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Genetic Breeding of L-Tyrosine Producer from *Brevibacterium lactofermentum*

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A wild-type parent of *Brevibacterium lactofermentum* was converted into an L-Tyr producer by three steps of genetic breeding. First, acquirement of *m*-fluoro-D,L-phenylalanine resistance (1,000 μg/ml) brought about MF1317 which produced 3.5 g/l of L-Tyr and a byproduct of 2.8 g/l of L-Phe. Second, increase in the drug resistance (5,000 μg/ml) gave MF358 that produced 6.4 g/l of L-Tyr and a byproduct of 6.0 g/l of L-Phe. Third, an L-Phe auxotrophic mutant (FT-1) derived from MF358 accumulated 16 g/l of L-Tyr. In FT-1, L-Phe was not accumulated at all, but a small amount of anthranilate (0.4 g/l) was. A key enzyme in the biosynthesis of L-Tyr, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, was free from synergistic feedback inhibition by L-Tyr and L-Phe in the producers, and so L-Tyr accumulation occurred independently of L-Phe concentration in the production medium.

A practical method for L-Tyr production can be described as follows: (a) protein hydrolyzation using soybeans as a raw material, (b) enzymatic synthesis using pyruvic acid or phenol as starting materials,[1] and (c) microbial production from a carbon source such as glucose.[2–5] Among them, the most effective is the microbial production directly from glucose, since the hydrolyzation of soybeans generates excessive byproducts and the starting materials in the enzymatic method are expensive.

To induce an L-Tyr-producing mutant from a wild-type strain of *Brevibacterium* or *Corynebacterium*, there are at least two barriers to be overcome. One is the regulation of a key enzyme in the biosynthesis of L-Tyr and L-Phe, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, catalyzing the synthesis of DAHP from phosphoenolpyruvate and erythrose-4-phosphate. This enzyme is susceptible to strong synergistic feedback inhibition by L-Phe and L-Tyr.[6,7] The other is prephenate dehydratase, which is specifically concerned with L-Phe biosynthesis at the expense of L-Tyr overproduction (Fig. 1).

An L-Tyr producer has been derived by overcoming one of these barriers. Hagino *et al.* isolated mutants resistant to L-Tyr analogues from an L-Phe auxotroph of *Corynebacterium glutamicum*. These mutants accumulated L-Tyr when supplied with sub-optimum levels of L-Phe. Their DAHP synthase was insensitive to the feedback control in the enzyme reaction system, but an excess of L-Phe in the production medium markedly restricted L-Tyr production.[5] Shiio *et al.*, on the other hand, obtained a mutant of *Brevibacterium flavum* that was resistant to an L-Phe analogue and had a DAHP synthase insensitive to the synergistic feedback inhibition by L-Phe and L-Tyr and that accumulated large amounts of both L-Tyr and L-Phe.[8]

For this paper, we induced a mutant of *Brevibacterium lactofermentum* in which DAHP synthase is not subject to synergistic

Abbreviations: L-Tyr, L-tyrosine; L-Phe, L-phenylalanine; L-Trp, L-tryptophan; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; mFP, *m*-fluoro-D,L-phenylalanine; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

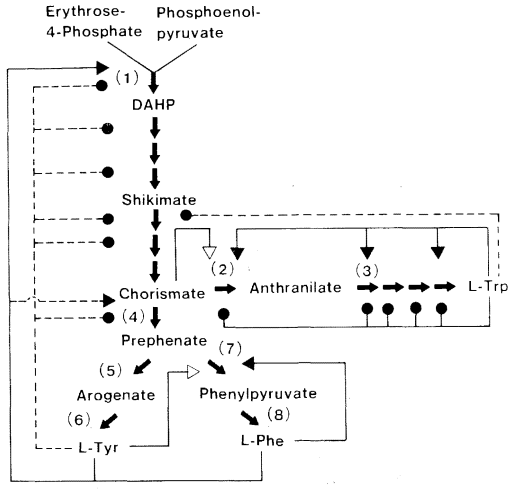

Fig. 1. Biosynthetic Pathway of Aromatic Amino Acids and their Regulatory Mechanism in *Brevibacterium*.

(1) DAHP synthase; (2) anthranilate synthase; (3) anthranilate phosphoribosyltransferase; (4) chorismate mutase; (5) arogenate aminotransferase; (6) arogenate dehydrogenase; (7) prephenate dehydratase; (8) phenylalanine aminotransferase; ◀——, inhibition (◀– – –, partial inhibition); ◁——, activation; ●——, repression (●– – –, weak repression).

feedback inhibition and the prephenate dehydratase is deficient in favor of L-Tyr accumulation.

Materials and Methods

Microbial strains. Brevibacterium lactofermentum No. 2256, which was used as the parent strain, has a potent ability to accumulate L-glutamic acid in biotin-insufficient medium.

Chemical agents. Amino acid analogues listed in Table I were purchased from Sigma Co.

Culture test detecting antimicrobial activity of amino acid analogues. B. lactofermentum No. 2256 was grown at 31.5°C overnight on an agar complete medium, CM-2G, composed of 0.5% glucose, 1.0% Polypeptone, 1.0% yeast extract, 0.5% NaCl, and 2% agar (pH 7.2). The cells were harvested and washed with 0.1 M phosphate buffer (pH 7.0). Then the cells were suspended in 4 ml of MM medium (glucose 2.0%, $(NH_4)_2SO_4$ 0.5%, urea 0.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, Fe^{2+} 2 ppm, Mn^{2+} 2 ppm, biotin 50 μg/ml, and VB_1–HCl 2,000 μg/ml, pH 6.6) containing 1.000 μg/ml of each amino acid analogue at the initial cell density of 0.05 OD (562 nm) and grown at 31.5°C in a test tube with shaking for 24 hr. Extent of the growth was measured by the optical density at 562 nm.

L-Tyr production test. Cultivation for L-Tyr production was done by modification of the method of Tsuchida *et al.*[9] as follows. A loopful of cells grown on the CM-2G agar medium for 24 hr at 31.5°C was inoculated into 20 ml of production medium SM-3 (fumaric acid 1.2%, acetic acid 3 ml/l, glucose 13%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, Fe^{2+} 2 ppm, Mn^{2+} 2 ppm, $(NH_4)_2SO_4$ 2.5%, biotin 200 μg/ml, vitamin B_1–HCl 2,000 μg/ml, soybean hydrolyzate (total nitrogen = 0.3%) 50 ml/l, and $CaCO_3$ 5%, pH 6.5) and cultured at 31.5°C in a 500-ml shaker flask for 72–96 hr. After the cultivation, 2 ml of 6 N KOH was added to the culture liquids to dissolve the L-Tyr produced.

Measurement of L-Tyr, L-Phe, and anthranilate. L-Tyr, L-Phe, and anthranilate were analyzed by HPLC (column; CPK-08). The mobile phase was 0.1 M Na_2HPO_4 (pH 4.2, adjusted with H_3PO_4) and the flow rate was 0.5 ml/min. These compounds were measured by their absorbance at 206 nm using a calibration curve.

Measurement of glucose. Glucose was analyzed enzymatically by the glucose oxidase–peroxidase method (Glucose C-Test; Wako Pure Chemical Industries, Ltd.).

Mutation procedures. The cell culture was made in 5 ml of SM-3 medium as described below with shaking at 31.5°C. Cells in late logarithmic phase were harvested, washed with 0.1 M phosphate buffer (pH 7.0), and resuspended in an equal volume of the same buffer containing 1,500 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). After 20–30 min of incubation at 30°C, cells were washed three times with the same buffer.

Selection of mFP-resistant mutants and L-Phe auxotrophs. For the selection of mFP-resistant mutants, NTG-treated cells (10^6) were spread on MM-agar plates containing mFP and cultured at 31.5°C for 3–7 days. mFP-resistant colonies thus appeared were used for the L-Tyr production test.

For the selection of L-Phe auxotrophs, NTG-treated cells were incubated on CM-2G agar medium. Among colonies that thus appeared, the ones which grew only in L-Phe-supplemented MM medium were isolated by the replica method.

Preparation of cell free extract. Cells were cultured in a 500-ml shaker flask containing 20 ml of SM-3 medium at 31.5°C for 24 hr, harvested, washed twice with 0.2% KCl, and suspended in 0.1 M Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol. Then the cells were disrupted by sonication (50 W, 8 min). The crude extract, obtained as the supernatant after centrifugation at $32,000 \times g$ for 20 min, was assayed immediately for enzyme activity.

Enzyme assay of DAHP synthase. Activity of DAHP synthase was measured by the method of Jensen *et al.*[10] and Shiio *et al.*,[6] measuring the DAHP formed in the reaction mixture.

Enzyme assay of prephenate dehydratase. Activity of prephenate dehydratase was measured by the method of Cotton *et al.*[11] and Sugimoto *et al.*,[12] measuring the phenylpyruvate formed in the reaction mixture.

Results and Discussion

(1) *Effects of Phe-, Tyr-, and Trp-analogues on the growth of B. lactofermentum*

Amino acid analogues inhibit key enzymes involved in the biosynthesis of corresponding amino acids. Therefore, a wild-type strain cannot grow in the presence of such an analogue since it cannot form the amino acid, but growth inhibition is restored when the organism is supplied with the amino acid. A mutant resistant to an analogue is expected to have a key enzyme which is no longer inhibited by the analogue, also suggesting that the enzyme is not subject to feedback inhibition by the amino acid. Therefore, such a mutant usually accumulates the amino acid due to the absence of control mechanisms.

From this point of view, as many as 50 kinds of amino acid analogues relating to aromatic amino acids were examined for their ability to inhibit growth of the parent strain as shown in Table I. *m*-Fluoro-D,L-phenylalanine (mFP), β-1-naphthylalanine, 4-fluoro-D,L-tryptophan, 5-fluoro-D,L-tryptophan, 6-fluoro-D,L-tryptophan, 5-methyl-D,L-tryptophan, and 5-methoxy-D,L-tryptophan were effective. Among them, mFP was considered to inhibit an enzyme involved in the biosynthesis of L-Phe (and L-Tyr), since growth inhibition due to mFP (1,000 μg/ml) was restored in the pres-

Table I. Effects of Amino Acid Analogues on Growth of *B. lactofermentum*

Relative growth in the presence of 1000 μg/ml of each analogue was expressed as a percentage of optical density observed without amino acid analogue.

Amino acid analogue	Relative growth	Amino acid analogue	Relative growth
m-Fluoro-D,L-phenylalanine	25	α-Methyl-D,L-tyrosine	100
o-Fluoro-D,L-phenylalanine	100	*m*-Fluoro-D,L-tyrosine	100
p-Fluoro-D,L-phenylalanine	90	*o*-Methyl-L-tyrosine	100
p-Amino-D,L-phenylalanine	100	α-Methyl-D,L-*m*-tyrosine	100
D,L-β-3-Thienylalanine	90	α-Methyl-D,L-*p*-tyrosine	100
D-Phenylalanine	95	D,L-Tyroxine	100
β-2-Thienylalanine	100	D,L-Tyronine	95
N-Benzoyl-β-alanine	100	2-Amino-3-methyl-benzoate	100
N-Methyl-D,L-alanine	95	4-Fluoro-D,L-tryptophan	10
p-Bromo-D,L-phenylalanine	100	5-Fluoro-D,L-tryptophan	5
p-Nitro-D,L-phenylalanine	100	6-Fluoro-D,L-tryptophan	10
p-Iode-D,L-phenylalanine	90	5-Methyl-D,L-tryptophan	10
p-Chloro-D,L-phenylalanine	95	6-Methyl-D,L-tryptophan	100
o-Chloro-D,L-phenylalanine	95	7-Methyl-D,L-tryptophan	80
N-Cyclohexyl-β-alanine	100	7-Benzyloxy-D,L-tryptophan	95
D,L-Phenylalanine-hydroxamate	90	α-Methyl-D,L-tryptophan	95
α-Methyl-D,L-phenylalanine	95	5-Methoxy-D,L-tryptophan	10
β-1-Naphthyl-alanine	30	5-Benzyloxy-D,L-tryptophan	100
L-Tyrosine-hydroxamate	95	L-Tryptophan-aminohydrochloride	100
3,5-Dibromo-L-tyrosine	100	*N*-Carbamyl-L-tryptophan	95
3-Iode-L-tyrosine	100	*N*-Carbobenzoxy-L-tryptophan	95
D,L-*m*-Tyrosine	100	*N*-*t*-Butoxycarbonyl-L-tryptophan	95
D,L-*o*-Tyrosine	100	*N*-Formyl-D,L-tryptophan	95
D-Tyrosine	100	*N*-Chloroacetyl-L-tryptophan	100
o-Benzyl-L-tyrosine	100	7-Aza-D,L-tryptophan	90

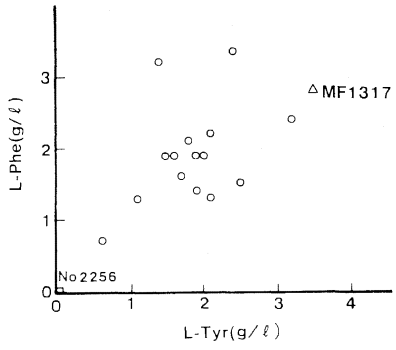

Fig. 2. Productivity of L-Tyr and L-Phe of mFP-Resistant (1,000 μg/ml) Mutants Derived from *B. lactofermentum*.
Cultivation was done in SM-3 medium at 31.5°C for 72 hr.

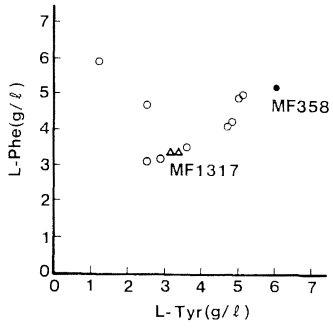

Fig. 3. Productivity of L-Tyr and L-Phe of mFP-Resistant (5,000 μg/ml) Mutants Derived from *B. lactofermentum*.
Cultivation was done in SM-3 medium at 31.5°C for 72 hr.

ence of L-Phe (100 μg/ml).

(2) *Derivation of mFP-resistant mutants and their L-Phe/L-Tyr productivity*

From a wild-type strain of *B. lactofermentum*, 16 mFP-resistant mutants were derived from mFP (1,000 μg/ml)-containing agar plates. Subsequent production study (Fig. 2) showed that all of the mutants accumulated both L-Phe and L-Tyr. Among them, the most potent producer of L-Tyr was MF1317 (3.5 g/l), which also accumulated 2.8 g/l of L-Phe as a byproduct.

Further, mFP resistance was increased to 5,000 μg/ml. Among the 10 strains obtained (Fig. 3), MF358 accumulated 6.0 g/l of L-Tyr and as much as 5.2 g/l of L-Phe was also excreted as a byproduct.

Table II. L-PHE AND L-TYR AUXOTROPHIC MUTANTS DERIVED FROM MF358, AND THEIR AMINO ACID PRODUCTIVITY

Cultivation was done in SM-3 medium supplemented with 200 mg/l of L-Phe (for L-Phe auxotrophs) or 300 mg/l of L-Tyr (for L-Tyr auxotrophs) at 31.5°C for 96 hr.

Strain	Phenotype	Accumulation (g/l)		
		L-Tyr	L-Phe	Anthranilate
B. lactofermentum				
MF358	—	6.4	6.0	0.4
FT-1	Phe^-	14.4	0	0.4
FT-2	Phe^-	0	0	7.5
FT-3	Phe^-	7.9	0	0.5
FT-4	Phe^-	8.9	0	0.2
FT-6	Phe^-	0.3	0	9.0
FT-7	Phe^-	11.7	0	0.5
FT-8	Phe^-	10.6	0	0.2
FT-12	Phe^-	12.1	0	0.2
FP-1	Tyr^-	0	5.1	7.2
FP-2	Tyr^-	0	4.8	5.8

(3) *Derivation of L-Phe auxotrophic mutants from mFP-resistant mutant, MF358*

In an attempt to convert MF358 into an L-Tyr producer without L-Phe excretion, L-Phe auxotrophic mutants were derived as shown in Table II. There were two types of the auxotrophic mutants: one was an L-Tyr-producer with weak anthranilate accumulation, and the other was an anthranilate-producer without L-Tyr excretion. L-Phe production was not observed at all in either type. The most potent producer of L-Tyr was FT-1, which accumulated 14.4 g/l of L-Tyr from 130 g/l of glucose with a production yield of 11.1%.

There can be two types of L-Phe auxotrophs. One is deficient in prephenate dehydratase which catalyzes the formation of phenylpyruvate from prephenate, and the other lacks the phenylalanine aminotransferase involved in the formation of L-Phe from phenylpyruvate (see Fig. 1). The former mutant is considered to accumulate L-Tyr exclusively as in the cases of FT-1, -3, -4, -7, -8, and -12. The latter case is complicated. Phenylalanine aminotransferase in *Brevibacterium* was reported to consist of two kinds of enzyme, $TA\text{-}P_I$ and $TA\text{-}P_{II}$.[13] In

addition to the formation of L-Phe, both aminotransferases reportedly also catalyzed the formation of arogenate from prephenate, leading to L-Tyr synthesis. One more aminotransferase is reported to exist for arogenate aminotransferase, TA-T.[13] The latter mutant is considered to be deficient in TA-P_I and TA-P_{II}, but to have TA-T. TA-T was supposed to be sufficient only to support the cell growth but not enough for L-Tyr overproduction. Therefore, the latter mutant could not accumulate L-Tyr and intracellular pools of intermediate metabolites would increase, including chorismate, thus leading to the accumulation of anthranilate as in the cases of FT-2 and FT-6.

In this experiment, two L-Tyr auxotrophs, FP-1 and FP-2, were also found. Both auxotrophs were first expected to accumulate L-Phe alone. But in addition to 4.8–5.1 g/l of L-Phe, they accumulated large amounts of anthranilate at concentrations of as much as 5.8–7.2 g/l as compared to 0.4 g/l for the parent strain, MF358.

This accumulation of anthranilate could be explained by the enzymatic properties of prephenate dehydratase, anthranilate synthase, and anthranilate phosphoribosyltransferase. Prephenate dehydratase is activated by L-Tyr and inhibited by L-Phe.[12] Therefore, in these L-Tyr auxotrophs, endogeneous L-Tyr was limited, but L-Phe was abundant, resulting in the inhibition of prephenate dehydratase. So endogeneous pools of intermediate metabolites such as chorismate increased. Anthranilate synthase, on the other hand, is strongly inhibited by L-Trp,[14] but excessive chorismate, a substrate of this enzyme, can set this inhibition free. In addition, anthranilate phosphoribosyltransferase, which converts anthranilate into phosphoribosylanthranilate, is inhibited by L-Trp even in the presence of excess amounts of anthranilate.[15] What was happening with FP-1 and FP-2 was that overproduction of L-Phe was restricted in favor of anthranilate accumulation.

These mutants can be used as parent strains for the genetic breeding of an L-Phe-producer lacking L-Tyr- and anthranilate-excreting activity as reported in our following paper.

(4) *Enzymatic properties of* L-*Tyr-producing mutants*

Enzymatic properties of DAHP synthase of the mFP-resistant mutant, MF358, and the L-Phe auxotrophic mutant, FT-1, were studied and compared to that of the wild-type strain of *B. lactofermentum* (Table III). The enzyme activity of the wild-type strain was significantly inhibited by L-Phe and L-Tyr, and the inhibition was more serious in the presence of both L-Phe and L-Tyr. The marked contrast was that the DAHP synthase of MF358 and FT-1 was not inhibited by L-Phe, and the inhibitions by L-Tyr alone and by L-Phe plus L-Tyr were also very slight. These properties suggest that mFP resistance is directly related to the desensitizing of DAHP synthase in favor of overproduction of both L-Phe and L-Tyr.

Enzymatic properties of prephenate dehydratase of the L-Tyr-producers were also studied and compared to that of the wild-type strain (Table IV). The enzyme activity of the wild-type strain was inhibited by L-Phe and activated by L-Tyr. Some L-Phe producers have prephenate dehydratase desensitized

Table III. Enzymatic Properties of DAHP Synthase of L-Tyr-Producing Mutants (MF358 and FT-1) as Compared to the Wild Type Strain

Cell extracts were prepared from cultures in SM-3. Two hundred mg/l of L-Phe was added to the FT-1 culture. DAHP synthase activity was measured in the presence and absence of the supplied amino acids under standard assay conditions. Relative activity was expressed as a percentage of that observed without amino acid. Specific activity was expressed as nmol of DAHP formed per min per mg protein.

Strain	Specific activity	Relative activity		
		L-Phe (1 mM)	L-Tyr (1 mM)	L-Phe (1 mM) L-Tyr (1 mM)
B. lactofermentum				
No. 2256	5.6	63	76	28
MF358	8.7	96	85	83
FT-1	7.5	99	88	88

Table IV. ENZYMATIC PROPERTIES OF PREPHENATE DEHYDRATASE OF L-Tyr-PRODUCING MUTANTS (MF358 AND FT-1) AS COMPARED TO THE WILD-TYPE STRAIN

Cell extracts were prepared from cultures in SM-3. Two hundred mg/l of L-Phe was supplemented to the FT-1 culture. Prephenate dehydratase activity was measured in the presence and absence of the supplied amino acids under standard assay conditions. Relative activity was expressed as a percentage of that observed without amino acid. Specific activity was expressed as nmol of phenylpyruvate formed per min per mg protein.

Strain	Specific activity	Relative activity		
		L-Phe (1 mM)	L-Tyr (1 mM)	L-Phe (1 mM) L-Tyr (1 mM)
B. lactofermentum				
No. 2256	3.2	48	274	242
MF358	4.9	31	219	188
FT-1	<0.1	—	—	—

to L-Phe feedback inhibition.[16,17] However, the prephenate dehydratase of MF358 which produced 6.0 g/l of L-Phe had no alteration from that of the wild-type strain. L-Phe production in MF358 is supposed to be due to the activation of prephenate dehydratase by L-Tyr produced by itself. Prephenate dehydratase activity in FT-1 was not detected at all.

(5) *L-Tyr accumulation by FT-1 independent of L-Phe concentration in production medium*

Usually the optimum concentration of a nutrient growth factor is the most important key when an auxotroph is employed for microbial production. An L-Tyr-producing L-Phe auxotroph derived by Hagino *et al.* from *C. glutamicum* required an L-Phe concentration which was sub-optimum for cell growth, and thus a control mechanism for aromatic amino acid biosynthesis still remained in the L-Tyr producer.[3,5] From this point of view, the effects of L-Phe concentrations on L-Tyr production by FT-1 were studied (Fig. 4). The production medium using soybean hydrolyzate as a component contained 400 mg/l of L-Phe, but an additional 200 mg/l of L-Phe was required to maintain sufficient growth

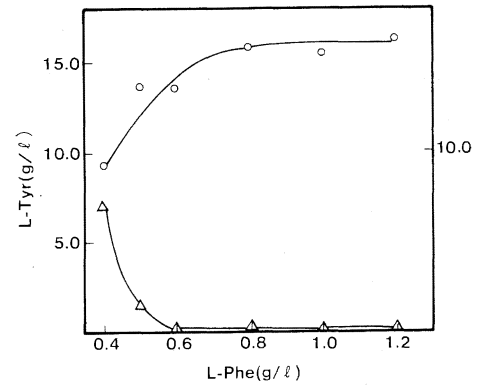

Fig. 4. Effects of L-Phe Concentration on L-Tyr Production of FT-1.

Cultivation was done in SM-3 medium containing the concentrations of L-Phe shown at 31.5°C for 96 hr. ○—○, L-Tyr; △—△, glucose.

of FT-1. Still further addition of L-Phe up to 800 mg/l did not cause a decrease in L-Tyr productivity, supposedly from the enzymatic property of the DAHP synthase of this strain in which the enzyme was free from the control mechanism exerted in a synergistic manner by L-Tyr and L-Phe.

In an L-Tyr producer derived from *C. glutamicum*, Hagino *et al.* suggested that DAHP synthase was released by the control mechanism and was chorismate-matase-sensitive.[17,18] On the other hand, with an L-Tyr and L-Phe producing *B. flavum* described by Shiio *et al.* it was reported that both DAHP synthase and chorismate mutase were desensitized.[19] They reported that DAHP synthase was chorismate mutase-A, a regulative subunit of chorismate mutase, in *Brevibacterium*, therefore both DAHP synthase and chorismate mutase were simultaneously desensitized to the feedback inhibition.[19] In the case of MF358 and FT-1, which were genetically induced in this study, both enzymes were considered to be free from the control mechanism.

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