Hanguk Nonghwahak Hoechi
(J.Korean Agric. Chem. Soc.)
34(2), 174~179(1991)

Production of L-arginine by intergeneric fusant MWF 9031 of coryneform bacteria

Chi-Young Ok, Chung Park, Min-Su Han and Hong-Kyu Choi
Research and Development Center, Miwon Co., Ltd, 720,
Banghak-dong Dobong-ku, Seoul 132-020, Korea

Abstract : Protoplast fusion was carried out between *Brevibacterium flavum* and *Corynebacterium glutamicum*. For the protoplast fusion, various mutants were isolated from *Brevibacterium flavum* ATCC 21493 and *Corynebacterium glutamicum* ATCC 21831. The optimum conditions for protoplast fusion of these mutants were examined. In the present work, the authors obtained a fusant, MWF 9031, by the intergeneric protoplast fusion between *Brevibacterium flavum* 108-12S and *Corynebacterium glutamicum* 41-214A, which was excellent in L-arginine fermentation. Fusant MWF 9031 was found to accumulate a large amount of L-arginine reached 32.5 mg/ml with a medium containing 10% glucose. The fusant possessed intermediate characteristics between the parental strains and the stability was found to retain for 60 days.

L-Arginine is a semi-essential amino acid with a guanidine group and it is especially important for infant animals on dietetics[1]. Since the production of L-arginine in an industrially practical amount was first reported by Kisumi et al[2], microbial production of much larger amount of L-arginine has been accomplished using traditional mutagenesis[3-6], and genetic techniques such as transduction, protoplast fusion and transformation[7-9].

The fusion process has been shown to generate large numbers of random recombinants. Furthermore because the fusion is neither strain specific nor species specific, this method of gene transfer may be advantageous for strain improvement[10]. Coryneform bacteria is highly important, as it is widely used in industry to produce various amino acids and nucleic acids. The protoplast fusion technique has been used successfully to the breeding of coryneform bacteria[11-14].

This paper describes the formation of intergeneric fusants between *B. flavum* and *C. glutamicum* by PEG-induced protoplast fusion for the production of L-arginine.

Key words : L-arginine, fusion, coryneform bacteria
Corresponding author : C. Park

Materials and Methods

Microorganisms

All mutants were derived from *Brevibacterium flavum* ATCC 21493 and *Corynebacterium glutamicum* ATCC 21831. Cells were mutagenized by treatment with N-methyl-N'-nitro-N-nitrosoguanidine and UV irradiation. In order to get convenient genetic markers for the protoplast fusion, antibiotic resistant mutants were isolated. The strains used in this study are listed in Table 1.

Media

The minimal medium (MM) was used for the isolation of mutants[15]. Dilution fluid, fusion fluid, buffer

Table 1. Bacterial strains used and their properties

Strains	Relevant properties*	L-arginine produced (mg/ml)**
B. flavum		
ATCC 21493	D-Arg^r, Can^r	3.0
MWB 1120	D-Arg^r, Can^r, Gua^{lea}	5.8
MWB 108-12S	D-Arg^r, Can^r, Gua^{lea} ArgHx^r, Homoarg^r, Str^r	25.1
C. glutamicum		
ATCC 21831	Can^r	5.3
MWC 93-360	Can^r, 2-TA^r, D-Arg^r, SG^r	8.7
MWC 41-214A	Can^r, 2-TA^r, D-Arg^r, SG^r SD^r, Amp^r	12.4

* D-Arg^r, Can^r, ArgHx^r, 2-TA^r, SG^r, SD^r, Str^r, and Amp^r denote resistance to D-arginine, canavanine, arginine hydroxamate, homoarginine, 2-thiazolealanine, sulfaguanidine, sulfadiazine, streptomycin, and ampicillin, respectively. Gua^{lea} : guanine leaky.

** Production of L-arginine was carried out as described under Materials and Methods.

solution and regeneration medium were prepared as described previously[14]. The standard medium for L-arginine production contained 10% glucose, 6% $(NH_4)_2SO_4$, 0.3% urea, 0.1% K_2HPO_4, 0.015% $MgSO_4 \cdot 7H_2O$, 50 μg/l biotin and 4% $CaCO_3$ (pH 7.2 with NaOH). The procedures used for protoplast formation and fusion were those of Kaneko and Sakaguchi[16].

Stability of fusant

The stability of the fusant obtained was examined by two ways. First, the fusant grown on a stock slant was transferred to a new slant. Secondly, the primary slant was preserved on a agar slant. The amount of L-arginine produced and cell growth of the fusant on each case were checked at ten day intervals for 60 days.

Analytical methods

The amount of L-arginine produced was determined by the method of Sakaguchi[17]. Bacterial growth was determined from the optical density at 610 nm (OD_{610}) of the culture broth diluted 100 times after dissolving $CaCO_3$ with HCl. Intracellular content of ATP was measured with a Luciferase-luciferin kit and an ATP photometer[18].

Results and Discussion

Protoplast formation and fusion

Protoplasts of the strains, *B. flavum* 108-12S and *C. glutamicum* 41-214A, were prepared by treatment with penicillin G and lysozyme[13-16]. Effect of penicillin G and lysozyme on the protoplast formation of *B. flavum* 108-12S and *C. glutamicum* 41-214A was investigated. As shown in Table 2, when 0.5 unit/ml of penicillin G was added at mid-exponential growth phase after 8 hrs of lysozyme treatment in *B. flavum* 108-12S and *C. glutamicum* 41-214A, the protoplasts were formed at an efficiency of 98.96 % and 99.87%, respectively.

Protoplast fusion was carried out between *B. flavum* 108-12S and *C. glutamicum* 41-214A. After polyethylene glycol (PEG) treatment of a mixture of protoplast of both strains, fusants were obtained at the frequency of about 10^{-3}-10^{-4}(Table 3). As shown in Table 3, effect of PEG concentration on fusion frequency was efficient at the concentration

Table 2. Effect of penicillin G treatment time during exponential growth phase on protoplast formation

Treated at	Strains	*B. flavum* 108-12S			*C. glutamicim* 41-214A		
	After treated lysozyme (hr)	0	4	8	0	4	8
Initial	Viable cell no.	1.2×10^{8}	3.0×10^{5}	2.8×10^{4}	5.0×10^{7}	2.2×10^{5}	2.4×10^{5}
	Protoplast no.	-	1.0×10^{8}	1.2×10^{8}	-	4.3×10^{7}	4.9×10^{7}
	Protoplast formation frequency (%)	-	83.08	98.31	-	85.56	97.50
Mid	Viable cell no.	7.8×10^{8}	4.8×10^{4}	4.1×10^{4}	8.0×10^{8}	3.2×10^{3}	3.0×10^{2}
	Protoplast no.	-	7.7×10^{8}	7.1×10^{8}	-	7.9×10^{8}	7.9×10^{8}
	Protoplast formation frequency (%)	-	98.71	98.96	-	98.62	99.87
Transit	Viable cell no.	2.5×10^{9}	2.3×10^{8}	5.6×10^{7}	2.4×10^{10}	6.8×10^{7}	7.9×10^{6}
	Protoplast no.	-	1.7×10^{9}	1.9×10^{9}	-	1.6×10^{10}	1.8×10^{10}
	Protoplast formation frequency (%)	-	58.8	73.76	-	66.38	74.97

* 0.5 unit/ml of penicillin G was added.

Table 3. Effect of polyvinyl pyrrolidone and polyethylene glycol concentration on fusion frequency

Cross	PEG conc. (%)	PEG (%)			
		1	2	3	4
B. flavum	0	1.0×10^{-7}	3.2×10^{-7}	3.1×10^{-7}	4.4×10^{-7}
MWB 108-12S	10	7.9×10^{-7}	3.0×10^{-6}	3.1×10^{-6}	2.0×10^{-6}
×	20	6.1×10^{-6}	2.0×10^{-4}	2.5×10^{-4}	2.4×10^{-5}
C. glutamicum	30	4.9×10^{-5}	5.2×10^{-3}	4.5×10^{-4}	2.5×10^{-5}
MWC 41-214A	40	3.0×10^{-6}	2.9×10^{-6}	2.0×10^{-6}	1.6×10^{-6}

Regeneration medium (RCG medium) used in this experiment was defined by Katsumata et al[22].

of 20~30%. Fusion frequency was calculated in terms of colony number per total regenerable protoplast number plated[14]. In general polyvinyl pyrrolidone (PVPI) has been widely used in regeneration medium. In this work, effect of PVPI on fusion frequency was examined. In the case of without treatment of PEG the fusant was formed at 10^{-7} frequency, and gradually increased with PVPI addition. 2% of PVPI treatment with 30% PEG 6000 the fusion frequency was 5.2×10^{-3}. From these results, PVPI may be a good fusogen with PEG.

Production of L-arginine by intergeneric fusants

A total of 403 fusants were tested for their L-arginine productivity in fermentation medium in comparison with the parental strains *B. flavum* 108-12S and *C. glutamicum* 41-214A. Fig. 1 shows the distribution of L-arginine productivity of the fusants. As shown in Fig. 1, almost of the fusants showed intermediate productivity between the parental strains. However, about 10% of fusants showed higher productivity than the parental strains.

L-arginine productivity of some fusants and parental strains was shown in Table 4. The best producer, MWF 9031, accumulated 32.5 mg/ml of L-arginine at 30℃. And this amount was 1.3 and 2.6 times higher than those of the parental strains *B. flavum* 108-12S and *C. glutamicum* 41-214A, respectively.

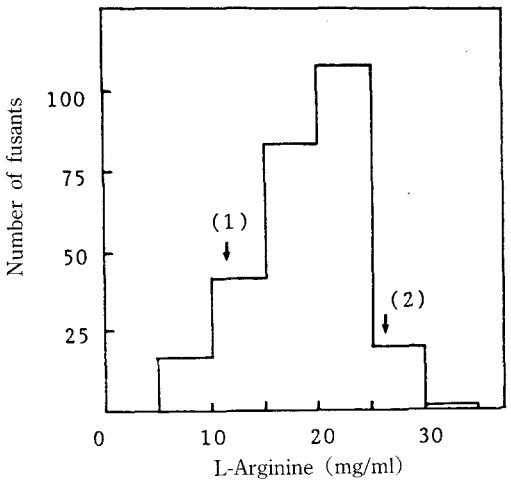

Fig. 1. Distribution of L-arginine productivities of the fusants.

The arrow indicate L-arginine production of the *B. flavum* 108−12S(1) and *C. glutamicum* 41−214A (2).

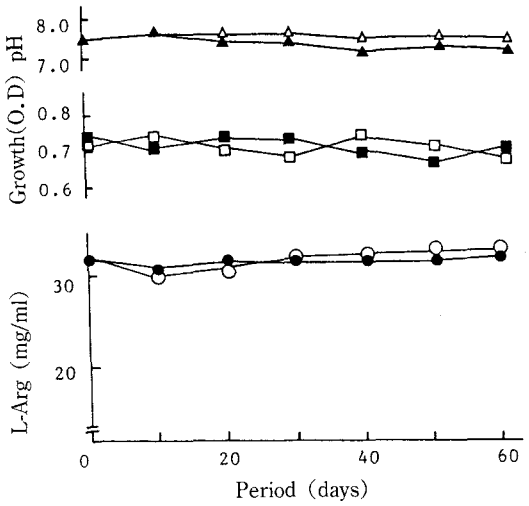

Fig. 2. Stability of the fusant MWF 9031.

The fusant grown on a stock slant containing antibiotics was transferred to a new slant (open symbols) and the primary slant was preserved for the 60 days (closed symbols).

Stability of the fusant MWF 9031

In the application of the protoplast fusion technique to the improvement of industrial microorganisms, the stability of the fusant is very important. For that reason, the stability of the fusant MWF 9031 was examined by two ways (Fig. 2). The amount of L-arginine accumulated, cell growth and pH of the culture broth were checked at ten day intervals for 60 days. As shown in Fig. 2, fusant on each case was found to maintain the L-arginine productivity, cell growth and pH of the broth. In these results, fusant MWF 9031 might be concluded to be sufficiently stable for the production of L-arginine.

Table 4. Accumulation of L-arginine by the fusants from *B. flavum* 108−12S and *C. glutamicum* 41−214A

Strains	Growth (O.D)	L-arginine produced(mg/ml)*	Relative index
Parental strains			
B. flavum 108−12S	0.681	25.1	1.00 —
C. glutamicum 41−214A	0.807	12.4	— 1.00
Fusants			
MWF 8875	0.871	28.6	1.14 2.30
MWF 8902	0.864	27.3	1.01 2.04
MWF 9005	0.773	31.7	1.26 2.56
MWF 9031	0.766	32.5	1.30 2.62
MWF 9163	0.889	31.8	1.27 2.56

* Production of L-arginine was carried out as described under Materials and Methods.

Intracellular content of ATP

Generally sulfa drugs prevent the formation of folic acid and inhibit the growth of many bacteria[19]. Several papers have been published so far in which the productivity of amino acids was improved by sulfa drugs resistant mutants[18, 20, 21]. This increase in production seemed to be due to an increase of intracellular content of ATP. The intracellular levels of ATP in some fusants and *C. glutamicum* were compared (Table 5). As shown in Table 5, the ATP content of sulfaguanidine and sulfadiazine resistant mutant *C. glutamicum* 41-214A was ca. 2 times higher than that of parental strain *C. glutamicum* ATCC 21831. Almost of the improved fusants showed the higher intracellular content of ATP than the parent. It was confirmed that the increase in intracellular content of ATP was very effective for the improvement of L-arginine production in fusants.

Time course of L-arginine production

Fig. 3 showed the typical time course of L-arginine production by intergeneric fusant MWF 9031 and the parental strains *B. flavum* 108-12S and *C. glutamicum* 41-214A. The fermentation, which usually occurs at 30℃, requires ca. 60 hrs to complete. As shown in Fig. 3, some differences were found in the growth and glucose consumption. In the fusant MWF 9031, the growth and glucose consumption were faster than parental strains.

Table 5. Intracellular contents of ATP in *C. glutamicum* and fusants

Strain	Cultivation(hr)	ATP content* ($x10^{-15}$g/cell)
C. glutamicum		
ATCC 21831	48	2.0
41-214A	48	3.8
Fusants		
MWF 9005	48	4.1
MWF 9031	24	5.6
	48	4.5
MWF 9163	48	4.4

* The strains were cultured at 30℃ in the standard medium. ATP extracted from viable cells was measured with a Luciferase-luciferin kit.

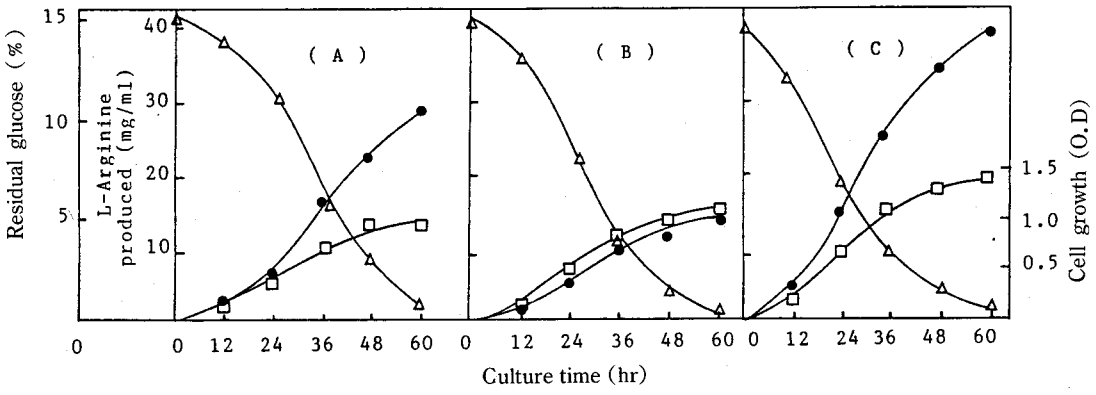

Fig. 3. Time course of L-arginine production.

Symbols : (●) L-arginine, (△) residual glucose, (□) cell growth. Strains : (A) *B. flavum* 108-12S, (B) *C. glutamicum* 41-214A, (C) Fusant MWF 9031

References

1. Aida, K., Chibata, I., Nakayama, K., Takinami, and Yamada, H. : In "Biotechnology of amino acid production' 24 : 131 (1986)
2. Kisumi, M., Kato, J., Sugiyama, M. and Chibata, I. : Appl. Microbial., 22 : 987 (1976)
3. Harwood, C.R. and Baumberg, S. : J. Gen. Microbial., 100 : 177 (1977)
4. Ramos, F., Thuriaux, P., Wiame, J.M. and Bechet, J. : Eur. J. Biochem., 12 : 40 (1971)
5. Yoshida, H., Araki, K. and Nakayama, K. : Agric. Biol. Chem., 43 : 105 (1979)
6. Kato, J., Kisumi, M., Takagi, T. and Chibata, I. : Appl. Environ. Microbiol., 34 : 689 (1977)
7. Kisumi, M., Takagi, T. and Chibata, I. : Amino Acid. Nucleic Acid., 35 : 118 (1977)
8. Japanese Patent 58—158185 (1983)
9. Margaret, C.M.S., Andrew, M. and Simon, B. : Mol. Gen. Genet., 205 : 176 (1986)
10. Okamoto, T., Fujita, Y. and Irie, R. : Agric. Biol. Chem., 49 : 1371 (1985)
11. Karasawa, M., Tosaka, O., Ikeda, S. and Yoshii, H. : Agric. Biol. Chem., 50 : 339 (1986)
12. Kyung, K.C., Lim, B.S., Lee, S.Y. and Chun, M.J. : Kor. J. Appl. Microbiol. Bioeng., 13 : 279 (1985)
13. Park, C., Lim, B.S. and Chun, M.J. : Kor.J. Microbiol., 23 : 265 (1985)
14. Park, C., Lim, B.S. and Chun, M.J. : Kor.J. Appl. Microbiol.Bioeng., 15 : 104 (1987)
15. Nakayama, K. and Yoshida, H. : Agric. Biol. Chem., 36 : 1675 (1972)
16. Kaneko, H. and Sakaguchi, K. : Agric. Biol. Chem., 43 : 1007 (1979)
17. Sakaguchi, S. : J.Biol. Chem., 86 : 217 (1925)
18. Tsuchida, T., Kubota, K., Yoshihara, Y., Kikichi, K. and Yoshinaga, F. : Agric. Biol. Chem., 51 : 2089 (1987)
19. Lehninger, A.L.: In'Biochemistry', 2nd Ed., Worth Publishers, p.732 (1975)
20. Tsuchida, T., Kubota, K. and Yoshinaga, F. : Agric. Biol. Chem., 50 : 2201 (1986)
21. Kubota, K., Onoda, T., Kamijo, H. and Yoshinaga, F. : J. Gen.Appl. Microbiol., 19 : 139 (1973)
22. Katsumata, R., Ozaki, A., Oka, T. and Furuya, A. : J. Bacteriol., 159 : 306 (1984)

Coryne형 세균의 이속간 융합주 MWF 9031에 의한 L-arginine생산

옥치영 · 박 청 · 한민수 · 최홍규((주) 미원 중앙연구소)

초록 : *Brevibacterium flavum* 과 *Corynebacterium glutamicum* 균주간의 원형질체 융합을 실시하였다. 원형질체 융합을 위하여, *Brevibacterium flavum* ATCC 21493과 *Corynebacterium glutamicum* ATCC 21831로부터 여러 변이주를 분리하고 융합의 최적조건을 검토하였다. 본 연구에서 저자 등은 *Brevibacterium flavum* 108—12S와 *Corynebacterium glutamicum* 41—214A 균주 간의 이속간 원형질체 융합주 MWF 9031의 L-arginine 발효능이 우수함을 확인하였다. 융합주 MWF 9031은 10% 포도당이 함유된 배지에서 32.5 mg/ml의 L-arginine을 생산하였다. 융합주의 생리적 성질은 두 모균주의 중간정도를 나타내고, 안정성이 60일 이상 유지되고 있음을 확인하였다.