration plates were incubated for 8 to 20 h at 30°C to allow expression of the resistance gene and then overlaid with 3 ml of nutrient broth (Difco Laboratories) containing 0.4% (wt/vol) agarose and kanamycin at a final concentration of 50 μg/ml.

DNA isolation and manipulation. Plasmid isolation from *B. lactofermentum*, agarose gel electrophoresis, and determinations of DNA fragment sizes were carried as described previously (10). Restriction endonucleases were obtained from Biolabs.

Development of plasmid vectors and host systems for transformation. Four plasmid vectors (pUL61, pUL62, pUL330, and pUL340) and four *B. lactofermentum* strains (BLO, BLOB, BLR31, and BL1035) were studied to choose the most efficient plasmid and the best host strain for transfor-

TABLE 1. Microbial strains and plasmids

Strain or plasmid	Genetic markers or phenotype[a]	Plasmid pBl1 presence	Origin or reference
B. lactofermentum			
BLO	*ser* Aec^r yellow	+	ATCC 21798
BLOB	Aec^r white	+	Derived from BLO
BL1035	Aec^r *thr* white	+	Derived from BLOB
BLR31	Mly^r Aec^r white	−	Derived from BLO
Plasmid			
pUL61	Kim^r Thio^r Ap^r		10
pUL62	Km^r Ap^r		10
pUL330	Km^r		This work
pUL340	Km^r		This work

[a] Abbreviations: Aec, *S*-aminoethylcysteine; Mly, methyllysine; Thio, the antibiotic thiostrepton (kindly provided by S. J. Lucania, E. R. Squibb & Sons, Princeton, N.J.); Km, kanamycin; Ap, ampicillin.

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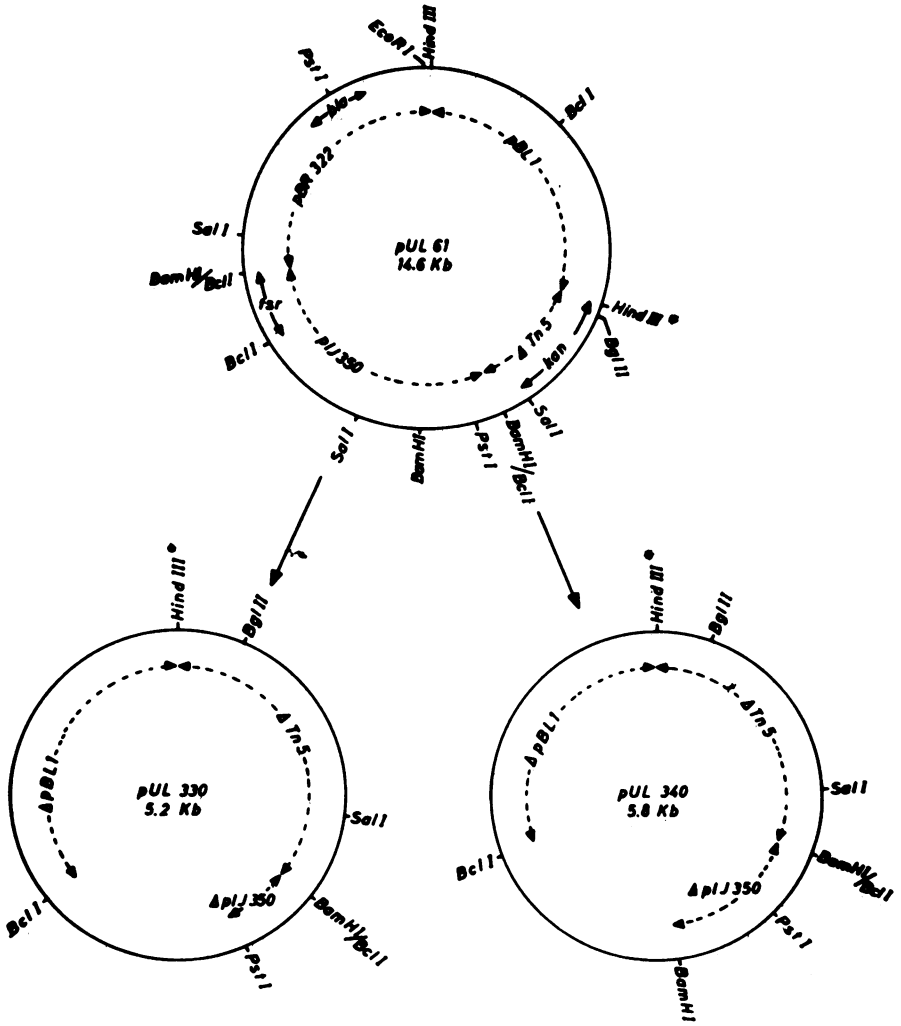

FIG. 1. Restriction maps of pUL61, pUL330, and pUL340. pUL330 and pUL340 arose as natural deletion plasmids from pUL61 when it was transformed into *B. lactofermentum*. The asterisks indicate the *Hin*dIII restriction site (of the two existing in pUL61) that was conserved in the deletion process.

mation. *B. lactofermentum* strains BLOB, BL1035, and BLO contain the resident plasmid pBL1, whereas *B. lactofermentum* BLR31 does not. Strain BLR31 was obtained by nitrosoguanidine treatment of *B. lactofermentum* BLO (Table 1). *B. lactofermentum* BL1035 is a threonine auxotroph obtained by nitrosoguanidine mutation of a prototrophic white mutant, strain BLOB (lacking the characteristic yellow pigment of *B. lactofermentum*). The four strains showed similar frequencies of protoplast formation and regeneration (46 to 61%).

Plasmids pUL330 (5.20 kilobases [kb]) and pUL340 (5.85 kb) carrying the kanamycin resistance marker of transposon Tn*5* were chosen from a number of small-sized derivatives of plasmid pUL61. They were isolated as natural deletion mutants of pUL61 when it was introduced by transformation into *B. lactofermentum* BLO and selection was made for kanamycin resistance. These plasmids had lost all of the pBR322 moiety of pUL61 and part of pBL1 and were very stable in *B. lactofermentum*. The frequency of transformation by these small plasmids was in the range 10^4 to 10^6 transformants per μg of DNA depending upon the experimental conditions, whereas for pUL61 and pUL62 the transformation frequency was 10^3 to 10^4 transformants per μg of DNA. With pUL330, the highest transformation frequency was obtained with BLR31 and BL1035 as host strains (8×10^5 to 1.1×10^6 in different experiments) as compared with BLO (1.5×10^4) and BLOB (2.5×10^4).

Effect of time of lysozyme treatment. *B. lactofermentum* BLR31 protoplasts were prepared as described previously (10), but portions of the protoplasts were kept in lysozyme for different times up to 24 h. The optimal transformation frequency (ca. 5×10^5 transformants per μg of DNA) was obtained with pUL330 DNA after 6 h of lysozyme treatment. However, the transformation frequency at time zero was 3×10^3 transformants per μg of DNA (the cells were actually in contact with lysozyme for 3 or 4 min during washing). At this time the cells were already osmotically sensitive but were not yet converted to true protoplasts.

Transformation frequencies with increased plasmid DNA concentrations and numbers of protoplasts. The same number of *B. lactofermentum* BLR31 protoplasts (3×10^8) was transformed with increasing concentrations of pUL330 DNA. The number of transformants obtained increased with increasing concentrations of plasmid DNA, but the frequency

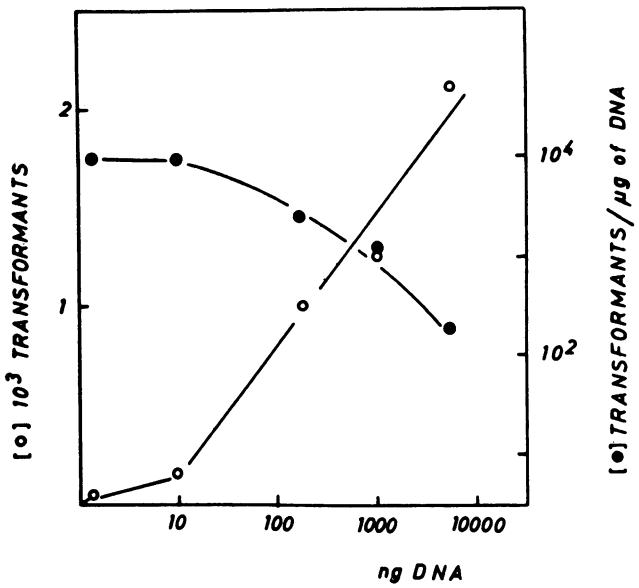

FIG. 2. Effect of increased amounts of pUL330 DNA on transformation frequency. Each transformation was carried out by using 3×10^8 protoplasts of *B. lactofermentum* BLR31.

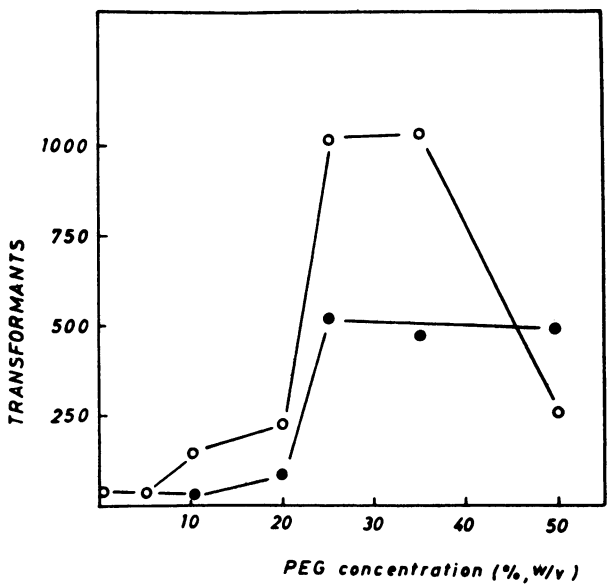

FIG. 4. Effect of the concentration of PEG 1000 (●) or PEG 6000 (○) on the transformation of *B. lactofermentum* BLR31 protoplasts; 30 ng of pUL330 and 10^8 protoplasts were used. Results are expressed as the number of transformants obtained per milliliter of transformation mixture.

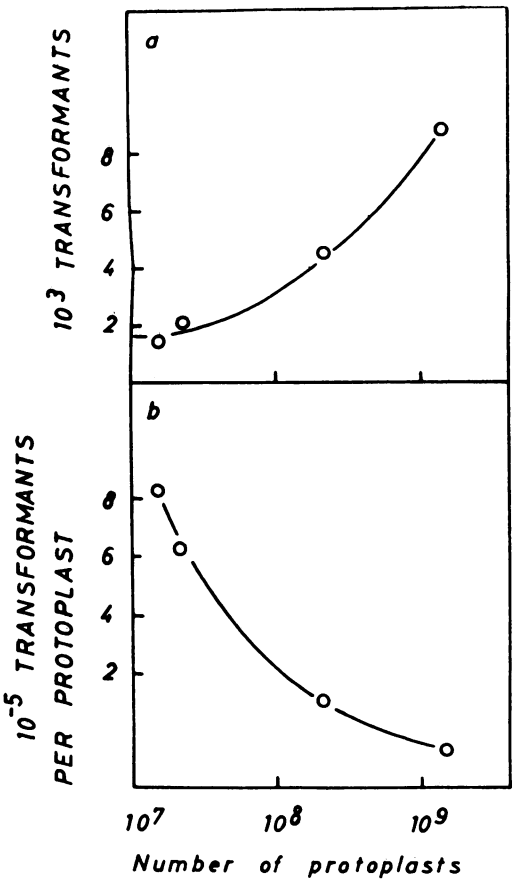

FIG. 3. Effect of the number of *B. lactofermentum* BLR31 protoplasts on their transformation by plasmid pUL330. (a) Number of transformants for different numbers of protoplasts. (b) Transformation frequency per protoplast in the transformation mixture.

of transformation was linear only below 10 ng of DNA. No saturation of the protoplast population with DNA was reached in the ranges used in these experiments (Fig. 2).

The total number of transformants increased when the number of protoplasts increased (Fig. 3a), but the efficiency of transformation per protoplast decreased (Fig. 3b).

Transformation by covalently closed circular and linear forms of plasmids. Linear forms of pUL340 were prepared by digestion with *Bam*HI and *Hin*dIII and isolated from low-melting-point agarose gels. The number of transform-

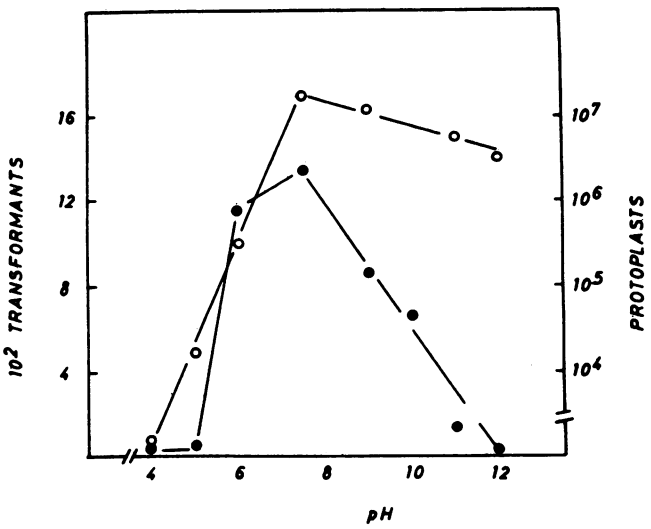

FIG. 5. Effect of the pH of the transformation buffer on the number of transformants recovered (●). The viability of protoplasts at different pH values (○) is also shown. The number of transformants is given as the number obtained per milliliter of transformation mixture.

ants obtained per microgram of DNA was 4×10^3 with the *Bam*HI-linearized plasmid and 6×10^3 with the *Hin*dIII-treated plasmid as compared with 5×10^5 transformants per μg of supercoiled DNA in the same experiment. Transformation with plasmid after ligation of the linear form yielded 3×10^4 transformants per μg of DNA.

Effect of PEG, Ca^{2+}, and Mg^{2+} concentrations. The effect of the concentration of PEG 6000 or PEG 1000 (Sigma) on the frequency of transformation of *B. lactofermentum* BLR31 protoplasts by pUL330 is shown in Fig. 4. Optimal transformation was obtained with 25 to 35% PEG 6000. PEG 1000 was less efficient than PEG 6000 except at very high concentration.

To study the effects of Ca^{2+} and Mg^{2+} on transformation, protoplast suspensions were washed with TSMC buffers containing different Ca^{2+} and Mg^{2+} concentrations and suspended in 0.1 ml of the corresponding buffer with pUL330 DNA (100 ng) and PEG 6000 (25% [wt/vol]). Concentrations of Ca^{2+} and Mg^{2+} in the range of 0 to 100 mM did not affect significantly the viability of the protoplasts or the transformation frequency, suggesting that Ca^{2+} and Mg^{2+} were not required for transformation.

Effect of transformation buffer TSMC pH. Protoplast suspensions were washed with TSMC at different pH values (4 to 12) before final suspension in 0.1 ml of TSMC at the corresponding pH containing plasmid DNA (100 ng) and PEG 6000 (25%). The results (Fig. 5) showed that both protoplast regeneration and transformation were optimal at pH 7.5.

Effect of temperature and time required for completion of transformation. Protoplast suspensions were incubated at different temperatures (from 4 to 37°C), and transformed separately with DNA and PEG at the same temperature. The results indicated that the temperature affected the efficiency of transformation only slightly.

The time required for irreversible binding of pUL330 DNA to *B. lactofermentum* BLR31 protoplasts before transformation was determined by sudden dilution of the DNA in the transformation mixture with TSMC at various times. Effective binding of the DNA was completed in 30 s, and incubation with DNA for longer periods (up to 30 min) gave no increase in the number of transformants.

Analysis of the efficiency of the transformation system. An efficient transformation system was developed in corynebacteria, as shown by the good expression of the kanamycin resistance gene from transposon Tn*5* in vector pUL61, pUL62, pUL330, and pUL340 derived from the *B. lactofermentum* endogenous plasmid pBL1.

Transformation of *B. lactofermentum* protoplasts was achieved reproducibly in the presence of either PEG 6000 or PEG 1000. Optimal transformation was obtained at 25% PEG, a concentration similar to that used with *Streptomyces* spp. (20%) for transformation or transfection of protoplasts (1, 11).

Two small plasmids, pUL330 and pUL340, were derived from shuttle vector pUL61 by deletion of all of the pBR322 moiety and part of the endogenous pBL1 plasmid of *B. lactofermentum*, with retention of the replication origin of pBL1. The reduction in size (from 14.65 kb in pUL61 to 5.20 and 5.85 kb in pUL330 and pUL340, respectively) resulted in a 10-fold increase in the frequency of transformation, apparently due to the removal of unnecessary and possibly deleterious pBR322 sequences.

The linear form of plasmid pUL340 (with *Hin*dIII- or *Bam*HI-generated cohesive ends) showed a 100-fold lower transformation efficiency than the supercoiled form. Similar results have been found in *Streptomyces* spp. (1) and *Bacillus subtilis* (3).

No restriction-deficient host strains of corynebacteria are known. However, mutant strains BLR31 and BL1035 differ considerably from strain BLO in their transformation efficiencies (10^6 versus 10^4 transformants per μg of DNA) obtained with the same plasmid (pUL330) under identical conditions. The presence of the endogenous plasmid pBL1 in the recipient strains appears not to affect their transforming ability. *B. lactofermentum* BLR31 did not harbor this plasmid but strain BL1035 contained it, and it was also present in strains BLO and BLOB, which showed lower transformation frequencies. Thus, homology between the vector and resident plasmid DNA in the recipient strain does not seem to be required for transformation (2, 6, 12). By using this transformation system, plasmid vectors pUL330, pUL340, and pUL61 were also introduced into *C. glutamicum* and *C. callunae*. In this regard, corynebacteria resemble *Streptomyces* strains, in which plasmid vectors can be established and replicate in a number of independently isolated strains (4, 8).

The number of transformants obtained per microgram of DNA was high with up to 10 ng of DNA and then decreased linearly (Fig. 2). With 10^8 protoplasts and 10 ng of DNA (equivalent to 17.2×10^8 plasmid molecules), the transformation frequency per plasmid DNA molecule ranged from 5.6×10^{-6} to 5.6×10^{-7}, similar to transfection frequencies reported for competent cells of *B. subtilis* (12) and *Streptomyces* protoplasts (11). This low frequency of transformation per plasmid molecule may be due either to nuclease activity in the host cells or to an ability of only a minor part of the plasmid DNA population to enter the protoplast and replicate in a stable form (5).

The number of transformants obtained seems to be adequate for shotgun cloning experiments, even if a 10- or 100-fold reduction in frequency occurs when the vectors are cleaved and ligated with exogenous DNA.

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