Protoplast Transformation of Glutamate-Producing Bacteria with Plasmid DNA

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A method for polyethylene glycol-induced protoplast transformation of glutamate-producing bacteria with plasmid DNA was established. Protoplasts were prepared from cells grown in the presence of penicillin by treatment with lysozyme in a hypertonic medium. The concentration of penicillin during growth affected the efficiency of transformation, regeneration, and polyethylene glycol-induced DNA uptake of protoplasts. Regeneration of protoplasts was accomplished on a hypertonic agar medium containing sodium succinate and yeast extract. The spectinomycin and streptomycin resistance plasmid pCG4, originally from Corynebacterium glutamicum T250, could transform various glutamate-producing bacteria such as C. glutamicum, Corynebacterium herculis, Brevibacterium flavum, and Microbacterium ammoniaphilum. The plasmid was structurally unchanged and stably maintained in new hosts. The transformation frequency of most competent protoplasts with pCG4 DNA isolated from primary transformants was high (ca. 10⁶ transformants per μg of covalently closed circular DNA) but was still two orders of magnitude below the frequency of transformation with modified DNA of the bacteriophage φCG1. The difference was ascribed to the involvement of regeneration in transformation.

The glutamate-producing bacteria, represented by Corynebacterium glutamicum, are industrially important microorganisms, since they are used in fermentative production of various amino acids, as well as glutamic acid. Although extensive studies on these bacteria have so far been devoted to biochemical analysis of the regulatory mechanisms in amino acid biosyntheses to improve their yields (16), there have been few studies on the gene transfer system, except for only two reports: one on protoplast fusion (12) and one on phage-mediated transduction (13) in Brevibacterium flavum, one of the typical glutamate-producing bacteria. The development of molecular cloning systems in these bacteria will not only enable us to study their genetic organization but also should provide a powerful new tool for future strain improvements. Despite the shortage of genetic information available for this approach, interest in both academic and industrial applications encouraged us to undertake the establishment of a gene cloning system with C. glutamicum as host.

One prerequisite for any cloning system is to develop a method for introducing DNA into the host organism. Since genetic transformations of polyethylene glycol-treated protoplasts have been successful in such diverse organisms as Bacillus subtilis (5), other Bacillus species (4, 11, 14), Streptomyces species (3), and Saccharomyces cerevisiae (9), a similar system might also be applicable to C. glutamicum. Actually, transformation of C. glutamicum protoplasts with phage DNAs occurred in the presence of polyethylene glycol plus divalent cations (A. Ozaki et al., submitted for publication). However, this DNA uptake system does not necessarily allow transformation with plasmid DNA at a frequency as high as that of transfection, because regeneration of protoplasts is involved in transformation. Therefore, we established conditions for preparation of protoplasts which possess high DNA uptake and regeneration abilities. This report provides the first plasmid transformation system available for glutamate-producing bacteria, including not only C. glutamicum but also other coryneform bacteria such as B. flavum and Microbacterium ammoniaphilum.

MATERIALS AND METHODS

Bacterial strains, plasmid, and phage. Eight independent wild-type glutamate-producing bacteria were used. The strains obtained from the American Type Culture Collection were as follows: C. glutamicum ATCC 13032, Corynebacterium herculis ATCC 13868, B. flavum ATCC 14067, and M. ammoniaphilum ATCC 15354. The other strains (T250, T218, T106, and KY9005), identified as C. glutamicum according to the proposal of Abe et al. (1), were from our collection. C. glutamicum T250 (ATCC 31830) harbors a plasmid of 29 kilobases named pCG4, which specifies resistance determinants to streptomycin and spectinomycin. The temperate bacteriophage φCG1 was originally isolated from its lysogen, C. glutamicum T36, and was infective to C. glutamicum T106.

Media and culture conditions. BY medium (0.3% NaCl, 0.5% yeast extract, 0.7% meat extract, 1% peptone) and BY agar (1.6%) medium were used for routine bacterial growth. MMYE medium [glucose, 20 g; (NH₄)₂SO₄, 10 g; urea, 3 g; yeast extract, 1 g; KH₂PO₄, 1 g; MgSO₄ · 7H₂O, 0.4 g; FeSO₄ · 7H₂O, 2 mg; MnSO₄ · 4H₂O to 6H₂O, 2 mg; NaCl, 50 mg; biotin, 50 μg; thiamine, 200 μg; deionized water, 1 liter (adjusted to pH 7.2)] was used for growth to prepare protoplasts and isolate the plasmid. For lysozyme digestion of intact cells and dilution of protoplasts, hypertonic medium RCG was used, which had the following composition: glucose, 5 g; Casamino Acids, 5 g; yeast extract, 2.5 g; KH₂PO₄, 3.5 g; MgCl₂ · 6H₂O, 0.4 g; biotin, 30 μg; thiamine · HCl, 2 mg; polyvinylpyrrolidone K15 (average molecular weight, 10,000; Tokyo Kasei Co., Tokyo, Japan), 30 g; sodium succinate, 135 g; trace element solution, 1 ml; agar, 16 g; and deionized water, 1 liter (adjusted to pH 7.6). The trace element solution contained 88 mg of Na₃B₂O₅ · 7H₂O, 37 mg of (NH₄)₆Mo₇O₂₇ · 4H₂O, 8.8 mg of

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ZnSO₄ 4H₂O, 270 mg of CuSO₄ 5H₂O, 7.2 mg of MnCl₂ 4H₂O, and 970 mg of FeCl₂ 6H₂O in a liter of deionized water. RCGA medium (RCG medium solidified with 1% agar) was inoculated with strain Kaneko (ca. 2 × 10⁶ cells per ml) was transferred to a tube, centrifuged, and suspended in 0.1 ml of cooled TSMC buffer. Plasmid or phage DNA in 100 μl of a mixture (1:1) of TES and two-fold-strength TSMC buffer was mixed with protoplasts, which was followed by addition of 1.8 ml of 20% polyethylene glycol 6,000 dissolved in TSMC buffer. The mixture was gently mixed, incubated on ice for 5 min, and then subjected to a heat pulse at 37°C for 3 min.

For transfection, serial dilutions of the above protoplasts were plated in PS soft-agar overlays together with intact indicator cells. Transfection efficiency was determined by the PFU per milliliter after overnight incubation. In transformation with pCG4, the protoplasts were harvested, washed once with 3 ml of TSMC buffer, and resuspended in 1 ml of RCG medium (pH 7.2). The suspension was incubated for 2 h to allow phenotypic expression of genetic determinants on the plasmid. Diluted or concentrated protoplasts were plated on RCGA medium containing 400 μg of spectinomycin per ml. After incubation for 10 to 14 days, spectinomycin-resistant colonies were scored and subsequently identified as pCG4 transformants by checking for resistance to spectinomycin (100 μg/ml) and streptomycin (12.5 μg/ml) on BY agar plates.

Analysis of plasmids. Digestion of plasmid DNA with restriction endonuclease BanHI (Takara Shuzo Co., Kyoto, Japan) was performed according to the specifications of the supplier. Agarose gel electrophoresis was carried out in a horizontal slab gel of 1.0% agarose in 40 mM Tris-acetate (pH 8.0)–20 mM sodium acetate–2 mM EDTA–0.6 mg of ethidium bromide per ml at 100 V for 3 h. Plasmids and their restricted fragments were detected under UV light and photographed.

Assay of streptomycin-inactivating enzyme. Streptomycin-inactivating activity was assayed by the microbiological method of Kawabe and Mitsuhashi (13). To facilitate disruption of cells, crude extracts were prepared from protoplasts as follows. After being washed with TSMC buffer, the protoplasts were suspended in 1/10 volume of the original culture by the addition of TMK buffer (100 mM Tris-hydrochloride [pH 7.8], 60 mM KCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol) and sonicated briefly. The extract was clarified by centrifugation at 30,000 × g for 30 min. The supernatant fluid was used as crude enzyme. The reaction mixture consisted of the following materials: 0.2 ml of crude enzyme solution (10 mg of protein per ml), 0.1 ml of 0.1 M ATP–Na₂ solution, 0.6 ml of TMK buffer,
and 0.1 ml of dihydrostreptomycin solution. The reaction was carried out at 35°C for 60 min and then stopped by heating at 80°C for 3 min. The activity of the antibiotic remaining in the reaction mixture was determined by bioassay with *B. subtilis* as the test organism.

**RESULTS**

**Effect of penicillin pretreatment on protoplast formation.**

The effect of Pen G on protoplast formation was investigated with *C. glutamicum* T106 (Table 1). Pen G inhibited growth at higher concentrations than 0.25 U/ml. Cells grown in the presence of Pen G were more or less converted to spherical forms by the subsequent lysozyme digestion in hypertonic RCG medium, whereas cells grown in the absence of Pen G remained rod shaped with the same treatment. Efficient conversion to spherical forms was observed in the cells treated with Pen G at concentrations over 0.5 U/ml. As the lysozyme digestion proceeded, the number of spherical forms gradually increased and reached a maximum (85 to 90%) by 8 to 10 h of incubation and then remained fairly constant until at least 24 h. The rest exhibited irregularly swollen shapes different from the original rod shape (data not shown). The proportion of osmotically stable cells fell to less than 10⁻³. These observations indicated that the spherical cells were true protoplasts and that the irregularly swollen cells were presumably spheroplasts. Only the exposure to 1.0 U of Pen G per ml, the highest concentration tested, appeared to cause partial lysis during lysozyme digestion.

Similar protoplast formations depending on Pen G treatment were also observed in other glutamate-producing strains (data not shown). When some strains were digested with lysozyme in a hypertonic solution containing 0.41 M sucrose, as was described by Kaneko and Sakaguchi (12), irreversible agglutination of cells occurred and disturbed further operations. The replacement of sucrose with 0.5 M sodium succinate eliminated the agglutination.

**DNA uptake and regeneration abilities of protoplasts from cells treated with various concentrations of penicillin.** Because transfectants have to be recovered through regeneration of protoplasts after uptake of DNA, transformation efficiency is affected by both DNA uptake and regeneration processes. Therefore, we assessed the above protoplasts of strain T106 with respect to their ability to take up DNA by transfection and regenerate on RCGA plates (Table 1). With increasing Pen G concentrations in pretreatment, the regeneration frequency of subsequently induced protoplasts decreased. The protoplasts from cells treated with 0.5 U of Pen G per ml gave the maximum transfection frequency (10⁷ PFU/ml), indicating the highest DNA uptake ability. These results suggest that treatment of cells with 0.5 U of Pen G per ml yielded the most favorable protoplasts for transformation. This concentration of Pen G was used throughout the subsequent experiments described herein.

**Requirements for regeneration of protoplasts.** Protoplast regeneration is also affected by the composition of a hypertonic medium. Therefore, we tested regeneration media for protoplasts of *C. glutamicum* T106, with special reference to those developed for *B. subtilis* (5) and *B. flavum* (12) (Table 2). The effect of osmotic stabilizers on protoplast regeneration was initially examined by using a semisynthetic medium. Sorbitol failed to support regeneration at 0.6 and 0.8 M. Sucrose with a concentration over 0.5 M gave rise to unstable translucent colonies with a frequency about 10-fold higher than that of osmotically resistant cells. These colonies appeared to grow in a protoplastic state, because they could not grow upon transfer to hypotonic plates. The addition of 0.3 to 0.7 M sodium succinate increased remarkably the efficiency of regeneration (2 to 6%). Colonies formed on plates with 0.3 M sodium succinate were osmotically sensitive, as was the case with sucrose, but those formed at concentrations higher than 0.5 M were osmotically resistant and judged to be regenerated intact cells. Colonies developed faster at 0.5 M than at 0.7 M sodium succinate. Therefore, we added 0.5 M sodium succinate as the osmotic stabilizer.

Although strain T106 was prototrophic, its protoplasts could not regenerate on the chemically defined medium which contained NH₄Cl as the sole nitrogen source. The substitution of yeast extract for the nitrogen source resulted in efficient regeneration, and the sizes of regenerated colonies depended on its concentration. Casamino Acids alone

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**TABLE 1.** Formation and characterization of *C. glutamicum* T106 protoplasts

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Protoplast formation</th>
<th>Spherical cells (%)</th>
<th>Proportion of osmotically stable cells (%)</th>
<th>Regeneration frequency (%)</th>
<th>Transfection (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 U/ml Pen G added</td>
<td>0/96</td>
<td>9.8 x 10⁻¹</td>
<td>97.4</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>0.1 U/ml Pen G added</td>
<td>96/96</td>
<td>1.7 x 10⁻¹</td>
<td>53.7</td>
<td>8.7 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>0.25 U/ml Pen G added</td>
<td>96/96</td>
<td>3.1 x 10⁻³</td>
<td>10.2</td>
<td>2.1 x 10⁷</td>
<td></td>
</tr>
<tr>
<td>0.5 U/ml Pen G added</td>
<td>103/97</td>
<td>4.2 x 10⁻⁴</td>
<td>6.4</td>
<td>1.4 x 10⁸</td>
<td></td>
</tr>
<tr>
<td>0.75 U/ml Pen G added</td>
<td>116/116</td>
<td>4.5 x 10⁻⁴</td>
<td>1.7</td>
<td>1.2 x 10²</td>
<td></td>
</tr>
<tr>
<td>1 U/ml Pen G added</td>
<td>114/101</td>
<td>3.8 x 10⁻⁴</td>
<td>0.1</td>
<td>6.2 x 10⁻⁶</td>
<td></td>
</tr>
</tbody>
</table>

* Cells from each culture were suspended in RCG medium containing lysozyme (1 mg/ml) and incubated at 30°C for 16 h.

* Added to MMYE culture early in the logarithmic phase of growth.

* The time required for doubling of OD₆₅₀.

* Percent of spherical cells counted under a phase-contrast microscope.

* Osmotically stable cells per initial viable cell subjected to the treatment with lysozyme.

* Percentage of colonies regenerated on RCGA plates to the initial viable cells subjected to the treatment with lysozyme.

* Protoplast preparation containing 1.5 x 10⁶ cells was used in each transfection with 0.05 µg of pCG1 DNA extracted from the phage grown on *C. glutamicum* T106. Transfection was determined by counting PFU on the indicator lawn with intact cells of *C. glutamicum* T106, as described in the text.
Table 3. Transformation of various glutamate-producing bacteria with pCG4 DNA

<table>
<thead>
<tr>
<th>Expt</th>
<th>DNA source</th>
<th>Recipient protoplastsa</th>
<th>Regeneration frequency (%)</th>
<th>Spc' clonesb/ml</th>
<th>Coinheritance of Sm' (%)c</th>
<th>Transformation frequencyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T250</td>
<td>C. glutamicum T106</td>
<td>5.3</td>
<td>9.2 x 10^4</td>
<td>100</td>
<td>1.2 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. glutamicum ATCC 13032</td>
<td>3.5</td>
<td>2.4 x 10^4</td>
<td>83</td>
<td>3.8 x 10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. glutamicum T218</td>
<td>6.7</td>
<td>1.9 x 10^2</td>
<td>100</td>
<td>1.8 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. glutamicum KY9005</td>
<td>3.1</td>
<td>9.0 x 10^4</td>
<td>98</td>
<td>1.9 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. hercules ATCC 13868</td>
<td>7.1</td>
<td>4.1 x 10^2</td>
<td>100</td>
<td>3.8 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. Flavum ATCC 14067</td>
<td>4.3</td>
<td>3.2 x 10^4</td>
<td>94</td>
<td>5.0 x 10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M. ammoniophilum ATCC 15354</td>
<td>4.5</td>
<td>2.6 x 10^4</td>
<td>69</td>
<td>2.7 x 10^-7</td>
</tr>
</tbody>
</table>

II T250 | C. glutamicum T106 | 6.2 | 1.1 x 10^2 | 100 | 1.2 x 10^-6 |
| T106 | C. glutamicum T106 | 6.2 | 3.5 x 10^4 | 100 | 4.6 x 10^-4 |

a Protoplasts were induced from cells pretreated with 0.5 U of Pen G per ml. A protoplast preparation containing about 1.5 x 10^7 input cells was used initially.
b Spc' clones were selected directly on RCGA plates containing 400 μg of spectinomycin per ml.
c All or 50 randomly selected Spc' clones were scored for coinheritance of streptomycin resistance (Sm').
d Number of Spc' Sm' clones per regenerated cell.

did not support regeneration, but their addition to the regeneration medium containing low concentrations of yeast extract increased the regeneration rate. Polyvinylpyrrolidone tended to make regeneration more reproducible. Based on these results, RCGA medium was established as a regeneration medium.

Regeneration of protoplasts was slow and asynchronous. Colonies became visible after 3 days and increased in number until day 10. Protoplasts of strain T106 and other glutamate-producing strains regenerated on RCGA medium at frequencies of 3 to 7% (Tables 2 and 3).

Transformation of protoplasts with plasmid pCG4. The success in efficient transfection and regeneration of protoplasts prompted us to try transformation with plasmid pCG4, which is indigenous to C. glutamicum T250. The plasmid encodes resistance to spectinomycin and streptomycin, which has enabled us to identify transformants easily.

Protoplasts of glutamate-producing bacteria were transformed with 0.1 μg of pCG4 DNA prepared from C. glutamicum T250. After incubation for phenotypic expression, protoplasts were plated on RCGA agar medium containing 400 μg of spectinomycin per ml. Spectinomycin-resistant (Spc') colonies developed in all the strains with frequencies of 10^-6 to 10^-7 (Table 3, experiment I). Each protoplast preparation plated on the same selective plates after the same treatment in the absence of pCG4 gave spontaneous Spc' colonies at frequencies of 10^-7 to 10^-8. These colonies were all sensitive to streptomycin, whereas a substantial part or all of the Spc' colonies arising from pCG4-treated protoplasts of each strain were resistant to streptomycin. All the double-resistant colonies derived from each strain showed the same MICs against spectinomycin and streptomycin as those for C. glutamicum T250, the original pCG4-carrying strain (Table 4). The streptomycin-inactivating enzyme of these clones had activity comparable with that found in C. glutamicum T250, but untransformed recipient cells did not show any activity. These results suggest that the double-resistant clones were pCG4 transformants.

The presumptive pCG4 transformants were examined for the presence of CCC DNA. All yielded CCC DNA bands in cesium chloride-ethidium bromide density gradient. When subjected to electrophoresis, CCC DNA preparations from double-resistant clones of M. ammoniophilum ATCC 15354, C. glutamicum KY9005 and C. glutamicum T218 gave two plasmids bands, one corresponding to the CCC pCG4 DNA and the other indigenous to each host (Fig. 1). Complete digestion of these plasmids with the BamHI restriction endonuclease gave seven common fragments which were found in the BamHI digest of pCG4. Judging from the amount of plasmid DNA preparation loaded and the intensity of the bands corresponding to the BamHI fragments of pCG4, pCG4 appeared to exist in all the transformants tested with a copy number similar to that in the original host. The stability of pCG4 in the transformants was checked by growth for about 10 generations in the absence of spectinomycin. No sensitive colonies were detected among 300

Table 4. Antibiotic resistance conferred by pCG4 in glutamate-producing bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>MICs (μg/ml)</th>
<th>Streptomycin-inactivating activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spectinomycin</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>C. glutamicum T250</td>
<td>&gt;1.600</td>
<td>200</td>
</tr>
<tr>
<td>C. glutamicum T106</td>
<td>25</td>
<td>3.2</td>
</tr>
<tr>
<td>C. hercules ATCC 13868</td>
<td>25</td>
<td>1.6</td>
</tr>
<tr>
<td>B. Flavum ATCC 14067</td>
<td>25</td>
<td>3.2</td>
</tr>
<tr>
<td>M. ammoniophilum ATCC 15354</td>
<td>25</td>
<td>1.6</td>
</tr>
<tr>
<td>C. glutamicum T106(pCG4)</td>
<td>&gt;1.600</td>
<td>200</td>
</tr>
<tr>
<td>C. hercules ATCC 13868(pCG4)</td>
<td>&gt;1.600</td>
<td>200</td>
</tr>
<tr>
<td>B. Flavum ATCC 14067(pCG4)</td>
<td>&gt;1.600</td>
<td>200</td>
</tr>
<tr>
<td>M. ammoniophilum ATCC 15354(pCG4)</td>
<td>&gt;1.600</td>
<td>200</td>
</tr>
</tbody>
</table>

a One loopful of each cell suspension diluted to ca. 10^6 cells was inoculated onto BY agar plates containing a series of twofold dilutions of the drug. MICs were determined after 24 h of incubation.
colonies tested for each strain. These results show that pCG4 was transferred, expressed, and stably maintained in a variety of glutamate-producing bacteria.

The transformation frequency of *C. glutamicum* T106 protoplasts with pCG4 prepared from *C. glutamicum* T250 was much lower than the transfection frequency with modified pCG1 DNA. This suggests the involvement of a restriction modification system in *C. glutamicum* T106. To test this, the T106 protoplasts were transformed with pCG4 prepared from T and T250 (Table 3, experiment II). The transformation frequency with pCG4 from strain T106 was two orders of magnitude higher than that with pCG4 from strain T250, indicating the presence of a restriction modification system in *C. glutamicum* T106.

The effect of DNA concentration on transfection and transformation of *C. glutamicum* T106 protoplasts was examined with modified pCG1 and pCG4 DNA. The frequencies of both transfection and transformation were linearly increased in response to increasing DNA concentration (0.1 to 10 ng) and saturated at around 50 ng/ml. The transformation frequency was two orders of magnitude lower than that of transfection at any concentration tested. Within a linear range, the frequencies of transfection and transformation per μg of DNA were about $10^6$ PFU and $5 \times 10^6$ transformants, respectively. The difference is explained by the additional requirement of the regeneration process for transformation.

**DISCUSSION**

Kaneko and Sakaguchi have reported that *B. flavum* cells grown below inhibitory concentrations of penicillin became sensitive to lysozyme and were converted to protoplasts under hypertonic conditions. These protoplasts were fused and regenerated to give fusions (12). Protoplasts of various glutamate-producing bacteria prepared by this method have been found to be transfected by phage DNA (Ozaki et al., submitted for publication). Extending the transfection procedure, we established a plasmid transformation system for these bacteria by using plasmid pCG4, which specifies resistances to spectinomycin and streptomycin. Although basically similar to those developed for protoplast transformation in *B. subtilis* and *Streptomyces* species (3), this system needs penicillin pretreatment, which was indispensable to protoplast formation, as noted by Kaneko and Sakaguchi (12), and critically affected the ability of resultant protoplasts to take up DNA and to regenerate, as demonstrated in this study.

Transformation requires both DNA uptake and regeneration, whereas transfection requires only the uptake. Therefore, we tried to maximize the product of the two successive events (DNA uptake and regeneration) by adjusting the concentration of penicillin in the pretreatment and the composition of the regeneration medium. The resultant protoplasts, however, still regenerated less efficiently compared with protoplasts of *B. subtilis* (5, 7) and *Streptomyces* species (2, 17). The possibility that colonies appearing faster interfere with the regeneration of nearby protoplasts, as observed with *B. subtilis* (6) and *Streptomyces* species (2), was ruled out, because the regeneration frequency did not change upon plating dilutions of transformation mixtures. Another possibility, that the regeneration medium was still inappropriate, cannot be denied completely but seems unlikely, because further manipulations of the medium, including increasing the amount of yeast extract (which was the most effective factor in it), failed to improve the frequency. Thus, we consider it more likely that pretreatment with penicillin resulted in its covalent binding to membrane proteins involved in cell wall synthesis, as found in all eubacteria examined (22), thereby impairing protoplast regeneration. This may also explain the relatively slow regeneration. In contrast, protoplasts of *B. subtilis* and *Streptomyces* species can be prepared without penicillin treatment and thus regenerate more efficiently.

The transfection frequency of protoplasts of *C. glutamicum* T106 with pCG1 DNA ($10^{-2}$ per input cell) is comparable to the transformation frequency with plasmid DNAs in *B. subtilis* and *Streptomyces* protoplasts (3, 5). This indicates that *C. glutamicum* T106 protoplasts are as competent as other protoplasts in DNA uptake. The transformation frequency of *C. glutamicum* T106 with pCG4, however, is more than two orders of magnitude less than the transfection frequency, even if the modified pCG4 was used. The possibility that 2 h of incubation is not sufficient for the expression of drug resistance is unlikely, because the transformation frequency in the direct selection used throughout this study was almost identical to that of the indirect selection, in which transformed protoplasts were first allowed to regenerate on a nonselective medium and then selected for spectinomycin resistance (data not shown). This lower frequency of transformation is probably due to the fact that it requires regeneration, which occurred with an efficiency just corresponding to the ratio between transformation and transfection. The poor regeneration inherent in the penicillin treatment is serious but still allows us to obtain the desired transformants at a reasonable frequency. To circumvent this defect, we obtained a mutant which can be converted to protoplasts without penicillin treatment (manuscript in preparation).

The transformation frequencies differed widely according to the source of pCG4 DNA. The frequency of *C. glutamicum* T106 with pCG4 isolated from the same host was over 100 times higher than that with pCG4 from *C. glutamicum* T250.
T250, the native host, indicating the presence of a restriction modification system in *C. glutamicum* T106. The relatively low transformation frequencies of other strains with pCG4 from *C. glutamicum* T250 may also be due to their restriction modification system.

To construct a recombinant DNA technology, we need a transformation procedure and vectors. The protoplast-mediated transformation system presented here will facilitate the construction of a plasmid-based cloning system for this industrially important group of glutamate-producing bacteria, which will eventually be used for gene cloning, as has been successfully done with a similar transformation system in *Streptomyces* species (20, 21). For cloning, a plasmid vector with a selectable marker is desirable, but such plasmids have not been reported for these bacteria. Plasmid pCG4 can stably replicate and express its spectinomycin and streptomycin resistance genes in these bacteria. Because of its large size (29 kilobases) and multiple sites for various restriction endonucleases (unpublished data), pCG4 itself is not a practical cloning vector. Instead, a smaller derivative of pCG4 and recombinant plasmids containing a part of pCG4, especially a fragment coding for spectinomycin or streptomycin resistance, or both, is more suitable as a cloning vector. The construction of these vectors will be reported.

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LITERATURE CITED