Cloning Vector System for *Corynebacterium glutamicum*

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A protoplast transformation system has been developed for *Corynebacterium glutamicum* by using a *C. glutamicum*-*Bacillus subtilis* chimeric vector. The chimera was constructed by joining a 3.0-kilobase cryptic *C. glutamicum* plasmid and the *B. subtilis* plasmid pBD10. The neomycin resistance gene on the chimera, pHY416, was expressed in *C. glutamicum*, although the chloramphenicol resistance gene was not. The various parameters in the transformation protocol were analyzed separately and optimized. The resulting transformation system is simple and routinely yields 10⁴ transformants per µg of plasmid DNA.

Coryneform bacteria are a taxonomically ill-defined group of gram-positive bacteria with a rod or club shape. They occupy a variety of ecological niches and display an even broader array of interesting and useful properties. Included among the coryneform bacteria are plant pathogens (6, 28), animal pathogens (2), and nonpathogenic soil bacteria used for the industrial production of amino acids (1). Other strains utilize hydrocarbons (5, 11), synthesize emulsifying agents (14, 30), produce antitumor activity (22), and carry out steroid conversions (10).

Despite the scientific and practical importance of coryneform bacteria, they remain relatively uncharacterized. Taxonomic classification of coryneform bacteria is steadily improving (15, 27, 28), but it is still difficult to predict the properties of a coryneform strain based on its genus or species designation. Two strains assigned to the same genus or even the same species sometimes have significantly different cell wall structures or guanine-plus-cytosine contents (4, 28). This taxonomic confusion has undoubtedly been a factor in thwarting attempts to develop classical genetic exchange systems or recombinant DNA systems in coryneform bacteria.

The lack of powerful genetic tools has placed severe limitations on the experimental approaches available to those studying coryneform bacteria, especially at the molecular level. However, it has been suggested in numerous reports that these restrictions could be overcome. Bacteriophages are known which infect certain plant pathogenic species, and plasmids occur in several coryneform species including the plant pathogen *Corynebacterium xerosis* (20) and the human pathogen *Corynebacterium diphtheriae*, in which some plasmids are associated with an increased resistance to erythromycin (25). These results offer promise for the development of both transduction and conjugation systems in coryneform bacteria.

Recently, transformation of coryneform bacteria has been reported with an endogenous 29-kilobase (kb) plasmid encoding spectinomycin and streptomycin resistance (18). This report describes the development of a cloning technology based on a polyethylene glycol (PEG)-mediated uptake of a 9.4-kb *Corynebacterium glutamicum*-*Bacillus subtilis* shut-

tle vector and represents the first report of the cloning and expression of foreign genes in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in this study are described in Table 1. The coryneform strains screened for the presence of plasmids are summarized in Table 2. *C. glutamicum* ATCC 19223 contains a 3.0-kb plasmid designated pSR1. *C. glutamicum* AS019 (Table 1) is a spontaneous rifampin-resistant derivative of *C. glutamicum* ATCC 13059 which was obtained by plating 0.1 ml of an overnight culture of strain ATCC 13059 on LB agar containing 10 µg of rifampin per ml. AS019 is resistant to 50 µg of rifampin per ml.

Table 1 also describes the plasmids used in this work. pBD10 is a *B. subtilis* vector. pHY416 and pHY47 are the products of recombinant DNA experiments described below. All other plasmids were newly identified in the strains listed in Table 2.

Media and buffers. All bacterial strains were grown in LB medium unless stated otherwise (23). LB agar is LB medium containing 15 g of agar per liter. TE is 10 mM Tris-1 mM Na₂EDTA (pH 8.0). SMMC buffer is 0.5 M sorbitol-20 mM maleate-20 mM MgCl₂-20 mM CaCl₂ (pH 7.0). SB medium is LB medium containing 0.5 M sorbitol, 20 mM MgCl₂, and 20 mM CaCl₂. SB medium was made by mixing equal volumes of twofold concentrated LB medium with 1 M sorbitol (pH 7.0) and then adding the appropriate volumes of 1 M MgCl₂ and 1 M CaCl₂ after sterilization. SB agar is SB medium containing 10 g of agar per liter. The agar was sterilized with the concentrated LB medium. SBK agar is SB agar containing 15 µg of kanamycin sulfate per ml. A 50% PEG solution was prepared in 0.5 M sorbitol and adjusted to pH 7.0 before sterilization.

Plasmid screening of coryneform strains. Two procedures were used to determine whether a coryneform strain harbored any plasmids. The first method was a modification of the one reported by Schiller et al. (25) for *C. diphtheriae*. Cultures (10 ml) were grown to early stationary phase at 30°C, washed with 5 ml of 10 mM Tris (pH 8.0), and suspended in 1 ml of 0.5 M sucrose-100 mM Tris (pH 8.0) containing 5 mg of lysozyme per ml. Lysozyme treatment was performed for 1 h at 37°C with shaking. Cells were then harvested and suspended in 280 µl of 10 mM Tris-10 mM EDTA (pH 8.0). Diethyl pyrocarbonate (2 µl) was added, and the cells were lysed by the addition of 30 µl of 10%

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TABLE 1. Bacterial strains and plasmids used for chimera constructions

Strain (plasmid)	Plasmid	Source
<i>B. subtilis</i>		
B-15306	None	Northern Regional Research Center, U.S. Department of Agriculture
B-15306	pBD10	Northern Regional Research Center, U.S. Department of Agriculture
<i>C. glutamicum</i>		
ATCC 19223	pSR1	American Type Culture Collection
ATCC 13059	None	American Type Culture Collection
AS019	None	Spontaneous Rif ^r isolate of ATCC 13059

sodium dodecyl sulfate in TE buffer. The cell suspension was mixed by gentle inversion and left for 15 min at room temperature. Sodium acetate (30 μ l; 5 M [pH 6.0]) was added, and the tubes were held on ice for 1 h to precipitate all debris. The precipitate was removed by centrifugation in an Eppendorf microcentrifuge (13,000 $\times$ g for 10 min), and the supernatant (approximately 100 μ l) was removed. This supernatant could be subjected to electrophoretic analysis directly on agarose gels or concentrated by ethanol precipitation before analysis on agarose gels.

In the second procedure, 10-ml cultures of coryneform bacteria were harvested, suspended in 350 μ l of E buffer (40 mM Tris, 2 mM EDTA, pH adjusted to 7.9 with acetic acid), and transferred to 1.5-ml Eppendorf tubes. PEG (50 μ l; 24%) was added, and then 60 μ l of a fresh lysozyme (100 mg/ml) solution was added. Cells were incubated with lysozyme for 15 min at 37°C with occasional mixing. EDTA (50 μ l; 0.5 M) and sodium dodecyl sulfate (120 μ l; 10%) were then added to each sample, and complete lysis was achieved by placing the tubes in a boiling water bath for 2 min. Samples were placed on ice for 5 min, and cellular debris was removed by centrifugation in a microcentrifuge for 15 min. The supernatant was recovered, extracted twice with phenol, and extracted once with chloroform, and nucleic acid was precipitated by the addition of an equal volume of isopropanol. The precipitate was suspended in 50 μ l of TE and subjected to electrophoretic analysis on agarose gels to detect plasmid DNA by standard procedure (19). Crude plasmid DNA preparations isolated by this second method were efficiently digested by a variety of restriction endonucleases.

Large scale isolation of plasmid DNA. pBD10, pHY47, and pHY416 were isolated from *B. subtilis* strains grown in the presence of 15 μ g of kanamycin per ml by the procedure of Guerry et al. (16). Plasmid DNA from coryneform strains was purified by a modification of the method used for *B. subtilis* (6). Cultures (1 liter) were grown to early stationary phase, harvested, and washed with 10 mM Tris (pH 8.2), and the cell pellets were frozen at -70°C for 1 h. After thawing at room temperature, the cells were suspended in 30 ml of 0.5 M sucrose-10 mM Tris (pH 8.0) and treated with 5 mg of lysozyme per ml for 1 h at 37°C. Cells were pelleted by centrifugation and suspended in 60 ml of 10 mM Tris-10 mM EDTA (pH 8.0). The removal of the osmotic stabilizer in the latter step was essential to efficient lysis. Sodium dodecyl sulfate (7 ml; 10%) was then gently mixed into the cell suspension; lysis was generally complete after 15 min at room temperature. NaCl (8 ml; 5 M) was added, and the mixture was placed at 0°C for 4 h or overnight. The resulting precipitate of cellular debris was removed by centrifugation, and the supernatant was directly added to a cesium chloride-

ethidium bromide equilibrium density gradient with a Beckman VTi50 rotor. Plasmid DNA was recovered from the gradient by standard procedure.

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out in a horizontal gel apparatus with TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.3]). Gels were 0.8 to 1.5% agarose depending upon the size of the DNA fragments to be analyzed. *Hind*III digests of lambda DNA and ϕ X174 *Hae*III fragments were used as standards for determining the sizes of linear fragments. The supercoiled forms of plasmids pBR322, pMB9, pSC101, and pCR1 were used as markers to determine the sizes of supercoiled plasmids.

Restriction enzyme analysis. Restriction endonuclease analysis was carried out as described in the instructions of the manufacturers. The double digestions were performed as follows: after digestion with the first enzyme, the sample was heated at 65°C for 5 min, and the buffer composition was adjusted to the required conditions of the second enzyme.

Purification of plasmid DNA from agarose gels. In strains containing more than one plasmid (e.g., ATCC 19223 and ATCC 15960), each plasmid was purified from a preparative agarose gel by electroelution before restriction analysis (21).

Transformation. Competent cells of *B. subtilis* strains

TABLE 2. Plasmids identified in various coryneform strains

Strain	Approx size (kb) of plasmid(s)
<i>Corynebacterium</i> spp.	
<i>C. glutamicum</i>	
ATCC 13032	3, >25
ATCC 13058	3, >25
ATCC 13059	ND ^a
ATCC 13060	ND
ATCC 19223	3, >25
ATCC 19225	ND
ATCC 15806	ND
<i>C. alkanolyticum</i> ATCC 21511	ND
<i>C. callunae</i> ATCC 15991	ND
<i>C. hydrocarboclastum</i>	
ATCC 15960	5.2, 15
ATCC 15961	5.2, 15
ATCC 15962	5.2, 15
ATCC 15963	ND
ATCC 15968	5.2, 15
<i>Bacillus</i> spp.	
<i>B. ammoniagenes</i> ATCC 13746	ND
<i>B. glutamigenes</i> ATCC 13747	ND
<i>B. roseum</i> ATCC 13825	ND
<i>B. flavum</i>	
ATCC 13826	ND
ATCC 15940	ND
<i>B. lactofermentum</i> ATCC 13869	4.4
<i>B. linens</i>	
ATCC 9174	7.0
ATCC 13931	7.0

^a ND, None detected.

were prepared and stored frozen at -70°C by the procedure of Dubnau and Davidoff-Abelson (13). Supercoiled plasmid DNA or ligation mixtures were added to 200 μl of thawed competent cells along with 4 μl of 50 mM EGTA. After 2 min of incubation at 37°C , the cell suspension was mixed with 5 ml of tryptose blood agar base (Difco) containing 0.5% agar and plated on tryptose blood agar base containing 1.5% agar. Plates were incubated for 90 min at 30°C , and transformants were selected by overlaying with the appropriate antibiotic.

An overnight culture of AS019 was inoculated (1:100) into LB broth containing 10 μg of rifampin per ml, 2% glucose, and 2% glycine. Cells were incubated with aeration at 30°C for 15 h, and then 10-ml samples of cells were harvested by centrifugation, washed once in 5 ml of SMMC buffer, and suspended in 1 ml of SMMC buffer containing 2.5 ml of lysozyme per ml. The cell suspension was incubated at 30°C for 90 min, and cells were harvested by centrifugation and suspended in 0.9 ml of SMMC buffer. Samples (0.3 ml) of cells ("protoplasted" cells) were placed in tubes (16 by 100 mm), plasmid DNA prepared in 0.5 M sorbitol was added, 0.7 ml of 50% PEG was added, and the contents of the tubes were mixed gently. SB broth (2.0 ml) was added, and the mixture was incubated without shaking at 30°C for 3 h. Transformants were plated on selective media. Modifications of this procedure are described in the figure legends.

RESULTS

Identification and characterization of coryneform plasmids.

The first step in the development of recombinant DNA technology for *C. glutamicum* was the identification of plasmid replicons which could be used in the construction of vectors. Various coryneform strains obtained from the American Type Culture Collection were screened for the presence of plasmids by two different procedures. Each method gave identical and reproducible results (Table 2). Plasmids were detected in three related *C. glutamicum* strains, four *Corynebacterium hydrocarboclastum* strains, one *Bacillus lactofermentum* strain, and two *Bacillus linens* strains. The existence of plasmids in strains ATCC 13058, ATCC 19223, ATCC 15961, ATCC 15962, ATCC 9174, and ATCC 19391 was further verified by purifying the plasmids from cesium chloride-ethidium bromide gradients. Since ATCC 13058 and ATCC 19223 were derived from ATCC 13032, it is not surprising that all three strains have the same plasmid profile, consisting of a small 3.0-kb plasmid and two larger plasmids. However, ATCC 13059 and ATCC 13060 are also derivatives of ATCC 13032 and do not harbor any plasmids. As discussed later, this fact was used in our choice of a suitable host for transformation experiments.

All four *C. hydrocarboclastum* strains containing plasmids have a 5.2-kb plasmid and a second plasmid of approximately 15.0 kb. Restriction analysis of the plasmids purified from ATCC 15961 and ATCC 15962 suggests that these two strains harbor identical plasmids.

Since smaller plasmids are generally more useful as vectors for recombinant DNA experiments, the smallest coryneform plasmids were chosen for restriction analysis. The restriction maps of the 3.0-kb plasmid from *C. glutamicum* ATCC 19223 (pSR1) and the 4.4-kb plasmid from *B. lactofermentum* ATCC 13869 (pWS101) are shown in Fig. 1. These two plasmids appeared to be unrelated, based upon restriction analysis results, and both plasmids contain several unique restriction sites potentially useful in cloning experiments.

Construction of *B. subtilis*-*C. glutamicum* shuttle vectors. To develop a *C. glutamicum* transformation system, we

FIG. 1. Restriction maps of pSR1 and pWS101.

needed to link one of the coryneform replicons identified in Table 2 to a selectable genetic marker which is expressed in *C. glutamicum*. Since initial experiments indicated that all of the coryneform plasmids were cryptic, we chose the strategy illustrated in Fig. 2 to clone two drug resistance genes into the coryneform plasmid pSR1. pSR1 was linearized by digestion with *Bcl*I, ligated into the large 6.3-kb *Bcl*I fragment of the *B. subtilis* vector pBD10, and transformed into *B. subtilis* 1A46. (The restriction map of the pBD10 obtained from the *B. subtilis* Genetic Stock Center differed from the published map of pBD10 in that it had two *Bcl*I sites located 0.6 kb apart instead of a unique *Bcl*I site.) Recombinant plasmids containing pSR1 were identified by analyzing the plasmids from $\text{Km}^r \text{ Cm}^r \text{ Em}^s$ transformants. Most of the transformants with this phenotype simply contained a deletion derivative of pBD10 corresponding to the large *Bcl*I fragment ligated upon itself. However, two of the 23 transformants screened contained a plasmid 9.3 kb in size. Further restriction analysis showed that these two plasmids, pHY47 and pHY416, contained a single copy of pSR1 cloned into the large *Bcl*I fragment of pBD10 (Fig. 2). pHY47 and pHY416 differ only in the orientation of pSR1 with respect to the pBD10 sequences.

Development of a protoplast transformation system for *C. glutamicum*. The potential *B. subtilis*-*C. glutamicum* shuttle vectors, pHY47 and pHY416, were used successfully in the development of a protoplast transformation system for *C. glutamicum*. The initial *C. glutamicum* host used for protoplast transformation experiments was ATCC 13059. This

FIG. 2. Strategy for the cloning of pSR1 into pBD10. Solid lines indicate pSR1 sequences; open lines indicate pBD10.

strain was chosen because (i) it does not contain any detectable plasmids, (ii) it is a descendant of ATCC 13032, which is known to function as a host for the coryneform replicon (pSR1) used in the construction of pHY47 and pHY416, and (iii) it is sensitive to low levels of kanamycin and chloramphenicol.

Before attempting transformation experiments with ATCC 13059, we first established conditions for the production and regeneration of protoplasts (osmotically sensitive) from ATCC 13059. These initial conditions were similar to those described by Chang and Cohen (7) for *B. subtilis*. The major modification was growing the cells in the presence of glycine before lysozyme treatment which resulted in greater than 99% protoplast formation (defined as the percentage of cells unable to form colonies on LB agar after treatment).

In initial attempts at transformation, protoplasts of ATCC 13059 were mixed with pHY47 and pHY416 DNAs and then treated with PEG by the procedure of Bibb et al. (3). Although we were not able to select transformants directly, we were able to obtain several putative transformants of both pHY47 and pHY416 by plating the protoplasts on nonselective regeneration medium (SB medium) and replica plating the resultant lawn onto LB agar containing 10 µg of kanamycin per ml. The existence of true *C. glutamicum* transformants was confirmed by showing that the Km^r cells contained a plasmid of the appropriate size and with the same restriction map as the input plasmid (data not shown) and that the plasmid DNA isolated from *C. glutamicum* transformants was capable of producing Km^r Cm^r transformants of *B. subtilis* 1A46. No deletions or rearrangements of pHY416 were observed in *C. glutamicum*.

Interestingly, ATCC 13059 transformed with pHY416 was very resistant to kanamycin (greater than 100 µg/ml) but was only slightly more resistant to chloramphenicol than was the

nontransformed strain. This result is not due to genetic alteration of the *cat* gene (conferring chloramphenicol resistance), since pHY416 DNA isolated from *C. glutamicum* transformants, when transformed back into *B. subtilis*, expressed Cm^r normally. One other important observation is that the Km^r phenotype conferred by pHY47 in ATCC 13059 was relatively unstable compared with pHY416 transformants. We have not yet determined whether this phenotypic instability was the result of plasmid loss, deletion events, or some other cause.

Optimization of conditions for *C. glutamicum* transformation. Although the first successful transformation experiment yielded fewer than 1 transformant per µg of plasmid DNA, it provided two critical pieces of information. First, it showed that the pSR1 replicon was still functional despite being interrupted at its *Bcl*I site. Second, it demonstrated that the *neo* gene (conferring kanamycin resistance) originally found in the *Staphylococcus aureus* plasmid pUB110 (19) functions efficiently in *C. glutamicum*. Given this information, we were able to use the shuttle vector pHY416 as a tool for optimizing conditions for *C. glutamicum* plasmid transformation. pHY416 was chosen over pHY47 because of its relative stability in ATCC 13059. Also, it should be noted that all of the following transformation experiments were performed with AS019 as the host organism. AS019 is a derivative of ATCC 13059 and has a Rif^r phenotype which provides an additional criterion for characterizing putative transformants. Lastly, the pHY416 DNA was isolated from the ATCC 13059 background to circumvent any problem due to a *C. glutamicum* restriction system.

The entire transformation process can be conveniently broken down into three stages: (i) preparation of protoplasts which are competent for DNA transformation, (ii) uptake of DNA into the cells, and (iii) selection and regeneration of transformed cells. We have systematically analyzed many of the variables which could affect the overall transformation efficiency during all three stages of this complicated process. The end result is the simple procedure summarized above which routinely yields 10⁴ to 10⁵ transformants per µg of plasmid DNA. The most important parameters in *C. glutamicum* transformation are discussed below, and representative data are presented in Table 3.

From our initial experiments aimed at generating *C. glutamicum* protoplasts, it was clear that *C. glutamicum* strains were much less sensitive to lysozyme than were other gram-positive bacteria such as *B. subtilis*. As a result, we attempted to increase the effectiveness of lysozyme treatment in two ways: (i) pretreatment of cells with inhibitors of cell wall synthesis or antibiotics which inhibit fatty acid synthesis, and (ii) treatment of cells with lipases or lytic enzymes other than lysozyme. The growth of cells in 2.0% glycine (a cell wall inhibitor) for 24 h before lysozyme treatment was essential for efficient transformation (Table 3). Cells which were not grown in the presence of glycine before lysozyme treatment seldom yielded transformants, and prolonged growth in glycine decreased transformation efficiency. The concentration of glycine used in these experiments reduced the growth rate of AS019 by approximately 30%.

We also tested the effects of penicillin and the fatty acid synthesis inhibitors cerulenin and isoniazid on *C. glutamicum* transformation. However, none of these antibiotics improved the transformation efficiency, whether used in combination with glycine or alone (data not shown). The rationale for testing fatty acid synthesis inhibitors was that the long-chain mycolic acids enveloping *C. glutamicum* cells

might be responsible for partially protecting cells from the action of lytic enzymes.

Table 3 also shows the effect of the length of lysozyme treatment on transformation. No transformants were obtained when the lysozyme step was omitted, and an optimum time of exposure to 2.5 mg of lysozyme per ml was 90 min. The decrease in the number of transformants observed with longer lysozyme treatments was due to the inability of the transformants to efficiently regenerate the protoplasts, because the number of transformants per regenerated cell actually increased even after up to 10 h of lysozyme treatment. We also analyzed the effects of lipases and the lytic enzymes mutanolysin and lysostaphin on *C. glutamicum* transformation, but none of these enzymes improved the transformation efficiency when combined with lysozyme treatment.

The last major variable discovered to affect transformation efficiency profoundly during protoplasting was agitation (Table 3). The surprising conclusion drawn was that *C. glutamicum* cells were much more competent for transformation when they were stirred vigorously during lysozyme treatment. This observation was a critical factor in the improvement of our *C. glutamicum* transformation system.

Temperature, PEG concentration and molecular weight, and addition of energy sources were among the variables we investigated for their effects on transformation efficiency during DNA uptake. Repeated experiments clearly demonstrated that PEG 3350 and PEG 8000 were superior to PEG

1000 in mediating transformation, and the optimum PEG concentration was 50% (Table 3). We analyzed the effect of temperature on transformation frequency by allowing the cells to stand at 0 or 25°C and then subjecting them to a variety of heat shock regimens at 37, 42, and 45°C before adding PEG. None of the heat shock treatments significantly increased the transformation frequency compared with that of cells held at room temperature for 5 to 10 min before addition of PEG (data not shown). Lastly, we observed that the presence of glucose at a final concentration of 10 mM during DNA uptake increased the transformation frequency by a factor of two in repeated experiments (Table 3). Whether the addition of glucose influenced the actual process of DNA uptake or affected subsequent regeneration of transformed protoplasts is unclear. Other energy sources such as pyruvate, ATP, and phosphoenolpyruvate were added during DNA uptake but had no discernable effect on transformation frequency.

We were able to devise a very simple method for the recovery of *C. glutamicum* transformants (see above). Unlike the procedures reported for *B. subtilis* and *Streptomyces* sp. protoplast transformation, in which PEG was removed from the protoplasts by centrifugation, we found that diluting *C. glutamicum* protoplasts with LB medium containing 0.5 M sorbitol was sufficient to eliminate any toxic effects of PEG.

Other important parameters in the selection of transformants are the regeneration medium and the time allowed for expression of the *neo* gene before exposure to kanamycin. Although incubation of the transformed protoplasts for 1 h at 30°C allowed the selection of a substantial number of Km^r transformants, a 3-h incubation period generally yielded more transformants. Longer intervals gave variable results; therefore, the 3-h incubation period was chosen as the standard condition. It should be noted that there was no increase in cell number during the 3-h incubation.

A variety of different media containing succinate, sucrose, or sorbitol as the osmotic stabilizer have been tested for their efficacy in regenerating *C. glutamicum* protoplasts. The medium which yielded the highest regeneration frequencies as well as the most rapid appearance of colonies was SB agar, which is described above. In addition to containing 0.5 M sorbitol as the osmotic stabilizer, this medium contains divalent cations which were also found to be critical for efficient regeneration of transformants. The regeneration frequency is approximately 20% as measured by the number of protoplasts recovered on SB agar compared with the number of cells recovered on LB agar. The addition of kanamycin to SB agar at a concentration of 15 µg/ml permitted the direct selection of *C. glutamicum* transformants. In general, the first transformant colonies were observed after 2 days at 30°C, and the number of Km^r colonies reached a maximum after 7 days. We have never observed a spontaneous Km^r colony of AS019 on the control plates.

DISCUSSION

The preceding results represent the initial stages in the development of recombinant DNA technology for *C. glutamicum* and hopefully for other coryneform bacteria as well. The availability of this technology for *C. glutamicum* is especially significant in view of the current lack of classical genetic exchange mechanisms in this organism. The previously reported transformation of *C. glutamicum* was accomplished by using an endogenous *C. glutamicum* plasmid (18). We have demonstrated the maintenance and integrity of foreign DNA upon transformation into *C. glutamicum*.

TABLE 3. Analysis of variables effecting transformation efficiency

Modification	Relative transformation efficiency ^a
Growth with glycine	
No	0
24 h	1.0
48 h	2.6
72 h	2.0
120 h	1.0
Treatment with lysozyme	
None	0
0.5 h	0.03
1.0 h	0.38
1.5 h	1.0
2.0 h	0.63
3.0 h	0.26
5.0 h	0.26
Static	0.05
Shaking	1.0
PEG addition	
50% PEG 1000	1.0
50% PEG 3350	6.2
Glucose addition	
None ^b	1.0
6 mM	1.9
14 mM	2.2
58 mM	1.7
140 mM	1.4

^a Transformation efficiency is shown relative to results obtained by the standard protocol, in which cells were grown for 24 h in 2% glycine and treated with lysozyme for 1.5 h and then with 50% PEG 1000 for 3 h. No glucose was added during resuscitation in SB medium. For more details, see the text.

^b Glucose was added during resuscitation.

The utility of any recombinant DNA system is ultimately dependent upon the flexibility of the vectors available and the efficiency of the transformation system. In this paper, we have described the construction of one useful plasmid vector for *C. glutamicum*, namely pHY416, in which a 3.0-kb replicon isolated from a *C. glutamicum* strain was cloned into the *B. subtilis* vector pBD10. Although pHY416 obviously lacks the sophistication of the vectors used in other well-developed systems, it has several unique restriction sites (e.g., *Bam*HI and *Pvu*II) which can be used in cloning experiments without interfering with essential plasmid functions. In addition, there are several more restriction sites within pSR1 sequences (e.g., *Eco*RI and *Sst*II) which may be useful. The other important property of pHY416 is that it is a *B. subtilis*-*C. glutamicum* shuttle vector. This represents a valuable connection between the recombinant DNA technology of *C. glutamicum* and a gram-positive species with more advanced genetic tools. For example, the link with *B. subtilis* could be used to expand our *C. glutamicum* repertoire more quickly to include transposon mutagenesis (29), protein identification (26), and regulatory probes (12).

At present, the lack of a second genetic marker which is expressed in *C. glutamicum* precludes a careful study of plasmid compatibility and also limits our ability to construct improved vectors. As a result, we are attempting to identify additional genetic markers for *C. glutamicum*. We do not have any evidence to explain why the *cat* gene from pBD10 is not efficiently expressed in *C. glutamicum*, whereas the *neo* gene is. One simple explanation is that transcription of the *neo* gene from pHY416 in *C. glutamicum* is initiated at a promoter within pSR1 rather than from the promoter used in *B. subtilis*. Although we cannot formally rule out this possibility, it is unlikely, since the *neo* gene is still expressed when placed in the opposite orientation relative to pSR1 (pHY47). Also, we have recently constructed additional vectors in which the *neo* gene is expressed in *C. glutamicum* despite having a variety of different relationships to the pSR1 replicon or even a coryneform replicon other than pSR1. The *neo* gene from Tn5 is also expressed in *B. lactofermentum* (24a), and the spectinomycin and streptomycin genes from a *C. glutamicum* plasmid are expressed in a wide variety of coryneform bacteria (18).

The protoplast transformation system described in this paper is similar to but not as efficient as those reported for other gram-positive species such as *B. subtilis* and *Streptomyces lividans* (3). As with *Streptomyces* sp., efficient protoplasting and, hence, transformation in *C. glutamicum* requires growth in a medium containing glycine before lysozyme treatment (17, 24). Enhanced protoplasting of *C. glutamicum* has also been reported after addition of penicillin G to the growth media (18), although we did not observe any significant benefit of penicillin treatment on transformation. Microscopic observation of cell growth during glycine and lysozyme treatment reveals little or no alteration in cell morphology. Despite the fact that these cells are extremely osmotically sensitive, they still retain their rod shape, suggesting that the cell wall has not been completely removed (data not shown). The retention of morphology and the apparent resistance to physical shock observed in lysozyme-treated *C. glutamicum* cells is likely due to the complex nature of the *C. glutamicum* cell wall. In addition to the peptidoglycan layer found in other gram-positive species, coryneform bacteria also contain an arabinogalactan layer and are enveloped by an unique and characteristic layer of mycolic acids (4, 8, 9). These unusual surface components may act to block lysozyme digestion peptidoglycan or to

limit DNA uptake (or both) despite the removal of the lysozyme-sensitive layer. Thus, our present inability to completely remove the cell wall may account for the lower efficiency of PEG-mediated transformation of *C. glutamicum* compared with that reported for other gram-positive species. Attempts to remove the mycolic acids by growing *C. glutamicum* in fatty acid biosynthetic inhibitors at growth-limiting concentrations or by treatment of the cells with lipase in conjunction with lysozyme have thus far not increased the transformation efficiency. Despite the relatively low transformation efficiency, the recombinant DNA system described in this paper has allowed the construction of a *C. glutamicum* chromosomal gene bank and the isolation of specific amino acid biosynthetic genes from this organism (M. Follettie and A. J. Sinskey, manuscript in preparation).

Several other minor differences between the *C. glutamicum* transformation system and other gram-positive transformation procedures serve to make it simpler and faster without causing a detectable loss in efficiency. For example, *C. glutamicum* protoplasts are centrifuged only once after lysozyme treatment, thereby eliminating an extra washing step which is usually used to remove lysozyme. Another common centrifugation step is avoided after PEG-mediated DNA uptake, since *C. glutamicum* protoplasts are unusually tolerant of relatively high PEG concentrations. For *C. glutamicum*, simple dilution of PEG to a final concentration of 12% is sufficient to allow survival of transformed protoplasts. The resistance of *C. glutamicum* protoplasts to the toxic effects of PEG is also indicated by the insensitivity of transformation frequency to the time of exposure to PEG during DNA uptake. Protoplasts can be exposed to a final PEG concentration of 35% for 15 min before dilution with SB medium without a significant effect on transformation frequency. In contrast, *B. subtilis* protoplasts are exposed to a lower PEG concentration for only 2 min before dilution and centrifugation.

An improvement in the regeneration medium for *C. glutamicum* protoplasts might also lead to a substantial increase in transformation frequency. Our current regeneration frequency of approximately 20% is well below the figure which has been achieved for some other organisms. A relatively modest increase in regeneration frequency could have a dramatic effect on transformation, since the protoplasts which are most competent for DNA uptake may also be the most difficult to regenerate.

Lastly, we hope that the rudimentary recombinant DNA system described in this paper will not only advance our knowledge of *C. glutamicum* molecular biology but will also contribute to the understanding of other important biological problems. For example, the capacity to use recombinant DNA technology in coryneform plant-pathogenic species would provide new experimental approaches to study the interactions between host plants and their bacterial pathogens. We are currently examining whether our simple protoplast transformation procedure for *C. glutamicum* is applicable to closely related coryneform species.

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