Production of L-Tryptophan by 5-Fluorotryptophan Resistant Mutants of *Bacillus subtilis*

Isamu SHIIO, Kenji ISHII and Kenzo YOKOZEKI

Central Research Laboratories of Ajinomoto Co., Inc., Kawasaki, Japan

Received December 23, 1972

The growth of *Bacillus subtilis* TR-44, a prototrophic transductant from one of inosine producers, was completely inhibited by 200 $\mu\text{g/ml}$ of 5-fluorotryptophan, a tryptophan analogue, and the inhibition was reversed by the addition of L-tryptophan.

Several mutants resistant to 5FT* produced L-tryptophan in the growing cultures. The best producer, strain FT-39, which was selected on a medium containing 1500 $\mu\text{g/ml}$ of 5FT, produced 2 g/liter of L-tryptophan, when cultured in a medium containing 8% of glucose but without any tryptophan precursors. In this mutant, anthranilate synthetase, a key enzyme of the tryptophan biosynthesis, had increased over 280-fold, presumably owing to a genetic derepression. From FT-39, mutants resistant to 7000 $\mu\text{g/ml}$ of 5FT were derived. Among them, strain FF-25 produced 4 g/liter of L-tryptophan, twice as much as did the parental strain. Since this strain produced large amount of L-phenylalanine as well as L-tryptophan, the genetic alteration seemed to be involved in some metabolic regulation of common part of the aromatic amino acid biosynthetic pathway.

Further, some auxotrophs derived from these 5FT resistant mutants produced more L-tryptophan than did the parental strains.

Relationships between the accumulation of L-tryptophan and the regulation mechanisms of the L-tryptophan biosynthesis were discussed.

Various attempts using tryptophan precursors such as indole and anthranilic acid have been made for the microbial production of L-tryptophan.^{1~5)} However, these methods are not advantageous as these precursors are very expensive. On the other hand, the previous study has revealed that 5-methyltryptophan resistant phenylalanine and tyrosine double auxotroph of *Brevibacterium flavum*, one of glutamate producers, accumulates 2 g/liter of L-tryptophan in a medium containing 10% of glucose but without any tryptophan precursors.⁶⁾ However, the yield of L-tryptophan by this mutant was still not so good for the industrial purpose.

Therefore, it was tried to use *Bacillus subtilis* as the parental strain for further derivation of L-tryptophan producers.

The fact that mutants which produce large amounts of inosine had already been derived

from *Bacillus subtilis*⁷⁾ suggests that this bacterium may have strong pentose cycle which relates to the biosynthesis of erythrose-4-phosphate and 5-phosphoribosyl pyrophosphate, precursors of L-tryptophan. On the other hand, regulation of the aromatic amino acid biosynthesis in *Bacillus subtilis* has been extensively studied as shown in Fig. 1.^{8~13)} These studies may provide useful suggestions for the derivation of tryptophan producing mutants. The present paper deals with the isolation of 5FT resistant tryptophan producing mutants of *Bacillus subtilis*, derivation of various auxotrophs from the 5FT resistant strains, cultural conditions for the tryptophan production, and with the relationships between the accumulation of L-tryptophan and the regulation of the aromatic amino acid biosynthesis.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* TR-44, a pro-

* The abbreviations used through this paper include: 5-fluorotryptophan, 5FT; N-methyl-N'-nitro-N-nitrosoguanidine, NG.

FIG. 1. Summary of Feedback Control in the Biosynthesis of Aromatic Amino Acids of *B. subtilis*.
—, feedback inhibition; -----, repression; ····, induction.

totrophic transductant derived from *Bacillus subtilis* J-No. 2 (ade⁻), was employed as the starting strain for the derivation of mutants (Table. I).

Phage. SP-10, a generalized transducing phage of *Bacillus subtilis*, was employed.

Culture media. Medium 1: Polypeptone, 10 g; yeast extract, 10 g; NaCl, 5 g; and distilled water to bring the total volume to 1 liter; adjusted to pH 7.0. Medium 2: Glucose, 5 g; KH₂PO₄, 8.65 g; KOH, 2.18 g; (NH₄)₂SO₄, 1 g; Na-citrate, 0.5 g; MgSO₄·7H₂O 0.2 g; the amino acid mixture of casein acid hydro-

lysate composition except that L-phenylalanine, L-tyrosine, and L-tryptophan* were omitted, 0.2 g; and distilled water to bring the total volume to 1 liter. Medium 3: Meat extract, 14 g; polypeptone, 8 g;

* The mixture was consisted of the following amino acids: L-Glutamic acid, 122.8 mg; L-methionine, 31.4 mg; L-lysine, 120.0 mg; L-serine, 80.0 mg; L-aspartic acid, 72.8 mg; L-cysteine, 0.4 mg; L-alanine, 40.0 mg; L-histidine, 25.6 mg; L-arginine, 41.4 mg; L-proline, 130.0 mg; L-valine, 84.4 mg; L-isoleucine, 64.4 mg; L-leucine, 95.6 mg; L-threonine, 55.8 mg; and glycine, 25.6 mg per 1 g.

TABLE I. *Bacillus subtilis* STRAINS

Strains	Description
K	Prototroph; wild type.
J-No. 2	Adenine auxotroph of strain K; inosine producer.
TR-44	Prototroph; transductant derived from J-No. 2 with phage SP-10.
FT-39	5FT resistant mutant derived from TR-44; tryptophan producer.
FF-25	5FT resistant mutant derived from FT-39; tryptophan and phenylalanine producer.
TT-4	Tryptophan auxotroph of TR-44.
FP-13	Phenylalanine auxotroph of FT-39; tryptophan producer.
FA-32, 39	Arginine auxotrophs of FT-39; tryptophan producers.
FL-97	Lysine auxotroph of FT-39; tryptophan producer.
FFA-3, 39	Arginine auxotrophs of FF-25; tryptophan producers.
FFL-5	Leucine auxotroph of FF-25; tryptophan producer.
FFL-90	Leucine bradytroph of FF-25; tryptophan producer.
FFU-6, 34, 80	Purine auxotrophs of FF-25; tryptophan producer.

yeast extract, 4 g; glucose, 1 g; and distilled water to bring the total volume to 1 liter; adjusted to pH 7.2. Medium 4: Glucose, 80 g; NH_4Cl , 20 g; KCl , 2 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; Fe^{2+} , 2 ppm; Mn^{2+} , 2 ppm; CaCO_3 , 25 g; the amino acid mixture,* 2 g; and distilled water to bring the total volume to 1 liter; adjusted to pH 7.0. Medium 5: Glucose, 80 g; NH_4Cl , 10 g; KCl , 2 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; Fe^{2+} , 2 ppm; Mn^{2+} , 2 ppm; CaCO_3 , 25 g; the amino acid mixture, 3 g; and distilled water to bring the total volume to 1 liter; adjusted to pH 7.0. Medium 6: Glucose, 80 g; NH_4Cl , 10 g; KCl , 2 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; Fe^{2+} , 2 ppm; Mn^{2+} , 2 ppm; CaCO_3 , 25 g; the amino acid mixture,* 4 g; and distilled water to bring the total volume to 1 liter; adjusted to pH 7.0. CaCO_3 was sterilized separately in medium 4, 5, and 6.

Chemicals. 5-Fluoro-DL-tryptophan, 6-fluoro-DL-tryptophan, 4-methyl-DL-tryptophan, 5-methyl-DL-tryptophan, 6-methyl-DL-tryptophan, 7-methyl-DL-tryptophan, α -methyl-DL-tryptophan, 5-methoxy-DL-tryptophan, 5-hydroxy-DL-tryptophan, N-acetyl-DL-tryptophan, DL-tryptophanhydroxamate, adenine, L-glutamine, and dithiothreitol were purchased from Sigma Corp., EDTA from Wako Junyaku Corp., and NG from Aldrich Chem. Corp. All the amino acids

used were the product of Ajinomoto Co., Inc. Chorismic acid was prepared by Mr. R. Miyajima of our laboratories according to the method of Gibson.¹⁴⁾

Culture conditions. For fermentation experiments, a loopful of cells grown on medium 1 agar plates for 1 day at 30°C was inoculated into 20 ml of a culture medium distributed in 500 ml flasks, or into 3 ml of medium distributed in test tubes, which had been sterilized at 115°C for 10 min. After cultivation on a shaker at 30°C, growth, pH, and L-tryptophan accumulated were determined. Growth was represented as optical density at 562 m μ after 26-fold dilution with 0.1 N HCl. L-Tryptophan accumulated in the culture broth was detected by paper chromatography with solvent system of butanol:acetic acid:water (4:1:2 by volume) using ninhydrin solution and Ehrlich solution (*p*-dimethylaminobenzaldehyde 1 g, acetone 90 ml, conc. HCl 10 ml) as the color-producing reagents. Quantitative determination of L-tryptophan was conducted by microbioassay with *Leuconostoc mesenteroides* P-60.

Isolation of 5FT resistant mutants. Cells of late logarithmic phase grown in 5 ml of medium 1 were washed with 0.05 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7.0), and resuspended in an equal volume of the same buffer containing 750 $\mu\text{g/ml}$ of NG. After standing for 20~30 min in an ice bath, cells were washed twice with the buffer.

The number of viable cells were reduced to 1~10% by NG treatment. After suitable dilution, one tenth ml of NG treated cell suspensions was spread on medium 3 agar plates containing various concentrations of 5FT, and cultured for 2 days at 37°C. 5FT resistant colonies appeared were tested for the tryptophan production.

Isolation of various auxotrophs. One tenth ml of cell suspensions which had been treated by NG washed and diluted as mentioned above was spread and cultured on medium 1 agar plates. Then, among colonies thus appeared, the ones which did not grow on medium 2 without the amino acids except for L-threonine, L-isoleucine, L-methionine, and L-glutamic acid were isolated by the replica method, and the auxotrophy was determined by the auxanographic techniques.

Enzyme assay. Anthranilate synthetase was assayed as follows.¹⁵⁾ The cells grown in 20 ml of medium 6 for 24 hr were suspended in a 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 5 mM dithiothreitol, and 0.5 M KCl, and sonically disrupted for 10 min at 10 kc. After centrifugation at 15,000 rpm for 20 min, the supernatant was used as the enzyme extract. The reaction mixture contained 50 μmoles of potassium phosphate buffer

(pH 7.0), 10 μ moles of MgSO_4 , 20 μ moles of L-glutamine, and 0.2 μ moles of chorismic acid in a final volume of 1 ml. The reaction was initiated by the addition of the enzyme extract to the reaction mixture and carried out for 30 min at 30°C. As soon as the reaction was stopped by the addition of 4 ml of cold water, the activity was determined with a Hitachi spectrophotofluorometer 203 by observing increase of fluorescence at excitation wave length of 320 m μ , and emission wave length of 395 m μ , and comparing the fluorescence to a standard curve for authentic anthranilate. Protein was determined by the method of Lowry *et al.*¹⁶⁾

Transduction procedure. 1 ml of culture broth ($10^8 \sim 10^9$ cells/ml) of the late logarithmic phase grown in 5 ml of medium 3 was mixed with 1 ml of phage SP-10 ($10^8 \sim 10^9$ plaque forming units/ml). After 30 min, the cells were washed and resuspended with the buffer. One tenth ml of the cells was spread on medium 2 agar plates, and cultured for 2 days at 37°C. Transductants appeared were isolated.

Phage sensitivity test. The cells were spread on medium 1 agar plates, and phage SP-10 was added dropwise to this medium.

After incubation for 1 day at 37°C, the lytic response was observed (drop test).

Lysogenicity test. A loopful of cells was inoculated into 5 ml of medium 3 and cultured for 16 hr at 37°C. The presence of the phage in the culture filtrate was assayed by the drop test.

RESULTS

Derivation of ade⁺ transductants from inosine producing bacterium, Bacillus subtilis J-No. 2

Bacillus subtilis K (final concentration, 1.5×10^7 cells/ml) and phage SP-10 (final concentration, 5.0×10^7 P.F.U./ml) was inoculated into 5 ml of medium 3. Growth of the cells infected with phage SP-10 reached the maximum after 4.5 hr and then, the cells were lysed subsequently. Titer of the phage SP-10 in the culture filtrate after 7.5 hr was 2.0×10^{10} P.F.U./ml. *Bacillus subtilis* J-No. 2 was transduced with the phage SP-10 as indicated above. The frequency of occurrence of ade⁺ transductants was not so different between multiplicity of infection 0.18 and 0.90.

After transductants thus obtained were purified by single colony isolation, they were

examined for growth rate in medium 4, phage sensitivity and lysogenicity. Strain TR-44 which satisfied these test was decided as the starting strain for derivating the mutants.

Growth inhibition of Bacillus subtilis TR-44 by various tryptophan analogues and its reversal by L-tryptophan

Growth inhibition of *Bacillus subtilis* TR-44 by various tryptophan analogues and its reversion by L-tryptophan was tested as in Table II. 5-Methyltryptophan, the most commonly used analogue as well as 6-fluorotryptophan had little inhibitory effect on the growth, while L-tryptophan had little reversing effect on the inhibition by 4-methyltryptophan, 6-methyltryptophan, and tryptophanhydroxamate. Although 7-azatryptophan strongly inhibited the growth and the inhibition was well reversed by L-tryptophan, frequency of occurrence of spontaneous resistant mutants was very high. As a result, 5-fluorotryptophan was the best analogue among tested. The growth of *Bacillus subtilis* TR-44 was completely inhibited in the presence of 200 μ g/ml of 5FT and almost completely recovered from the inhibition by the addition of 200 μ g/ml of L-tryptophan (Table II). 5-Methoxytryptophan, 7-methyltryptophan, α -methyltryptophan, and N-acetyltryptophan had no inhibitory effect. The inhibitory test of 5FT in liquid culture agreed with the result in the plate culture as shown in Table II (Fig. 2 (a)).

Isolation of L-tryptophan producing 5FT resistant strains

NG treated cells of *Bacillus subtilis* TR-44 were spread onto medium 2 agar plates containing various concentrations of 5FT and incubated for 2 days at 37°C. As shown in Table III, frequency of appearance of 5FT resistant colonies decreased at concentrations more than 400 μ g/ml of 5FT, and was not changed up to 5000 μ g/ml of 5FT. On the other hand, percentage of mutants producing more L-tryptophan than parental strain TR-44 (produced 50 μ g/ml of L-tryptophan in medium 6) among 5FT resistant strains was about 10%.

TABLE II. GROWTH INHIBITION OF *Bacillus subtilis* TR-44 BY VARIOUS ANALOGUES AND ITS REVERSAL BY L-TRYPTOPHAN

0.1 ml of overnight culture of *Bacillus subtilis* TR-44 in medium 1 was inoculated on medium 2 agar plates containing the indicated amount of analogues. Cultures incubated at 37°C were recorded as (+++) very good growth, (++) good growth, (+) medium growth, (±) poor growth, (−) no growth. a, 8.4×10^5 cells/ml; b, 8.4×10^4 cells/ml.

Tryptophan (μg/ml)		0				200			
Cultivation time (hr)		24		48		24		48	
Number of cells/ml		a	b	a	b	a	b	a	b
Analogues (μg/ml)	None	+++	+	+++	++	+++	+	+++	++
	4-Methyl-tryptophan { 200	—	—	+	±	++	+	+++	+
	500	—	—	+	—	+	—	++	+
	1000	—	—	—	—	—	—	—	—
	5-Methyl-tryptophan { 200	++	±	+++	++	+++	+	+++	++
	500	++	—	+++	+	+++	+	+++	++
	1000	+	—	+	—	+++	—	+++	—
	6-Methyl-tryptophan { 200	±	—	+	+	++	+	++	+
	500	—	—	±	—	—	—	+	±
	1000	—	—	—	—	—	—	—	—
	5-Fluoro-tryptophan { 200	—	—	—	—	+++	+	+++	+
	500	—	—	—	—	+++	+	+++	+
	1000	—	—	—	—	++	+	++	+
	6-Fluoro-tryptophan { 200	+	±	++	+	+	+	+++	+
	500	+	±	++	+	+	+	+++	+
	1000	+	—	++	+	+	+	++	+
	7-Azatryptophan { 200	—	—	—	—	+++	+	+++	++
	500	—	—	—	—	+++	+	+++	++
	1000	—	—	—	—	+	+	++	++
	5-Hydroxy-tryptophan { 200	—	—	+	±	++	+	+++	+
	500	—	—	±	—	+	±	++	+
	1000	—	—	—	—	±	±	++	+
	Tryptophan hydroxamate { 200	—	—	±	—	—	—	±	±
	500	—	—	—	—	—	—	—	—
	1000	—	—	—	—	—	—	—	—

FIG. 2. Growth of Strain TR-44, (a) and FT-39, (b) in Medium 2 Containing 5FT and 5FT plus L-Tryptophan.

Overnight cultures of TR-44 and FT-39 in 5 ml of medium 2 were harvested and washed with potassium phosphate buffer and resuspended in 5 ml of medium 2. A 0.1 ml of the cell suspensions was added to series of 6 ml of medium 2 containing various concentration 5FT and incubated for 24 hr at 30°C. The growth was determined by measuring optical density at 562 mμ after dilution with water.

○—○, 5FT; ●—●, 5FT plus L-tryptophan (200 μg/ml).

TABLE III. APPEARANCE OF TRYPTOPHAN PRODUCING 5FT RESISTANT MUTANTS FROM NG TREATED *Bacillus subtilis* TR-44 AT VARIOUS CONCENTRATION OF 5FT

NG treated cells of *Bacillus subtilis* TR-44 were spread onto medium 2 plates containing various concentration of 5FT and incubated for 2 days at 37°C. Appeared colonies were purified and cultured at 30°C for 72 hr in 3 ml of medium 4 and L-tryptophan produced were determined.

NG conditions	Viable cells/ml	Concentration of 5FT(μ g/ml)	Number of cells treated	Number of resistant colonies	Frequency of resistance	Number of tested colonies	Number of tryptophan producer
750 (μ g/ml) 0°C 30 min	2.9×10^8 ↓ 1.0×10^6	200	1.0×10^5 1.0×10^4	2.1×10^2 2	2.1×10^{-3} 2.0×10^{-4}	180	24
		300	1.0×10^5 1.0×10^4	1.2×10^2 1	1.2×10^{-3} 1.0×10^{-4}	144	19
500 (μ g/ml) 0°C 30 min	2.5×10^9 ↓ 7.5×10^7	400	7.5×10^6 7.5×10^5	4.6×10^2 10	6.1×10^{-5} 1.3×10^{-5}	220	12
		600	7.5×10^6 7.5×10^5	4.0×10^2 8	5.3×10^{-5} 1.1×10^{-5}	110	11
500 (μ g/ml) 0°C 30 min	1.6×10^9 ↓ 8.0×10^6	1500	8.0×10^5	4.1×10^2	5.1×10^{-4}	110	16
		2000	8.0×10^5	2.9×10^2	3.6×10^{-4}	110	18
		3000	8.0×10^5	70	8.7×10^{-5}	110	10
750 (μ g/ml) 0°C 30 min	2.9×10^8 ↓ 1.0×10^6	5000	1.0×10^5	47	4.7×10^{-4}	50	10

when selected on 300 μ g/ml of 5FT plates, while about 5% when selected on plates containing more than 400 μ g/ml of 5FT. The best strain FT-39 which excreted 0.9 g/liter of L-tryptophan in medium 4 was isolated from the plates containing 1500 μ g/ml of 5FT. As shown in Fig. 2(b), the growth of FT-39 was completely free from the inhibition by 5FT at the concentration up to 3,000 μ g/ml.

Culture conditions for L-tryptophan production

Although medium 4 had been used as the screening medium, a preliminary experiment indicates better tryptophan production with medium 5 than medium 4. Therefore, effects of concentration of components of the cultural medium on L-tryptophan production were examined using medium 5 as the basal medium.

The results with strain FT-39 are shown in Fig. 3. As 0.4% of the amino acid mixture was better than the concentration of the amino acid mixture in the basal medium, this concentration of the amino acid mixture was used for the L-tryptophan production.

The reproducible maximum amount of

FIG. 3. Effect of Concentration of Components in Culture Medium on L-Tryptophan Production by Strain FT-39.

Strain FT-39 was cultured in media as indicated in the figures for 72 hr at 30°C in flasks on a shaker. Closed circles show the amount of L-tryptophan accumulated in the basal medium (medium 5).

L-tryptophan produced was 2.0 g/liter in medium 6.

Anthranilate synthetase in FT-39

As shown in Table IV, anthranilate synthetase activity in FT-39 was elevated to 280-fold than that in TR-44, while the activity in a tryptophan auxotroph, TT-4, derived from TR-44 was derepressed 200-fold under a limiting condition as reported with various strains of *Bacillus subtilis*.¹¹⁾ Therefore, anthranilate synthetase seems to have been genetically derepressed in FT-39. On the other hand,

TABLE IV. ANTHRANILATE SYNTHETASE LEVELS IN TRYPTOPHAN PRODUCING 5FT RESISTANT MUTANT

TR-44 and FT-39 were grown for 24 hr in medium 6 without tryptophan, and TT-4 were grown with a limited amount of tryptophan (25 $\mu\text{g/ml}$). Extracts obtained by sonic oscillation were assayed as described in the text.

Strain	Addition of tryptophan ($\mu\text{g/ml}$)	Growth	Specific activity ^{a)}
TR-44	0	0.69	0.14
TT-4	25	0.42	29.00
FT-39	0	0.61	39.70

^{a)} Milimicromoles anthranilate formed for 30 min per mg protein.

feedback inhibition by L-tryptophan of anthranilate synthetase in FT-39 was almost the same as in TT-4 (Fig. 4). 50% inhibition of anthranilate synthetase activity in FT-39 was shown at 7.5×10^{-6} M of L-tryptophan.

FIG. 4. Effect of L-Tryptophan on Anthranilate Synthetase from Mutant and the Tryptophan Auxotroph from Parental Strain.

Activities were measured under the standard assay conditions and L-tryptophan was added as indicated. ●—●, TT-4; ○—○, FT-39; L-tryptophan producer.

Derivation of mutants resistant to higher concentration of 5FT from strain FT-39

Although FT-39 was resistant to 3,000 $\mu\text{g/ml}$ of 5FT in Fig. 2(b), the growth was still inhibited at a concentration more than 4000 $\mu\text{g/ml}$ of 5FT (Table V). Thus, about a hundred mutants resistant to 7000 $\mu\text{g/ml}$ of 5FT were derived from FT-39 by NG treatment. Distribution of these mutants with respect to the L-tryptophan production is shown in Fig. 5. Among tested mutants, about thirty strains produced more L-tryptophan than did the parental strain. L-Tryptophan productivities in medium 6 of the best three strains, FF-25, FF-65, FF-74 were 4.0, 3.4, 3.3 g/liter, respectively. Time course of growth, pH, and L-tryptophan production in strain FF-25

TABLE V. GROWTH INHIBITION OF *Bacillus subtilis* FT-39 BY VARIOUS CONCENTRATION OF 5FT

A 0.1 ml of overnight culture of *Bacillus subtilis* FT-39 in medium 1 was inoculated on medium 2 agar plates containing the indicated amount of 5FT. Cultures incubated at 37°C were recorded as (+++) very good growth, (++) good growth (+), medium growth, (±) poor growth, (−) no growth. a, 1.9×10^6 cells/ml; b, 1.9×10^5 cells/ml.

Tryptophan ($\mu\text{g/ml}$)		0				200			
Cultivation time (hr)		24		48		24		48	
Inoculated cells/ml		a	b	a	b	a	b	a	b
5FT ($\mu\text{g/ml}$)	0	++	±	+++	++	++	+	+++	++
	1500	±	±	++	+	±	±	++	+
	2000	—	—	+	±	±	—	+	±
	3000	—	—	±	—	—	—	±	—
	4000	—	—	—	—	—	—	—	—
	5000	—	—	—	—	—	—	—	—

FIG. 5. Distribution of 5FT Resistant Mutants Derived from Strain FT-39 with Respect to Their L-Tryptophan Production.

Mutants resistant to 7000 ($\mu\text{g/ml}$) of 5FT were isolated from NG treated FT-39 and cultured in 3 ml of medium 6.

L-Tryptophan was determined after culturing for 72 hr at 30°C on a shaker. The arrow shows the amount of L-tryptophan produced by the parental strain, FT-39.

FIG. 6. Time Course of Growth, pH, L-Tryptophan and L-Phenylalanine Accumulation of Strain FF-25.

A loopful of cells grown on medium 1 plate for 24 hr at 30°C were inoculated into 20 ml of medium 6 distributed in 500 ml flasks.

$\triangle-\triangle$, pH; $\square-\square$, growth; $\bullet-\bullet$, L-tryptophan accumulation; $\circ-\circ$, L-phenylalanine accumulation.

was investigated using medium 6 as shown in Fig. 6. The maximum level of L-tryptophan accumulation was 4.0 g/liter after 48 hr. More remarkable was the fact that large amount of L-phenylalanine as well as L-tryptophan was produced in strain FF-25 which was scarcely done in strain FT-39. As specific activity of anthranilate synthetase

as well as level of the feedback inhibition in strain FF-25 was almost identical with those in strain FT-39, it is likely that this strain may be altered in some properties involved in the common pathway of the aromatic amino acid biosynthesis.

Derivation of auxotrophs from 5FT resistant mutants

Various auxotrophs were derived from strain FT-39 and FF-25, 5FT resistant mutants. Among them, producers better than the parental strains were selected and tested for the optimum concentrations of the supplements to the culture media.

Tables VI and VII shows the result with phenylalanine, arginine, leucine, lysine, and purine auxotrophs and a leucine bradytroph which produced more L-tryptophan than did the parental strains. A strain FFL-90, leucine bradytroph, was the best strain which produced 6.15 g/liter of L-tryptophan in medium 6 containing of 300 $\mu\text{g/ml}$ of L-leucine. Although histidine auxotrophs produced in a histidine supplemented medium, more than did the parental strains in the medium without supplement, addition of L-histidine (500 $\mu\text{g/ml}$) markedly increased the accumulation of L-tryptophan in strain FT-39 and FF-25, parental strains. Thus, the accumulation of L-tryptophan increased from 2.01 g/liter to 3.44 g/liter in FT-39, and from 4.00 g/liter to 4.44 g/liter in FF-25 by the addition of 500 $\mu\text{g/ml}$ of L-histidine. Therefore, increase of L-tryptophan in histidine auxotrophs is not due to their auxotrophy but the histidine addition. This promotive effect of L-histidine has been reported by Kane and Jensen.¹⁷⁾

DISCUSSION

It has been known that L-tryptophan inhibits anthranilate synthetase and represses enzymes of the tryptophan specific biosynthetic pathway in *Bacillus subtilis*. Further, chorismic acid and prephenic acid, common precursors of aromatic amino acids, inhibit 3-deoxy-D-arabinoheptulosonic acid 7-phos-

TABLE VI. L-TRYPTOPHAN PRODUCTION OF VARIOUS AUXOTROPHS FROM STRAIN FT-39

Cultivation was carried out using 20 ml of medium 6 distributed in 500 ml flasks supplemented with the described concentrations of supplements for 48 hr at 30°C. Parental strain FT-39 produced 2.06 g/liter of L-tryptophan.

Strain	Auxotrophy	Supplements ($\mu\text{g/ml}$)		O.D.	L-Tryptophan (g/liter)
FP-13	Phe ⁻	L-Phenylalanine	50	0.72	2.56
			200	0.53	2.93
FA-32	Arg ⁻	L-Arginine	200	0.44	2.59
			400	0.51	2.87
FA-39	Arg ⁻	L-Arginine	200	0.53	2.57
			400	0.69	2.44
FL-97	Lys ⁻	L-Lysine	400	0.66	2.54
			500	0.65	2.09

TABLE VII. L-TRYPTOPHAN PRODUCTION OF VARIOUS AUXOTROPHS FROM STRAIN FF-25

Cultivation was carried out using 20 ml of medium 6 distributed in 500 ml flasks supplemented with the described concentration of supplementes for 48 hr at 30°C. Parental strain FF-25 produced 4.13g/liter of L-tryptophan.

X⁻, X auxotroph; X[±], X bradytroph.

Strain	Auxotrophy	Supplements ($\mu\text{g/ml}$)		O.D.	L-Tryptophan (g/liter)
FFA-3	Arg ⁻	L-Arginine	300	0.52	2.45
			500	0.58	5.20
FFA-39	Arg ⁻	L-Arginine	300	0.43	5.23
			500	0.44	4.00
FFL-5	Leu ⁻	L-Leucine	200	0.70	5.30
			300	0.66	5.25
FFL-90	Leu [±]	L-Leucine	200	0.60	5.66
			300	0.49	6.15
FFU-6	Pur ⁻	Adenine	200	0.59	4.56
			300	0.60	4.75
FFU-34	Pur ⁻	Adenine	200	0.37	5.00
			300	0.59	4.49
FFU-80	Pur ⁻	Adenine	200	0.52	4.28
			300	0.43	5.41

phate synthetase (DAHP synthetase) and shikimate kinase in the common pathway of the aromatic amino acid biosynthesis. In this type of regulation which has been known as the sequential feedback inhibition, it is expected that a release of the feedback regulation in the tryptophan specific pathway may easily give an accumulation of tryptophan. In fact, strain FT-39, which was resistant to 5FT and genetically derepressed in the tryptophan specific pathway, overproduced 2 g/liter of L-tryptophan. In strain FF-25, a mutant resistant to higher concentration of 5FT, in

which the feedback inhibition and derepression of anthranilate synthetase were almost identical with those in FT-39, 4.0 g/liter of L-tryptophan and 6.0 g/liter of L-phenylalanine were simultaneously accumulated. Therefore, this strain would have been altered in the regulation of the common pathway of the aromatic amino acid biosynthesis.

In *Bacillus subtilis*, the first enzyme of the common pathway, DAHP synthetase, which is inhibited by chorismate and prephenate is repressed by L-tyrosine and more strongly by L-tyrosine plus L-phenylalanine, while the

first enzyme of the L-phenylalanine specific pathway, prephenate dehydratase is inhibited by L-phenylalanine (Fig. 1). Therefore, desensitization of the latter enzyme to the feedback inhibition would be essential for the overproduction of L-phenylalanine. In fact, β -thienylalanine resistant mutant of *Bacillus subtilis* WB-740, whose prephenate dehydratase is completely feedback resistant has been reported to excrete L-phenylalanine.¹⁸⁾ Thus, it is possible that strain FF-25 producing L-phenylalanine may have desensitized prephenate dehydratase, similar to strain WB-740. Moreover, as suggested by Nester *et al.*,¹¹⁾ an excess production of L-phenylalanine in addition to L-tryptophan may cause a limitation of L-tyrosine, which, in turn, may derepress key enzymes of the common pathway. Therefore, it is likely that higher production of L-tryptophan in FF-25 than FT-39 may come from phenotypic derepression of key enzymes of the common pathway. Although desensitization of DAHP synthetase to the feedback inhibition by chorismate may increase the L-tryptophan production, it is not clear whether this alteration simultaneously causes an overproduction of L-phenylalanine or not.

Furthermore, the yield of L-tryptophan was increased in the following nutritional mutants derived from strain FF-25, that is, 5.23 g/liter in strain FFA-39, an arginine auxotroph; 5.30 g/liter in strain FFL-5, a leucine auxotroph; 6.15 g/liter in strain FFL-90, a leucine bradytroph; and 5.41 g/liter in strain FFU-80, a purine auxotroph.

The enhancement of L-tryptophan production by leucine auxotroph and bradytroph is very interesting if compared with the fact that leucine activates prephenate dehydratase and reverses the inhibition by tryptophan.¹⁹⁾ All the tryptophan producing mutants obtained in the present study have been not altered with respect to the feedback inhibition of

anthranilate synthetase, although desensitization to this feedback inhibition may be very important for the better production of L-tryptophan.

Acknowledgements. The authors are indebted to Director Dr. T. Tsunoda and Assistant Director Dr. M. Takahashi of their laboratories for the encouragement during the course of this work. They also wish to thank Miss K. Furuya for her skillful technical assistance.

REFERENCES

- 1) B. Marlin and J. Westhead, *J. Biochem. Microbiol. Technol. Eng.*, **1**, 49 (1959).
- 2) Chugai Seiyaku Co., French Patent 1437993 (1966).
- 3) G. Terui and T. Enatsu, *Amino Acid and Nucleic Acid*, **3**, 71 (1961).
- 4) A. Donald, Japanese Patent 2384 (1960).
- 5) P. G. Lim and R. I. Mateles, *Science*, **140**, 338 (1963).
- 6) I. Shiio, H. Sato and M. Nakagawa, *Agr. Biol. Chem.*, **36**, 2315 (1972).
- 7) R. Aoki, H. Momose, Y. Kondo, N. Muramatsu and Y. Tsuchiya, *J. Gen. Appl. Microbiol.*, **9**, 387 (1963).
- 8) R. A. Jensen and E. W. Nester, *J. Mol. Biol.*, **12**, 468 (1965).
- 9) R. A. Jensen and E. W. Nester, *J. Biol. Chem.*, **241**, 3373 (1966).
- 10) E. W. Nester and R. A. Jensen, *J. Bacteriol.*, **91**, 1594 (1966).
- 11) E. W. Nester, R. A. Jensen and D. S. Nasser, *ibid.*, **97**, 83 (1969).
- 12) D. S. Nasser, G. Henderson and E. W. Nester, *ibid.*, **98**, 44 (1969).
- 13) J. F. Kane and R. A. Jensen, *Biochem Biophys. Res. Commun.*, **38**, 1161 (1970).
- 14) F. Gibson, *Biochem. J.*, **90**, 256 (1964).
- 15) I. Shiio and R. Miyajima, *J. Biochem.*, **72**, 1447 (1972).
- 16) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randal, *J. Biol. Chem.*, **193**, 265 (1951).
- 17) J. F. Kane and R. A. Jensen, *ibid.*, **245**, 2384 (1970).
- 18) J. H. Coats and E. W. Nester, *ibid.*, **242**, 4948 (1967).
- 19) J. L. Rebello and R. A. Jensen, *ibid.*, **245**, 3378 (1970).