The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation

JILL A. HAYNES¹ and MARGARET L. BRITZ*²

¹ Commonwealth Serum Laboratories, 45 Poplar Road, Parkville, Victoria 3052, Australia

² Division of Biotechnology, Commonwealth Scientific and Industrial Research Organization, Clayton Victoria 3168, Australia

(Received 23 March 1989; revised 12 September 1989; accepted 29 September 1989)

A rapid and efficient method of transforming *Corynebacterium glutamicum* by electroporation is described. A number of factors are important in determining the level of transformation obtained. Cells grown in the presence of glycine and isonicotinic acid hydrazide and harvested in early exponential growth phase were much easier to transform. The recovery medium on which transformants were isolated also had a significant effect on the number of transformants obtained because cells were osmotically or electrochemically sensitive following electroporation. Transformation efficiencies of up to 5×10^5 transformants per μg plasmid DNA with homologically derived DNA and 2×10^3 transformants per μg of DNA derived from *Escherichia coli* were obtained.

Introduction

Non-pathogenic saprophytic coryneform bacteria, including species such as *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*, are widely used for industrial production of amino acids and nucleotides. As classical mutation and selection programmes for strain improvement can be complemented by recombinant DNA approaches, efforts have been made to develop efficient transformation systems for these species (Martin *et al.*, 1987).

Most published protoplast transformation protocols are based on methods developed for *Bacillus subtilis* and *Streptomyces* species and involve lysozyme treatment followed by PEG-stimulated uptake of DNA. However, the unusual nature of their cell wall structures, which include corynemycolic acids, make the corynebacteria resistant to lysozyme unless they have been grown in the presence of cell wall inhibitors which modify these structures. For example, 0.3–0.5 U penicillin G ml⁻¹ (Katsumata *et al.*, 1984; Santamaria *et al.*, 1984) or 0.5–2.5% (w/v) glycine (Yoshihama *et al.*, 1985; Thierbach *et al.*, 1988) have been used during growth as a prerequisite to protoplast or spheroplast generation. Generally, strains of *Brevibacterium lactofermentum* are more sus-

ceptible to lysozyme treatment than are species of *C. glutamicum* (Martin *et al.*, 1987). Using such approaches, reported transformation frequencies have varied between 10^2 and 10^5 transformants ($\mu\text{g DNA}$)⁻¹ in *C. glutamicum* (Serwold-Davis *et al.*, 1987; Thierbach *et al.*, 1988; Yoshihama *et al.*, 1985), and up to 10^6 transformants ($\mu\text{g DNA}$)⁻¹ in *B. lactofermentum* (Santamaria *et al.*, 1985), depending on the methodology used, the species or strains, the plasmid and the source of the DNA. DNAs isolated from heterologous species transform at much lower frequencies, suggesting the presence of restriction and modification systems in the corynebacteria which differ from those found in donor species: for example, Serwold-Davis *et al.* (1987) reported 0.2 transformants per μg of DNA purified from *Escherichia coli*. Indeed Thierbach *et al.* (1988) suggested that temporary inactivation of restriction systems by heat treatment improves frequencies for heterologous DNA transformation but these frequencies remained low [40–60 transformants ($\mu\text{g DNA}$)⁻¹ for *C. glutamicum* spheroplasts]. Despite the success of these methods, spheroplast or protoplast transformation remains a tedious and lengthy procedure and with this in mind we have investigated an alternative transformation system using electroporation. Electroporation involves the application of a brief, high-voltage pulse to a suspension of cells and DNA which results in transient membrane permeability and subsequent uptake of DNA. Most previous studies on electroporation carried out with *E. coli*

Abbreviations: EPB, electroporation buffer; INH, isonicotinic acid hydrazide; LAG, Luria agar; LB(G) Luria broth (supplemented with 0.5% glucose); NAG, nutrient agar.

strains such as LE392 and DH5 α (Dower *et al.*, 1988) have shown that high levels of transformation are possible [up to 4×10^6 transformants ($\mu\text{g DNA}$) $^{-1}$].

Many Gram-positive bacteria, including *Lactobacillus* (Chassy & Flickinger, 1987), *Streptococcus* (Powell *et al.*, 1988) and *Clostridium* (Allen & Blaschek, 1988), have been transformed successfully by electroporation. The reported transformation efficiencies vary greatly, from 10^2 – 10^3 transformants ($\mu\text{g DNA}$) $^{-1}$ for *Cl. perfringens* (Allen & Blaschek, 1988) to 1×10^4 – 5×10^5 transformants ($\mu\text{g DNA}$) $^{-1}$ for *S. lactis* (Powell *et al.*, 1988). However, the latter workers found that a mild lysozyme pre-treatment of the cells was necessary to achieve these levels and, without this, only 10^2 transformants ($\mu\text{g DNA}$) $^{-1}$ were obtained. Chassy & Flickinger (1987) obtained up to 8.5×10^4 transformants ($\mu\text{g DNA}$) $^{-1}$ with species of *Lactobacillus*.

In this paper, we describe an efficient and rapid method of transforming *C. glutamicum* using electroporation. A number of factors which were found to influence the transformation efficiency and frequency were investigated and are reported here.

Methods

Bacterial strains and plasmids. The *C. glutamicum* strains used were: AS019, a spontaneously rifampicin-resistant mutant of ATCC 13059 (Yoshihama *et al.*, 1985) obtained from A. J. Sinskey, Massachusetts Institute of Technology, USA; CG2, obtained from Queensland University Culture Collection (no. 2704); and MLB131, an auxotrophic derivative of AS019 (Best & Britz, 1986). The *B. lactofermentum* strain BL1 was a derivative of ATCC 21798 obtained from P. Rogers, University of New South Wales, Australia. *E. coli* strain HB101 has been described previously (Maniatis *et al.*, 1982). This strain lacks some of the *E. coli* restriction and modification systems (Raleigh, 1987).

Three plasmids were used: pHY416 Cm/Km (9.1 kb) is a *Bacillus subtilis*–*C. glutamicum* shuttle vector (Britz & Best, 1986; Yoshihama *et al.*, 1985); pCSL17 (7.2 kb) is an *E. coli* shuttle vector constructed by sub-cloning the *C. glutamicum* *ori* from pY416 Cm/Km into pBR322 and inserting Tn903 (A. Hodgson and others, unpublished); pUL340 (5.8 kb) is a *B. lactofermentum* plasmid kindly provided by J. F. Martín, University of Leon, Spain (Santamaria *et al.*, 1984). All three plasmids express kanamycin resistance in *C. glutamicum*.

Culture conditions and media. *C. glutamicum* and *B. lactofermentum* were routinely grown in Luria broth (LB; Miller, 1972) supplemented with 0.5% glucose (LBG), at 30 °C and 200 r.p.m. Growth was monitored by measuring optical density at 600 nm. Glycine (0.5–6.5%) and isonicotinic acid hydrazide (INH) (4–8 mg ml $^{-1}$) were added from sterile stock solutions [20% (w/v) glycine and 100 mg INH ml $^{-1}$].

To obtain the total viable count before and after electroporation, cells were diluted in SMMC buffer (0.7 M-sucrose; 50 mM-maleic acid; 10 mM-MgCl $_2$ ·6H $_2$ O; 10 mM-CaCl $_2$; pH 6.5) and spread onto nutrient agar [NAG; Oxoid Nutrient Broth no. 2 supplemented with 0.5% glucose and solidified with 1.0% (w/v) agar] or ET agar (Best & Britz, 1986). Presumptive transformants were isolated on ET agar, NAG or Luria agar (LAG; LBG solidified with 1.0% agar), all supplemented with kanamycin sulphate (50 $\mu\text{g ml}^{-1}$).

E. coli HB101 was grown in LBG at 37 °C and 200 r.p.m. and was harvested when the OD $_{600}$ was approximately 1.0.

Plasmid isolation. Plasmid DNA was isolated from *E. coli* by an alkaline lysis method and purified by CsCl $_2$ density gradient centrifugation (Maniatis *et al.*, 1982). *C. glutamicum* and *B. lactofermentum* DNA was isolated using a modification of the method of Santamaria *et al.* (1984). Cells were grown for approximately 18 h at 30 °C in a rotary shaker at 200 r.p.m. in 400 ml LBG supplemented with kanamycin (50 $\mu\text{g ml}^{-1}$) and glycine (2.5%). When the OD $_{600}$ had reached ≥ 1.0 , the cells were collected by centrifugation, resuspended in 20 ml lysis buffer (2 mg lysozyme ml $^{-1}$ in 10 mM-Tris, pH 8.0; 0.7 M-sucrose; 10 mM-CaCl $_2$; 10 mM-MgCl $_2$ ·6H $_2$ O; 0.05% KH $_2$ PO $_4$; 0.01% BSA) and incubated for 2 h at 30 °C with gentle shaking (100 r.p.m.). Then 5 ml 0.5 M-EDTA, pH 8, was added and the mixture was incubated at 30 °C for 30 min before adding 50 ml 1% SDS/0.1 M-NaOH and heating at 56 °C for approximately 10 min. Chromosomal DNA was precipitated using potassium acetate and plasmid DNA was subsequently collected following 2-propanol precipitation prior to purification on CsCl $_2$ density gradients, as described by Maniatis *et al.* (1982).

Transformation protocols. *E. coli* HB101 was transformed either by the CaCl $_2$ -induced competence method, as described by Maniatis *et al.* (1982), or by electroporation as detailed by Dower *et al.* (1988). Protoplasts of *C. glutamicum* AS019 and MLB131 were prepared as described previously (Best & Britz, 1986). A volume of suspension (1 ml, containing up to 10^9 cells) was centrifuged and cells were resuspended in 50 μl TSMC buffer (10 mM-MgCl $_2$ ·6H $_2$ O; 30 mM-CaCl $_2$ ·2H $_2$ O; 500 mM-sodium succinate; 50 mM-Tris; pH 7.5), and then 30 μl DNA in TSMC was added. After 5 min on ice, 0.5 ml PEG 6000 (25%, w/v, in TSMC) was added, the contents mixed by inversion and the tube placed on ice for 2 min, then at 37 °C for 2 min. The suspension was diluted 10-fold in LB/TSMC and incubated at 30 °C for 90 min with gentle shaking. Cells were collected by centrifugation, concentrated 15-fold and viable counts performed on ET and ET-Km plates after diluting in SMMC. Transformants regenerated after 2–10 d at 30 °C.

Transformation by electroporation was carried out using the Gene Pulser apparatus (Bio-Rad) with either 0.2 cm or 0.4 cm cuvettes (Bio-Rad). The method used with 0.4 cm and 0.2 cm cuvettes varied in the washing and suspension solutions and in the volumes used. Cells were routinely harvested by centrifugation at OD $_{600}$ between 0.15 and 0.25, unless otherwise stated.

The first method, employing 0.4 cm cuvettes, was essentially that described by the manufacturers for use with *E. coli*. The cells were washed in one culture volume of ice-cold electroporation buffer (EPB) (0.5 M-sucrose; 1 mM-MgCl $_2$; 7 mM-K $_2$ HPO $_4$ /KH $_2$ PO $_4$; pH 7.4), resuspended in approximately 0.02 culture volumes of ice-cold EPB and held on ice until required. Plasmid DNA (up to 50 μl) was thoroughly mixed with 0.8 ml of cell suspension (about 10^9 cells) in a chilled cuvette. Cells were exposed to a single pulse (2.5 kV, 25 μF) unless otherwise stated.

When 0.2 cm cuvettes were used the cells were washed in one culture volume of 15% (v/v) glycerol and resuspended in 0.002 culture volumes of 15% glycerol. The concentrated cells (40 μl containing approximately 10^9 cells) were transferred to a cold 1.5 ml polypropylene tube; 1–2 μl DNA was added and the suspension was mixed. Cells were transferred to the cuvette and exposed to a single pulse (2.5 kV, 25 μF) unless otherwise stated. The output of the pulse generator was directed through a Pulse Controller unit containing two 10 Ω resistors in series and two 100 Ω resistors in parallel circuit with the sample.

Time constants for both methods were 4.5–5.0 ms. Immediately following the pulse, cells from 0.4 cm cuvettes were diluted 1:10 in LBG and the cells from 0.2 cm cuvettes were diluted 1:25 in LBG. They were then incubated for 1 h at 30 °C before plating for viable counts and selection of kanamycin-resistant cells on ET, NAG and LAG plates.

Characterization of transformed cells. Transformants were scored initially after 48 h at 30 °C, then after 4 d of growth; duplicate samples

were scored and means calculated. Kanamycin resistance of presumptive transformants was confirmed by replica plating onto LAG containing kanamycin ($50 \mu\text{g ml}^{-1}$). The presence of plasmid DNA in presumptive transformants was confirmed by agarose gel electrophoresis of crude extracts. Restriction endonuclease patterns of CsCl_2 density gradient-purified plasmids were used to confirm the structural integrity of transformed plasmid DNA. Restriction endonuclease digestions were performed as specified by the manufacturers (Bethesda Research Laboratories) with a series of single and multiple restriction digests. DNA fragment sizes were compared with digests of *Hind*III-digested λ DNA (BRL).

Transformation efficiency was calculated as c.f.u. per μg of plasmid DNA added. To account for variations in the number of cells treated, transformation frequencies were calculated as transformants per number of cells pulsed.

Results and Discussion

Initial attempts at obtaining transformants of *C. glutamicum* by electroporation involved using late-exponential-phase cells grown in LBG and pCSL17 which had been isolated from strain AS019: 0.4 cm cuvettes were used with a pulse of 6.25 kV cm^{-1} , $25 \mu\text{F}$. This approach yielded disappointing results, as only 10–100 transformants $(\mu\text{g DNA})^{-1}$ were obtained. However, this prompted the following investigations which showed that a combination of several parameters was important in achieving a greatly improved efficiency of transformation. The majority of experiments were performed using the 0.4 cm cuvettes but when the 0.2 cm cuvettes became available, these were also used.

Influence of glycine and INH in the growth medium on electroporation efficiency

The addition of both glycine (2.5%, w/v) and INH (4 mg ml^{-1}) to *C. glutamicum* during growth has been found to improve the frequency of transformation in protoplast transformation using PEG (M. L. Britz, unpublished). Using electroporation, we found that growth in the presence of glycine (2.5%) increased the number of cells transformed with pCSL17 DNA approximately 10-fold and that the further addition of INH produced almost another 10-fold increase (Table 1). Similar results were obtained later when the same experiment was carried out using 0.2 cm cuvettes. Transformants were not obtained in the absence of plasmid DNA or the pulse.

In order to determine whether the efficiency of transformation could be increased further, different concentrations of glycine and INH were added to the growth medium, separately and in combination (Figs 1 and 2). Fig. 1 shows that when glycine alone (up to 6.5%) was added to the growth medium, the number of transformants obtained following electroporation with pUL340 was never as high as when cells were grown with glycine plus 4 mg INH ml^{-1} . As the concentration of

Fig. 1. Effect of glycine in the growth medium on the number of transformants obtained. *C. glutamicum* AS019 was cultured in LBG containing 0–6.5% glycine (■) or LBG supplemented with 4 mg INH ml^{-1} plus 0–6.5% glycine (□). Cells were harvested when OD_{600} was 0.2 and then subjected to electroporation in a 0.4 cm cuvette in the presence of 1 μg plasmid pUL340.

Table 1. Effects of growth in LBG containing glycine and INH on electroporation transformation frequencies

C. glutamicum AS019 cells were harvested when the OD_{600} was 0.2. After electroporation transformants were recovered on ET-Km plates.

Growth supplement	No. of transformants $(\mu\text{g DNA})^{-1}$	
	0.4 cm cuvette*	0.2 cm cuvette†
None	2.0×10^2	1.4×10^4
Glycine 2.5% (w/v)	1.8×10^3	7.2×10^4
Glycine 2.5% (w/v) plus INH (4 mg ml^{-1})	9.9×10^3	5.2×10^5

* pCSL17 DNA (1 μg) isolated from *C. glutamicum* AS019 was added to 0.8 ml cells ($\sim 10^9$ cells) suspended in electroporation buffer prior to pulsing at 2.5 kV and 25 μF .

† pCSL17 DNA (0.1 μg) isolated from *C. glutamicum* AS019 was added to 40 μl cells ($\sim 10^9$ cells) suspended in 15% glycerol prior to pulsing at 2.5 kV and 25 μF .

glycine in the growth medium increased, the efficiency of transformation (transformants per 10^9 cells treated) increased, with the highest number of transformants seen following growth in 2.5–4.5% glycine plus 4–8 mg INH ml^{-1} . However, increasing the concentration of glycine above 4.5% resulted in a decrease in the number of transformants obtained. Supplementing LBG with INH alone also improved transformation efficiency but even at the highest concentration tested (12 mg ml^{-1}), INH was not as effective alone as when it was used in combination with glycine. As growth was severely inhibited at high glycine and INH concentrations, LBG

Fig. 2. Effect of INH concentration in the growth medium on the number of transformants obtained. *C. glutamicum* AS019 was cultured in LBG (■) or LBG containing 2.5% glycine (□) in the presence of 0–12 mg INH ml⁻¹. Growth and electroporation conditions were as Fig. 1.

was supplemented routinely with 2.5% glycine and 4 mg INH ml⁻¹ in subsequent experiments.

The growth inhibition and presumed increase in cell surface permeability produced by glycine and INH are probably due to different mechanisms. Glycine is incorporated into the Gram-positive cell wall in place of D-alanine. However, its incorporation is less efficient and leads to less cross-linking and a looser wall structure (Hammes *et al.*, 1973). It has been suggested that this looser structure is a better substrate for lysozyme and our data suggest that this may facilitate increased uptake of DNA by *C. glutamicum* during electroporation. Cells of *C. glutamicum* grown in the presence of glycine elongate and bulge at sites where the cell wall is apparently weakened or damaged (Best & Britz, 1986; Serwold-Davis *et al.*, 1987). The concentration of glycine used in the growth medium for corynebacteria prior to lysozyme treatment and protoplast transformation has varied greatly between laboratories (0.5%–2.5%). Serwold-Davis *et al.* (1987) found that when the levels of glycine inhibited cell growth substantially, corresponding to the formation of exaggeratedly misshapen cells, then these cells were resistant to the effects of lysozyme. We have found that when *C. glutamicum* was grown in very high concentrations of glycine, the efficiency of electroporation increased, suggesting that further modifications to cell surface structures may have been occurring which were important for DNA uptake.

INH only inhibits the growth of mycobacteria and other mycolic-acid-containing bacteria. With *Mycobacterium*, *Nocardia* and *Rhodococcus* strains, concentrations of INH from 1 µg ml⁻¹ to 100 µg ml⁻¹ have been shown to cause significant inhibition of growth (Tomiyasu & Yano, 1984). In these bacteria, the incorporation of INH

in the growth medium decreases both the ratio of mycolic to non-mycolic fatty acids and the mean carbon atom length of the mycolic acids (Tomiyasu & Yano, 1984; Maekura, 1984). However, at levels of INH up to 100 µg ml⁻¹, neither growth inhibition nor change in the corynomycolic acid composition was observed in *Corynebacterium xerosis* and *Rhodococcus rhodochrous* (Tomiyasu & Yano, 1984).

Yoshihama *et al.* (1985) reported that the incorporation of INH into the growth medium had no effect on protoplast formation or transformation in *C. glutamicum*. However, the concentration used was not stated and it is possible that much lower concentrations than we have used were tested. It is significant that the levels we have found to be useful as a pretreatment for electroporation with *C. glutamicum* are much higher (i.e. 4 mg ml⁻¹) than those used by Tomiyasu & Yano (1984): at lower concentrations of INH, growth of *C. glutamicum* is not affected (M. L. Britz, unpublished). Although the effects of high concentrations of INH on mycolic acid synthesis have not been evaluated in *C. glutamicum*, our observations using phase-contrast microscopy have shown that cells grown in the presence of INH exhibit distorted shapes (longer, rounded cells) consistent with the postulated modification of the cell surface.

It appears that the optimum electroporation transformation obtained by growing cells in the presence of glycine and INH arises from a combination of two effects: the glycine producing a less cross-linked peptidoglycan and the INH possibly causing formation of mycolic acids of shorter chain-length. Both probably decrease the physical barrier to the uptake of DNA.

Effect of growth phase on electroporation transformation efficiency

Cultures of *C. glutamicum* grown in LBG (supplemented with glycine and INH) were harvested after 16–18 h growth when the OD₆₀₀ ranged between 0.075 and 4.5. It was found that early exponential phase cells were more easily transformed with pCSL17 DNA and that as the OD₆₀₀ increased, there was a significant reduction in the number of transformants obtained (Fig. 3). This was true whether 0.4 cm or 0.2 cm cuvettes were used. In most experiments, cells were routinely harvested when OD₆₀₀ was between 0.15 and 0.25.

Cells harvested in the early to mid-exponential growth phase have generally been found to be optimal for electroporation (Miller *et al.*, 1988; Powell *et al.*, 1988; Dower *et al.*, 1988). Powell *et al.* (1988), working with lactic streptococci, found that the number of transformants recovered was not affected in the optical density range of 0.25–0.7. However, when cells were harvested at an OD₆₀₀ of 0.9, the number of transformants recovered

Fig. 3. Effect of harvesting cultures at different growth phases on the number of transformants obtained using electroporation. *C. glutamicum* AS019 was grown in LBG supplemented with 2.5% glycine and 4 mg INH ml⁻¹ and subjected to electroporation in the presence of pCSL17 DNA in 0.2 cm (□) or 0.4 cm (■) cuvettes as described in Methods.

decreased by 15%. The stage of growth required for efficient electroporation transformation of *C. glutamicum* appears to be more critical than found for other genera to date. This may be related to the composition of the cell surface, as we have found that the mycolic acid composition of strain AS019 and auxotrophic mutants (Best & Britz, 1986) varies during the growth cycle (D. Pierotti and others, unpublished).

Effect of the concentration of DNA and cells on the number of transformants

The relationship between DNA concentration and number of recombinants recovered for all three plasmids tested was linear (Fig. 4). The transformation efficiencies (no. of transformants µg⁻¹) for these points were constant within experimental variation: means ± SD were pCSL17, $1.5 \pm 0.5 \times 10^4$ µg⁻¹; pUL340, $9.2 \pm 1.2 \times 10^3$ µg⁻¹ and pHY416, Cm/Km $5.9 \pm 1.4 \times 10^3$ µg⁻¹. The size of the plasmid appeared to have no effect on the relative ease of transformation but the size range of the plasmids tested was small. No clear-cut relationship has been observed between plasmid size and transformation by electroporation, and large plasmids, exceeding 25 kb, have been introduced efficiently into *Lactobacillus* and *Streptococcus* spp. (Powell *et al.*, 1988). With *E. coli* (Dower *et al.*, 1988) and *Campylobacter jejuni* (Miller *et al.*, 1988), the relationship between DNA concentration and the number of transformants is also linear over several orders of magnitude and a constant efficiency of transformation is obtained. DNA saturation has not been reported with any bacteria using electroporation

Fig. 4. Relationship between the number of transformants and the amount of DNA used. *C. glutamicum* AS019 cells were electroporated in the presence of pCSL17 (■), pHY416 Cm/Km (□) or pUL340 (◆) in 0.4 cm cuvettes, and pUL340 in 0.2 cm cuvettes (◇). For conditions of growth and electroporation see Methods.

Fig. 5. Effects of voltage on transformation efficiency. *C. glutamicum* AS019 cells were electroporated in the presence of pCSL17 DNA in the 0.4 cm (■) and 0.2 cm (□) cuvettes. For growth and electroporation conditions see Methods.

and it appears that the level at which saturation occurs is much greater than that observed with other transformation procedures.

A linear relationship was also observed when 1 µg pCSL17 DNA was pulsed in the presence of cells at concentrations of approximately 1×10^6 c.f.u. to 1×10^9 c.f.u. (data not shown).

Effect of voltage (kV cm⁻¹) on the number of cells transformed

C. glutamicum cells and pCSL17 were exposed to voltages from 3.1 to 6.25 kV cm⁻¹ in 0.4 cm cuvettes and voltages from 5.0 to 12.5 kV cm⁻¹ in 0.2 cm cuvettes (Fig. 5).

Table 2. Effect of selection medium and recovery time following electroporation on the number of transformants obtained

Recovery medium	Diluent	Recovery time	Transformants* ($\mu\text{g DNA}$) ⁻¹	Percentage of maximum
ET-Km	SMMC	1 h†	5.8×10^4	100
NAG-Km	SMMC	1 h†	1.1×10^4	19
ET-Km	SMMC	None	1.2×10^4	21
NAG-Km	SMMC	None	$<1.5 \times 10^1$	<0.03
ET-Km	DW‡	None	9.3×10^2	1.6
NAG-Km	DW‡	None	$<1.5 \times 10^1$	<0.03

* *C. glutamicum* AS019 was grown to an OD₆₀₀ of 0.2 in LBG containing 2.5% glycine plus 4 mg INH ml⁻¹, harvested, then electroporated in a 0.4 cm cuvette in the presence of 1 μg of pUL340 DNA.

† Cells were incubated at 30 °C in LBG for 1 h with gentle shaking. Viable counts did not increase during this period.

‡ DW, distilled water.

Although the buffer and the volume of cells were different when using the 0.4 cm and 0.2 cm cuvettes, Fig. 3 shows that there appears to be a good correlation between the two methods once account is made for the different amounts of DNA supplied. The best results were obtained at 12.5 kV cm⁻¹ and 25 μF , the maximum possible values obtainable with the Bio-Rad Gene Pulser, conditions found to be the most efficient for other bacteria (Dower *et al.*, 1988). However, one exception is *Streptococcus*: using 0.4 cm cuvettes, Powell *et al.* (1988) found that 2.0 kV (4 kV cm⁻¹) gave higher numbers of transformants than 2.5 kV (6.25 kV cm⁻¹). •

Effect of the recovery medium used to isolate transformants

Following electroporation of cells with pUL340 DNA, and incubation at 30 °C for 1 h, the cells were diluted and spread onto three selective media: ET-Km, LAG-Km and NAG-Km. Transformants grew faster on the osmotically protective medium (ET-Km) and this was also the medium which consistently supported the highest number of colonies (data not shown). The degree of superiority of ET-Km over NAG-Km and LAG-Km showed some variability from experiment to experiment but counts on ET-Km were usually at least 70% higher than those on NAG-Km and LAG-Km. This was true whether cells were pulsed at 6.25 kV cm⁻¹ (0.4 cm cuvette) or 12.5 kV cm⁻¹ (0.2 cm cuvette).

Total viable counts were carried out on both ET and NAG plates before and after electroporation. There was no significant difference in the viable counts obtained on the two media or from cells diluted in distilled water prior to plating on NAG. This suggests that most of the cells were not osmotically fragile following growth in

LBG/glycine/INH or after electroporation. However, the data presented in Table 2 show that transformants arising following electroporation were osmotically or electrochemically fragile, as dilution in distilled water or plating directly onto NAG-Km after diluting in SMMC resulted in substantially fewer transformants. This phenomenon was not caused by a lack of expression time, although the number of transformants did increase five-fold after a recovery period, as selection onto high ionic strength, osmotically-protective medium (ET-Km) increased the number of transformants 10- to 10³-fold. This fragility was lost after a recovery period (compare counts on NAG-Km before and after recovery). Osmotic or electrochemical fragility has not, to our knowledge, been reported for any other electroporation system. However, Powell *et al.* (1988) showed that over 90% of the transformants recovered were derived from osmotically fragile cells following mild lysozyme treatment of *Streptococcus lactis*. We have found that mild lysozyme treatment (1 kU ml⁻¹) of *C. glutamicum* improved electroporation transformation efficiency approximately five-fold.

Comparison of 0.4 cm and 0.2 cm cuvettes

Initial experiments were carried out using the 0.4 cm cuvettes and with cells suspended in a sucrose/phosphate buffer according to methods supplied by Bio-Rad. However, when the 0.2 cm cuvettes became available, these were used instead and the cells were suspended in 15% glycerol. The latter method was found to increase the transformation efficiency from approximately 10⁴ ($\mu\text{g DNA}$)⁻¹ to 10⁵ ($\mu\text{g DNA}$)⁻¹ and the transformation frequency (no. of transformants per survivor) from approximately 1.0×10^{-6} to 5.0×10^{-5} , using

Table 3. Efficiency and frequency of transformation of *C. glutamicum*, *B. lactofermentum* and *E. coli* with homologously and heterologously derived DNA

Plasmid	Source of DNA	<i>C. glutamicum</i> *		<i>B. lactofermentum</i> †		<i>E. coli</i> ‡	
		Efficiency (transformants μg^{-1} per 10^9 cells)	Frequency§	Efficiency (transformants μg^{-1} per 10^9 cells)	Frequency§	Efficiency (transformants μg^{-1} per 10^9 cells)	Frequency§
pCSL17	<i>C. glutamicum</i> AS019	5.0×10^5	5.0×10^{-5}	1.2×10^3	1.2×10^{-7}	2.6×10^7	2.6×10^{-3}
pCSL17	<i>E. coli</i> HB101	2.0×10^3	2.0×10^{-7}	2.2×10^3	2.2×10^{-7}	7.3×10^8	7.3×10^{-2}
pUL340	<i>C. glutamicum</i> AS019	4.5×10^5	4.5×10^{-5}	1.2×10^3	1.2×10^{-7}		
pUL340	<i>B. lactofermentum</i> BL1	6.3×10^2	6.3×10^{-8}	1.0×10^6	1.0×10^{-4}		
pHY416	<i>C. glutamicum</i> AS019	3.9×10^5	3.9×10^{-5}	8.0×10^3	8.0×10^{-7}		

* *C. glutamicum* AS019 was grown in LBG containing 2.5% (w/v) glycine and 4 mg INH ml^{-1} . Cells were harvested at an OD_{600} of 0.2 and pulsed in the presence of DNA at 12.5 kV cm^{-1} in a 0.2 cm cuvette.

† *B. lactofermentum* BL1 was grown in LBG containing 2.0% (w/v) glycine. Cells were harvested at an OD_{600} of 0.2 and pulsed in the presence of DNA at 12.5 kV cm^{-1} in a 0.2 cm cuvette.

‡ *E. coli* HB101 was grown in LBG. Cells were harvested at an OD_{600} of 1.0 and pulsed in the presence of DNA at 12.5 kV cm^{-1} in a 0.2 cm cuvette.

§ Transformation frequency is the number of transformants obtained divided by the total number of cells pulsed in the presence of 0.1 μg plasmid DNA.

homologously isolated DNA. Two other *C. glutamicum* strains, CG2 and MLB131, gave similar results. We have found that using the same strains and plasmids, electroporation represents at least a 10- to 100-fold improvement in transformation efficiency over our spheroplast transformation methods. We have used a similar approach in developing an electroporation-transformation method for *B. lactofermentum* (see Table 3 for transformation efficiencies), where the pre-treatment conditions differ from those required for *C. glutamicum* (J. A. Haynes & M. L. Britz, unpublished).

The number of cells killed during electroporation was variable. Using the 0.4 cm cuvettes at 6.25 kV cm⁻¹, less than 30% of cells were killed, whereas when the 0.2 cm cells were used at 12.5 kV cm⁻¹, up to 50% of cells were killed. With *E. coli* the maximum transformation frequencies were obtained when 50–75% of cells were killed (Dower *et al.*, 1988).

Restriction barriers to transformation

Table 3 indicates that restriction barriers exist between *C. glutamicum*, *B. lactofermentum* and *E. coli*. *C. glutamicum* and *B. lactofermentum* are sufficiently related to enable both to use the origins of replication of pUL340 and pCSL17. However, the barrier to transformation was as great between these two species as that seen for *E. coli*-derived DNA.

Other workers have also found that restriction barriers exist between *E. coli*, *B. lactofermentum* and *C. glutamicum*. Yeh *et al.* (1986) found that *B. lactofermentum* could be transformed at a frequency of 1.5×10^5 transformants μg^{-1} with homologously isolated DNA whereas DNA isolated from *C. glutamicum* only gave 2.8×10^1 transformants ($\mu\text{g DNA}$)⁻¹. Serwold-Davis *et al.* (1987) found that *C. glutamicum* was transformed with homologous DNA at 10^3 transformants μg^{-1} but with heterologous DNA from *C. diphtheriae* and *E. coli* only 10^1 transformants μg^{-1} and 10^{-1} transformants ($\mu\text{g DNA}$)⁻¹ were obtained, respectively.

Conclusions

We have found electroporation to be an efficient and rapid method of transforming *C. glutamicum*. The frequency and efficiency of transformation are comparable with those obtained by other authors using spheroplast or protoplast transformation (Yoshihama *et al.*, 1985; Thierbach *et al.*, 1988). Using the same strains and plasmids, we have found an increase in transformation efficiency of 10- to 100-fold using electroporation, relative to spheroplast or protoplast transformation methods.

Several factors influence the number of transformants obtained. There is a linear relationship between both the concentration of the cell suspension and the amount of DNA added and number of cells transformed. The maximum voltage and capacitance available using the Bio-Rad Gene Pulser were required to achieve high levels of transformation. The addition of glycine (2.5–4.5%) and INH (4 mg ml⁻¹) to the growth medium and harvesting cells in early exponential phase were found to be important prerequisites for efficient electroporation. Finally, the recovery medium on which the transformants were isolated had a significant effect on the number of transformants obtained. The osmotically protective (ET-Km) medium was better than NAG-Km or LAG-Km as a recovery medium suggesting that cells successfully transformed by electroporation were electrochemically or osmotically sensitive.

We have found that, despite the restriction barriers which exist between *C. glutamicum*, *B. lactofermentum* and *E. coli*, we were able to transform heterologously isolated DNA between species, although at a reduced frequency.

We are grateful to Gregory Kemp for capable technical assistance. We also thank Professors J. F. Martín, A. Sinskey and P. Rogers for providing some of the corynebacterial plasmids and strains.

References

- ALLEN, S. P. & BLASCHEK, H. P. (1988). Electroporation-induced transformation of intact cells of *Clostridium perfringens*. *Applied and Environmental Microbiology* **54**, 2322–2324.
- BEST, G. R. & BRITZ, M. L. (1986). Facilitated protoplasting in certain auxotrophic mutants of *Corynebacterium glutamicum*. *Applied Microbiology and Biotechnology* **23**, 288–293.
- BRITZ, M. L. & BEST, G. R. (1986). Expression of chloramphenicol resistance specified by plasmid pHY416 hosted in *Corynebacterium glutamicum*. *Current Microbiology* **14**, 13–17.
- CHASSY, B. M. & FLICKINGER, J. L. (1987). Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiology Letters* **44**, 173–177.
- DOWER, W. J., MILLER, J. F. & RAGSDALE, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* **16**, 6127–6145.
- HAMMES, W., SCHLEIFER, K. H. & KANDLER, O. (1973). Mode of action of glycine on the biosynthesis of peptidoglycan. *Journal of Bacteriology* **116**, 1029–1053.
- KATSUMATA, R., OZAKI, A., OKA, T. & FURUYA, A. (1984). Protoplast transformation of glutamate-producing bacteria with plasmid DNA. *Journal of Bacteriology* **159**, 306–311.
- MAEKURA, R. (1984). Effect of isonicotinic acid hydrazide on mycolic acid synthesis in *Nocardia rubra*. *Kekkaku* **59**, 435–440.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARTIN, J. F., SANTAMARIA, R., SANDOVAL, H., DEL REAL, G., MATEOS, L. M., GIL, J. A. & AGUILAR, A. (1987). Cloning system in amino acid-producing corynebacteria. *Biotechnology* **5**, 137–147.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- MILLER, J. F., DOWER, W. J. & TOMKINS, L. S. (1988). High voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 856–860.
- POWELL, I. B., ACHEN, M. G., HILLIER, A. J. & DAVIDSON, B. E. (1988). A simple and rapid method for genetic transformation of lactic streptococci by electroporation. *Applied and Environmental Microbiology* **54**, 655–660.
- RALEIGH, E. A. (1987). Restriction and modification *in vivo* by *Escherichia coli* K12. *Methods in Enzymology* **152**, 130–141.
- SANTAMARIA, R., GIL, J. A., MESAS, J. M. & MARTIN, J. F. (1984). Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. *Journal of General Microbiology* **130**, 2237–2246.
- SANTAMARIA, R. I., GIL, J. A. & MARTIN, J. F. (1985). High-frequency transformation of *Brevibacterium lactofermentum* by plasmid DNA. *Journal of Bacteriology* **162**, 463–467.
- SERWOLD-DAVIS, T. M., GROMAN, N. & RABIN, M. (1987). Transformation of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Corynebacterium glutamicum*, and *Escherichia coli* with the *C. diphtheriae* plasmid pNG2. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 4964–4968.
- THIERBACH, G., SCHWARZER, A. & PUHLER, A. (1988). Transformation of spheroplasts and protoplasts of *Corynebacterium glutamicum*. *Applied Microbiology and Biotechnology* **29**, 356–362.
- TOMIYASU, I. & YANO, I. (1984). Isonicotinic acid hydrazide induced changes and inhibition in mycolic acid synthesis in *Nocardia* and related taxa. *Archives of Microbiology* **137**, 316–323.
- YEH, P., OREGLIA, J., PREVOTS, F. & SICARD, A. M. (1986). A shuttle vector system for *Brevibacterium lactofermentum*. *Gene* **47**, 301–306.
- YOSHIHAMA, M., HIGASHIRO, K., RAO, E. A., AKEDO, M., SHANABRUCH, W. G., FOLLETTIE, M. T., WALKER, G. C. & SINSKEY, A. J. (1985). Cloning vector system for *Corynebacterium glutamicum*. *Journal of Bacteriology* **162**, 591–597.