Breeding of Phenylalanine-producing *Brevibacterium flavum* Strains by Removing Feedback Regulation of Both the Two Key Enzymes in Its Biosynthesis

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The growth of a *mFP*-resistant *Brevibacterium flavum* mutant, No. 221-43, having PDT^R was synergistically and completely inhibited by *mFP* plus Tyr-Glu, but not by *mFP* plus tyrosine or *pFP* plus Tyr-Glu, whereas that of a mutant having DS^R was only partially inhibited by *mFP* plus Tyr-Glu. Tyr-Glu could replace tyrosine required for the growth of a tyrosine auxotroph. The phenylalanine uptake was competitively inhibited by tyrosine and the tyrosine uptake by phenylalanine. The phenylalanine uptake was also inhibited by *mFP*, but not by Tyr-Glu. Mutants having both PDT^R and DS^R derived from strain No. 221-43 were effectively selected by the resistance to *mFP* plus Tyr-Glu, and produced much larger amounts of phenylalanine, with small amounts of tyrosine, than the parent. By the same method, mutants having DS^R and PDT^R , which produced 23.4 g/l of phenylalanine at maximum, were obtained from a *pFP*-resistant tyrosine auxotroph having PDT^R which produced 18 g/l. Similar mutants were also obtained from a tryptophan-producing strain, but produced smaller amounts of tryptophan than the parent, whereas the total amounts of tryptophan and phenylalanine produced were increased.

Brevibacterium flavum has two feedback regulations in its phenylalanine biosynthetic pathway, the synergistic inhibition of DS by phenylalanine and tyrosine,¹⁾ and the inhibition of PDT by phenylalanine²⁾ (Fig. 1). As reported in the previous paper,³⁾ mutants resistant to one of the feedback inhibitions were obtained as phenylalanine analogue (*e.g.*, *mFP*)-resistant mutants. Mutants having DS^R derived from the wild strain accumulated both phenylalanine and tyrosine, while those having PDT^R accumulated only phenylalanine.³⁾ Whereas mutants having both DS^R and PDT^R seemed to be better phenylalanine producers, it was difficult to obtain them, because the mutants having either DS^R or PDT^R were already resistant to phenylalanine analogues. Furthermore, a preliminary experiment show-

ed that the sensitivities to *mFP* of mutants having PDT^R and feedback-sensitive DS were not increased by the further addition of tyrosine, which stimulated the *mFP* inhibition of DS.¹⁾ This seems to be due to competition between *mFP* and tyrosine in the transport system common to aromatic amino acids.⁴⁾ Sato *et al.*⁵⁾ have shown that in the presence of *pFP*, a phenylalanine analogue, tyrosine auxotrophs of *Brevibacterium lactofermentum* did not grow with tyrosine but did with a tyrosine-containing dipeptide, Tyr-Glu. This suggests that Tyr-Glu was taken up without competition with *pFP* and tyrosine was generated inside the cells through its hydrolysis. Thus, it is likely that the addition of *mFP* and Tyr-Glu to the medium may increase the intracellular concentrations of both *mFP* and tyrosine enough

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DS, DAHP synthase; PDT, prephenate dehydratase; CM, chorismate mutase; DS^R , DS resistant to feedback inhibition; PDT^R , PDT resistant to feedback inhibition; *mFP*, *m*-fluorophenylalanine; *pFP*, *p*-fluorophenylalanine; Tyr-Glu, tyrosylglutamate; Tyr-Ala, tyrosylalanine; Phe-Ala, phenylalanylalanine; TES, *N*-tris(hydroxymethyl)methyl-aminoethane sulfonic acid.

FIG. 1. Regulation of Aromatic Amino Acid Biosynthesis in *Brevibacterium flavum*.

⇒, feedback inhibition; (1), DS; (2), CM; (3), PDT; (4), anthranilate synthase.

for them to inhibit DS synergistically, and, therefore, the growth of mutants having feedback-sensitive DS.

This paper reports confirmation of the suggestions described above, development of a method for the selection of mutants having both DS^R and PDT^R with *mFP*-resistance in the presence of Tyr-Glu among those derived from strains having only PDT^R, and application of this method to the breeding of phenylalanine producers and tryptophan producers.

MATERIALS AND METHODS

Organisms. *Brevibacterium flavum* No. 2247 (ATCC 14067) and its *mFP*-resistant mutants,³⁾ No. 221-43 having PDT^R and No. 482-18 having DS^R, were used. Strain No. 239 is a phenylalanine-producing *pFP*-resistant tyrosine-methionine-double auxotroph.^{6,7)} Tryptophan-producing sulfaguanidine-resistant mutant S-179 was derived from strain A-100, which was an azaserine- and 5-fluorotryptophan-resistant mutant derived from strain No. 239.^{6,8)}

Media. The compositions of the glucose-salts media, Medium 20,⁹⁾ 30,¹⁰⁾ and 41,¹¹⁾ and complex medium Medium 7¹²⁾ were given in the previous papers. The following media were used for amino acid production, Medium 3,¹³⁾ containing 100 g/l of glucose as a carbon source, and Medium 31,¹⁰⁾ containing 130 g of glucose, 12 g of fumaric acid, and 3 ml of acetic acid per liter.

Chemicals. DL-*mFP*, DL-*pFP*, L-Tyr-L-Glu, L-Tyr-L-Ala,

and L-Phe-L-Ala were purchased from Sigma Chemical Co. L-[U-¹⁴C]Phenylalanine and L-[U-¹⁴C]tyrosine were from Amersham International plc.

Enzyme preparation. *mFP*-Resistant mutants derived from strain No. 221-43, strain S-179, and strain No. 239 were cultured at 30°C for 24 hr in Medium 3, Medium 31, and Medium 31 but containing 400 mg/l of L-tyrosine, respectively. The cells were harvested, washed twice with 0.2% KCl, suspended in 0.1 M TES-NaOH buffer, pH 7.0, containing 5 mM MgCl₂, 2 mM dithiothreitol and 1 mM tryptophan, and then sonically disrupted at 10 kc for 20 min. The supernatant solutions of the sonicates obtained on centrifugation at 32,000 × *g* for 30 min were gel-filtered on a Sephadex G-25 column (1.5 × 10 cm) and then used as the enzymes. The buffers used for equilibration of the column were 0.05 M TES-NaOH buffer, pH 7.0, containing 5 mM MgCl₂, 1 mM dithiothreitol, and 1 mM tryptophan for assaying DS, 0.05 M TES-NaOH buffer, pH 7.0, containing 5 mM MgCl₂ for assaying CM, and 0.05 M Tris-HCl buffer, pH 7.5, for assaying PDT.

Assay methods. The assay methods for DS,¹¹⁾ CM,¹⁴⁾ and PDT²⁾ were described previously. The uptake of phenylalanine or tyrosine was assayed by a modification of the method described previously⁸⁾: strain No. 2247 was cultured at 30°C for 16 hr in Medium 20. The standard assay mixture contained 10 mM Tris-HCl buffer, pH 7.5, and 0.05 ml of a suspension of the washed cells (absorbancy at 562 nm after 750-fold dilution, 0.20), in a total volume of 0.45 ml, and was preincubated at 30°C for 3 min, and then 0.05 ml of labeled phenylalanine or tyrosine (specific activity; 10 μCi/μmol) was added to a final concentration of 0.02 mM. After incubation for 2.5 min, 0.1 ml aliquots of the mixture were placed on membrane filters (Toyo TM-2, with a pore size of 0.45 μm) and then washed 8 times with 1 ml of cold 0.01 M Tris-HCl buffer, pH 7.5. The radioactivity of the dried cells was measured.

Derivation of *mFP*-resistant mutants. Strain No. 221-43 grown in Medium 41, and strain S-179 and No. 239 grown in Medium 7 supplemented with L-tyrosine and L-methionine (200 mg/l each) for 16 hr were harvested, washed once with 10 ml of 0.1 M sodium phosphate buffer, pH 7.0, and then treated with 750, 300, and 750 μg/ml of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine at 30°C for 15 min to give 19.5, 1.0, and 34% survivors, respectively. Then, the cells spread on selection agar plates containing Medium 41 supplemented with 300 mg/l of Tyr-Glu and *mFP* for strain No. 221-43, Medium 30 supplemented with 30 mg/l of monosodium glutamate, 200 mg/l of L-threonine, 200 mg/l of L-lysine, 50 mg/l of L-cysteine, 300 mg/l of Tyr-Glu and *mFP* instead of tyrosine for strain S-179, and Medium 30 supplemented with 300 mg/l of Tyr-Glu and *mFP* instead of tyrosine for strain No. 239. Colonies appearing during 8 to 10 days' incubation at 30°C were picked up as *mFP*-resistant mutants.

Production of L-phenylalanine or L-tryptophan. Mutants derived from strains No. 221-43, S-179, and No. 239 were aerobically cultured at 30°C for 72 hr in 500 ml-flasks (20 ml/flask) or test tubes (3 ml/tube) containing Medium 3, Medium 31, and Medium 31 but containing 500 mg/l of tyrosine, respectively. Phenylalanine and tryptophan were determined microbiologically as described previously.¹³⁾

RESULTS AND DISCUSSION

Competition in the uptake of aromatic amino acids and their derivatives

As shown in Fig. 2, the phenylalanine uptake was competitively inhibited by tyrosine,

FIG. 2. Competitive Relationship in Cellular Transport Systems between Phenylalanine and Tyrosine.

The phenylalanine or tyrosine uptake was assayed under the standard conditions except for the phenylalanine or tyrosine concentration, respectively. To the assay mixture, 0.2 mM tyrosine (A, ▲), 0.2 mM phenylalanine (B, □), or nothing (●) was added.

TABLE I. EFFECTS OF *m*FP AND DIPEPTIDES CONTAINING TYROSINE OR PHENYLALANINE ON PHENYLALANINE UPTAKE

Addition	Concentration (mM)	Relative uptake rate (%)
None	—	100 ^a
Tyrosine	0.4	17
<i>m</i> FP	1.0	9.8
Tyr-Glu	1.0	120
Tyr-Ala	1.0	111
Phe-Ala	1.0	127

^a 2951 Δ cpm/2.5 min.

and the tyrosine uptake was competitively inhibited by phenylalanine in *B. flavum*, indicating that both are taken up into the cells with a common transport system. Table I also shows the inhibition of phenylalanine uptake by *m*FP. This suggests that they are taken up by the common system. On the other hand, 3 tyrosine-containing dipeptides did not inhibit the phenylalanine uptake, suggesting that they were taken up systems other than that for phenylalanine, tyrosine, and *m*FP. Furthermore, Tyr-Glu did not inhibit glutamate uptake (data not shown). A dipeptide transport system, distinct from those for its component amino acids, has been reported in *Escherichia coli*¹⁵⁾ and *Salmonella typhimurium*.¹⁶⁾ These results indicate that *m*FP competes in the cellular transport system with tyrosine, but not with Tyr-Glu. Therefore, it seems difficult to increase the intracellular concentrations by the addition of *m*FP and tyrosine to the medium, but it seems possible to do so by the addition of *m*FP and Tyr-Glu. The Tyr-Glu uptake and formation of tyrosine from Tyr-Glu inside *B. flavum* cells were confirmed by the growth of a tyrosine auxotroph, S-179, in the presence of Tyr-Glu instead of the required amino acid, tyrosine. It showed maximum growth at 300 μg/ml of Tyr-Glu.

Sensitivities to phenylalanine analogues and effects of tyrosine-containing dipeptides

As shown in Fig. 3 (A), strain No. 221-43 having PDT^R and feedback-sensitive DS, a *m*FP-resistant mutant derived from wild strain No. 2247, was resistant to *m*FP. That is, its growth was only inhibited 20% by 5000 μg/ml of *m*FP. This resistance to *m*FP was not influenced by the addition of tyrosine, which synergistically inhibited DS with *m*FP. However, the simultaneous presence of *m*FP and a tyrosine-containing dipeptide, Tyr-Glu or Tyr-Ala, which did not compete with *m*FP in the transport system, strongly inhibited the growth of the strain. The simultaneous presence of *m*FP, tyrosine and glutamate did not make it sensitive to *m*FP. Moreover, the growth inhibition by *m*FP and Tyr-Glu was

FIG. 3. Effects of Tyrosine-containing Peptides, *mFP*, and *pFP* on the Growth of *PDT*^R-, *DS*^R-, or *DS*^R *PDT*^R-Type Mutants.

Strains No. 221-43 (A and B), No. 482-18 (C), No. 239 (D), and M-145 (E and F) were cultured at 30°C for 16, 40, 24, and 24 hr in test tubes containing 5 ml of Medium 41 with various additions, respectively. Relative growth was determined as the relative increase in absorbancy at 562 nm after 26-fold dilution, as to that in the absence of *mFP* or *pFP*. Nothing (○), 300 mg/l of Tyr-Glu (△), 300 mg/l of Tyr-Ala (■), 150 mg/l of tyrosine (□), 150 mg/l of tyrosine and 150 mg/l of monosodium glutamate (●), 300 mg/l of Tyr-Glu and 200 mg/l of phenylalanine (▲), and the indicated concentrations of *mFP* or *pFP* were added to the medium. In (A), the absorbancies at 562 nm in the absence of *mFP* were 0.500 (○), 0.540 (△), 0.430 (■), 0.500 (□), 0.540 (●), and 0.500 (▲). In (D), 150 mg/l of methionine was further added.

completely recovered by the further addition of phenylalanine. Therefore the growth inhibition seems to be due to depression of phenylalanine biosynthesis through the synergistic inhibition of *DS*¹⁾ by *mFP* and tyrosine formed from Tyr-Glu intracellularly.

pFP, another phenylalanine analogue, which inhibits *PDT*²⁾ but not *DS*¹⁾ with tyrosine synergistically, did not strongly inhibit the growth of strain No. 221-43 in the presence of Tyr-Glu, unlike *mFP* (Fig. 3(B)). Furthermore, as shown in Fig. 3(C), the sensitivity to *mFP* of strain No. 482-18¹⁾ having *DS*^R, another *mFP*-resistant mutant derived from wild strain No. 2247, was not affected by

the presence of Tyr-Glu. These results support that the growth inhibition in the presence of both *mFP* and Tyr-Glu was due to the inhibition of *DS* by *mFP* and tyrosine. The partial growth inhibition of strain No. 482-18 at high *mFP* concentrations will be discussed later.

Derivation of mutants having both DS^R and PDT^R

From the results described above, it seems that mutants having both *DS*^R and *PDT*^R, which were suggested to accumulate larger amounts of phenylalanine than those having only *PDT*^R or *DS*^R, can be selected by *mFP*-

TABLE II. PHENYLALANINE AND TYROSINE PRODUCTION AND ENZYMATIC ALTERATION IN *mFP*-RESISTANT MUTANTS DERIVED FROM PDT^R-TYPE STRAIN No. 221-43

Production (g/l)	Parent and mutant strain No.					
	221-43	M-51	M-144	M-145	M-154	M-211
L-Phe	2.1	4.5	4.0	6.0	4.0	5.2
L-Tyr	<0.01	0.14	0.11	1.4	0.22	0.11

Enzyme activity (%)							
Enzyme	Addition (1 mM)						
DS	None	100 (10.5)	100 (28.3)	100 (13.9)	100 (9.1)	100 (13.3)	100 (12.4)
	Phe + Tyr	29	94	91	93	99	90
CM	None	100 (2.1)	100 (0.78)		100 (2.1)		100 (1.3)
	Phe	9.7	280		173		242
	Tyr	13	183		148		224
	Trp	257	414		218		275
PDT	None	100 (2.6)			100 (4.2)		100 (5.0)
	Phe	202			179		210
	Tyr	255			240		313
	Trp	101			100		99

The specific activities in nmol/min/mg protein are given in parentheses.

Abbreviations: Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan.

resistance in the presence of Tyr-Glu from among mutants derived from strains having PDT^R. After mutagenesis of strain No. 221-43, *mFP*-resistant mutants were isolated as colonies appearing on agar plates containing 600 to 4000 µg/ml of *mFP* and 300 µg/ml of Tyr-Glu. Among 271 mutants, 14 accumulated more than 3.5 g/l of phenylalanine in a test tube culture, in which the parent produced 2.0 g/l of phenylalanine. The best production was 4.6 g/l of phenylalanine. Table II shows the phenylalanine production of the best 5 strains in flask cultures after single colony isolation. The best producer, strain M-145, accumulated 6.0 g/l of phenylalanine, that is, 2.9-times more than the parent strain. These strains accumulated tyrosine in addition to phenylalanine (Table II). Tyrosine accumulation is a characteristic of mutants having DS^R.³⁾

Enzyme alterations of these mutants were examined, as shown in Table II. All the strains

tested had DSs resistant to synergistic inhibition by phenylalanine and tyrosine, and CMs resistant to inhibition by phenylalanine or tyrosine, in addition to PDT^R inherited from the parent. As reported previously, DS and CM form a bifunctional enzyme complex with common regulatory sites for phenylalanine and tyrosine. Therefore, the inhibitions of DS and CM are simultaneously removed on mutation.¹⁷⁾ It is of interest enzymologically that phenylalanine and tyrosine, like tryptophan, stimulate the activity of feedback-resistant CMs.

Thus, mutants having both PDT^R and DS^R were obtained from strain No. 221-43 having only PDT^R. As shown in Fig. 3 (E,F), the sensitivities of strain M-145 having DS^R and PDT^R to *mFP* and *pFP* were not affected by the addition of Tyr-Glu, in contrast to in the case of parent strain No. 221-43 having PDT^R. This result again supports that the growth inhibition of strains having PDT^R alone by

TABLE III. PHENYLALANINE AND TRYPTOPHAN PRODUCTION AND ENZYMATIC ALTERATION IN *mFP*-RESISTANT MUTANTS DERIVED FROM STRAINS No. 239 AND S-179

Production (g/l)		Parent and mutant strain No.							
		239	M-87	M-48	M-245	M-257	M-128	S-179	M-423
L-Phe		18.1	23.4	23.2	22.8	22.4	22.1	1.6	9.1
L-Trp		—	—	—	—	—	—	18.9	15.2
L-Phe + L-Trp								20.5	24.3
Enzyme activity (%)									
Enzyme	Addition (1 mM)								
DS	None	100 (16)	100 (18)	100 (19)	100 (15)	100 (12)	100 (18)	100 (47)	100 (54)
	Phe + Tyr	30	98	94	99	98	99	53	90
CM	None	100 (5.6)	100 (3.5)	100 (0.6)	100 (0.8)	100 (3.9)	100 (1.3)	100 (4.1)	100 (5.4)
	Phe	8.0	177	510	112	177	294	7.9	152
	Tyr	15	143	286	194	140	182	12	123
	Trp	161	213	928	356	217	547	189	205

The specific activities in nmol/min/mg protein are given in parentheses.

—, not determined.

mFP and Tyr-Glu is due to the synergistic inhibition of DS. It was also clarified that the weak growth inhibition by *pFP* and Tyr-Glu of strain No. 221-43 was also caused by the DS inhibition and that the partial growth inhibition of strain No. 482-18 by a high concentration of *mFP* was caused by the inhibition of PDT by *mFP*.

Application to the breeding of phenylalanine producers

The method used for the derivation and selection of mutants having both DS^R and PDT^R was applied for the improvement of phenylalanine producers. A phenylalanine-producing strain, No. 239, was used as the parent strain. In this strain, whereas DS was sensitive to the inhibition, similar to that in the wild strain (Table III), PDT with a specific activity of 0.9 nmol/min/mg protein, was not inhibited at all but activated 7.7- or 10.9-times by 1.0 mM phenylalanine or tyrosine, respectively, the former of which completely inhibited PDT of the original wild strain, No. 2, with a specific activity of 0.4 nmol/min/mg protein. As shown in Fig. 3(D), growth of strain No.

239 was almost completely inhibited by more than 600 µg/ml of *mFP* in the presence of Tyr-Glu, similar to that of a simple PDT^R-type strain, No. 221-43. Then, 1100 *mFP*-resistant mutants were isolated from agar plates containing 1000 to 4000 µg/ml of *mFP* after independent mutagenizing treatment of strain No. 239 three times. Mutants producing more than 20 g/l of phenylalanine in a test tube culture were obtained at a high frequency, for example, 33 out of 300 mutants in the second experiment. The production by the best 5 strains in flask cultures after single colony isolation is shown in Table III. The best production, 23.4 g/l of phenylalanine, was shown by strain M-87, while the parent, No. 239, produced 18.1 g/l. The DSs and CMs of these 5 mutants were resistant to the inhibition, similar to those derived from strain No. 221-43 (Table III). Thus, mutants having both DS^R and PDT^R could be derived from phenylalanine-producing strain No. 239 and the phenylalanine production was increased by 5.3 g/l at maximum.

Application to the breeding of tryptophan-producing mutants

The sulfaguanidine-resistant tryptophan-producing strain, S-179, used as the parent in this experiment was derived, similar to strain S-225,⁶⁾ from strain A-100,⁸⁾ which is a 5-fluorotryptophan- and azaserine-resistant tryptophan-producing mutant derived from a phenylalanine-producing strain, No. 239. Strain S-179 has PDT^R inherited from strain No. 239 and partially feedback-resistant DS inherited from strain A-100 (Table III). Since DS is a biosynthetic enzyme common to tryptophan and phenylalanine, complete removal of the feedback inhibition of DS could possibly lead to an increase of the tryptophan production. Since the growth of strain S-179 was completely inhibited by more than 400 µg/ml of *mFP* in the presence of Tyr-Glu, *mFP*-resistant mutants were isolated from agar plates containing 400 to 1000 µg/ml of *mFP*. Contrary to our expectation, none out of the 481 mutants obtained showed higher productivity than the parent in a test tube culture. However, mutants showing higher phenylalanine accumulation were found. Strain M-423, selected from among them, accumulated larger total amounts of phenylalanine and tryptophan than the parent, as shown in Table III. The synergistic inhibition of DS by phenylalanine and tyrosine was almost completely removed in strain M-423 and the inhibitions of CM were also removed. Thus, a mutant having both DS^R and PDT^R could be derived from a tryptophan-producing strain, S-179, as in the cases of strain No. 221-43 and No. 239. DS^R provided by this mutation seems to have increased the total amounts of tryptophan and phenylalanine accumulated, whereas the decreased tryptophan production and the increased phenylalanine production seem to be due to the feedback-resistant CM, because CM is the enzyme on the phenylalanine-

side at the chorismate-branch point of the tryptophan and phenylalanine biosynthetic pathway. In order to remove the phenylalanine by-product, lacking of CM activity or complete removal of the feedback inhibition of anthranilate synthase at the tryptophan-branch seems necessary.

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