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Electrotransformation of intact cells of *Brevibacterium flavum* MJ-233

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SUMMARY

Electroporation allowed transformation of intact cells of *Brevibacterium flavum* MJ-233. The two plasmids used for electroporation were pCRY2 (6.3 kilobases) and pCRY3 (8.2 kilobases). Both plasmids contain the chloramphenicol-resistance gene and the autonomous replication origin in *B. flavum* MJ-233. The efficiency of electrotransformation was optimal with cells harvested at the middle log phase of growth, and was improved by the addition of 1.0 U/ml of penicillin G to the culture medium. The optimum yield of transformants per μg DNA was 5×10^4 when the cell suspension was pulsed at a cell density of 1×10^{10}/ml and at a DNA amount of 1.0 μg.

INTRODUCTION

Brevibacterium flavum MJ-233, belonging to the nonpathogenic so-called coryneform group, has some useful characteristics, including the ability to metabolize ethanol as a carbon source, a rapid growth rate, and failure to autolyze under starvation conditions. Therefore, we have been studying the practical production of such chiral chemicals as L-aspartic acid [20,25,27,28], L-isoleucine [26] and L-malic acid [29].

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To expand the application of *B. flavum* MJ-233, we have focused our efforts on construction of a recombinant DNA system in this strain.

There have been some reports about host-vector systems for coryneform bacteria. Available methods for transformation are classified into two groups: (a) transfection of protoplasts [15,23] and (b) addition of plasmid DNA to protoplasts in the presence of polyethylene glycol (PEG) [12]. These two methods, however, are still associated with some problems, including low tranformation frequency, difficulties of protoplast formation and regeneration, and a period of more than 10 days required to obtain the transformants.

In this study we attempted to apply the electroporation method for transformation in *B. flavum* MJ-233. Electroporation stimulates the uptake of DNA into living cells by inducing transient pores in their outer membranes. This technique has been used to transfer DNA into a variety of eucaryotic cells [16,22] and a few kinds of bacteria [3,8,13,18].

In this report, we demonstrate that electroporation is an efficient transformation method in intact cells of *B. flavum* MJ-233 with plasmid DNA.

MATERIALS AND METHODS

Bacterial strains

Coryneform strains obtained from IFO (Institute for Fermentation Osaka, Osaka, Japan) and *B. flavum* MJ-233 were used for screening of plasmids.

Host strain preparation

A cured strain of *B. flavum* MJ-233 was obtained by incubation with novobiocin. The cured strain showed a similar phenotype to the parental strain in resistance to antibiotics, heavy metals, and fermentation sugars. The cured strain was used for both PEG-mediated and electroporation-assisted transformation.

Culture condition

AR medium [glucose, 5 g; $(NH_4)_2SO_4$, 7 g; urea, 2 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract, 1.0 g; casamino acids, 1.0 g; $FeSO_4 \cdot 7H_2O$, 6 mg; $MnSO_4 \cdot 4$–$6H_2O$, 6 mg; biotin, 200 μg; thiamine-HCl, 200 μg; deionized water, 1 liter (adjusted to pH 7.5 with NaOH)] was used as the culture medium for preparation of protoplasts and plasmid isolation. For *B. flavum* MJ-233, ethanol was used as the carbon source (initial conc. 2% v/v). HAR medium contained 171 g of sucrose, 30 g of polyvinylpyrrolidone (PVP) K15 (average molecular weight 10 000; purchased from Tokyo Kasei Co., Ltd.), and 10 mg of thiamine-HCl per liter. Cultivation was carried out by shaking at 30°C for coryneform strains obtained from IFO and 33°C for *B. flavum* MJ-233.

Plasmid isolation

Plasmid DNA was isolated by the alkaline lysis procedure [2]. The plasmid of pHSG398 [19] carrying the chloramphenicol-resistance gene from *E. coli* was purchased from Takara Shuzo Co., Ltd.

Transformation

(1) PEG-mediated protoplast transformation

Protoplast formation. Protoplasts of *B. flavum* MJ-233 were prepared by a modification of the method of Kaneko and Sakaguchi [9] as follows. A 0.5% inoculum from an overnight culture of *B. flavum* MJ-233 cells in AR medium was inoculated into the same medium and cultured with shaking. When OD_{610} reached 0.5, penicillin G was added to a final concentration of 0.2 U/ml. The cells were harvested by centrifugation after 2-h incubation at 33°C. The harvested cells were washed twice with 'osmotic hyper solution' (OHS: 0.5 M sodium succinate added to AR medium without yeast extract and casamino acids) and suspended in OHS containing lysozyme and other enzymes in one-eighth volume of the original culture. The suspension was incubated overnight at 33°C with shaking to form protoplasts. The number of osmotically sensitive cells was determined by diluting the suspension with OHS and plating on AR agar plates.

Transformation and regeneration. The protoplasts were harvested and washed once with OHS and resuspended in one-fifth volume of OHS. Plasmid DNA was added to the protoplast suspension, and was then mixed with a 9-fold volume of 20% PEG 6000 dissolved in OHS. The mixture was gently stirred, incubated on ice for 5 min, and 3 volumes of OHS were added. The harvested protoplasts were resuspended in HAR medium and incubated at 33°C for 2 h without shaking. Regeneration was carried out for several days on HAR agar plates containing chloramphenicol at 33°C. Efficiency of regeneration was calculated as the ratio of the number of cells on HAR agar plates and on AR agar

plates against total cells before protoplast treatment.

(2) Transformation by electroporation

The basic protocol used was based on the Gene Pulser's Manual (Bio-Rad Lab; Richmond, CA). Cells of *B. flavum* MJ-233 were grown in AR medium at 33°C with shaking. Cells harvested from 10 ml of culture broth were washed twice with 1 ml of the transformation buffer containing 272 mM sucrose, 1 mM $MgCl_2$, and 7 mM sodium phosphate, pH 7.4, and then suspended in the same buffer at a final volume of 0.8 ml (cell concentration approximately 10^{10} cells per ml). One μg of plasmid DNA was added, followed by dipping in a cold water bath (about 0°C). The Gene Pulser apparatus was used at 2000 to 2500 V and 25 μFD capacitance. The cuvette containing the cell suspension was placed in the Gene Pulser apparatus chamber, exposed to a single pulse, then returned to an ice bath for an additional 10 to 30 min. The sample was diluted in an 8-fold volume of AR medium and incubated at 33°C for 1 h. Aliquots were plated on AR agar plates containing chloramphenicol as the selective marker (3 μg per ml).

RESULTS AND DISCUSSION

Identification and characterization of coryneform plasmids

To develop a *B. flavum* MJ-233 transformation system, we tried to obtain plasmids that could propagate in the MJ-233 strain. Fifteen coryneform strains obtained from IFO and *B. flavum* MJ-233 were screened for the presence of plasmids. *B. flavum* MJ-233 contained a 45 kb plasmid, designated pBY502, and *Brevibacterium stationis* IFO-12144 also contained a15 kb plasmid, designated pBY503. In the other 13 strains (IFO-3730, -12077, -12127, -12143, -12146, -12154, -12160, -12471, -12674, -12676, -12684, -12974, and -13762), no plasmid was detected. The two plasmids pBY502 and pBY503 remain cryptic, as we have not found any correlation between these plasmids and resistance to antibiotics, ability to use sugars as a carbon source, and resistance to heavy metals (data not shown). Thus far, restriction mapping only of pBY503 has been performed (Fig. 1).

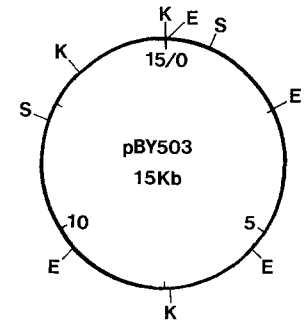

Fig. 1. Physical map of plasmid pBY503 from *B. stationis*. The abbreviations used are: K, *Kpn*I; E, *Eco*RI; S, *Sma*I.

Construction of E. coli–Brevibacterium shuttle vectors

To isolate the fragments of pBY502 that were capable of autonomous replication, pBY502 DNA and pHSG398 were digested separately with *Hind*III, mixed, and ligated. The ligated DNA was then used to transform *B. flavum* MJ-233 to a chloramphenicol-resistant form (Cm^r). Ten Cm^r transformants were characterized, and all were found to carry recombinant plasmids of the 4.1 kb fragment of pBY502 DNA and pHSG398 DNA. This hybrid plasmid was named pCRY2. A similar experiment was also carried out with a *Kpn*I-digested fragment of pBY503. Recombinant plasmid of 6.0 kb *Kpn*I fragment of pBY503 DNA and pHSG398 DNA was obtained, and was named pCRY3. Both hybrid plasmids could propagate and express the chloramphenicol-resistant gene in both *B. flavum* MJ-233 and *E. coli* K12 derivatives (Fig. 2).

PEG-mediated protoplast transformation of B. flavum MJ-233 with pCRY2 and pCRY3

As previous reports have described [1,9,17], protoplast formation in coryneform bacteria was accelerated by the addition of lysozyme, however, some reports have demonstrated that in some strains protoplasts could not be generated by the addition of lysozyme alone. In *C. glutamicum* ATCC 39019,

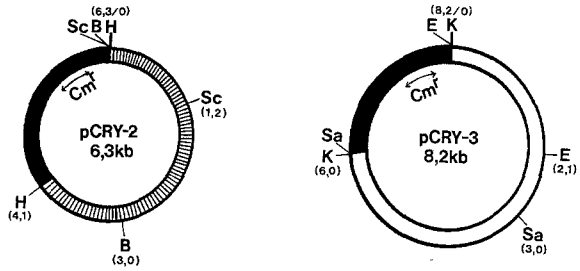

Fig. 2. Characterization of hybrid plasmids. The solid segments indicate plasmid pHSG398 and the striped and open segments represent the 4.1 kb *Hind*III fragment from pBY502 and the 6.0 kb *Kpn*I fragment from pBY503, respectively. The abbreviations used are: H, *Hind*III; B, *Bam*HI; Sc, *Sac*I; Sa, *Sal*I; E, *Eco*RI; K, *Kpn*I; Cm^r, chloramphenicol resistance.

protoplasts were generated by using a lysozyme sensitive strain [11], and in *C. glutamicum* ATCC 13032, protoplasts were generated by treatment with a mixture of lysozyme and achromopeptidase [21]. In *B. flavum* MJ-233, protoplast formation was not observed microscopically after addition of lysozyme alone. Therefore, we investigated other enzymes, achromopeptidase [14] and *N*-acetylmuramidase [24], in addition to lysozyme. Protoplast formation in the *B. flavum* MJ-233 strain occurred only after addition of both lysozyme and achromopeptidase/*N*-acetylmuramidase (Table 1).

Agents used as osmotic stabilizers might have an influence on protoplast formation. As shown in Table 1, 0.5 M sodium succinate was most effective.

To increase transformation frequency, we tested several regeneration media based on the results obtained with *B. subtilis* [4] and *C. glutamicum* [12]. In a previous report [5], PVP and BSA (bovine serum albumin) were described as agents that promoted protoplast regeneration. Therefore, we investigated the effect of regeneration-promoting agents in addition to the effect of osmotic stabilizers and the type of agar used. Transformation was carried out as described in Materials and Methods. Transformants could be directly selected on HAR plates containing 6.6 μg of chloramphenicol per ml, and could be detected after 4 to 6 days of incubation at 33°C. The most effective medium for protoplast regeneration included 0.5 M of sucrose as osmotic stabilizer, 0.8% of DIFCO Purified Agar, and 3.0% of PVP as the protoplast regeneration-promoting agent (Table 2). The efficiency of transformation with pCRY2 and pCRY3 was the same, about 5×10^4 transformants per 1 μg of plasmid DNA.

Table 1

Effects of enzymes[a] and osmotic stabilizer on protoplast formation

Osmotic stabilizer	Conc. [M]	Lysozyme[b]	Lysozyme achromopeptidase[c]	Lysozyme *N*-acetylmuramidase[d]
Succinate	0.5	−[e]	+++	++
	0.7	−	++	++
Sucrose	0.5	−	++	+
	0.7	−	++	+
Sorbitol	0.5	−	+	+
	0.7	−	++	+
Lactose	0.3	−	++	+
Raffinose	0.3	−	+	+

[a] Penicillin G treated cells were suspended in HAR medium containing each enzyme (see b, c and d).
[b] Lysozyme (Egg White, SEIKAGAKU KOGYO, 4 mg/ml).
[c] Lysozyme + achromopeptidase (purified (TBL-1), WAKO, 1000 U/ml).
[d] Lysozyme + *N*-acetylmuramidase SG (SEIKAGAKU KOGYO, 200 U/ml).
[e] Protoplast formation was observed microscopically. −: not formed, +: approximately 30% formed, ++: 70%, +++: >90%.

Table 2

Effects of osmotic stabilizer, kind of agar and promotive agent on protoplast regeneration

		Concentration	Regeneration[a] frequency [%]
Osmotic stabilizer	Sucrose	0.5 [M]	0.5
	Succinate	0.5	<0.01
	Sorbitol	0.7	0.4
	Lactose	0.3	0.1
Kind of agar	TAKARA, Agarose LO3 [TAKARA]	0.5 [%]	0.5
		0.8	0.1
	DIFCO, Agar Purified	0.5	0.8
		0.8	1.7
	WAKO, Agar Powder E.P.	0.5	0.5
		0.8	0.2
Promotive agent	TOKYOKASEI, PVP (K15)	1.5 [%]	2.1
		3.0	3.2
		4.5	3.0
	SIGMA, BSA	1.0	1.8
		2.0	2.0

[a] Percentage of colonies regenerated on HAR plates from initial viable cells subjected to treatment with lysozyme. Protoplasts prepared from *B. flavum* MJ-233 cells grown in the presence of 0.2 U/ml of penicillin G were used.

Transformation by electroporation

Several reports [7,8,13] have appeared concerning the high effectiveness for transformation by electroporation using log phase cells. Transformation efficiency might be closely related to the physiological condition of the cell wall. Therefore, we studied the effects both of the growth phase and of penicillin G, a known inhibitor of cell wall synthesis. Transformants by electroporation could be detected after about 40 h incubation at 33°C.

Effect of growth phase. As shown in Fig. 3, the maximum of 2×10^3 transformants per μg DNA was obtained at the middle log phase. On the other hand, at either the very early or the very late log phase, transformation efficiency decreased by approximately 90 percent.

Effect of penicillin G concentration. As shown in Fig. 4, addition of penicillin G to a concentration of 1.0 U/ml improved the transformation efficiency by more than one order of magnitude in comparison with the control. Therefore, the effectiveness of penicillin G might be related to changes in the characteristics of the *B. flavum* MJ-233 cell wall.

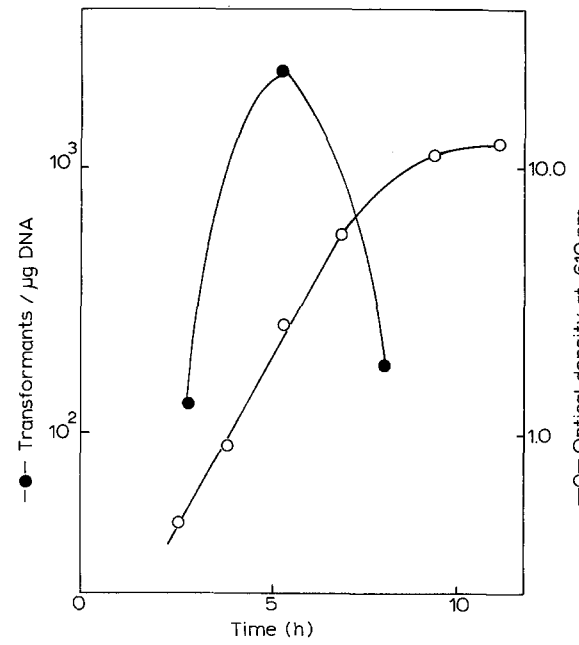

Fig. 3. Effect of growth phase on transformation efficiency. Each transformation was carried out by using approximately 10^{10} cells of *B. flavum* MJ-233, which were grown in AR medium at 33°C (see text for details).

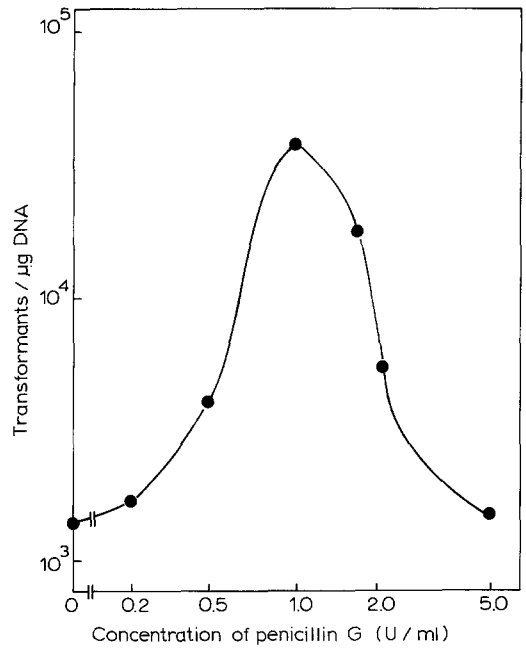

Fig. 4. Effect of penicillin G concentration on transformation efficiency. Penicillin G was added to AR culture in the early log phase of growth.

Effects of DNA amount and cell density. The effects of DNA amount and cell number on transformation efficiency were also investigated.

Using amounts of DNA ranging from 20 ng to 10 μg, as shown in Fig. 5, the number of transformants was shown to increase with increasing amounts of DNA. Thus, saturation was observed at amounts of DNA above 1.0 μg.

Transformation efficiency also increased up to a density of 10^{10} cells per ml (see Materials and Methods for standard protocol), which was the highest cell density obtained.

Electrotransformation of *B. flavum* MJ-233 occurred at the optimum frequency of 5×10^4 transformants per 10^{10} intact cells.

In transformation of *E. coli,* the competent cells could be stored at −80°C for several months [6]. Therefore, we tested the conditions of cell storage and found that cells stored at −80°C for at least 2 weeks could be used as host cells.

This report demonstrates that a transformation efficiency of more than 10^5 transformants per 10^{10} cells could be achieved. We believe that this method can be widely used for other coryneform bacterial strains. However, it is advisable to investigate various modes of cell pretreatment, such as the addition of penicillin G or glycine to the culture medium as was done in the present study.

This method has the advantage that intact cells can be used as host cells without the need for protoplast formation and regeneration. Moreover, for the selection of transformants a minimal medium can be used, and therefore the complementary selection of transformants may be possible.

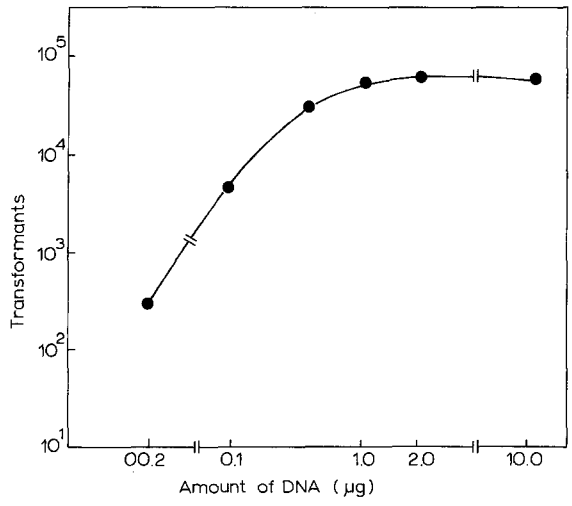

Fig. 5. Effect of amount of DNA on transformation efficiency.

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