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Development of L-Lysine Producing Strains by Intergeneric Protoplast Fusion of *Brevibacterium flavum* and *Corynebacterium glutamicum*

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*Brevibacterium flavum*과 *Corynebacterium glutamicum*의 이속간 원형질체 융합에 의한 L-라이신 생산균주 개발

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As a method of breeding L-lysine producing strains, the intergeneric protoplast fusion between *Brevibacterium flavum* and *Corynebacterium glutamicum* was performed.

As a results, *Brevibacterium flavum* ATCC 21528 R showed 99% of protoplast formation and 10% of regeneration frequencies when treated with 400 μg/ml of lysozyme for 12 hrs. In *Corynebacterium glutamicum* ATCC 21514 S, 99% and 12% were obtained by treatment of 300 μg/ml lysozyme for 12 hrs.

In intergeneric protoplast fusion between *Brevibacterium flavum* ATCC 21528 R and *Corynebacterium glutamicum* ATCC 21831 S, 1.0×10^{-6} of recombinant frequency per regenerable cells was observed by use of PEG 6000, 30% (w/v). Among the strains obtained KR_{43} strain showed 12% higher productivity of L-lysine than the parental cell. Then, the activity of aspartokinase of KR_{43} was about 13% higher than the parental cell.

Brevibacterium and *Corynebacterium* spp. were developed up to now through mutagenesis as L-lysine producing strains,[1-7] but this method is now considered having been reached to its limit. Therefore, protoplast fusion method was recently tried for the improvement of the strains because only some gene transfer system has been found.[8-12]

In genus *Brevibacterium*, Kaneko and Sakaguchi[13] first made an attempt on intraspecific protoplast fusion. And after, interspecific and intraspecific protoplast fusion of *B.flavum* and *B.lactofermentum* were tried,[14] and intergeneric protoplast fusion between *B.flavum* and *C.glutamicum* was also succeeded by Zhdanova et al.[15] Recently, there is a report improving for L-threonine production by using of protoplast fusion method.[16]

In the present study, intergeneric protoplast fusion between *B.flavum* and *C.glutamicum* was tried for the development of L-lysine producing strains, and L-lysine productivity and aspartokinase activity, as a key enzyme of L-lysine biosynthetic pathway, of the fusants were compared with those of parents.

MATERIALS AND METHODS

Bacterial strains

The strains used in this experiments are listed in Table 1. streptomycin and rifampicin resistant mutants were obtained spontaneously.

Media

Complete medium (CM) used for seed culture, and MMYE (minimal medium supplemented with 0.2% yeast extract) was used as the ordinary culture medium.[17] Regeneration medium (RCG) defined by Katsumata et al.[18] was used for

Table 1. Lists of bacterial strains and their mutants

Bacterial strains	properties
B. flavum ATCC 21528	thr^-, AEC^r
C. glutamicum ATCC 21514	thr^-, met^-
C. glutamicum ATCC 21831	can^r
B. flavum ATCC 21528 R	thr^-, AEC^r, rif^{r*}
C. glutamicum ATCC 21514 S	thr^-, met^-, str^{r**}
C. glutamicum ATCC 21831 S	can^r, str^r

*rif^r; rifampicin resistance to 100 $\mu g/ml$ and streptomycin sensitive to 5 $\mu g/ml$
**str^r; streptomycin resistance to 100 $\mu g/ml$ and rifampicin sensitive to 5 $\mu g/ml$

cell wall regeneration. Lysis fluid (LF), including 0.03-0.1% lysozyme, was used for digestion of the cell wall. Dilution fluid (DF) was used for dilution of protoplasts, and fusion fluid (FF) was supplemented with 5 mM EDTA and 100 mM Ca^{++} ion. The medium compostion for L-lysine production was as follows (%); glucose 10, $(NH_4)_2SO_4$ 4, K_2HPO_4 0.06, $MgSO_4 \cdot 7H_2O$ 0.04, $FeSO_4 \cdot 7H_2O$ 0.001, $MnSO_4 \cdot 6H_2O$ 0.001, soybean protein acid hydrolysate 0.04, corn steep liquor 0.6, thiamine·HCl 100 r, biotin 50 r, peptone 0.2, $CaCO_3$ 5, homoserine 50 mg, leucine 100 mg, threonine 100 mg, methionine 200 mg.

Protoplast formation and cell wall regeneration

An overnight culture of bacteria, 0.4ml, was inoculated into 40ml of fresh MMYE medium and the culture was incubated at 30°C with shaking. In the early mid-exponential phase, penicillin G was added at the rate of 0.3 unit/ml. After 2 hours more shaking, cells were harvested and resuspended into 20ml of LF in a 100ml conical flask. Protoplasts were observed under the phase contrast microscope. Protoplasts were diluted with DF, and plated on RCG agar medium. Colonies from non protoplasted cells were examined by diluting protoplasts in TM buffer and plating on CM solid agar medium.

Protoplast fusion

Protoplasts of two strains were mixed and centrifuged (3000 rpm, 20min, 4°C). The pellet was resuspended in a one-tenth volume of FF. This dense suspension was then diluted 10-fold with PEG 6000 solution. After incubation for 15 min at 30°C, the suspension was twice diluted with FF. After centrifuge, the pellets were resuspended in FF and plated onto the direct selection medium. After 10 days incubation at 30°C, colonies in the selection medium were isolated, and their auxotrophic properties were examined.

Measurement of L-lysine productivity

Seed cultured cells were inoculated in the L-lysine production media at the rate of 1:100. After shaking culture for 72 hrs in 30°C, the cultured media were centrifuged and the supernatant was used for L-lysine productivity test.

Enzyme assay

The activity of aspartokinase was measured by estimating the quantity of aspartate hydroxamate formed in the presence of hydroxylamine by the method of Black.[20] The specific activity of aspartokinase was determined by the number of nmole of the β-hydroxamate of L-Aspartate formed in 1 min per mg protein of the enzyme preparation.

Table 2. Effect of lysozyme treatment concentration and time in *C. glutamicum* and *B. flavum*.

Strains	Concentration of added lysozyme ($\mu g/ml$)	Treatment time of lysozyme (hrs)	Colony forming unit/ml			Pff* (%)	Rf** (%)
			Intact cell number	Hypertonic medium	Hypotonic medium		
C. glutamicum ATCC 21514 S	300	12	4.0×10^8	4.7×10^7	8.5×10^5	99	12
		20		4.0×10^6	5.0×10^4	99	1
	1000	12	4.0×10^8	1.0×10^7	5.5×10^4	99	2.5
		20		2.5×10^6	1.0×10^4	99	0.7
B. flavum ATCC 21528 R	300	12	3.8×10^8	3.0×10^7	5.7×10^5	99	8
		20		3.8×10^6	1.4×10^4	99	1
	400	12	3.8×10^8	4.1×10^7	6.9×10^5	99	10
		20		3.6×10^6	4.5×10^4	99	1
	1000	12	3.8×10^8	1.7×10^7	4.3×10^4	99	5
		20		2.9×10^6	1.1×10^4	99	0.8

*Pff; protoplast formation frequency **Rf; regeneration frequency

Table 3. Effect of PEG concentration on fusion frequency between *Brevibacterium flavum* ATCC 21528 R and *Corynebacterium glutamicum* ATCC 21514 S

Cross	Fusion frequency (str^r, rif^r)					
B. flavum ATCC 21528 R (thr^-, AEC^r, rif^r) × *C. glutamicum* ATCC 21514 S (thr^-, met^-, str^r)	Without PEG	Concentration of PEG 6000 (w/v)				
		10%	20%	30%	40%	50%
	1.0×10^{-7}	1.0×10^{-6}	1.0×10^{-6}	1.2×10^{-5}	5.0×10^{-6}	1.0×10^{-5}

Results

Protoplast formation and cell wall regeneration

The conditions stable for protoplast formation were studied by penicillin G and lysozyme treatment. Mild penicillin G treatment of the cells, 0.3 unit/ml of penicillin G concentration, which gave no growth inhibition, had a good effect on protoplast formation. Penicillin G was added at the concentration of 0.3 unit/ml in early-, mid-, and late-exponential growth phase and lysozyme was treated after 2 hrs more culture. In the case of initial-, and mid-exponential growth phase, protoplast were formed an efficiency of 99% after 12 hrs of lysozyme treatment, but only 75% in late-exponential growth phase. Thus, treatment of penicillin G in early mid-exponential growth phase ($O.D_{580}$ = 1.5-2.0, 2.0×10^8 cell/ml) was desirable in protoplast formation.

Effect of lysozyme concentrations were examined (Table 2). In *C. glutamicum*, lysozyme concentration of 300 μg/ml was more effective than that of 1 mg/ml considering the protoplast formation and cell wall regeneration at the same time. After 12 hrs incubation, the cells treated with the concentration of 300 μg/ml lysozyme were protoplasted at the rate of 99% and colonies were formed at an efficiency of 12%. In *B. flavum*, the optimum lysozyme concentration was observed at 400 μg/ml, and at that concentration, protoplasts were formed at the rate of 99% and regenerated at an efficiency of 10%.

Protoplast fusion

Effect of PEG concentration on recombinant frequency was observed by intergeneric protoplast fusion between *Brevibacterium flavum* ATCC 21528 R and *Corynebacterium glutamicum* ATCC 21514 S. Fusion frequency was calculated in terms of colony number per total regenerable protoplast number plated. 30% or more concentration of PEG 6000 was efficient, and at 30% of PEG 6000, fusion frequency was 1.2×10^{-5} of plated protoplasts (Table 3).

Intergeneric protoplast fusion between *B. flavum* ATCC 21528 R and *C. glutamicum* ATCC 21831 S was induced with 30% of PEG 6000 and the fusion frequency was ca. 1.0×10^{-6} (Table 4).

When the selection agar medium was used as control, the induction of spontaneous resistance was not detected either of the two parents. The composition of selection agar medium was RCG medium supplemented 50 μg/ml of streptomycin and 50 μg/ml of rifampicin.

Auxotrophic properties

Fusants obtained by intergeneric protoplast fusion between *B. flavum* ATCC 21528 R and *C. glutamicum* ATCC 21514 S were characterized by their auxotrophic and analog resistant marker (Table 5). In the case of No. 4, the fusant was valid because it was confirmed by antibiotic resistant, auxotrophic, and analog resistant characterization. The fusant named KR_{43} by the order was obtained. With some other fusants, it was measured L-lysine productivity and aspartokinase activity.

Measurement of L-lysine productivity and aspartokinase activity

The activity of aspartokinase was some relative to the productivity of L-lysine (Table 6). In the case of KR_{43}, the productivity of L-lysine had ca. 12% enhancement and the specific activity of aspartokinase was ca. 13% increased.

Discussion

In genus *Brevibacterium* and *Corynebacterium*, only some genetic transfer system were known. The discovery of the genetic transfer system to raise the number of recombinants is valuable in connection with the industrial importance of this group of bacteria.

Table 4. Fusion frequency between *Brevibacterium* flavum ATCC 21528 R and *Corynebacterium glutamicum* ATCC 21831 S

Cross	Fusion frequency (str^r, rif^r)	
B. flavum ATCC 21528 R (thr^-, AEC^r, rif^r) × *C. glutamicum* ATCC 21831 S (can^r, str^r)	Without PEG	With PEG 6000 (30% w/v)
	1.0×10^{-7}	1.0×10^{-6}

Table 5. Fusant formation by protoplast fusion between *B. flavum* ATCC 21528 R(thr⁻, AEC^r, rif^r) and *C. glutamicum* ATCC 21514 S(thr⁻, met⁻, str^r)

No.	RCG SR*	MM**	MM+thr	MM+met	MM+thr+met	NA+AEC***	No. of colonies obtained
1	R	−	+	−	+	+	2
2	R	−	−	−	+	+	36
3	R	−	−	−	+	−	4
4	R	−	−	+	+	+	1

* RCG SR was RCG supplemented 50μg/m*l* of streptomycin and 50μg/m*l* of rifampicin
** MM was minimal medium
*** NA+AEC was nutrient agar supplemented 2mg/m*l* of aminoethyl cysteine

In order to use protoplast fusion for generic recombination, efficient protoplast formation and regeneration of cell walls are essential steps. Strains varied in their ability to form protoplasts. In *B. flavum*, protoplasts were formed after treatment with 0.3 unit/ml of penicillin G during early mid-exponential gowth phase followed by treatement of 400 μg/ml lysozyme in a hypertonic medium. In *C. glutamicum*, protoplasts were obtained at an efficiency of 99% by treatment of 300 μg/ml lysozyme.

Protoplast fusion can be induced by various polyol (sorbitol, mannitol, ethylene glycol, sucrose) and divalent cations (Ca^{++}, Mg^{++}).

Table 6. Test of L-lysine productivity and aspartokinase enzyme activity in parental cells and their fusants

Strains	Productivity of L-lysine (g/*l*)		Specific activity of aspartokinase (nmole/min/mg)
	Medium A	Medium B	
B. flavum ATCC 21528 R	25.8 (100)*	23.6 (100)	20.38 (100.00)**
C. glutamicum ATCC 21514 S	20.08 (81)	19.8 (84)	17.26 (84.69)
KR_2	20.1 (78)	− (−)	− (−)
KR_6	24.6 (95)	16.3 (69)	17.83 (87.49)
KR_{43}	28.7 (112)	25.8 (109)	23.07 (113.20)

* The number in ( ) showed the relative percentage of L-lysine productivity for *B. flavum* ATCC 21528 R
** The number in ( ) showed the relative activity of aspartokinase for *B. flavum* ATCC 21528 R

Protoplast fusion offers an opportunity to improve industrial strains. In antibiotics producting *Streptomyces*,[21] protoplast fusion led to a new antibiotics producing recombinant. In amino acid producing strains, there is a report improving L-threonine production by using of protoplast fusion method.[16] In this study, intergeneric protoplast fusion between L-lysine producing strains led to a fusnat, KR_{43}, enhancing a little L-lysine productivity compared to parental cells.

Protoplast fusion between L-lysine producing strain and other amino acid producing strains will be very interesting because the objective materials in the biosynthetic pathway differ. And, considering the productivity of L-lysine, the induction of protoplast fusion by using of analog resistant mutants will be desirable.

요 약

L-Lysine 생산균주 육종의 한 방법으로, ***Brevibacterium flavum***과 ***Corynebacterium glutamicum***의 이속간 원형질체 융합을 실시하였으며, 이들 균주에 대한 원형질체 형성과 재생의 최적 조건을 조사하였다.

그 결과, ***Corynebacterium glutamicum*** ATCC 21514 S의 경우, lysozyme을 300μg/m*l* 의 농도로 처리하였을 때 12시간 경과 후 99%의 원형질체 형성과 12%의 재생율을 보였으며, ***Brevibacterium flavum*** ATCC 21528 R은 lysozyme을 400 μg/m*l* 로 처리했을 때 12시간 경과 후 99%의 원형질체 형성과 10%의 재생율을 보였다.

Brevibacterium flavum ATCC 21528 R과 ***Corynebacterium glutamicum*** ATCC 21514 S의 이속간 원형질체 융합에서 PEG 농도별 실험을 하여본 결과 PEG 6,000, 30% (w/v)를 사용함으로써 재생세

포당1.2×10^{-5}의 재조합 빈도를 얻었으며, 여기에서 얻어진 재조합주들 가운데 KR$_{43}$ 주는 L-lysine 생성능이 모균보다 12% 증가를 나타내었으며, aspartokinase효소 활성 측정치는 모균보다 13% 높은 것으로 나타났다.

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