

A Host–Vector System for an *Arthrobacter* Species

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An efficient host–vector system has been developed for an industrial strain of *Arthrobacter* sp. (NRRL B3728) used for glucose isomerase production. Protoplasts of *Arthrobacter* were generated by treating the cells with 0.5 mg lysozyme ml⁻¹ for 60 min in a solution containing 0.5 M-sucrose. Around 30% of the protoplasts regenerated on agar containing 0.5 M-sodium succinate as osmotic stabilizer. Three hybrid vectors, pBL2100, pCG1100 and pCG2100, were constructed by combining the *Escherichia coli* plasmid pBR322, a kanamycin-resistance gene from pNCAT4 and a cryptic plasmid from either *Brevibacterium lactofermentum* NCIB 9567 or *Corynebacterium glutamicum* NCIB 10026. These vectors transformed the protoplasts and expressed the kanamycin-resistance gene for screening. They contain a number of unique restriction sites for cloning of foreign DNA. The transformation frequency of this system was 10⁵–10⁶ transformants per µg of input plasmid and was constant up to 5 µg of DNA. The probability of a plasmid transforming a protoplast was in the range 10⁻⁵–10⁻⁶. The copy number of pBL2100 was around 5 per cell and those of pCG1100 and pCG2100 were around 33 per cell. Deletion mutants were generated from pCG2100. One of them, pCG2120, was able to transform protoplasts of strain NRRL B3728. Plasmids pBL2100 and pCG2100 were structurally stable in cells of NRRL B3728 but could not be maintained in non-selective medium. They segregated at a rate of 12.2 and 2.3% per generation respectively.

INTRODUCTION

Arthrobacter is a member of the Gram-positive coryneform bacteria that have useful properties such as production of erythromycin (Roberts *et al.*, 1985) or glucose isomerase (Reynolds, 1973), or degradation of nicotine (Brandsch *et al.*, 1986) or benzonitrile (Bandyopadhyay *et al.*, 1986). A major obstacle to manipulating these bacteria genetically has been the lack of a transformation system and suitable vectors. Heterologous complementation of *Escherichia coli* mutants may be employed, but many *Arthrobacter* genes are not expressed in *E. coli* (Dart *et al.*, 1981). Recently, transformation systems for some of the amino-acid-producing coryneform bacteria have been developed (Santamaria *et al.*, 1985; Katsumata *et al.*, 1984; Yoshihama *et al.*, 1985; Yeh *et al.*, 1986). These systems are based on polyethylene glycol (PEG)-mediated uptake of plasmid vectors by protoplasts and their subsequent regeneration on medium containing an osmotic stabilizer. A transformation system for *Arthrobacter* sp. H13A has also been developed in a similar way (Singer, 1986).

In this report, we describe the construction of hybrid vectors and the development of a transformation system for *Arthrobacter* NRRL B3728, a strain used for industrial production of glucose isomerase. These vectors may prove useful for the genetic manipulation of other coryneform bacteria, as have similar systems for introducing a cloned α-amylase gene into *Brevibacterium lactofermentum* (Smith *et al.*, 1986), and improving phenylalanine biosynthesis in *Corynebacterium glutamicum* (Ozaki *et al.*, 1985) or threonine biosynthesis in *B. lactofermentum* (Nakamori *et al.*, 1987).

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METHODS

Bacteria and plasmids. These are listed in Table 1.

Media and culture conditions. L medium (1%, w/v, Difco Bacto-tryptone; 0.5%, w/v, Difco Bacto-yeast extract; 0.5%, w/v, NaCl) was used for routine growth of bacteria. Difco Bacto-agar (1.2%, w/v) was added for plates.

E. coli HB101 was incubated at 37 °C and coryneform bacteria at 30 °C.

Preparation, regeneration and transformation of protoplasts. Protoplasts of *Arthrobacter* sp. NRRL B3728 (referred to as B3728) were generated by a method based on Chang & Cohen (1979). B3728 was cultured in 100 ml L medium at 30 °C until the OD₆₀₀ reached 0.5 ($1-2 \times 10^8$ c.f.u. ml⁻¹). The culture was split into two (40 ml each) and harvested by centrifugation at 1380 g for 10 min at 10 °C. The pellet in each tube was resuspended in 5 ml SMMLBV medium consisting of equal volumes of SMM medium (1.0 M-sucrose; 0.04 M-sodium maleate; 0.04 M-MgCl₂; pH 6.5) and LBV medium (2%, w/v, Difco Bacto-tryptone; 1%, w/v, Difco Bacto-yeast extract; 1%, w/v, NaCl). Lysozyme was added to 0.5 mg ml⁻¹ and the cells were incubated at 30 °C for 1 h. SMMLBV medium (10 ml) was then added to each tube and protoplasts were centrifuged at 4830 g for 15 min at 10 °C. The pellets in each tube were resuspended gently in 0.5 ml SMMLBV medium, pooled and incubated at 30 °C for 1-2 h. Samples (0.1 ml) were serially diluted and plated onto DM3 plates (per litre: 8 g Difco Bacto-agar, 5 g Difco Casamino acids and 5 g Difco Bacto-yeast extract; made up to 350 ml and autoclaved). The following items were added separately: 50 ml 3.5% (w/v) K₂HPO₄; 50 ml 1.5% (w/v) KH₂PO₄; 25 ml 20% (w/v) glucose; 20 ml 1 M-MgCl₂; 5 ml 2% (w/v) filter-sterilized serum albumin and 500 ml 1 M-sodium succinate (Sigma). Sodium succinate from another supplier (Fisons) did not give satisfactory results. Colonies of regenerated protoplasts appeared after incubation at 30 °C for 3-4 d.

An equal volume of SMMLBV medium was added to the plasmid DNA solution and around 10 µl of this solution containing 100 ng DNA was added to 0.1 ml protoplasts; this was followed by 0.3 ml 40% (w/v) PEG 6000 in 0.5 × SMM. The solutions were mixed by gently vortexing and the mixture left at room temperature (about 20 °C) for 2-3 min. SMMLBV medium (1 ml) was added and, after mixing, the protoplasts were centrifuged at 9980 g for 1 min at room temperature. The pellet was resuspended in 0.2 ml SMMLBV medium and incubated at 30 °C for 1-2 h. Samples (0.1 ml) were plated onto DM3 plates supplemented with 4 mg kanamycin sulphate ml⁻¹.

Susceptibility to antibiotics. Kanamycin sulphate (100 µg ml⁻¹) was used in medium for the maintenance of plasmid-bearing B3728 cells. On DM3 agar, kanamycin sulphate (4 mg ml⁻¹) was required to inhibit the regeneration of plasmid-free protoplasts.

Ampicillin (100 µg ml⁻¹) was used for the maintenance of plasmid-bearing HB101 cells. On DM3 agar, ampicillin could not inhibit the regeneration of B3728 protoplasts.

Transformation of E. coli. Cells of HB101 were made competent and transformed by plasmid DNA according to the standard protocol of Hanahan (1983).

Extraction of plasmid DNA. Plasmid DNA from HB101 was extracted by the methods described in Maniatis *et al.* (1982). Plasmid DNA from coryneform bacteria was extracted by a method adapted from Hunter (1985) for *Streptomyces*. Lysis of *C. glutamicum* NCIB 10026 was not effected by using lysozyme alone, and good lysis was obtained only after the addition of ampicillin (0.6 mg ml⁻¹) 2 h before harvesting the cells. After harvesting, the remaining steps were according to Hunter (1985). Small amounts of plasmid DNA were prepared from HB101 and B3728 by the rapid alkaline lysis method described in Maniatis *et al.* (1982).

Digestion of DNA by restriction enzymes. Restriction enzymes were purchased from Amersham or New England Biolabs. Buffer and reaction condition for specific enzymes were according to Maniatis *et al.* (1982) or as recommended by the manufacturers.

Agarose gel electrophoresis. This was done in a horizontal slab TAE gel (0.8%, w/v, agarose; 0.04 M-Tris/acetate; 0.002 M-EDTA pH 8.0; 0.5 µg ethidium bromide ml⁻¹). DNA was viewed and photographed under UV light (254 nm).

Recovery of DNA fragments from gels. DNA fragments were separated by agarose gel electrophoresis in the presence of ethidium bromide. The region containing the required DNA was located by UV light of 366 nm, removed, and the DNA was recovered by use of an electroelution tank (H. W. Erdmann Co., USA).

5' end-filling and ligation of DNA. The 5' protruding ends of the vectors and the gene for kanamycin resistance were filled in by the following procedures. To 1 µg DNA in 12 µl H₂O, 1.5 µl each of dA, dTdc, dGTPs (1 mM) were added. Then, 2 µl of 10 × repair buffer (0.5 M-Tris-HCl pH 7.4; 70 mM-MgCl₂; 10 mM-dithiothreitol) and finally, 1 µl (about 3 U) DNA polymerase I (Klenow enzyme) (Amersham). The mixture was left at room temperature for 30 min. A portion of this blunt-ended DNA was used in subsequent ligation reactions.

A typical ligation reaction contained 10 or 20 µl ligation buffer (66 mM-Tris/HCl pH 7.5; 5 mM-MgCl₂; 5 mM-dithiothreitol; 1 mM-ATP), with insert and vector DNA having a molecular ratio of 1 to 1. T4 DNA ligase (0.5-1 U, Boehringer) was added and the mixture incubated at 15 °C for 16 h prior to transforming into competent HB101 cells.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Property	Use	Reference	Source
Bacteria				
<i>Arthrobacter</i> sp. NRRL B3728 (B3728 for short)	Glucose isomerase producer	Host for development of host-vector system	Reynolds (1973)	Our collection
<i>Escherichia coli</i> HB101	<i>hsdS20</i> ($r_{\text{B}}m_{\text{B}}$), <i>recA</i>	Intermediate host for plasmid construction	Maniatis <i>et al.</i> (1982)	Our collection
<i>Brevibacterium lactofermentum</i> NCIB 9567 (ATCC 13869)		Source of pAM330 (renamed pBL100)	Miwa <i>et al.</i> (1984)	NCIB
<i>Corynebacterium glutamicum</i> NCIB 10026 (ATCC 13058)		Source of pHM1519 (renamed pCG100)	Miwa <i>et al.</i> (1984)	NCIB
Plasmids				
pBR322	Ap ^r Tc ^r	Part of the shuttle vector	Bolivar <i>et al.</i> (1977)	Our collection
pNCAT4	Ap ^r Km ^r	Extraction of Km ^r gene	Herrera-Estrella <i>et al.</i> (1983)	C. Lichtenstein (Imperial College, London)

ATCC, American Type Culture Collection; NCIB, National Collection of Industrial Bacteria, UK.

Nick-translation. DNA was nick-translated with [α -³²P]dCTP (Amersham; 3000 Ci mol⁻¹, 111 TBq mol⁻¹) using the nick-translation kit obtained from the same company.

In situ hybridization. Regenerated B3728 protoplasts that had been treated with DNA and were resistant to kanamycin were lifted onto nitrocellulose filter paper (Schleicher and Schuell, BA85). The filter, with the colonies side up, was placed on a stack of Whatman 3MM papers saturated with 4 mg lysozyme ml⁻¹ and 20 mM-EDTA pH 8.0 and incubated at 37 °C for 15 min. Further treatments were as described in Maniatis *et al.* (1982).

Southern hybridization. DNA was digested by restriction enzyme and the digested fragments were separated by agarose gel electrophoresis. They were then transferred to a piece of nitrocellulose paper and hybridized with nick-translated radioactive DNA probe according to Maniatis *et al.* (1982).

Measurement of plasmid loss. Strain B3728(pBL2100) was grown overnight in L medium, centrifuged and resuspended in L medium to remove the kanamycin. The resuspended cells were added to a 100 ml flask containing L medium prewarmed at 30 °C. The viable count was measured every hour.

Strain B3728(pCG2100) was grown overnight and inoculated into L medium as in the above experiment. The culture was grown and subcultured to a fresh flask containing pre-warmed and pre-aerated L medium when the OD₆₀₀ reached 0.5-0.7. At each subculture, a sample was taken and plated out to determine plasmid loss.

Determination of plasmid copy number. Strains B3728(pBL2100) and B3728(pCG2100) were grown in 500 ml L medium with 100 μ g kanamycin ml⁻¹ until the OD₆₀₀ reached 0.7; 40 ml of each culture was concentrated to 5 ml in SMMLBV medium, lysozyme was added (0.5 mg ml⁻¹) and the number of protoplasts counted. The cells from 400 ml were pelleted, washed once with 0.01 M-Tris/HCl pH 8.0 and resuspended in 8.5 ml PM (20% w/v, sucrose; 20 mM-MgCl₂; 20 mM-Tris/HCl pH 7.5). Lysozyme (1 ml of 10 mg ml⁻¹) was added and the cells were incubated at 37 °C for 45 min. Then, 0.5 ml 20% (w/v) SDS and 0.5 ml 5 mg proteinase K ml⁻¹ (Boehringer) were added. The mixture was incubated overnight at 55 °C. Samples (1.5 ml) were removed for DNA assay (Giles & Myers, 1965). The remaining 9 ml sample was extracted twice with phenol/chloroform and dialysed in 4 l 10 mM-Tris/HCl pH 8.0 for 5 h.

The dialysed nucleic acid (1 μ g) was digested by *SalI* restriction enzyme, serially diluted and loaded onto a 0.8% agarose gel. A known amount of *SalI*-digested pBL2100 or pCG2100 DNA was used as standard. After electrophoresis, Southern transfer was performed and DNA hybridized to nick-translated pBR322. By use of autoradiographs the radioactive bands on the filters were identified, cut out and counted by a scintillation counter.

By comparison with the standard DNA, the amounts of pBL2100 and pCG2100 in the crude nucleic acid preparations were calculated. The copy number of plasmid in a sample of crude nucleic acid preparation was estimated by use of the following equation:

$$\text{Plasmid no. per genome} = \frac{\text{Amount of plasmid}}{\text{Amount of B3728 DNA}} \times \frac{\text{Molecular mass of B3728 DNA}}{\text{Molecular mass of plasmid}}$$

in which the molecular masses of pBL2100 and pCG2100 are 6.8×10^6 Da and 5.8×10^6 Da respectively. The molecular mass of the B3728 genome was estimated to be 2.6×10^9 Da from measurements of the renaturation rate of single-stranded DNA according to Seidler & Mandel (1971).

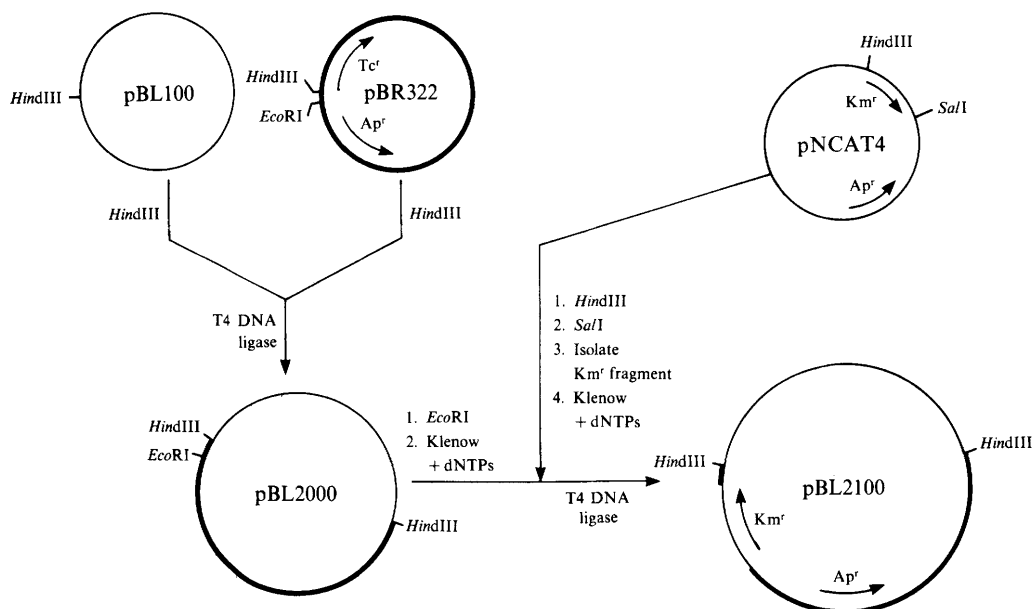


Fig. 1. Construction of pBL2100. A hybrid plasmid was obtained by ligating pBL100 and pBR322 through their *Hind*III sites to form pBL2000. The *Km*^r gene from pNCAT4 was inserted into the filled-in *Eco*RI site. The drawing is not to scale.

RESULTS

Protoplast formation and regeneration. *Arthrobacter* sp. B3728 was sensitive to lysozyme treatment. The process of protoplast formation was nearly complete in 60 min. The efficiency of protoplast regeneration was calculated as the percentage of regenerants on DM3 plates from the total protoplasts applied. The figure varied between 15 and 30% among different experiments. In subsequent transformation experiments, the cells were treated with lysozyme for 1 h.

Construction of hybrid vectors. Hybrid vectors between pBR322 and the cryptic plasmids pBL100 and pCG100 were constructed as shown in Figs 1 and 2. Since restriction maps of the cryptic plasmids have been reported (Miwa *et al.*, 1984), two or three restriction enzymes were chosen to digest the plasmids at unique sites in different regions. This increased the probability that the replicative origins were not destroyed during linearization. The linearized forms were ligated to suitably digested pBR322. The ligated mixtures were transformed to *E. coli* HB101.

Many reports have shown that ampicillin-resistance genes do not function properly in coryneform bacteria; however, kanamycin-resistance genes work well (reviewed in Batt *et al.*, 1985; Martín *et al.*, 1987). Therefore, a gene coding for the aminoglycoside 3'-phosphotransferase II enzyme and conferring kanamycin resistance was isolated from pNCAT4 (Herrera-Estrella *et al.*, 1983). This gene was refilled and ligated to linearized and refilled hybrids. The mixture was transformed to HB101.

DNA from the transformants was extracted and analysed by restriction digestion to confirm that there were no large deletions or rearrangements. Restriction maps of the hybrid plasmids are shown in Fig. 3.

Transformation of hybrid plasmids to protoplasts of B3728. The constructed vectors, pBL2100, pCG1100 and pCG2100, were transformed to protoplasts of B3728. All three hybrid plasmids transformed the protoplasts successfully and colonies resistant to kanamycin were found. The existence of plasmids was confirmed by *in situ* hybridization using the excised *Km*^r gene as a radioactive probe. Strong signals were obtained from cells treated with pCG1100 and pCG2100,

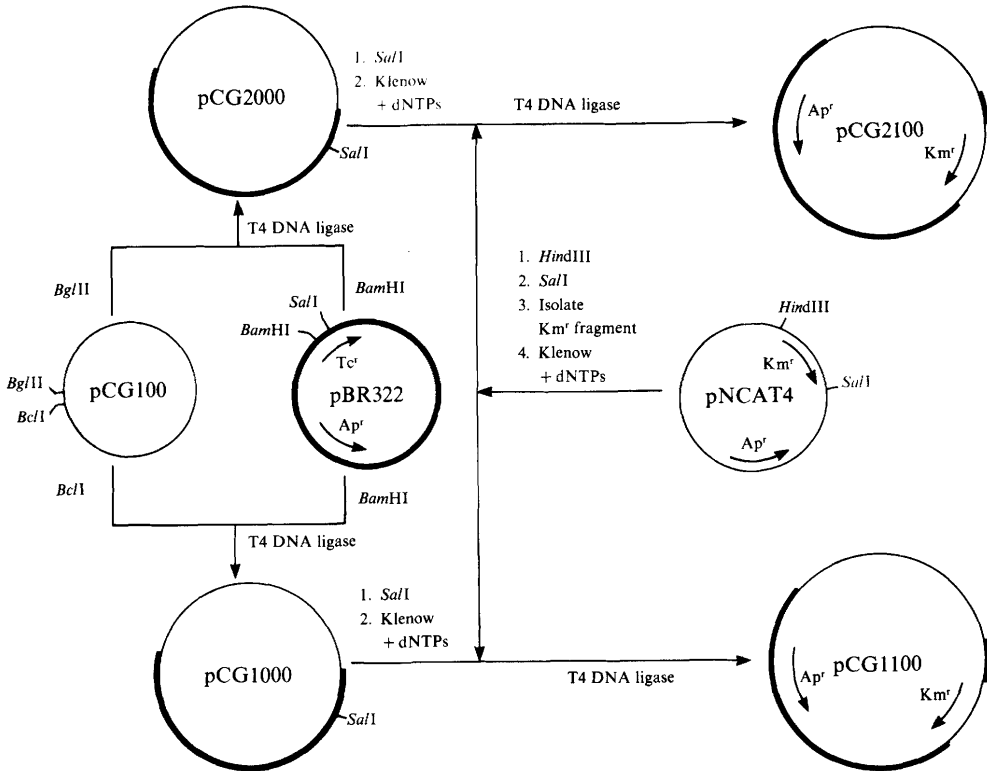


Fig. 2. Construction of pCG1100 and pCG2100. Two hybrid plasmids were obtained by digesting pCG100 with *BclI* or *BglII*, respectively, and ligating the linearized plasmid to pBR322 digested by *BamHI*. The *Km^r* gene from pNCAT4 was inserted into the filled-in *SalI* site. The drawing is not to scale.

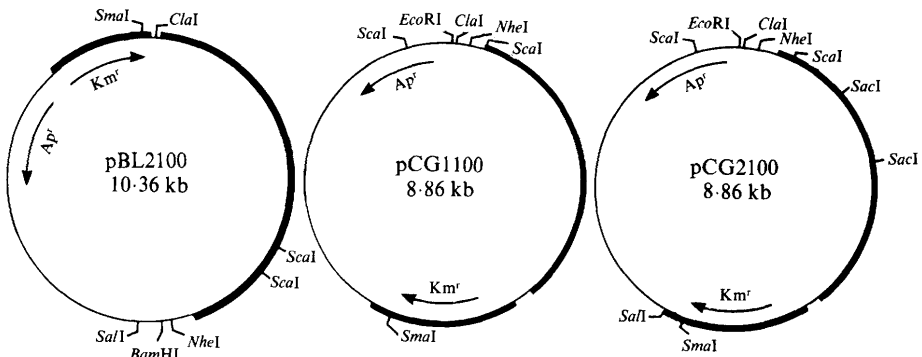


Fig. 3. Restriction maps of pBL2100, pCG1100 and pCG2100. These plasmids were analysed by different restriction enzymes. Thick lines represent either the cryptic plasmid or the *Km^r* gene; thin lines represent pBR322. Orientations of the genes for antibiotic resistance are indicated by arrows.

and weak signals from pBL2100-treated cells. The kanamycin-resistant colonies were streaked onto L plates containing ampicillin or kanamycin (25–1000 µg ml⁻¹). They did not grow on L plates with ampicillin but grew well on plates with this range of kanamycin concentrations.

Transformation frequency. Plasmid pBL2100 extracted from HB101 transformed B3728 cells to a frequency of 10⁴–10⁵ transformants per µg DNA. pCG1100 and pCG2100 extracted from HB101 gave a frequency of 10²–10³ transformants per µg DNA. However, when pCG1100 and

Table 2. Amount of DNA and number of cells in the 400 ml culture for copy number determination

Cell numbers were estimated by total count of protoplasts. The values shown are means of several determinations in a single experiment.

Culture	Total cell count	Amount of DNA (μg)	Calculated no. of genomes	No. of genomes per cell
B3728(pBL2100)	2.32×10^{11}	4515	1.05×10^{12}	4.5
B3728(pCG2100)	2.96×10^{11}	3675	8.51×10^{11}	2.9

pCG2100 were extracted from B3728, the transformation frequency increased to 10^5 – 10^6 per μg DNA. This suggests that pCG1100 and pCG2100 contain site(s) sensitive to the attack of a B3728 restriction endonuclease. These sites are protected if the plasmids pass through the *Arthrobacter* host.

Linearity of response. Plasmid DNA in the range 1–5000 ng from pBL2100 and pCG2100 was used to transform 4×10^9 protoplasts of B3728 in 0.1 ml SMMLBV medium. The relationship between the amount of DNA used and number of protoplasts transformed was positive and linear. No saturation by plasmid DNA was reached with the range of DNA concentrations used, although the transformation frequency appeared to decrease slightly at 1000 and 5000 ng of input plasmid DNA. Both pBL2100 and pCG2100 followed a similar pattern in the frequency of transformation. The probability of a plasmid transforming a protoplast was in the order of 10^{-5} – 10^{-6} . This figure remained quite constant for the various amounts of input DNA.

Effect of protoplast concentration. Different amounts of B3728 protoplasts were transformed by 10 ng of pCG2100. The number of transformants obtained increased linearly with the amount of protoplasts used and began to level off at around 5.8×10^8 protoplasts. Therefore, to obtain an optimal number of transformed cells, it is necessary to keep the number of protoplasts higher than this.

Effect of duration of lysozyme treatment. No transformants were scored if B3728 cells were not treated with lysozyme. Cells treated with lysozyme for 15, 45 and 60 min gave similar frequencies of transformation. This shows that once some of the cell wall was removed, the cells could take up DNA with the same efficiency.

Assay of copy number. The copy numbers of pBL2100 and pCG2100 were determined to be 1.2 and 11.1 per genome by comparing the radioactivity of the plasmid bands from the crude extract with those from the standard. Since the amount of DNA and the number of cells in the culture were known (Table 2), the copy numbers of these two plasmids per cell could be calculated: 5.4 for pBL2100 and 33.1 for pCG2100.

Stability of hybrid plasmids in B3728. pBL2100 and pCG2100 in B3728 were extracted by the preparative method, linearized, and up to 2×10^8 copies were applied to an agarose gel. After electrophoresis, DNA was transferred to a nitrocellulose paper and hybridized with radioactive pBR322. Only a full-length discrete band was found for these DNAs, showing that there was no change in size during their replication.

Loss of pBL2100 and pCG2100 in B3728 under non-selective conditions was determined. Loss of these two plasmids (and/or reduction in copy number below a threshold allowing kanamycin resistance) followed an exponential pattern as shown in Fig. 4. The apparent segregation of pBL2100 per generation was 12.2% and that of pCG2100 was 2.3%.

Deletion mutants of pCG2100. Derivatives of pCG2100 were generated by digesting the plasmid with appropriate restriction enzymes; the required fragments were separated by gel electrophoresis and extracted by electroelution. They were then self-ligated and transformed to competent HB101 cells (Fig. 5). The derivatives generated were transformed to protoplasts of

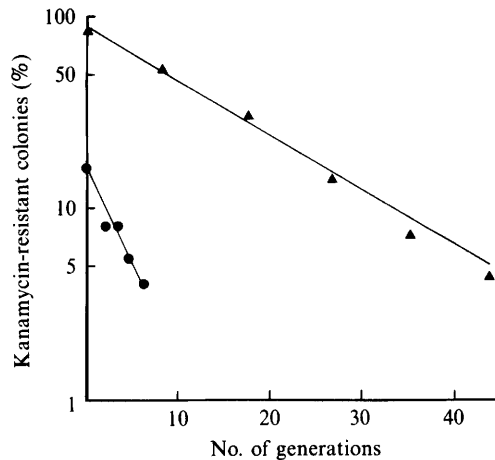


Fig. 4. Rate of loss of kanamycin resistant colonies during non-selective growth. Plasmid-containing cells growing in L medium were diluted and spread on L plates with or without 100 µg kanamycin ml⁻¹. ●, B3728(pBL2100); ▲, B3728(pCG2100).

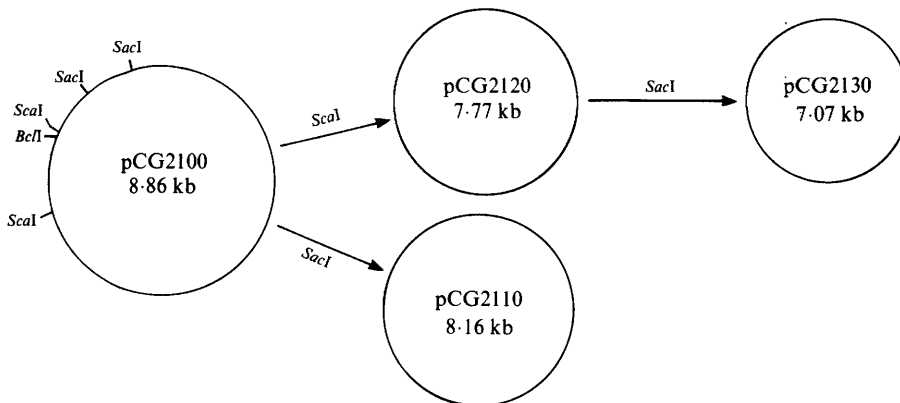


Fig. 5. Construction of deletion derivatives of pCG2100. Plasmid pCG2100 was digested with the indicated restriction enzymes and the large fragments were isolated from agarose gels by electroelution. After self-ligation, the fragments were transformed to competent HB101 cells.

B3728. B3728 was transformed by pCG2120 but not by pCG2110 or pCG2130. This indicates that the 0.7 kb *SacI* fragment originating from the cryptic plasmid pCG100 is important for the maintenance of the hybrid plasmid in B3728. It may be a region concerned with replication.

DISCUSSION

Transformation of protoplasts of *Arthrobacter* sp. B3728 has been achieved by the hybrid plasmids constructed. These plasmids contain unique restriction sites outside the cryptic plasmid DNA and can be used as cloning vectors for transferring foreign DNA into the *Arthrobacter* host.

The transformation frequency of 10⁵–10⁶ transformants per µg plasmid DNA is comparable to that of other efficient transformation systems for coryneform bacteria (Katsumata *et al.*, 1984; Santamaria *et al.*, 1985) and is sufficient for shotgun cloning experiments. The probability of a plasmid transforming a protoplast was around 10⁻⁵–10⁻⁶. This figure is higher than that in a similar transformation system for *Brevibacterium lactofermentum* (Santamaria *et al.*, 1985), in

which a frequency of 5.6×10^{-6} – 5.6×10^{-7} was achieved. Chang & Cohen (1979) achieved a higher frequency of around 10^{-4} for *Bacillus subtilis*.

Of the three hybrid plasmids that have been constructed for this host–vector system, pBL2100 has a low copy number (about 5 copies per cell) and pCG2100 a high copy number (about 33 copies per cell). The copy number of pCG1100 is similar to pCG2100 since both gave similar intensities of hybridization to a radioactive probe. pCG2120, a deletion mutant of pCG2100, has also been constructed. It is about 1 kb smaller than the parental plasmid and provides an alternative choice of vector.

Unlike the cryptic plasmids in their natural hosts, these hybrid plasmids cannot be maintained stably in B3728 under non-selective conditions. Hence, they are not suitable for continuous-culture fermentation unless a selective pressure is applied. This could be achieved by addition of antibiotic to the culture medium or by inserting into these vectors a gene essential for the growth of the host.

Despite the taxonomic confusion of the coryneform group, requirements for plasmids to function in different members of this group are strikingly similar. (a) A plasmid from one member can function in various other members. For example, Katsumata *et al.* (1984) reported that plasmid pCG4, originating from *C. glutamicum*, transforms into various coryneform species. (b) Kanamycin-resistance genes function in different members of coryneform bacteria. (c) Ampicillin-resistance genes do not make various coryneform species resistant to this antibiotic. Ozaki *et al.* (1984) found β -lactamase activity in plasmid-transformed *C. glutamicum* even though the cells were ampicillin sensitive. They proposed that this was due to the incorrect location of β -lactamase in the cell.

The hybrid plasmids we constructed may also be used as vectors for *C. glutamicum* and *B. lactofermentum*. Miwa *et al.* (1985) reported the use of cryptic plasmids pBL100 and pCG100 (pAM330 and pHM1519) to construct shuttle vectors and succeeded in transforming these two genera. Hence, it is likely that our hybrid plasmids will function in other coryneform species as well and become a useful tool in industrial microbiology.

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