Characterization of an Endogenous Plasmid and Development of Cloning Vectors and a Transformation System in *Brevibacterium lactofermentum*

By RAMÓN SANTAMARÍA, JOSÉ A. GIL, JUAN M. MESAS
AND JUAN F. MARTÍN*

Departamento de Microbiología, Facultad de Biología, Universidad de León, León, Spain

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A cryptic plasmid, pBL1 of 4.3 kb, has been found in lysine-producing *Brevibacterium lactofermentum* strains BL0, BL70, BL74 and BL77. pBL1 had single restriction sites for *BalI*, *BclI*, *HaeII*, *HindIII* and *HpaI*. It had four sites for *AvaI*, seven for *HaeIII*, eight for *MboI* and a very large number for *AluI*, but no sites were found for *PstI*, *EcoRI* or *BamHI*. The estimated copy number was 30. Three different pBL1–pBR322 hybrids named pUL1, pUL10 and pUL20 were constructed. Transposon Tn5 was inserted by transposition into either the pBR322 or the pBL1 components of plasmid pUL1, pUL10 and pUL20. A shuttle vector able to replicate in *Escherichia coli*, *Streptomyces lividans* and *B. lactofermentum* was constructed by cloning pBL1 into the plasmid pIJ860, a bifunctional *E. coli*–*S. lividans* vector carrying the *tsr*, *bla* and *kan* genes. A polyethylene glycol-assisted transformation system for *B. lactofermentum* protoplasts was developed. Transformation frequencies of 10^2 transformants ($\mu\text{g DNA}$)⁻¹ were obtained. The *kan* resistance gene from Tn5 was expressed very efficiently in *B. lactofermentum* (up to 200 $\mu\text{g ml}^{-1}$). A smaller plasmid, pUL62, was constructed in which the *tsr* (thiostrepton resistance) gene of pUL61 was deleted.

INTRODUCTION

The corynebacteria are Gram-positive bacteria widely distributed in nature. Many species inhabit the soil and a number cause disease in plants and animals (Vidaver, 1982). Some non-pathogenic species belonging to the genera *Corynebacterium* and *Brevibacterium* are currently used in the industrial production of amino acids (Yamada *et al.*, 1972), nucleotides (Ogata *et al.*, 1976) and other useful metabolites.

Preliminary evidence has been reported on the presence of extrachromosomal genetic elements in pathogenic and non-pathogenic corynebacteria. A 37 MDal plasmid has been described in *Brevibacterium lactofermentum* 2256 (Kaneko *et al.*, 1979) but without physical or genetic maps. In *Corynebacterium diphtheriae* lysogenization by phage β causes the synthesis and secretion of the extremely potent heat-labile diphtheria toxin. A 78 MDal plasmid occurs in virulent strains of the plant pathogen *Corynebacterium fascians* (Lawson *et al.*, 1982) and 30 and 41 MDal plasmids have been reported in the human pathogen *Corynebacterium xerosis* (Kono *et al.*, 1983). Pathogenicity of *C. fascians*, *C. xerosis* and *C. diphtheriae* and the large size of the *B. lactofermentum* plasmid prevent their use for development as vectors for cloning in industrial corynebacteria. The development of such vectors would be extremely useful for the study of genetic expression in these micro-organisms and for amplification of amino acid biosynthesis in corynebacteria which have the important characteristic of being able to excrete large amounts of amino acids. Recently a low molecular weight plasmid (pCC1) has been isolated in this laboratory from a non-pathogenic strain of *Corynebacterium callunae* (Sandoval *et al.*, 1984). The aim of this work was the development of cloning vehicles and a transformation system in amino acid-producing strains of *B. lactofermentum*.

Abbreviations: LA, Luria agar; LB, Luria broth.

METHODS

Bacterial strains. The strains of bacteria used and their phenotypes are listed in Table 1. Plasmids are listed in Table 2.

Culture conditions. Culture media for *Brevibacterium* and *Corynebacterium* strains were trypticase soy agar (TSA) (Difco), Luria agar (LA) (Miller, 1972), and minimal medium (MM) Kaneko & Sakaguchi 1979). Protoplasts were regenerated in modified R2-medium (Okanishi *et al.*, 1974) containing 0.25 M-sucrose, 0.25 M-sodium succinate and 50 µg ml⁻¹ diaminopimelic acid (hereafter referred to as RS medium).

Escherichia coli strains were grown on Luria broth (LB) or LA. *Streptomyces lividans* strains were grown in YEME plus 34% (w/v) sucrose, or R2YE (Chater *et al.*, 1982).

Plasmid isolation. Several *B. lactofermentum* strains were tested for the presence of plasmid DNA by a modification of the procedure of Birnboim & Doly (1979). Cells were grown for 18 h at 30 °C in a rotary shaker at 250 r.p.m. in 100 ml trypticase soy broth (TSB; Difco), collected by centrifugation, resuspended in 20 ml lysis buffer (25 mM-Tris, 10 mM-EDTA, 10.3%, w/v, sucrose, pH 8.0 containing 1 mg ml⁻¹ lysozyme) and incubated at 37 °C for 3 h. Cell lysis was achieved by adding 40 ml of hot (55 °C) 1% (w/v) SDS/0.1 M-NaOH followed by incubation at room temperature for 5 min.

After lysis, 30 ml of 3 M-sodium acetate, pH 4.6 cooled to 4 °C was added, the mixture homogenized and then incubated at 4 °C for 1 h. The mixture was centrifuged at 15000 g for 20 min in a refrigerated centrifuge and the DNA precipitated from the supernate by addition of an equal volume of 2-propanol. The precipitate was collected by centrifugation at 2300 g for 5 min and suspended in 8 ml TE (10 mM-Tris/HCl, 1 mM-EDTA, pH 8) buffer. Caesium chloride (8.28 g) and ethidium bromide (0.3 ml of a 10 mg ml⁻¹ solution) were added and the solution was centrifuged at 110000 g for 48 h at 15 °C in a Beckman LB-70 ultracentrifuge. Plasmid DNA was detected by fluorescence in UV light at 320 nm and collected with a sterile syringe. The ethidium bromide was removed by filtration through a Dowex AG 500W-X4 ion exchange column and the DNA precipitated, and re-precipitated twice, with 2.2 vols ethanol.

Rapid screening of plasmid DNA. An alternative method was used for quick screening of plasmid DNA. *Brevibacterium* cells from 1 ml cultures or from agar plates (patches) were suspended in 200 µl lysis buffer, incubated for 3 h at 37 °C, and lysed by addition of 400 µl 1% SDS in 0.1 M-NaOH. After 5 min at room temperature, 300 µl 3 M-sodium acetate, pH 4.8, was added and the lysate was kept in ice for 15 min and centrifuged in an Eppendorf centrifuge for 5 min. Plasmid DNA was precipitated from the supernate by adding 1 vol. 2-propanol. The pellet was rinsed with ethanol, dried and resuspended in 100 µl TE buffer. RNAase (10 µl; Sigma; 1 mg ml⁻¹ in 10 mM-citrate buffer, pH 4.0, pre-boiled for 10 min) was then added. After incubation at 37 °C for 30 min the mixture was extracted with 1 vol. phenol/chloroform (500 g phenol, 200 ml water, 500 ml chloroform and 0.5 g hydroxy-quinoline) and the DNA in the upper (aqueous) phase was precipitated with 2-propanol, rinsed with ethanol, dried and resuspended in 100 µl TE buffer.

Plasmid DNA was isolated from *E. coli* by a modification of the procedure of Birnboim and Doly (Gil & Hopwood, 1983).

Phenotypic markers of cryptic plasmids. Plasmids found in *Brevibacterium* were tested for their ability to confer (a) resistance to antibiotics, (b) resistance to mercury and (c) fermentation of sugars. Sensitivity of strains to 24 different antibiotics was tested in Mueller-Hinton (Difco) plates using paper discs with antibiotic concentrations ranging from 1 to 30 µg per disc. Resistance to mercury was tested using HgCl₂ (5 to 20 µg ml⁻¹). Tests were also made to study utilization of different sugars. Plasmid-free strains were obtained by supplementing the culture medium with acridine orange (2.5 to 10 µg ml⁻¹).

DNA manipulation. Restriction endonucleases were obtained from Biolabs and BRL. Digestions were carried out using the buffers and the concentrations suggested by the manufacturers and incubating at 37 °C for 3 h, except for *BclI* which was incubated at 55 °C for 1 h. Reactions were terminated by heating to 70 °C for 10 min, except for *BclI* where diethylpyrocarbonate was used to stop the reaction. T4 DNA ligase was obtained from Biolabs and used as described by Hepburn & Hindley (1979).

Agarose gel electrophoresis was performed with horizontal 20 × 20 cm slab gels in either TBE (90 mM-Tris/borate, 2 mM-EDTA) or in TAE buffers (40 mM-Tris, 5 mM-sodium acetate, 1 mM-EDTA) adjusted to pH 7.8 with glacial acetic acid. Agarose concentrations of 0.7, 1.0 and 1.6% were used, depending on the fragment sizes to be resolved.

After electrophoresis, the gels were stained in ethidium bromide (1 µg ml⁻¹) for 30 min and the DNA bands were visualized under UV light (302 nm; C-63 Mineralight; UVP International). Fragment sizes were determined by the method of Southern (1979) using phage lambda DNA digested with *HindIII* as size standards (Daniels *et al.*, 1980).

Transposition with Tn5. Tn5 was introduced into hybrid pBR322-*Brevibacterium* plasmids by transforming the plasmids into *E. coli* strain UNF510 harbouring Tn5 in the chromosome.

Transformants were selected in plates of LA containing 200 µg ampicillin ml⁻¹. Eight randomly selected ampicillin-resistant colonies were grown in liquid LB-medium supplemented with 200 µg ampicillin ml⁻¹ and

Table 1. *Microbial strains*

Micro-organisms	Genetic markers*	Origin†
<i>B. lactofermentum</i> BL0	<i>ser</i> Aec ^r	ATCC 21798
<i>B. lactofermentum</i> BL0/6	<i>ser</i> Aec ^r	Derived from BL0 by protoplast regeneration
<i>B. lactofermentum</i> BL70	Wild-type	CECT 70
<i>B. lactofermentum</i> BL74	Wild-type	CECT 74
<i>B. lactofermentum</i> BL77	Wild-type	CECT 77
<i>B. lactofermentum</i> BL18	<i>ser arg</i> Aec ^r	Derived from BL0 (NTG mutagenesis)‡
<i>C. glutamicum</i> Cg 210	<i>hom leu</i> Aec ^r	This laboratory
<i>C. glutamicum</i> Cg 13032	Wild-type	ATCC 13032
<i>E. coli</i> HB101		Boyer & Roullard-Dussoix (1969)
<i>E. coli</i> V517		Macrina <i>et al.</i> (1978)
<i>E. coli</i> UNF510	Harbours Tn5	Merrick <i>et al.</i> (1978)
<i>S. lividans</i> 66 (JI1326)	Wild-type	Lomovskaya <i>et al.</i> (1972)

* Aec^r, aminoethyl-L-cysteine-resistant.

† CECT, Spanish type culture collection.

‡ NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.Table 2. *Plasmids used*

Plasmids	Phenotypic characteristics	Source
pBL1	Cryptic	This work
pBR322	Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pIJ860	Ap ^r Km ^r Thio ^r	J. A. Gil and D. A. Hopwood
pUL1	Ap ^r	This work
pUL10	Ap ^r Tc ^r	This work
pUL20	Ap ^r	This work
pUL1::Tn5 (pUL51, pUL52)	Ap ^r Km ^r	This work
pUL10::Tn5 (pUL53, pUL54)	Ap ^r Tc ^r Km ^r	This work
pUL20::Tn5 (pUL55, pUL56)	Ap ^r Km ^r	This work
pUL61	Km ^r Thio ^r Ap ^r	This work
pUL62	Km ^r Ap ^r	This work

15 µg kanamycin ml⁻¹. After incubation at 37 °C for 8 h they were plated in LB medium with 200 µg of ampicillin ml⁻¹ and 200 µg of kanamycin ml⁻¹. Colonies that were able to grow were pooled and the DNA was used to transform *E. coli* HB101. Selection for transformants carrying the transposon was made on LB plates containing 200 µg of ampicillin ml⁻¹ and 200 µg of kanamycin ml⁻¹. The transformants were lysed separately and the insertion of Tn5 as well as its orientation was established by digestion with restriction endonucleases.

Protoplasting conditions. Protoplasts of *B. lactofermentum* were prepared by a modification of the method of Kaneko & Sakaguchi (1979) as follows. *Brevibacterium lactofermentum* cultures were grown overnight in an orbital shaker at 30 °C in minimal medium supplemented with 0.1% (w/v) yeast extract and 0.05% (w/v) Casamino acids (MMYC). Overnight culture (2 ml) was used to inoculate a 500 ml flask containing 100 ml MMYC and the incubation was followed until the OD₆₀₀ was approximately 0.6 (10⁸ cells ml⁻¹). Then 0.3 units of penicillin G ml⁻¹ was added and incubation continued for 3 h before cells were harvested by centrifugation and resuspended in 10 ml of protoplasting medium (MMYC diluted twice and supplemented with 0.41 M-sucrose, 0.01 M-MgSO₄ and 300 µg lysozyme ml⁻¹). During incubation (4 h) at 35 °C, protoplast formation was monitored by microscopy or by plating in hypertonic and hypotonic medium.

Transformation of *B. lactofermentum* protoplasts. Protoplasts obtained from 25 ml of culture were collected by centrifugation for 5 min at 1200 g and gently resuspended in 2 ml TSMC buffer (MgCl₂·6H₂O, 10 mM; CaCl₂·2H₂O, 30 mM; sodium succinate, 500 mM; Tris, 50 mM) pH 7.5. After centrifugation, protoplasts were again resuspended in 0.1 ml TSMC buffer. DNA (1 µg) in TSMC buffer and 0.7 ml polyethylene glycol 6000 (25%, w/v, solution in TSMC) were added. After incubation for 3 min at room temperature, samples of protoplasts were plated in RS regeneration medium and incubated at 30 °C for 4 d. After 24 h of incubation, transformants were directly selected by overlaying the RS plates with 3 ml nutrient broth (Difco) containing 0.4% (w/v) agarose and kanamycin at a final concentration of 50 µg ml⁻¹.

Transformation in E. coli and S. lividans. Transformation of *E. coli* HB101 cells was done according to Dagert & Erlich (1979). Transformants were selected on LA supplemented with ampicillin (50 to 200 µg ml⁻¹) and kanamycin (15 to 200 µg ml⁻¹) depending on the experiments. Preparation of *S. lividans* protoplasts, transformation and regeneration were done according to Chater *et al.* (1982).

RESULTS

Plasmids of Brevibacterium and Corynebacterium. Agarose gel electrophoresis of cleared lysates of different strains of *B. lactofermentum* showed the presence of a low molecular weight plasmid in strains BL0, BL70, BL74 and BL77 but no plasmid was detected after repeated tests in strain BL18. The plasmid detected in BL0 was named pBL1. It had a size of 4.3 kb as calculated by comparing the mobility of the covalently closed circular form of pBL1 with that of the eight plasmids (covalently closed circular forms) of *E. coli* V517 (Macrina *et al.*, 1978). No plasmids were found in different experiments using *Corynebacterium glutamicum* Cg 210 and *C. glutamicum* ATCC 13032, other amino acid-producing corynebacteria.

Lack of phenotypic characters and plasmid removal. Strains carrying pBL1 plasmids were compared to strain BL18 (a strain obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutation in which no plasmid was detected) in regard to antibiotic resistance, sugar fermentation and heavy metal resistance. Plasmid-carrying and plasmid-free strains did not differ in these phenotypic characteristics.

Since pBL1 might be integrated in strain BL18, 'cured' strains were isolated by growing *B. lactofermentum* BL0 in acridine orange (10 µg ml⁻¹) (see Methods). Cell viability of cultures grown in presence of the dye decreased by four orders of magnitude. Of colonies obtained after treatment with acridine orange 25% had lost plasmid pBL1. However the phenotypic characteristics of these strains were identical to those of plasmid-carrying BL0 strains.

Restriction map of pBL1. Plasmid pBL1 purified by CsCl-ethidium bromide ultracentrifugation was digested with restriction endonucleases. pBL1 had single restriction sites for the endonucleases *Ba*II, *Bc*II, *Hae*II, *Hind*III and *Hpa*I. It had four sites for *Ava*I, seven for *Hae*III, eight for *Mbo*I and a very large number for *Alu*I, whereas no sites were found for *Pst*I, *Eco*RI or *Bam*HI. Double digestions were carried out with combinations of two different endonucleases, both having single, or single and multiple sites. The restriction map of plasmid pBL1 is shown in Fig. 1. The size was 4.3 kb, obtained by comparing the mobility of the DNA fragments generated by different restriction enzymes with that of fragments of phage λ DNA digested with *Hind*III (Southern, 1979).

Copy number. The number of copies of pBL1 was estimated by comparing the amount of plasmid in the covalently closed circular form with the amount of chromosomal DNA in total DNA preparations obtained according to Chater *et al.* (1982). Assuming that the size of chromosomal DNA of corynebacteria is 6000 kb (Sandoval *et al.*, 1984), the estimated copy number is about 30 per cell.

Construction of pBL1-pBR322 hybrids. With the aim of introducing antibiotic-resistance markers into the cryptic plasmid pBL1 and at the same time developing putative bifunctional vectors able to work in *E. coli* and *B. lactofermentum*, three different types of hybrid plasmids were constructed between pBR322 and pBL1. In order to construct these hybrid plasmids, pBR322 and pBL1 were digested with restriction enzymes that cut both plasmids only once (Fig. 2). pBR322 (1 µg) was digested with *Hind*III, *Ba*II or *Bam*HI and ligated with 5 µg pBL1 digested with *Hind*III, *Ba*II or *Bc*II, respectively.

The three ligation mixtures were transformed separately into *E. coli* HB101 and selection was made on ampicillin-containing plates. Several clones containing hybrid plasmids of each type were obtained, with a plasmid molecular weight of 8.6 kb, corresponding to the sum of pBR322 and pBL1.

The hybrid plasmid of pBR322 and pBL1 (*Hind*III-digested) was named pUL1. Plasmid pUL10 was obtained after digestion of pBR322 with *Ba*II and ligation to pBL1 also digested with *Ba*II. Finally plasmid pUL20 was found after *Bam*HI/*Bc*II digestion and ligation.

Fig. 1. Simplified restriction map of pBL1. Single restriction sites for *Ba*II, *Bc*II, *Hae*II, *Hind*III and *Hpa*I and four sites for *Ava*I are shown. The seven sites for *Hae*III, eight for *Mbo*I and >10 *Alu*I sites are not shown. No sites were found for *Pst*I, *Eco*RI or *Bam*HI.

Fig. 2. Construction of hybrid plasmids pUL1, pUL10 and pUL20. Three different hybrid plasmids were obtained by digestion of pBR322 with *Hind*III, *Ba*II and *Bam*HI, and of pBL1 with *Hind*III, *Ba*II and *Bc*II, respectively. The orientations of the *bla* (ampicillin resistance) and *tet* (tetracycline resistance) genes are indicated by arrows.

Fig. 3. Insertion of Tn5 into pUL1 by transposition. Tn5 was inserted into pBL1 (right) or into pBR322 (left) in both orientations. *kan*, kanamycin resistance; *bla*, ampicillin resistance. ■—■ Indicates Tn5.

Insertion of Tn5. Tn5 carries a gene coding for resistance to the aminoglycoside antibiotics neomycin and kanamycin (Berg *et al.*, 1975; Beck *et al.*, 1982). The hybrid plasmids pUL1, pUL10 and pUL20 were introduced by transformation into *E. coli* UNF 510 and selection was made for Tn5 insertions into the hybrid plasmids according to the procedure described in Methods. Several kinds of Tn5 insertions were found including: (1) those in which Tn5 was inserted into the pBR322 component of pUL1 (named pUL51), pUL10 (named pUL53), and pUL20 (named pUL55) and (2) those with Tn5 inserted into the pBL1 fragment of the hybrid plasmids pUL1 (named pUL52), pUL10 (named pUL54) and pUL20 (named pUL56) (Fig. 3). In both cases, Tn5 was inserted in both orientations, as established by restriction analysis. However no differences were found in expression of the kanamycin-resistance marker in *E. coli*.

Development of a transformation system for *Brevibacterium* protoplasts. No transformation system for protoplasts of corynebacteria was known prior to this work. Based on procedures described for *Streptomyces* protoplasts (Chater *et al.*, 1982; Martin & Gil, 1984), a transformation system of *Brevibacterium* protoplasts was developed as indicated in Methods using the previously constructed hybrid plasmids.

Construction of new hybrid plasmids. Hybrid plasmids pUL51 to pUL56 were transformed into *B. lactofermentum* protoplasts with selection for kanamycin resistance. *Brevibacterium lactofermentum* did not produce spontaneous Km^r mutants ($<10^{-9}$). In all the transformation experiments, Km^r clones (at up to $20 \mu\text{g}$ kanamycin ml^{-1}) were obtained, but plasmid DNA was not detected in cleared lysates of these 'putative' transformants. Total DNA of these Km^r colonies was also used to transform *E. coli* HB101 competent cells, but no Ap^r or Km^r colonies were found (see Discussion).

To avoid the natural instability of plasmids containing the whole Tn5, pBL1 was cloned into a stable bifunctional *E. coli*-*Streptomyces* vector containing the Km^r gene from Tn5. This plasmid, named pIJ860, was constructed by J. A. Gil and D. A. Hopwood (unpublished work) and its structure is represented in Fig. 4. pBL1 DNA ($3 \mu\text{g}$) digested with *Hind*III was ligated with $1 \mu\text{g}$ pIJ860 DNA also digested with *Hind*III. The ligation mixture was transformed into *E. coli* HB101 competent cells and transformants were selected on LA plates containing ampicillin ($200 \mu\text{g ml}^{-1}$) and kanamycin ($200 \mu\text{g ml}^{-1}$). Eight plasmids of 14-65 kb were found. One of these plasmids, named pUL61, was purified by CsCl-ethidium bromide ultracentrifugation and mapped by digestion with restriction enzymes. Its structure is represented in Fig. 4.

PUL61 was used to transform *B. lactofermentum* protoplasts and Km^r transformants (up to $200 \mu\text{g}$ kanamycin ml^{-1}) were isolated. The frequency of transformation was initially 10^2

Fig. 4. Construction of multifunctional plasmid pUL61. pIJ860 and pBL1 were digested with *Hind*III, ligated and transformed into *E. coli* HB101. Plasmid pUL61 transforms and replicates in *E. coli*, *B. lactofermentum* and *S. lividans*. ΔTn5 indicates a fragment of Tn5 containing the kanamycin resistance gene. *kan*, kanamycin resistance; *tsr*, thiostrepton resistance; and *bla*, ampicillin resistance.

transformants ($\mu\text{g DNA}^{-1}$) (average of six experiments). Cleared lysates of these Km^r transformants showed a clear plasmid band; DNA from this band give a restriction pattern identical to that of the original pUL61. This plasmid was used to re-transform *B. lactofermentum* and we obtained the same frequency of transformation as when plasmid DNA isolated from *E. coli* was used. Plasmid pUL61 transformed *E. coli* competent cells and *S. lividans* protoplasts, and could be re-isolated both from *E. coli* and *S. lividans* Km^r transformants. The ampicillin resistance (*bla*) encoded by pUL61 was not expressed in *B. lactofermentum*, but was expressed in *E. coli* after passing the plasmid through *B. lactofermentum*. Similarly, the thiostrepton resistance (*tsr*) encoded by pUL61 was not expressed in *B. lactofermentum*, but plasmids isolated from *Brevibacterium* did express thiostrepton resistance in *Streptomyces*.

Fig. 5. Restriction map of plasmid pUL62 derived from pUL61 by total *SalI* digestion and ligation. The two small *SalI* fragments (Fig. 4) were eliminated. pUL62 transforms and replicates in *E. coli* and *B. lactofermentum* but not in *S. lividans*.

pUL61 seems to be a good cloning vector for corynebacteria but attempts were made to reduce its size and hence improve its usefulness. Since the thiostrepton resistance (*tsr*) from *Streptomyces azureus* was not expressed in *Brevibacterium*, most of the *Streptomyces* DNA was eliminated from pUL61 by a *SalI* digestion followed by ligation and transformation into *E. coli* HB101. A new plasmid, pUL62 was obtained (Fig. 5) and transformed into *B. lactofermentum* protoplasts. The frequency of transformation in *B. lactofermentum* protoplast was also 10^2 transformants ($\mu\text{g DNA}$) $^{-1}$.

Host strains for transformation experiments. More studies are needed to establish an efficient transformation system in corynebacteria. The frequency of transformation was 10 to 100 times greater in *B. lactofermentum* BL0, BL70 and BL77 containing the pBL1 endogenous plasmid than in *B. lactofermentum* BL18 (lacking the plasmid). However, a strain BL0/6 'cured' by protoplasting, retained the high efficiency of transformation.

When strains BL0, BL70 and BL77 were transformed with pUL61 or pUL62, the original pBL1 was no longer present in the Kan^r transformants, suggesting that pBL1 is eliminated from the host strain.

DISCUSSION

A cryptic multicopy low molecular weight plasmid pBL1 was found in *B. lactofermentum*, an industrial strain used in the production of lysine. The restriction pattern of this plasmid was different from that of plasmid pCC1 of *C. callunae* (Sandoval *et al.*, 1984). pBL1 is easily lost when *B. lactofermentum* is incubated in culture media supplemented with acridine orange. These plasmid-free strains showed similar phenotypes in relation to resistance to antibiotics, resistance to mercury and fermentation of sugars. It should be noted that although no plasmid DNA was observed in lysates of these plasmid-free strains, the possibility that the plasmid had become integrated into the chromosome cannot be excluded.

The pBL1-pBR322 hybrids containing Tn5 were used to transform *B. lactofermentum* protoplasts, and kanamycin-resistant colonies were obtained, but no plasmid DNA was found in these 'putative' transformants. It is possible that the hybrid plasmids containing Tn5 were integrated into the *B. lactofermentum* chromosome, or alternatively Tn5 itself may have become

inserted into the chromosome. It was clear, however, that the kanamycin resistance encoded by Tn5 could be expressed in *B. lactofermentum*. This result was not a surprise, because the Km^r encoded by Tn5 is expressed in *E. coli*, *Streptomyces* (Gil & Hopwood, 1983), and *Saccharomyces cerevisiae* (Jimenez & Davies, 1980).

Hybrid plasmids pUL61 or pUL62 derived from pIJ860 are functional in *B. lactofermentum* and can be used as cloning vectors for corynebacteria.

Of four different resistance markers studied (*kan*, *bla*, *tet*, and *tsr*), only the kanamycin resistance encoded by Tn5 seems to be expressed in corynebacteria. Further resistance markers (chloramphenicol, viomycin, streptomycin) and other markers (tyrosinase, β -galactosidase) are being cloned into pUL61 and pUL62 in order to obtain a plasmid vector for corynebacteria similar to pBR322 for *E. coli* (Bolivar *et al.* 1977) or pIJ41 for *Streptomyces* (Thompson *et al.*, 1982).

Plasmid pUL61 and pUL62 are shuttle vectors that may be isolated from *E. coli* (where the *kan* and *bla* resistance markers are expressed) or from *Brevibacterium* where only the *kan* resistance marker is expressed. Moreover, pUL61 can be introduced into and isolated from *S. lividans* where the *tsr* and *kan* resistance markers are expressed.

Using this cloning system it should be possible to clone 'limiting steps' in amino acid production in corynebacteria in order to overproduce these commercially important products. Corynebacteria are specially suited for amino acid excretion due to the lack of efficient uptake systems for amino acids under conditions of biotin limitation in these biotin-requiring micro-organisms (Shiio, 1983).

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