Accumulation of L-Histidinol by a Histidine Auxotroph of *Corynebacterium glutamicum* and Its Conversion into L-Histidine by an *Escherichia coli* Strain†

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A histidine auxotroph of *Corynebacterium glutamicum* was found to accumulate L-histidinol in the culture medium. The accumulation of L-histidinol reached a level of 10~11 mg as dihydrochloride per ml with a cane molasses medium containing 15% sugar (as glucose), 2~3% (NH₄)₂SO₄ and limited amount of L-histidine. When an *Escherichia coli* strain was mix-cultured with a later phase-culture of the above L-histidinol-producer, 4 mg/ml of L-histidine was produced with the reduction of the L-histidinol accumulation.

Vogel *et al.*¹⁾ found the excretion of L-histidinol by a histidine auxotroph of *Escherichia coli* and observed that this compound could support the growth of another histidine auxotroph. At present, there have been many informations on the excretion of L-histidinol by fungal^{2~5)} and bacterial^{6~9)} histidine auxotrophs, and it has been known that L-histidinol is a terminal member of a sequence of imidazole intermediates on the histidine biosynthetic pathway.²⁰⁾

Kubota *et al.*¹⁰⁾ reported that more than 9 mg/ml of L-histidinol (as dihydrochloride) is accumulated in the culture medium of a histidine auxotroph of *Brevibacterium flavum*. These investigators also described the accumulation of a considerable amount of L-histidine in the culture medium of various kinds of microorganisms, when L-histidinol was supplemented to the growth medium.

The present paper describes the production of L-histidinol by a histidine auxotroph of *Corynebacterium glutamicum* and conversion of the produced L-histidinol to L-histidine by an *Escherichia coli* strain. A part of the data on these subjects has been described in our previous paper¹¹⁾ and patent descriptions.^{12,13)}

MATERIALS AND METHODS

Microorganisms. A histidine auxotroph employed in the experiment for L-histidinol production was derived from a wild-type *Corynebacterium glutamicum* strain by the mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine. This mutant strain has been deposited to the American Type Culture Collection with an accession number of ATCC 21339. It has been found to be defective¹¹⁾ in histidinol dehydrogenase (L-histidinol: nicotinamide adenine dinucleotide oxidoreductase, EC 1.1.1.23). All the microorganisms employed for the conversion test of L-histidinol into L-histidine were stock cultures of our laboratory.

Culture method for L-histidinol production. Forty milliliters of a seed medium in a 250-ml Erlenmeyer flask was inoculated with a loopful of the cells of *C. glutamicum* ATCC 21339 grown on a bouillon agar slant, and incubated at 28°C on a rotary shaker operated at 220 rpm. After incubation for 24 hr, one milliliter of the seed culture was transferred into a same-typed flask containing 10 ml of a fermentation medium. The flask was incubated for 5 days in the same manner as that with the seed culture. The seed medium contained glucose 4 g, peptone 2 g, yeast extract 0.5 g, urea 0.3 g, KH₂PO₄ 0.15 g, K₂HPO₄ 0.05 g, MgSO₄·7H₂O 0.05 g and biotin 3 µg per 100 ml. The fermentation medium contained cane molasses 15 g (as glucose), (NH₄)₂SO₄ 2 or 3 g, KH₂PO₄ 0.15 g, K₂HPO₄ 0.05 g, MgSO₄·7H₂O 0.05 g and CaCO₃ 3 g per 100 ml as basal ingredients. L-Histidine and several natural nutrients were added to the fermentation medium at variable levels. In the experiment shown in Table V, FeSO₄·7H₂O and MnSO₄·4H₂O were also added to the medium. The hydrogen ion concentration of the medium was

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adjusted to pH 7.4 with NH_4OH , then the medium was sterilized at 120°C for 10 min. The fermentation-test shown in Fig. 2 was carried out with a 5-liter jar fermenter principally according to the method of the flask-test described above. The details of the test are described in the legend of Fig. 2.

Culture method for the conversion of the accumulated L-histidinol into L-histidine. The microorganisms to be used for the conversion of the L-histidinol accumulated by *C. glutamicum* ATCC 21339 into L-histidine were cultured in the same manner with the seed culture of the *C. glutamicum* mutant. After the cultivation for 24 hr, the cells of each microorganism were collected by centrifugation and suspended in 4 ml of 60% sterilized glucose solution. An aliquot of the cell-suspension was transferred into the flask containing 5-days culture of *C. glutamicum* ATCC 21339 and the flask was incubated for further 2 days in the same manner as that described for L-histidinol production.

Preparation of acid-hydrolyzate of soybean meal and of C. glutamicum cells. Five hundred g of soybean meal or *C. glutamicum* cells were suspended in 2 liters of 6 N H_2SO_4 and hydrolyzed for 5 hr at 120°C , then the hydrolyzates were filled up to 3 liters with water. After neutralization with NH_4OH , they were used as the L-histidine source in the fermentation medium. The ammonium concentration in the medium was adjusted by adding appropriate amount of $(\text{NH}_4)_2\text{SO}_4$ to make the final concentration to be 3% as $(\text{NH}_4)_2\text{SO}_4$, when these acid-hydrolyzates were used at variable levels.

Analysis. L-Histidine and L-histidinol were determined by the method of McPherson,¹⁵⁾ after paper-chromatographic separation as described in our previous paper.¹⁶⁾ The growth of the microorganisms was measured by reading the absorbancy at 660 nm of the culture broth in the cuvette with a light-path of 10 mm using a Hitachi Colorimeter Model 101.

Reagents. L-Histidine was a product of Kyowa Hakko Kogyo Co., Ltd. L-Histidinol·2HCl was purchased from Sigma Chemical Co.

RESULTS

1. Identification of L-histidinol

During the course of the studies on the fermentative production of amino acids with auxotrophic mutants of *C. glutamicum*, it was found that a histidine auxotroph, ATCC 21339 accumulated a substance which is positive to both the ninhydrin-reaction and the diazosulfanilic acid-reaction. The separated

band of the substance in a paperchromatography of an aliquot of the culture broth with *n*-propanol-0.2 N NH_4OH (3:1 by volume) as solvent was extracted with hot water. After concentration *in vacuo*, the extract was re-chromatogramed in a solvent system of *n*-butanol-acetic acid-water (5:2:2 by volume). The extraction of the substance and the concentration of the extract were repeated again in the same manner as described above. The sample thus obtained gave the same *Rf*-values in paperchromatography with 5 different solvent systems with authentic L-histidinol, as shown in Table I, and supported the growth of *S. typhimurium* hisG46 as effectively as authentic L-histidinol. From these results, we concluded the substance produced by *C. glutamicum* ATCC 21339 as L-histidinol.

2. Effect of L-histidine on L-histidinol production

Because ATCC 21339 requires L-histidine for growth,¹⁸⁾ the effect of L-histidine on L-histidinol production was tested in a cane molasses medium containing 15% sugar (as glucose). As shown in Fig. 1A, the L-histidinol production increased with the increase of L-histidine concentration upto 1 mM of the amino acid. At the concentration of L-histidine lower than 0.24 mM, the level of the growth of the mutant was rather higher than the case with excess of L-histidine. This may be ascribable to the appearance of non-auxotrophic revertant from the mutant during the fermentation, as has been assumed in the citrulline fermentation with an arginine auxotroph of *C. glutamicum* where a similar abnormal growth response to limited amounts of L-arginine was observed.¹⁷⁾ A similar response of L-histidinol production and the growth of the mutant to L-histidine concentration was observed in another medium containing 0.5% corn steep liquor as a basic ingredient (Fig. 1B). In this case, the L-histidinol production responded more sharply to L-histidine concentration than that in the above case. At the optimal L-histidine concentration *i.e.* 0.71 mM, 10~11 mg/ml of L-histidinol (as dihydrochloride) accumulated.

TABLE I. PAPERCHROMATOGRAPHIC IDENTIFICATION OF THE SUBSTANCE PRODUCED BY *C. glutamicum* ATCC 21339 AS L-HISTIDINOL

Solvent system ^{a)}	Substance produced by ATCC 21339	Authentic L-histidinol
A	0.72	0.72
B	0.81	0.82
C	0.47	0.49
D	0.37	0.37

^{a)} Solvent system (ratio by volume): A, *n*-propanol-0.2N NH₄OH (3:1); B, isobutyric acid-28% NH₄OH (100:20); C, *n*-butanol saturated with 2N NH₄OH; D, *n*-propanol-N acetic acid (3:1). Rf-values of the spots located with diazosulfanilic acid-reaction are given.

3. Effect of natural nutrients on L-histidinol production

In the fermentative production of amino acid on an industrial scale, various natural nutrients are used as the source of growth factors of the microorganisms employed. Among the natural nutrients, acid-hydrolyzate of soybean meal, acid-hydrolyzate of *C. glutamicum* cells, meat extract, corn steep liquor and peptone are the ones which are most favourably used for this purpose, because of their low cost. To establish an economical process for L-histidinol production, the effect of the above 5 natural nutrients on L-histidinol production by ATCC 21339 was investigated.

TABLE II. EFFECT OF ACID-HYDROLYZATE OF S.B.M.^{a)} AND OF *C. glutamicum* CELLS ON THE L-HISTIDINOL PRODUCTION BY *C. glutamicum* ATCC 21339

The fermentation medium described in MATERIALS AND METHODS supplemented with indicated levels of the natural nutrients was used.

	Natural nutrients added (%) ^{b)}	L-Histidinol produced (mg/ml) ^{c)}	Growth (O.D. _{660nm} 1/100)
Acid-hydrolyzate of S.B.M.	(0.5)	4.8	0.45
	(1.0)	7.0	0.38
	(1.5)	7.6	0.35
Acid-hydrolyzate of the cells of <i>C. glutamicum</i>	(0.5)	5.4	0.33
	(1.0)	6.0	0.35
	(1.5)	6.2	0.41

^{a)} Soybean meal.

^{b)} w/v %, with respect to dry weight of S.B.M. and *C. glutamicum* cells.

^{c)} As dihydrochloride.

As shown in Table II, when 0.5~1.5% of the hydrolyzate of soybean meal and the acid-hydrolyzate of *C. glutamicum* cells were added to the fermentation medium, 6~7.6 mg/ml of L-histidinol (as dihydrochloride) was produced. In the medium basically supplemented with 1.5% of the hydrolyzate of soybean meal or of *C. glutamicum* cells, further addition of meat extract reduced the L-histidinol production extensively, but signi-

FIG. 1. Effect of L-Histidine on the L-Histidinol Production by *C. glutamicum* ATCC 21339 in the Presence or Absence of Corn Steep Liquor.

●—●, L-histidinol; ×---×, growth at 5 days.

The fermentation medium described in MATERIALS AND METHODS supplemented with the indicated levels of L-histidine was used. In B, 0.5% corn steep liquor was added to the medium.

TABLE III. EFFECT OF MEAT EXTRACT, PEPTONE AND CORN STEEP LIQUOR ON L-HISTIDINOL PRODUCTION BY *C. glutamicum* ATCC 21339, WHEN ADDED IN COMBINATION WITH ACID-HYDROLYZATES OF S.B.M. AND OF THE CELLS OF *C. glutamicum* TO THE MEDIUM

The fermentation medium supplemented with each of 1.5% (with respect to dry weight) acid-hydrolyzates of S.B.M. (in I) and *C. glutamicum* cells (in II) in addition to the indicated natural nutrients was used.

	Addition (%, w/v)	L-Histidinol produced (mg/ml) ^{a)}	Growth (O.D. _{660nm} 1/100)
I	Meat ext. (1.5)	trace	0.52
	(0.75)	trace	0.46
	Corn steep (3.0)	7.4	0.35
	liquor (1.5)	7.4	0.35
	Peptone (1.5)	6.1	0.46
II	(0.75)	6.4	0.35
	Meat ext. (1.5)	trace	0.47
	(0.75)	trace	0.53
	Corn steep (3.0)	10.1	0.47
	liquor (1.5)	9.3	0.40
	Peptone (1.5)	7.9	0.50
	(0.75)	7.6	0.40

^{a)} As dihydrochloride.

ificantly stimulated the growth of the mutant (Table III). Further addition of 1.5 or 3% of corn steep liquor to the medium supplemented with 1.5% acid-hydrolyzate of *C. glutamicum* cells gave 9~10 mg/ml of L-histidinol (as dihydrochloride).

4. L-Histidinol production in jar fermenter

The time course of L-histidinol fermentation by ATCC 21339 was investigated in a 5-liter jar fermenter with a medium containing 15% (as glucose concentration) cane molasses, 3% (NH₄)₂SO₄, 1.5% acid-hydrolyzate of *C. glutamicum* cells and 1% corn steep liquor. As shown in Fig. 2, in the earlier phase of the fermentation until 16 hr, the growth and the consumption of sugar were rapid. However, the analysis of the culture in 40 hr-incubation revealed that the growth and the sugar consumption were extremely sluggish. Then, meat extract, which stimulated the growth of the mutant in the preceding experiment, was fed to the culture at the concentration of 0.2%, as indicated with an

FIG. 2. Time Course of L-Histidinol Fermentation with *C. glutamicum* ATCC 21339.

●—●, L-histidinol; ×---×, growth; △—△, sugar. Fermentation was carried out in a 5-liter jar fermenter containing 3 liters of a fermentation medium described in MATERIALS AND METHODS supplemented with 1.5% (with respect to dry weight) acid-hydrolyzate of *C. glutamicum* cells and 1% corn steep liquor. After incubation for 40 hr, 0.2% meat extract was added. Culture conditions: aeration, 4.5 liters/min; temperature, 28°C; agitation, 600 rpm. Hydrogen ion concentration was automatically adjusted to pH 7.0 with NH₄OH during fermentation.

arrow in Fig. 2. After the addition of meat extract, the growth level slowly increased upto 108 hr at which the level of L-histidinol reached 11.6 mg as dihydrochloride per ml.

5. Conversion of L-histidinol into L-histidine by microorganisms

Kubota *et al.*¹⁰⁾ have described that many microorganisms, including *B. flavum* ATCC 14067 and *Escherichia coli* ATCC 13070, could convert L-histidinol supplemented to their growth medium into L-histidine. The best yield of L-histidine has been obtained with *B. flavum* ATCC 14067, which produced 1.8 mg/ml of L-histidine in the medium containing 0.5% L-histidinol dihydrochloride.

To provide more direct method for L-histidine production, we sought the microorganisms which were capable of converting the L-

TABLE IV. PAPERCHROMATOGRAPHIC IDENTIFICATION OF L-HISTIDINOL FORMED BY *E. coli* KY-3592 FROM L-HISTIDINOL PRODUCED BY *C. glutamicum* ATCC 21339

Rf-values of the spots located with diazosulfanilic acid-reaction are given. With the solvent C, two spots were detected.

Solvent system ^{a)}	L-Histidine formed by KY-3592	Authentic L-histidine
A	0.18	0.18
B	0.17	0.17
C	{ 0.17 0.23	{ 0.17 0.24
D	0.19	0.19
E	0.41	0.42

^{a)} Solvent system (ratio by volume): A, *n*-propanol-0.2 N NH₄OH (3: 1); B, *n*-propanol-N acetic acid (3: 1); C, *n*-butanol-acetic acid-water (5: 2: 2); D, methylethylketone-*n*-butanol-28% NH₄OH-water (100: 100: 5: 60); E, phenol-water (3: 1).

TABLE V. CONVERSION OF L-HISTIDINOL INTO L-HISTIDINE BY *E. coli* KY-3592

C. glutamicum ATCC 21339 was cultured for 5 days in 7 separate flasks each containing a fermentation medium. Then the cultures were inoculated with the indicated inoculum sizes of *E. coli* KY-3592 cells suspended in 60% glucose solution, and continued to cultivate for further 2 days. Two of the flasks were feeded with urea or CH₃COONH₄ simultaneously with the inoculation of the *E. coli* cells. The fermentation medium used was the one described in MATERIALS AND METHODS, supplemented with 1.5% peptone, 0.02% FeSO₄·7H₂O and 0.02% MnSO₄·4H₂O; the concentration of (NH₄)₂SO₄ was 2%.

Inoculum size of <i>E. coli</i> KY-3592(ml)	Addition(%)	L-Histidine produced (mg/ml)	Residual L-histidinol (mg/ml) ^{a)}
0		0	7.3
0.5		1.9	5.5
1.0	None	2.6	3.5
1.5		2.2	3.8
2.0		1.8	5.0
0.5	Urea (0.3)	4.3	2.6
0.5	CH ₃ COONH ₄ (0.3)	2.5	5.4

^{a)} As dihydrochloride.

histidinol accumulated by ATCC 21339 into L-histidine directly in the culture of the L-histidinol producer. The cells of 25 microorganisms, including *Arthrobacter paraffineus*

KY-4303, *Aspergillus niger* KY-1556, *Brevibacterium ammoniagnes* KY-3454, *C. glutamicum* KY-9632 and KY-10025, *E. coli* KY-3592 and KY-8227, and *Torulopsis candida* KY-5801, were inoculated into the 5-days culture of ATCC 21339, in which 6~7 mg/ml of L-histidinol (as dihydrochloride) has been accumulated, and cultured for further 2 days. Paperchromatographic analysis of the cultures thus obtained revealed that *E. coli* KY-3592 reduced the L-histidinol and accumulated L-histidine. L-Histidine was identified on the basis of the *Rf*-values in paperchromatography and supporting of the growth of *S. typhimurium* hisG46, with a sample prepared by the procedure described in the previous section for the identification of L-histidinol. The results on the paperchromatographic analysis are presented in Table IV. Trace amount of imidazoleacetic acid was detected in the culture broths of *E. coli* KY-3592, *B. ammoniagenes* KY-3454 and *T. candida* KY-5801 in a paperchromatography accomplished in a manner as described in our previous paper¹⁹⁾ for the identification of this imidazole.

The conversion of L-histidinol into L-histidine by *E. coli* KY-3592 was further investigated in some details. When the inoculum size of the *E. coli* strain was varied, the accumulation of L-histidine was not so extensively changed as expected. Addition of 0.3% urea stimulated the accumulation of L-histidine, and led to the accumulation of 4.3 mg/ml of L-histidine (Table V).

DISCUSSION

A histidine auxotroph of *C. glutamicum*, ATCC 21339 was found to accumulate a large amount of L-histidinol in the culture medium. Limited supply of L-histidine to the mutant gave the maximal yield of L-histidinol. When the histidine biosynthetic pathway of *C. glutamicum* is identical with that of other microorganisms,²⁰⁾ these results are reasonably explained in terms of the defect of histidinol dehydrogenase in this mutant and the lack of the feedback regulation on the

histidine pathway due to the limitation of L-histidine in the medium. In fact, it has been verified that ATCC 21339 has a defect in histidinol dehydrogenase and is subject to both of feedback inhibition control and feedback repression control by L-histidine at the reaction of phosphoribosyl-ATP pyrophosphorylase (EC 4.2.1c), the first enzyme of histidine pathway.¹⁸⁾

Cultivation of *E. coli* KY-3592 by mixing with a later phase-culture of *C. glutamicum* ATCC 21339 resulted in the reduction of L-histidinol, which had been accumulated by ATCC 21339 in the culture, and the accumulation of 4 mg/ml of L-histidine. The yield of L-histidine is much lower than that given by a *C. glutamicum* mutant which produces 15 mg/ml [10% (w/w) of the initial sugar] of L-histidine by itself.²¹⁾ Further work is required to improve the yield of L-histidine in the two-step method for the L-histidine production described here.

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